CHARACTERIZATION OF OUTER MEMBRANE VESICLES FROM *ESCHERICHIA COLI* BZB1107 AND BL21-DE3 STRAINS

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การตรวจสอบสมบัติของถุงเยื่อหุ้มเซลล์ชั้นนอกจาก *Escherichia coli* สายพันธุ์ BZB1107 และ BL21-DE3

น.ส.สุนิสา หาญกัน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	CHARACTERIZATION OF OUTER MEMBRANE VESICLES FROM *ESCHERICHIA COLI* BZB1107 AND BL21-DE3 STRAINS
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Field of Study	Pharmaceutical Sciences and Technology
Thesis Advisor	WANATCHAPORN ARUNMANEE, Ph.D.

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สุนิสา หาญกัน : การตรวจสอบสมบัติของถุงเยื่อหุ้มเซลล์ชั้นนอกจาก *Escherichia coli* สายพันธุ์ BZB1107 และ BL21-DE3. (CHARACTERIZATION OF OUTER MEMBRANE VESICLES FROM *ESCHERICHIA COLI* BZB1107 AND BL21-DE3 STRAINS) อ.ที่ปรึกษาหลัก : อ. ดร.วนัชพร อรุณมณี

วัคซีนคือผลิตภัณฑ์ทางชีวภาพที่ช่วยให้ระบบภูมิคุ้มกันของร่างกายจดจำและสร้างภูมิคุ้มกันเพื่อป้องกันเชื้อโรค โดยในปัจจุบันถุงเยื่อหุ้มเซลล์ชั้นนอก (Outer Membrane vesicles; OMVs) ถูกใช้สำหรับพัฒนาระบบนำส่งวัคซีน อย่างไรก็ตาม OMVs ยังมีโปรตีนเยื่อหุ้มชั้นนอก (OMPs) ซึ่งอาจมีผลต่อภูมิคุ้มกันและความเสถียรได้ โดยวัตถุประสงค์หลักของการศึกษานี้คือเพื่อตรวจสอบคุณสมบัติของ OMVs สำหรับระบบนำส่งวัคซีน โดยทำการเปรียบเทียบ OMVs ที่ผลิตจากสายพันธุ์ BL21-DE3 และ BZB1107 ของ *Escherichia coli* ที่มีและไม่มีการแสดงออกของโปรตีนเยื่อหุ้มชั้นนอก (OMPs) ตามลำดับ การศึกษาเริ่มจากการแทรกยีน ClyA-GFP-6xhistidine tag เข้าสู่เวกเตอร์ โดยที่ green fluorescent proteins (GFP) เป็นตัวแทนของแอนติเจนต่อกับโปรตีนเยื่อหุ้มเซลล์ชั้นนอกคือ cytolysin A (ClyA) และใช้ 6xHistidine tag สำหรับการตรวจจับ โดยผลการทดสอบด้วย Sanger sequencing ของพลาสมิดที่มีการดัดแปลงพันธุกรรมพบลำดับเบสที่ถูกต้องของพลาสมิดที่มี ClyA-GFP-6xHistidine tag หลังจากการผลิต OMVs ที่มีการแทรกโปรตีน ClyA-GFP แล้ว Dynamic light scattering (DLS) แสดงให้เห็นการกระจายขนาดของ OMVs ของ BZB1107 และ BL21-DE3 พบว่ามีเส้นผ่านศูนย์กลางตั้งแต่ 40 ถึง 200 นาโนเมตร และ 43 ถึง 300 นาโนเมตรตามลำดับ นอกจากนี้การตรวจสอบด้วยวิธีการ SDS-PAGE และ western blot พบแถบของโปรตีน ClyA-GFP-6xHistidine ขนาด 62 kDa ใน OMVs ทั้งสองสายพันธุ์ และผลการตรวจสอบจากภาพถ่ายภายใต้กล้องจุลทรรศน์อิเล็กตรอนชนิดส่องผ่าน(TEM) แสดงให้เห็นสัณฐานวิทยาและขนาดของ OMVs ทั้งสอง ซึ่งบ่งชี้ว่าขนาดของ OMVs จาก BZB1107 นั้นมีขนาดเล็กกว่า BL21DE3 ในส่วนของการทดสอบด้วย ELISA assay พบว่า ClyA-GPF-6xhistidine tag นั้นมีการแสดงออกที่บริเวณเยื่อหุ้มชั้นนอกของ OMVs ทั้งสองสายพันธุ์ การทดสอบความเสถียรแสดงให้เห็นว่า OMVs จาก BZB1107 และ BL21-DE3 มีความทนต่อสภาวะที่มีการเปลี่ยนแปลงอุณหภูมิ แต่มีเพียง OMVs จาก BZB1107 เท่านั้นที่ไม่ได้รับผลกระทบจากความไม่เสถียรที่เกิดจาก EDTA ซึ่งบ่งชี้ว่า OMP อาจเกี่ยวข้องกับการรักษาความเสถียรของ OMVs แม้ว่าจะมีข้อแตกต่างบางประการ OMV แพลตฟอร์มใหม่นี้ ยังจำเป็นต้องมีการตรวจสอบคุณสมบัติเพิ่มเติม และเนื่องจากการทดสอบแล้วในเบื้องต้นนี้อาจมีความเป็นไปได้ที่ OMVs ที่ผลิตจากสายพันธุ์ BZB1107 ที่ไม่มีการแสดงออกของโปรตีนเยื่อหุ้มชั้นนอก (OMPs) อาจเป็นตัวแทนของแพลตฟอร์มใหม่ของ OMV ที่ไม่มีปฏิกิริยาตอบสนองต่อภูมิคุ้มกันมากเกินไปอีกทั้งมีความเสถียรในสภาวะที่มีการเปลี่ยนแปลงอุณหภูมิ ซึ่งอาจจะพัฒนาให้กลายเป็นวัคซีนที่มีประสิทธิภาพในการกระตุ้นระบบภูมิคุ้มกันต่อไปได้ในอนาคต

