

Formulations of liposomal vaccine containing AdhO36 antigen and β -glucan as immunoadjuvant

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ABSTRACT

Humans are the most crucial host of *Salmonella typhi* as it has limited ability to reproduce outside the human body. *S. typhi* infections caused typhoid fever which can be prevented with vaccines. This study aims to develop an effective typhoid fever oral vaccine using a liposomal delivery system. Several studies showed that the immunization of AdhO36 protein from *S. typhi* orally provides significant protection in inhibiting the *S. typhi* to the mice intestine. β -Glucan is a potent immunostimulant for macrophages activations. Meanwhile, the method used to produce Liposome formulations was using thin-film hydration. The liposomal products had good characteristics based on their particle size. The particle size results met the requirement for optimum intestinal absorption 399.220 \pm 28.095 nm Liposome+AdhO36 and 383.360 \pm 8.594 nm Liposome+ β -glucan. Based on the zeta potential measurement, the electrical charges of the three formulations were positive, meaning that the liposome is cationic because of the DDAB (dimethyl dioctadecyl ammonium bromide) component. The positive charge will facilitate the internalization of antigen to the immunity cells.

Keywords: liposome, AdhO36, β -glucan, *Salmonella typhi*, oral vaccine, dimethyl dioctadecyl ammonium bromide (DDAB), cationic liposome

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INTRODUCTION

The World Health Organization (WHO) stated that each year an estimated 11 to 21 million illnesses and 128,000 to 161,000 deaths are related to typhoid fever (Gotuzzo, 2018). The increasing cases and relapses show that current treatment methods have not been effective (Ministry of Health, 2006). The resistance worsens the problem to highly used antibiotic drugs and multidrug resistance worldwide (Ugboko and De, 2014). Oral Ty21a vaccine and parenteral Vi capsular polysaccharide (ViCPS) Vaccine are considered as the available, safe, effective, and recommended typhoid fever vaccines based on several clinical trials by CDC (Center for Disease Control and Prevention, USA) (Slayton et al., 2013). The Ty21a used weakened organism and administered orally, which make it is technically easier and caused less pain. However, the attenuated antigen can be virulent to the individual with lower immune responses.

On the other hand, the ViCPS vaccine is an independent T-cell antigen administered parenterally (MacLennan, 2015). Based on the field research, the efficacy was only between 51% to 67%, while preparations in enteric capsules only had 43% efficacy (Slayton et al., 2013). Due to the relatively low protection of these products, we still need to develop a new generation of typhoid vaccines.

S. typhi has a fimbria adhesin protein called AdhF36 with type I fimbriae and AdhO36 (afimbrial adhesin). AdhF36 and AdhO36 proteins are proven to be virulent in adhesion and colonization. They have the potential to be immunogenic to stimulate the formation of protective S-IgA so that they can become vaccine candidates to prevent the early stages of infection (Kundera et al., 2012). Several studies showed that the immunization of AdhO36 protein from *S. typhi* orally provides significant protection in inhibiting the *S. typhi* to the mice intestine. β -Glucan is a natural polysaccharide in the cell walls of cereals, bacteria, and fungi with significantly different physicochemical properties depending on the source. β -Glucan is a potent immunostimulant or a stimulator of cellular immunity activating macrophages. Based on several studies, β -Glucan has been proven to provide biological effects, for example, the immune potentiation in anti-infection (Vetvicka and Vetvickova, 2007).

Cationic liposomes are widely used in vaccine delivery research because of their efficiency in increasing the immune response. The primary mechanism of DDAB cationic liposomes as an adjuvant is to target APC cell membranes so that they can increase antigen uptake and presentation. (Korsholm et al., 2014) DDAB Liposomes with cationic properties will aggregate after the administration because they interact with proteins in the interstitial space, preventing clearance and giving depot effect. (Perrie et al., 2013) The development of a liposomal vaccine vehicle (VV) with active ingredients AdhO36 and β -Glucan obtained from *Candida albicans'* cell wall needs to be further investigated and characterized.

MATERIALS AND METHOD

Materials

S. typhi and *C. albicans* were obtained from the Laboratory of Microbiology, Medical Faculty, Brawijaya University. Dimethyl dioctadecyl ammonium bromide (DDAB) and cholesterol from Sigma-Aldrich, pro-analytical grade methanol from Merck, Soy-Phosphatidylcholine from Lipoid. The researchers used TEM (JEM 1400) and DLS (Malvern Zeta-sizer) to characterize the liposomal products.