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Sunisa Hankan : CHARACTERIZATION OF OUTER MEMBRANE VESICLES FROM *ESCHERICHIA COLI* BZB1107 AND BL21-DE3 STRAINS. Advisor: WANATCHAPORN ARUNMANEE, Ph.D.

Vaccine is a prerequisite enhancer of acquired immunity used to protect against pathogen. The outer membrane vesicles (OMVs) are currently used for developing vaccine delivery system. Nevertheless, OMVs also contains outer membrane proteins (OMPs), which may be able to alter immunogenic effects and stability. The main objective of this study was to characterize OMVs for vaccine delivery system. Here, we compared OMV produced from BL21-DE3 and BZB1107 strains of *Escherichia coli* with or without expression of outer membrane proteins (OMPs), respectively. ClyA-GPF-6xhistidine tag gene was inserted into the vector. GFP was used as a representative antigen and an outer membrane protein, cytolysin A (ClyA)whereas 6xhistidine tag was used for detection. Sanger sequencing of engineered plasmid revealed a correct sequence of plasmids encodingClyA-GFP-6xHistidine tag. After the production of OMVs containing ClyA-GFP, dynamic light scattering (DLS) revealed the size distribution of OMVs from BZB1107 and BL21-DE3 were 40 to 200 nm and 43 to 300 nm in diameter, respectively. In addition, SDS-PAGE and western blot analysis were able to detect band of ClyA-GFP-6xHistidine tag at 62 kDa in both types of OMVs. Transmission electron microscope revealed the morphology and size of both OMVs, indicating that size of OMVs from BZB1107 were smaller than those from BL21DE3. ELISA assay indicated that ClyA-GPF-6xhistidine tag localized on the surface of both types of OMVs. Stability assay showed that OMVs isolated from BZB1107 and BL21-DE3 were resistant to temperature-challenged conditions, but only OMVs from BZB1107 was not affected by EDTA-induced destabilization, suggesting that OMPs may be involved in maintaining OMV stability. Although there are some differences and a new OMV platform need to more characterized, it is possible that OMV from BZB1107(without OMP expression) may represent a new platform of OMV without immunogenic hyper-reactivity. In addition, stability of OMVs from BZB1107 was stable in response to various temperature exposure in which this property may be further developed to be an efficient vaccine in the future.

Field of Study:	Pharmaceutical Sciences and Technology	Student's Signature .....
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CHAPTER 1
INTRODUCTION

1.1 Rationale and significant

Vaccine is a biological product that is capable of promoting the active acquired immunity to protect against pathogens such as virus and bacteria. Generally, vaccine can be divided into several types based on their manufacturing methods for example toxoid, live-attenuated, and killed (inactivated) vaccines. However, there are some disadvantages of these types of vaccines. In some severe cases, patients who received vaccine may die due to their adverse effects. At present, based on biomolecular fundamental biotechnology and genetic engineering, some pathogenic structure of microorganisms can be modified and used as antigens for the development of subunit vaccine, also called recombinant subunit vaccine. Therefore, the recombinant subunit vaccine is able to stimulate immune response better than other types of vaccine. Indeed, recombinant subunit vaccines have been considered having predominant beneficial impacts over live inactivated and attenuated vaccines in term of its efficiencies in stimulating cell- and humoral-mediated immune responses, and the pathogenic risk factors related to pathogen exposure is eliminated (Lidder and Sonnino 2012). In addition, recombinant subunit vaccine is also safer than live-attenuated vaccine with low side effects(Wang, Jiang et al. 2016). The production of subunit vaccine requires pure antigen such as toxoid, pathogen cell membrane components or molecules located on cell membrane. Indeed, the degree of immune response induced by subunit vaccine is different based on types of antigen. In fact, protein and polysaccharide antigens can trigger adaptive and innate immune responses, respectively. Of note, both types of antigen can also activate T cell function (Baxter 2007). In addition to effectiveness, vaccine delivery system is also important for improving vaccine-induced immune response by maintaining the stability of antigen, enhancing vaccine delivery to target cells, and controlling the continuity of vaccine release. Hence, the vaccine delivery system can stimulate specific immune response by presenting antigen to immune cells. Of note, subunit vaccine-mediated immune response always requires adjuvants to potentiate its effect, it may usually not be able solely to activate immune response (Schulze and Zuckert 2006, Wang, Jiang et al. 2016). Indeed, Alum, MF59 and AS03, AS04 that can be used to trigger inflammatory signals are well-known licensed adjuvants for vaccination (Coffman, Sher et al. 2010).

This study is focusing on the outer membrane vesicles (OMVs) to develop as vaccine delivery system. Currently, the bacteria-derived OMVs have been of interests for vaccine development and drug delivery. OMVs are capable of natively displaying membrane surface expression of antigens and having a self-adjuvant impact (Tan, Li et al. 2018). Importantly, fusion capability of OMVs to target immune cells is better than traditional form of vaccine (Wang, Gao et al. 2019, Balhuizen, Veldhuizen et al. 2021). Indeed, the OMV-producing bacteria is suitable for genetic modification that can be used to enhance their effectiveness as vaccines. Several lines of evidence

focusing on *meningitis* B vaccines indicated that genetic alteration of OMVs by removing or reactivating lipopolysaccharide, to generate a safely effective OMV product. Moreover, overexpression or producing simultaneous expression of multiple antigenic inducers as OMV applications for vaccine platform. In addition, alteration of OMV product may increase the yield and can be combined with particular processes to obtain high quality of well-characterized, stabilized and uniform OMV-based vaccine (van der Pol, Stork et al. 2015) Of particular importance, OMVs can easier penetrate into lymph system as it is lipid-soluble, encapsulated platform of vaccine. Hence, developing OMVs as a platform of vaccines and its delivery system have been fascinating research idea. The OMVs produced from gram-negative bacteria, with ~ 20 – 200 nm in diameter, can permeate to lymphatic vessels and can be captured by antigen-presenting cells (Kuehn and Kesty 2005, Baxter 2007, Bachmann and Jennings 2010). Of particular importance, there are several biological roles of OMVs including cell-to-cell communication, surface modification, and expulsion of components. The OMVs consist of phospholipid, lipopolysaccharides, and outer membrane proteins (OMP) (Kulp and Kuehn 2010), which can activate both humoeral and cell-mediated immune response (Ellis and Kuehn 2010). At present, some vaccine products from OMVs can be used for prevention of *Neisseria meningitides* serogroup B (BEXSERO®) or *Meningitis* B Vaccine that is now commercially available (Gorringe and Pajon 2012). Nevertheless, the main issue in vaccine development from OMVs is LPS-mediated hyperimmune reactivity, unwanted OMV-associated immunogens, batch-to-batch consistency of OMV production, and biodiversity of each strain that has various specific antigen. Therefore, genetic engineering of OMVs may be beneficial for the improvement of the effective vaccine delivery system for human diseases.

As mentioned above, OMP is one of the main components of OMVs. With undefined functions, it has never been fully understood whether OMPs affect OMV-based vaccine delivery system in term of immune-modulatory efficiency and OMV stabilization. OMPs composes of membrane domain, the OMP protein, substrate-specific porins (LamB, ScrY), porins (OmpF, PhoE), phospholipase A, and the TonB-dependent iron siderophore transporters (Koebnik, Locher et al. 2000). The primary members of the OMP are, for example, the porins including OmpC, OmpF, OmpD, PhoE, and the heat-modifiable protein (Omp A) as well as the Braun's lipoprotein. In general, functions of OMPs have been known as an immune-modulatory mediator that is associated with bacterial biofilm formation, antibiotic resistance, eukaryotic cell infection (Nie, Hu et al. 2020). In perspective of vaccine development, it is better and necessary to be known possible side effect and role in enhancing vaccine's efficiency of OMPs. Importantly, designing OMV platform to having less their membrane component is one of the fascinating concerns because it is easy to control OMV quality and can produce OMV without expression of other non-specific or non-antigenic components. For example, OmpF, one of the major OMPs, should be removed from the OMVs to eliminate non-antigenic factors from this platform. However, it is still unknown whether removing major OMP from OMVs affect the

characteristics of OMV. In this research project, ClyA-GFP fusion protein will be inserted into OMVs. Indeed, ClyA-GFP fusion protein can be a representative antigen for the future vaccine development. Furthermore, OMVs used in this study will be made from BZB1107 and BL21-DE3 strains of Escherichia coli. Of interest, previous study suggested that OMVs was able to activate innate immune responses. It is necessary to identify and compare the effects of OMP-expressing OMVs and OMVs alone that may influence to vaccine effectiveness. The main aim of this work is to characterize OMV containing ClyA-GFP produced from BZB1107 (without OMP expression) and BL21-DE3 (having OMP expression) strains. The knowledge from this study may lead to the better version of OMV displaying the safety and efficacy vaccine in the future.

1.2 Research Question

Are characteristics of OMVs produced by *E. coli* BZB1107 and BL21-DE3 different?