Method

Isolation of AdhO36

S. typhi bacteria propagation temperature on McConkey medium was 37°C, and the duration was 18-24 hours. After that, this culture was transferred to liquid broth medium and thiaprolone, pThe liquid culture was then transferred to the centrifugation tubes while adding TCA (trichloro acetate) until 3% concentration. Centrifugation was done at 4° C at 6000 rpm for 15 minutes. The precipitate was suspended with pH 7.4 PBS, and the fimbria fraction was separated with a modified pili cutter at

Formulations of liposomal ... (Rachmawati et al.,)

4° C and centrifuged at 4° C with 12000 rpm for 15 minutes. The fimbria was isolated from the sediment and repeated five times. Separated cell fractions were suspended with PBS and added with 5% concentration CHAPS detergent. The mixture was processed using vortex for 5 minutes and then centrifuged again at 4° C, 12000 rpm for 15 minutes. The filtrate containing crude OMP was dialyzed using pH 7.4 PBS (Santoso, 2018; Winarsih et al., 2017).

Protein Profiling of AdhO36

The dialysate was electrophoresed using SDS-PAGE. The electrophoregram band at position 36 kDa was separated from the rest and collected. The gel pieces were electrocut and dialyzed using Phosphate Buffer Saline (PBS) solution to obtain the uncontaminated AdhO36 protein (Megayasa et al., 2016; Winarsih et al., 2017)

Isolation of β -glucan from *Candida albicans*

The *Candida albicans* culture was harvested and separated by centrifugation (3000g rpm) using pH 7.4 PBS. The cell pellet was collected and washed five times with lysis buffer. The lysis buffer is prepared by mixing ten mM Tris-HCl and 1 mM phenylmethylsulphonyl Fluoride fluoride [PMSF] and then centrifuged at 3000xg for 10 minutes. The residue was collected and suspended in lysis buffer at 4° C then lysed with 1 mm glass beads (Omni Mixer Homogenizer) at 6000 x g for 10 minutes. Centrifugation at 3000 x g for 10 minutes was done to separate the cells. The cell wall fraction was obtained in the precipitate.

β -glucan characterization

The precipitate was then spray-dried and characterized using FTIR (Fourier Transform Infrared, Panalytical) and SEM (Scanning Electron Microscope, Hitachi). FTIR characterization was performed by comparing samples of isolates with standards. The samples were mixed with KBr and pressed to form pellets. Furthermore, pellets were measured using FTIR. Morphological testing was carried out using SEM tools. Several samples were placed on the adhesive and support membrane surface, then put on the instrument and observed.

Preparations of liposomal formulations

The thin-film hydration approach was used to produce liposomes based on liposome formulations with the ovalbumin antigen model (Yusuf et al., 2019). DDAB, SPC (Soy Phosphatidylcholine), and cholesterol were dissolved in 5 mL methanol during the production procedure. All formulations had the same lipid phase, which consisted of 0.025 g DDAB, 0.091 g SPC, and 0.005 g cholesterol. The organic methanol solvent was then evaporated using a rotary evaporator at 450°C for 60 minutes, until a thin coating of lipid phase formed on the round bottom flask's walls. A solution of the active component, either β - Glucan or AdhO36 in PBS pH 7.4, was used to hydrate the thin film layer. The dosage of AdhO36 was 100 g in 1 mL, while the concentration of β - Glucan was 300 g. the liposomal suspension was sonicated for 15 minutes to decrease the particle size. Particle Size Analyzer (PSA) and Transmission Electron Microscope (TEM). morphological analyses were used to characterize the liposomal products

Particle Size and Potential Zeta Determination

Dynamic light scattering (DLS) was used to measure liposome size and polydispersity index (PDI). The Nanosizer ZS (Malvern Instruments) was utilized as the instrument. The zeta potential of liposomes was measured using a Doppler velocimetry laser and a Nanosizer ZS with a universal dip cell after a 10-fold dilution in water.

TEM analysis

Transmission electron microscopy (TEM, JEOL JEM-1400) was used to observe the morphology of the samples. The liquid samples were prepared on a copper grid at room temperature and examined using negative staining.