1.3 Objectives

To produce and characterize decorated OMVs from *E. coli* BZB1107 and BL21-DE3

1.4 Hypothesis

- Successful development of an outer membrane vesicle that has been inserted with ClyA-GFP-6XHistidine tag from *E. coli* BZB1107 and BL21-DE3 strains.
- Characteristics of the inserted outer membrane vesicle with ClyA-GFP-6XHistidine tag, which are produced from *E. coli* BZB1107 and BL21-DE3 strains are different.

1.5 Benefit of this study

This study can provide information on production of Outer membrane vesicles with having designed antigen in vaccine delivery system.

1.6 LITERATURE REVIEW

1.6.1 *Escherichia coli* (*E. coli*)

Escherichia coli (*E. coli*) is a group of gram-negative bacteria that is generally found in environment including in food and intestine in both human and animals. Even though, most of *E. coli* are not harmful to human body, some types of *E. coli* are able to induce diarrhea and is considered as a cause of infection in urinary and respiratory tracts that promote pneumonia and other diseases. The ability of *E. coli* or other types of gram-negative bacteria to the pathogenesis of diseases depend on the components that is located on the bacterial cell wall such as lipopolysaccharides (LPS), peptidoglycans, or endotoxins, which are the large molecules that compose of lipid and polysaccharides. All of these components are linked with O-antigen on the outer membrane layers of gram-negative bacteria, which is able to stimulate immune system by inducing a production of cytokines, main mediators of inflammation that are toxic to the cells.

The B strain of *E. coli* has an important role for the study of phage sensitivity, restriction modification system, bacterial evolution, and protein expression (Jeong, Barbe et al. 2009). In this research study, B strains of *E. coli* including BZB1107 and BL21-DE3. Indeed, BZB1107 is one of the derivatives of classical *E. coli* BE. These strains have Tn5 insertion at the original fragment of OmpF gene. In addition, it was found that there was no expression of OmpC porin and sequence of OmpF (Rosenbusch 1974). On the other hand, *E. coli* BL21-DE3 is a derivative of BL21, which is widely used for the study of recombinant protein expression because this strain highly expresses the recombinant protein. In fact, this strain obtains bacteriophage λ that has T7 RNA polymerase under lacUV5 promoter. Furthermore, *E. coli* BL21 can be used for non-T7 expression and can be modified for the production of plasmid DNA vaccine (Phue, Lee et al. 2008).

1.6.2 Outer membrane vesicle (OMVs)

The characteristics of OMVs can be described as the circular lipid bilayers in a form of vesicles. Generally, OMVs can be produced from the gram-negative bacteria. In addition, this type of vesicle has a diameter around 20 – 200 nm, which is small and can pass through the lymphatic vessels and is consequently captured by antigen presenting cells (APC) (Kuehn and Kesty 2005, Bachmann and Jennings 2010, Kulp and Kuehn 2010). Inside OMV lumen, there are metals (including iron and zinc), some LPS, unfolding protein, antibiotic-resistance proteins, and sulfatase (Schwechheimer and Kuehn 2015). Over the past several years, the main aim of the study of OMVs has been aimed at investigating an association of bacterial pathogenesis. Moreover, OMVs have been used in the investigations that are closely related to genetic and biochemical analyses as well. All of these studies can lead to the explanation of the mechanisms of the production of OMVs that includes OMV production from non-pathogenic bacteria. Of note, OMVs should be from non-pathogenic bacteria, which functionally forms extracellular vesicles such as the mediator of the cellular communication. Moreover, components located on the surface

of the OMVs can be modified in order to insert or eliminate required or non-essential components of OMVs (Deatherage and Cookson 2012), respectively. Due to OMVs are from cell membrane of the gram-negative bacteria. Of note, Budding process generally occurs in all stages of growth phase of bacteria. (Orench-Rivera and Kuehn 2016) (Rueter and Bielaszewska 2020).Therefore, important to be considered regardless the special structure and understanding mechanisms of budding and releasing OMVs.

In fact, cell membrane of the gram-negative bacteria composed mainly of outer membrane and cytoplasmic membrane. Cytoplasmic membrane has phospholipid bilayer whereas outer membrane composes of interior leaflet of phospholipids and exterior leaflet of lipopolysaccharide (LPS). In general, LPS contains lipid A oligosaccharide and O antigen. In periplasmic space (space in between two layers of bacterial membranes, there are peptidoglycan (PG) andperiplasmic proteins in which PG has disaccharide N-acetylglucosamine–Nacetylmuramic acid (NAG–NAM) that forms peptide bridges including traditional 4–3 (D-Ala–meso-diaminopimelic acid (mDAP)) crosslinks and non-traditional 3–3 (mDAP– mDAP) crosslinks. In addition, envelope proteins have the capacity to be dissolved. Envelope stability depends mainly on the differences of crosslinks including covalent crosslinking of Braun's lipoprotein (Lpp) in outer membrane and PG These interactions are non-covalent interactions between PG and porin outer-membrane protein A (OmpA) and non-covalent interactions between PG and Tol–Pal (peptidoglycan-associated lipoprotein) complex, which compose of TolA, TolB, TolQ, TolR and Pal as well as envelope from cytoplasmic membrane interact with periplasm and outer membrane.