RESULT AND DISCUSSION

Protein Profiling of AdhO36 from *Salmonella typhi*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is widely employed to produce high-resolution separation of complex combinations in proteins (Nowakowski et al., 2014). Proteins are separated using SDS-PAGE based on their size. After heating under denaturation and reduction conditions, the protein unfolds and is coated with SDS detergent molecules, resulting in a significant negative charge proportional to the length of the polypeptide chain. When negatively charged protein molecules are deposited on the gel matrix and placed in an electric field, they will migrate towards the positively charged electrode and be separated by molecular sieve action (Suvra Roy, 2014).

Figure 1A shows the identification of pilli and outer membrane protein (OMP) fragments containing protein with 36 KDa molecular weight. Furthermore, crude OMP was dialyzed to purify the AdhO36 protein. The dialysate electrophoresis results showed AdhO36 protein (Figure 1B) to be used as an antigen. AdhO36 protein was proven to be virulent in adhesion and colonization. It has the potential to be immunogenic to stimulate the formation of protective S-IgA, so it can be the candidate for vaccines to prevent the initial stages of infection (Rachmawati et al., 2019)

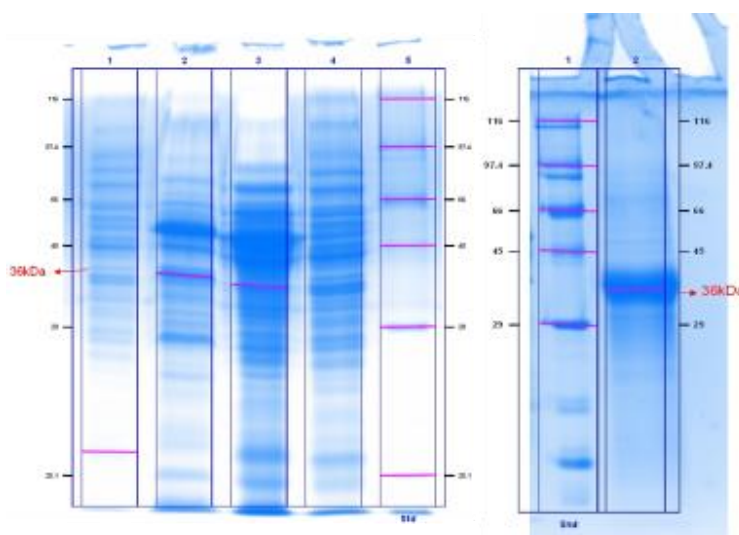


Figure 1. A. Profiling of protein outer membrane protein (OMP) (1. Whole-cell, 2. Pilli, 3. OMP, 4. Whole-cell after pilli cutting, 5. Marker); B. Single-band (1. Marker, 2. OMP)

Characterization of β -glucan isolated from *Candida albicans*

In the standard or sample (Figure 2, Table 1), there were -OH groups or hydrogen bonds, then there were also bonds between C-H atoms and C-O-C bonds in the pyranose ring (Novak and Vetvicka, 2008; Wulandari et al., 2016). There were similarities in functional groups between samples isolated from *C. albicans* with β -glucan standards indicating the existence of β -glucan in the model.

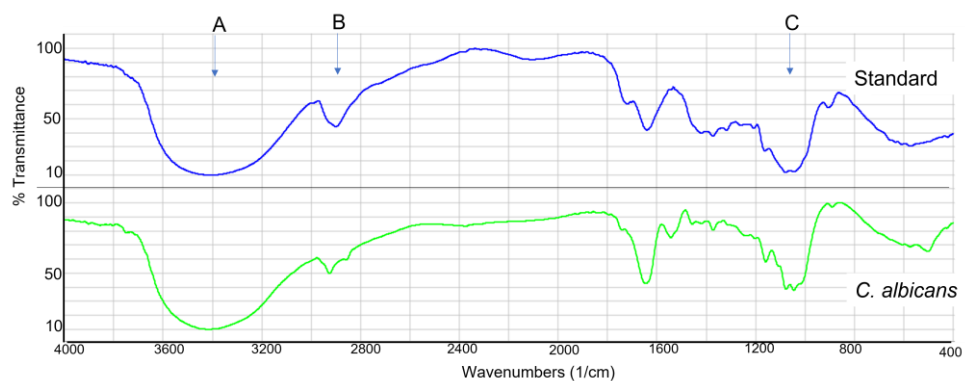


Figure 2. FTIR spectra of β -Glucan isolated from *Candida albicans* (Green) compared to the standard (Blue)

Table 1. Functional group analysis of β -Glucan isolated from *Candida albicans* (Green) compared to the standard (Blue)

No	Functional Groups	Standard	Sample
A	O-H	3387	3418
B	C-H	2899	2924
C	C-O-C in pyranose ring	1044	1044

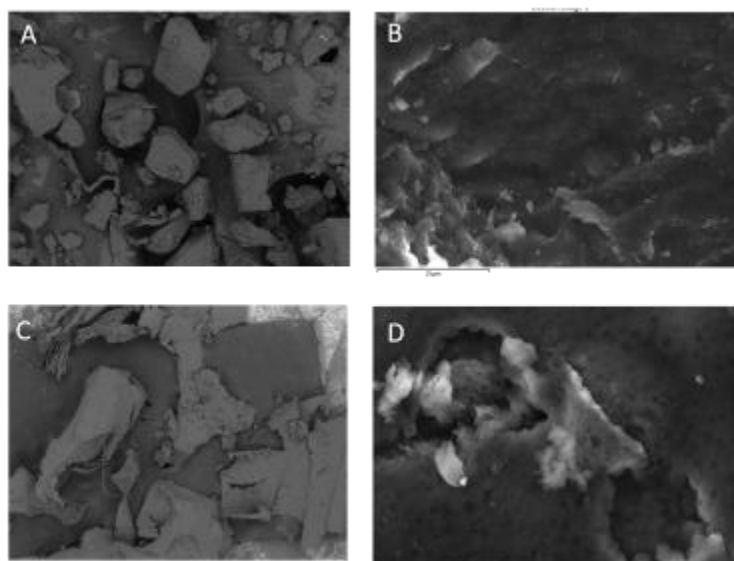


Figure 3. Morphological analysis of standard β -Glucan (A 250x and B 1500x) and isolated β -Glucan (C 40x and D 1500x) respectively

Surface analysis with SEM instruments showed uneven surfaces (Figure 3) at the microscopic level caused by the drying process and suspensions containing inhomogeneous cell wall fragments

during solidification (Novák et al., 2012). These results supported crystallographic analysis with XRD showing that both the sample and standard were amorphous.

Particle size analysis and zeta potential

The dynamic light scattering method is the most often used for particle size investigation in the nanoscale range. Brownian motion of distributed particles governs Dynamic Light Scattering (DLS). Particles dispersed in a liquid travel in all directions at random. Particles clash with solvent molecules all the time due to Brownian motion. This collision causes a certain amount of energy to be transferred, therefore inducing the movement of particles. The Stokes-Einstein equation explains the relationship between particle velocity and particle size, as the essential requirement for the Stokes-Einstein equation is the Brownian motion. There will be no random movement if there is sedimentation, which will result in incorrect findings.

Table 2. particle size analysis of liposomal formulations

Replication	Liposome+AdhO36		Liposome+β-glucan		Blank Liposome	
	Particle Size (nm)	Polydispersity Index (PdI)	Particle Size (nm)	Polydispersity Index (PdI)	Particle Size (nm)	Polydispersity Index (PdI)
1	440.700	0.958	395.200	0.857	446.700	1.000
2	400.600	0.872	386.800	0.647	488.100	0.938
3	388.800	0.934	371.900	0.695	520.100	0.897
4	403.000	0.979	383.000	0.639	584.200	0.638
5	363.000	1.000	379.900	0.797	605.500	0.552
AVERAGE	399.220	0.949	383.360	0.727	528.920	0.805
Standard of Deviation	28.095	0.049	8.594	0.096	66.001	0.198

Particle size affects the per-oral bioavailability of the formulations. The smaller the liposome size, the more effective it is in increasing the oral bioavailability of the active ingredient delivered up to three times higher than the larger-sized formulations. This study provided results following the expected specifications, 400 nm (Table 2) because further downsizing to ≤ 400 nm did not increase the uptake (Ong et al., 2016). The results met the particle size requirement for optimum intestinal absorption, namely 399.220+28.095 nm Liposome+AdhO36 and 383.360+8.594 nm Liposome+β-glucan.