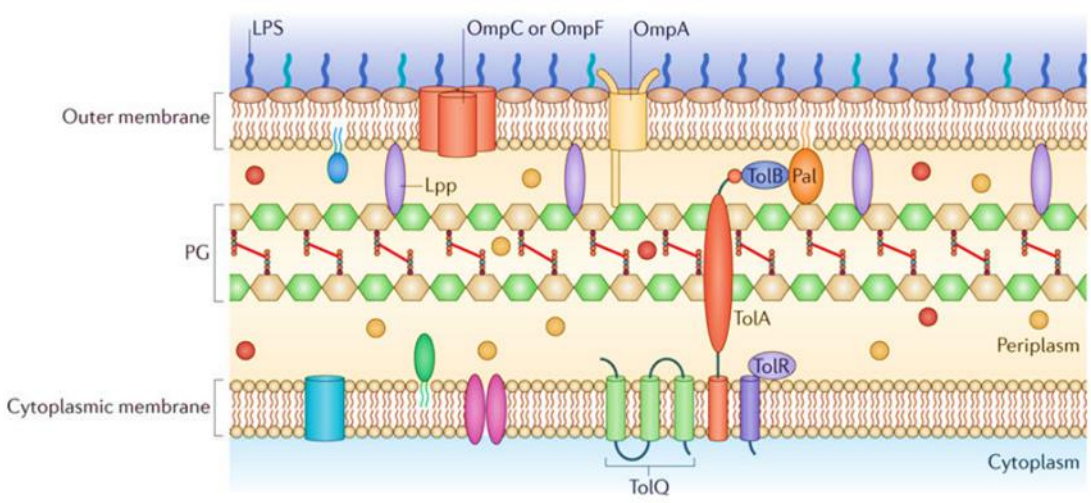

Figure 1.1 Gram-negative cell envelope composition (Schwechheimer and Kuehn 2015)

(periplasmic; orange and red sphere), transmembrane proteins (pink oval and blue cylindrical) segments attached to both membranes via covalent bonds to the lipid segments. (lipoproteins; green and blue ovals)

In addition, OMVs have long been used as a vaccine platform as well because of its ability to mimic a nonreplicating immunogenic effect and have properties of natural adjuvant, which can stimulate immune reactions in both innate and adaptive immune response. Of particular importance, OMVs have some predominant advantages in development of vaccine rather any other lipid nanoparticles because it contains phospholipids, LPS, and outer membrane protein (OMPs), which are capable of activating both humeral and cell-mediated immune responses (Ellis and Kuehn 2010). Furthermore, OMPs expressed on the surface of the gram-negative bacterial cell wall can be recognized by host immune system as a foreign body. Then, immune response was generated according to exposure to OMPs. It seems that OMPs can function similar to either antigen or adjuvants for efficient inducers of innate immune responses (Galdiero, Falanga et al. 2012). Consistently, Sharma and co-workers identified that OmpF epitope (66-88) was designed to be linked with carrier protein is used as vaccine to protect against antibiotic-resistant A. hydrophila. In case of no expression of OMPs, there is some possible effects on releasing mechanisms of OMVs. There was a study that reported the lack of OmpA enhanced the rate of OMV release and can reduce the viability of Acanthamoeba castellanii (Valeru, Shanan et al. 2014).

1.6.3 Outer membrane proteins (OMPs)

Membrane proteins are structurally like backbone that are locally and functionally regulated. Biologically, mechanistic insights into their respective insertions through the Sec translocon, inner membrane proteins and outer membrane proteins are all α-helical and are almost all β-sheets (Osborne, Rapoport et al. 2005, Dong, Beis et al. 2006, Hagan, Silhavy et al. 2011), respectively. There is, nowadays, increasing interest in outer membrane proteins (OMPs) due to its effect on antibiotic resistance and their useful applications as biosensors, and their localization, which makes them accessible to the outside of the bacterial cells. The illustrative structure of OMPs is ~100 heterogenous structural pattern of OMPs in the PDB. β-barrel strands are amphipathic and are basically introduced antiparallel to each other. The subcellular architecture of the OMPs that are linked mainly by hydrogen bound through the backbone resulting in the alteration of side chains in direction between those facing the pore and those facing the membrane. Each loop of OMPs is linked to the strands of protein sequence, with larger loops in the extracellular space and smaller one in periplasmic space. In certain situation, the loops generate plugs in the barrels before linking to the next strand (Slusky 2017). In most of outer membrane β-barrels, they have been structurally characterized as monomeric with one chain generating one barrel. Nevertheless, some barrels can oligomerize. In some cases, they can form as trimers although dimers are still visualized. In every single barrel, many of protein chains contribute to forming the complicated topologies(Slusky 2017). In addition to antibiotic effect of OMPs, they can be channels for exchange between nutrient, ion, and waste product. Several lines of evidence also reported the immune-modulatory effects of OMPs.