Zeta potential is an electrical charge at the interface surface of a solid or colloidal surface with its liquid medium measured in millivolts (mV). Zeta potential shows the potential difference between the dispersing medium and the stationary layer of liquid that surrounds the dispersed particles. The zeta potential measurement is the easiest and most straightforward way to directly characterize the charge on the colloidal surface (Honary and Zahir, 2013). Particle surface charge is crucial in determining the efficiency of particle transmission into cells because it can determine the interaction process with negatively charged cell membranes and initiate endocytosis. Furthermore, the releasing process of particles from the endo lysosome is also influenced by the surface charge of particles (Sumadi, 2020).

Table 3. Zeta potential and conductivity analysis of liposomal formulations

Replication	Liposome+AdhO36		Liposome+β-glucan		Blank Liposome	
	Zeta Potential (mV)	Conductivity (mS/cm)	Zeta Potential (mV)	Conductivity (mS/cm)	Zeta Potential (mV)	Conductivity (mS/cm)
1	7.52	14.50	20.70	13.60	20.50	11.50
2	6.13	16.10	22.30	14.90	24.30	14.20
3	7.83	16.30	16.70	15.10	21.60	14.50
4	5.78	16.40	17.60	15.30	26.70	14.70
5	7.18	16.40	18.60	15.50	26.80	14.80
Average	6.89	15.94	19.18	14.88	23.98	13.94
Standard of Deviation	0.89	0.81	2.29	0.75	2.88	1.38

Based on Table 3, it can be seen that the zeta potential for the three formulations had a positive value, meaning that this liposome was cationic as a result of the DDAB component (Giddam et al., 2012). However, there was a decrease in the charge from the Liposome Blank (VV) ($+23.98 \pm 2.88$) mV compared to VV + Adho36 ($+6.89 \pm 0.89$) mV. This result is presumably because the AdhO36 protein is naturally negatively charged. Thus, reducing the potential difference. However, because it was cationic, it can still facilitate the delivery of antigens into APC (Antigen Presenting Cell) cells, increasing the immune responses (Baxa, 2018). The physical properties of the liposome system, significantly the charge and fluidity of the bilayer membrane, are mainly determined by the composition of the lipid bilayer. The physical stability of DDAB is low because it is aggregated quickly in salty liquids, even at low concentrations (Nugraheni et al., 2020). Our previous studies showed the effectiveness of the combination between liposomes containing β-Glucan from *C. albicans* and AdhO36 *S. typhi*, which potentially affects cellular and humoral immune response (Rachmawati et al., 2020).

Morphological analysis

Transmission electron microscopy (TEM) is a method that creates an image by passing electron beams through a material. The specimen was made very thin with a less than 100 nm thickness or a lattice suspension. The image was formed from the interaction of electrons with the sample when the beam was transmitted through the specimen. TEM is an essential method for characterizing the size and shape of nanoparticles because it can directly visualize a single particle and its inner structure.

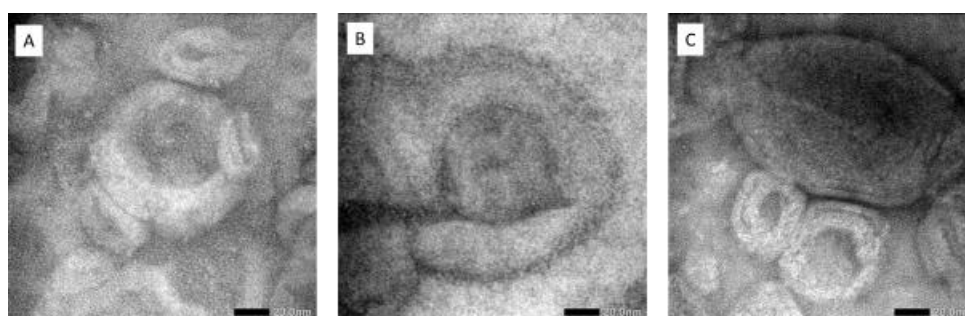


Figure 4. TEM imaging of liposomal formulations: A. Liposome+AdhO36; B. Liposome+β-glucan; C. Blank Liposome. Magnitude 200.000x

Imaging results with the TEM instrument showed that liposomes were spherical in shape (Figure 4). The phospholipid bilayer membrane was multilamellar or had several layers indicated by red arrows. This multilamellar wall serves to release antigens slowly or gradually (Agnihotri et al., 2010).

CONCLUSION

It can be concluded that the AdhO36 protein and β -glucans have the potential to be developed into liposomal dosage forms and can be given orally due to their small particle size.

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