1.6.4 CytolysinA (ClyA)

The cytolysin A (ClyA) belongs to a group of proteinaceous bacterial hemolysin mostly found in OMVs (Wai, Lindmark et al. 2003, Kouokam JC 2006). ClyA is highly abundant in *E. coli*, especially in periplasmic space (Atkins, Wyborn et al. 2000). Furthermore, there was a report for the mechanisms underlying the transport of ClyA to extracellular medium and release to form the ClyA pore complexes at OMVs (Kouokam JC 2006). According to the two mechanisms mentioned above, ClyA that is released into medium of OMVs and transport of ClyA to periplasm have currently been difficult to understand because little is known about the roles of ClyA in mediating bacterial signal sequence (Atkins, Wyborn et al. 2000, Kouokam JC 2006). ClyA can be synthesized in a form of soluble protein with a molecular size of ~ 34 kDa that is encoded from ClyA gene. However, regulatory mechanisms of gene expression of this gene are now still complicated and difficult to understand and related to several transcription factors. At present, there are no evidence supporting the signal sequence cleavage and posttranslational modification of ClyA. In some investigations, ClyA accumulates in the space of periplasm. When ClyA is increasingly produced, it translocates this toxin to periplasm and there is releasing process from *E. coli* by the process of blebbing of OMVs. There is an investigation of genetic engineering of ClyA and other proteins that can modify the translocation process of those heterologous proteins to OMVs (Zhou, Srisatjaluk et al. 1998). It is still unclear about the mechanism of ClyA membrane insertion but its stabilization on membrane surface has been reported to be cholesterol-dependent mechanism (Sathyanarayana, Maurya et al. 2018). Moreover, there is no evidence indicating interfering effect of ClyA on bacterial membrane budding process. In addition, Galen and co-workers indicated that genetic-modified, ClyA-linked antigen can enhance translocation process of antigen into the OMVs. This modification was performed in vaccine/OMVs platform produced from Salmonella can improve the efficiency of vaccination (Galen, Zhao et al. 2004).

1.6.5 Roles of OMVs in the process of vaccine development

Research studies that have long been focusing on using OMVs as the development of platforms for vaccine and vaccine/delivery system have been consecutively increased for the past several years (Robbins and Morelli 2014). Recently, beneficial roles of OMVs in protecting against human pathogens including *Neisseria meningitidis, Francisellatularensis subspnovicida, Burkholderiapseudomallei* and *Mannheimiahaemolytica* have been discovered (Nokleby, Aavitsland et al. 2007, Ayalew, Confer et al. 2013, McCaig, Koller et al. 2013, Nieves, Petersen et al. 2014). Based on antigens located on the bacterial surface and adjuvant e.g., LPS, efficiency of OMVs was proposed to generate an effectiveness of adaptive immune response. However, an idea that have attempted to modify OMVs to increase the immune responsiveness by molecular techniques related to genetic engineering approach that can incorporate antigen into OMVs and reduce toxicity of LPS is still challenging. Roles of OMVs in the process of vaccine

development for inducing activation of adaptive immune response have been currently in focus. Indeed, OMVs can alter the process of infection. For example, based on the study using *V. cholerae*, injection of *V. Cholerae* OMVs was able to attenuate disease severity, hyperinfection, and reduced mortality rate in mice induced by *V. Cholerae* infection (Schwechheimer and Kuehn 2015). In general, vaccine is a biological product that can activate immune system for prevention of disease and for enhancing and sustaining immune response to infection. This approach can trigger both innate and adaptive immune systems. Therefore, vaccine products should have characteristics similar to pathogen but should not be able to initiate pathogenesis of related diseases (Singh, Chakrapani et al. 2007, Scheerlinck and Greenwood 2008, Bachmann and Jennings 2010, Zepp 2010). In fact, vaccine product should have an optimal molecular size and should also have both PAMPs and specific antigen of pathogen of interest. In addition, the component that cause disease and certain surface components mimicry host components should not be in the melody of vaccine. Moreover, several characteristics that may lead to increasing evolutionary response of disease should be avoid, for example, components that can induce an increase in protease production and biofilm production. To overcome these possible issues in OMV development, genetic engineering method can be used to eliminate unwanted toxic components or to increase the number of surface antigens in OMVs. This is designed to make surface antigen exposure and differential designs of antigen expression as shown in Figure 1.2. Indeed, surface exposed antigen can be captured by B cells specifically whereas luminal antigen induces a response of cytotoxic T-cell (Galen and Curtiss 2014). Therefore, approach to design OMVs and their antigen localization is a key process to induce host immune response.

At present, some vaccine products from OMVs can be used for prevention of *Neisseria meningitides* serogroup B (BEXSERO®) or *Meningitis* B Vaccine that is now commercially available (Gorringe and Pajon 2012). There are four main components of Bexsero that play major role in targeting lifecycle of *N. meningitidis* in human host, from colonization in nasopharynx, to survival, function, and virulence in blood stream and cerebrospinal fluid. Firstly, factor H binding protein (fHbp), GNA2091 protein, binds human factor H, the gene encoding fHbp is nmb1870. The second component, NadA, a major adhesion protein involved in colonization, invasion, and induction of pro-inflammatory cytokines. The third component is a heparin-binding protein that increases resistance against the bactericidal activity of human serum and is virtually present in all strains. NHBA is fused with protein GNA1030. The forth component is OMV. In this combination, it has successfully demonstrated tolerability and effectiveness in actual use (in the case of the New Zealand serogroup B outbreak) (Carry and Benichoux 1969). Other OMV-based vaccines, are still being developed in preclinical studies most of which utilize OMV antigen binding (Table 1.1).

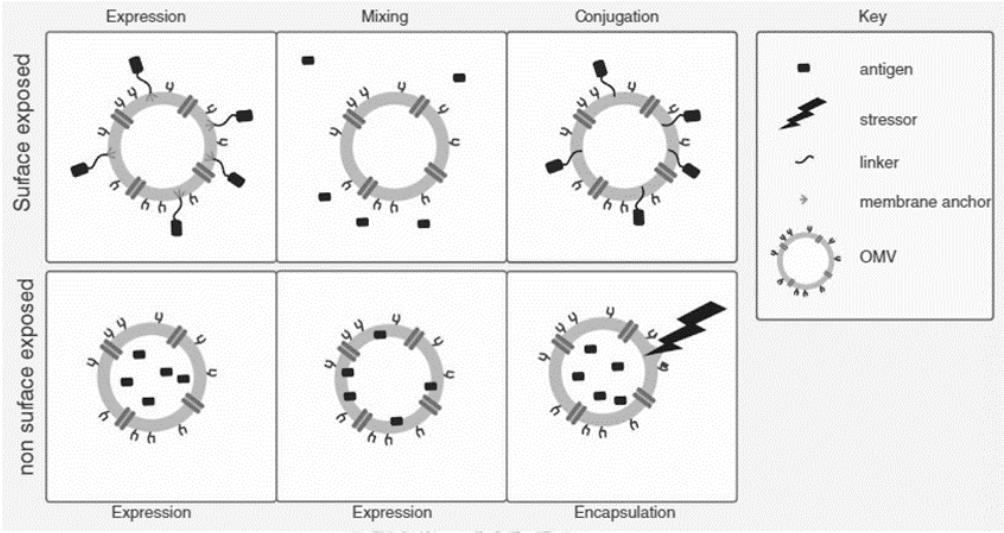

Figure 1.2 Antigen expression at different locations on the outer membrane vesicle (Gerritzen, Martens et al. 2017)

Table 1.1 Outer membrane vesicle-based vaccine in preclinical

OMV Source	Model Establishment	Animal Model	Adjuvant Used	References
B. burgdorferi	Infection	New Zealand White Rabbits	Alum	(Shang, Champion et al. 2000)
B. pseudomallei	Septicemic infection	Female BALB/c Mice (8–10 weeks of age)	None	(Nieves, Asakrah et al. 2011)
E. coli	Sepsis	Both C57BL/6 and BALB/c Mice (5 weeks of age)	None	(Kim, Hong et al. 2013)
E. coli expressing streptococcal antigen	Sepsis (group A streptococci)	CD1 female Mice	Alum	(Fantappie, de Santis et al. 2014)
E. coli expressing Apx fusion antigen	Porcine pleuropneumonia	Female BALB/c Mice (4–5 weeks of age)	Alum	(Xu, Zhao et al. 2018)
N. meningitides with inactivated RSV	Infection (RSV)	Female BALB/c Mice (5–8 weeks of age)	OMVs	(Etchart, Baaten et al. 2006)

The OMVs can be obtained from either nature or artificial method using detergent or by sonication. The components of OMVs derived from outer membrane layer of bacteria are different from those derived from detergent-induced approach. In fact, detergent generally eliminates lipoproteins in which its PRR can be modified based on experimental design. Based on the study of Van der Ley and co-workers, types of OMVs can categorized according to the production methods including spontaneous OMVs (sOMVs) by which this type of OMVs is produced bacteria naturally. On the other hand, there is another type of OMVs including detergent OMVs (dOMVs), which is the OMVs from detergent-induced method. For the third one, native OMVs (nOMVs), which is produced by sonication method (Gnopo, Watkins et al. 2017).

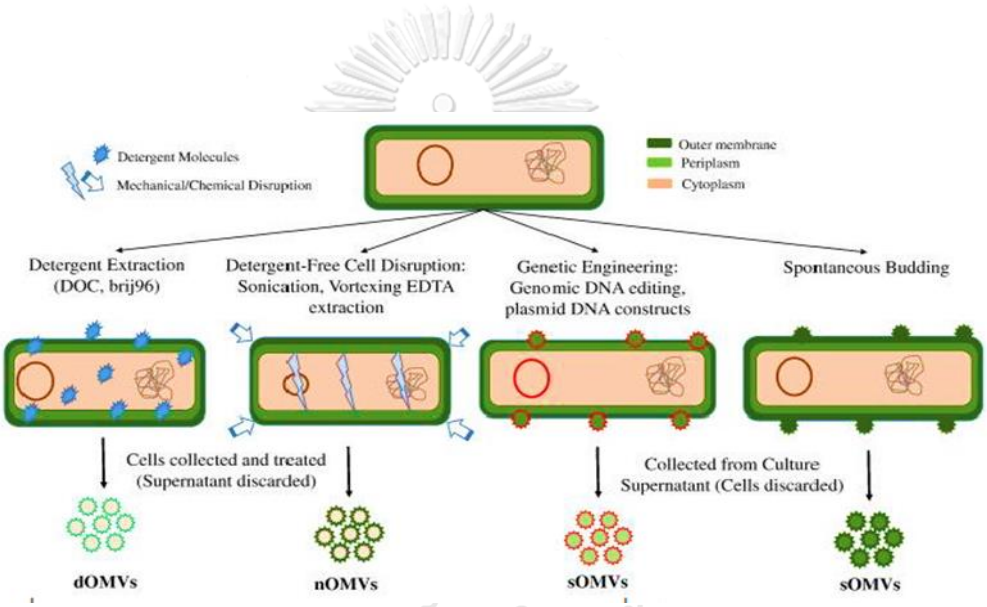

Figure 1.3 Classification of OMVs (Gnopo, Watkins et al. 2017)

In this thesis, spontaneous OMVs (sOMVs) have been in-focus. In general, sOMVs are released from the cell membrane of bacterial by natural way. Although it was reported that sOMVs can be released by the natural way but most of research works have tried to know the mechanism of the release of OMVs. Despite the cell membrane as a source of OMVs, proteomics approach indicated that membrane surface of OMVs was not 100% similar to cell membrane of intact bacterial cell wall. Indeed, some proteins were increased, and some proteins were decreased. For example, ClyA protein from *E. coli* as mentioned above, this protein was highly abundant in OMVs rather than in an intact cell membrane of live bacteria. In addition, disulfide bond formation A (DsbA) protein was found in a small amount in the intramembrane space of OMVs but highly abundant in the periplasm of the cells. At present, there are several lines of evidence that investigate the expression diversity of

antigens on sOMVs in the development of system for efficient vaccine delivery as shown in Table 1.2.

Table 1.2 Antigen expressions of sOMVs in the development of vaccine delivery systems.

Type	**Antigen location**	**Antigen display**	**Antigen**	**Target**
sOMVs	Surface exposed	ClyA fusion	Omp22 M2e	*Acinetobaterbaumannii* Influenza A
		Hbp fusion	ESAT6	Tubercolosis
	OMV lumen	OmpA fusion	SpyCEP	Group A Streptococcus disease
			SAM_1372	Group B Streptococcus disease
	Mixing	Mixing	O-antigen	Shigellosis
			AnAPN1	Malaria

CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Table 2.1 Chemical reagents

chemical	brand
pET15b	EMD Millipore
pMS119	Sent from Newcastle university
Gene ClyA-GFP-6XHistidine tag in pUC57	GenScript
LB broth	Hardy diagnostic
LB agar	Hardy diagnostic
TRYPTONE-R	TM Media
YEAST EXTRACT POWDER TYPE 1	TM Media
Ampicillin	AppliChem GmbH
Glycerol	Vivantis
Presto mini Plasmid Kit Quick	Geneaid
CutSmart Buffer 10X Concentrate	NEB
EcoRI-HF	NEB
HindIII-HF	NEB
Agarose	Vivantis
Gel Loading Dye Purple (6X)	BioLabs
HyperLadder 1kb	Bioline
Precision Plus Protein Dual Color Standards	Biorad
RedSafe Nucleic Acid Staining Sulotion	Intron
QIAquick Gel Extraction Kit	Qiagen
T4 DNA Ligase Buffer 10X concentrate	NEB
T4 DNA Ligase	NEB
5X Q5 Buffer	NEB
Q5 DNA polymerase	NEB
MgCl2	Vivantis
dNTP	Vivantis
Taq	Vivantis
Isopropyl B-D-1-thiogalactopyranoside (IPTG)	Bio Basic Cannada inc.
Tris-HCl	Sisco Research Laboratories Pvt. Ltd.
SDS	Biorad
Acrylamide/bis-acrylamide	Merck
TEMED	Merck
Extrathick blote filter paper	Biorad
Nitrocellulose membrane	Biorad
Skim milk	Hardy diagnostic
Goat anti-mouse secondary antibodies	ThermoFisher Scientific

6X-His tag Monoclonal Antibody (HIS HB)	Invitrogen
HRP Goat anti-mouse IgG (minimal x-reactivity) Antibody	BioLegend
Tween-20	Lobachemie
25X alkaline phosphatase development buffer	Biorad
AP colour reagent A	Biorad
AP colour reagent B	Biorad
PBS 10X	Vivantis
TMB chromogen solution	ThermoFisher Scientific
Albumin, from bovine serum	Sigma

2.1.2 Bacterial strains for experiments

Main strains of bacteria used in study are BZB1107 and BL21-DE3 strains. BZB1107 is a derivative of *E. coli* B^E containing Tn5 insertion at the beginning of the ompF gene. This strain does not express the porin OmpC. In addition, BL21-DE3 is a derivative of BL21, which is widely used in high-level expression of recombinant proteins, there are prophage DE3 derived from bacteriophage λ carries T7 RNA polymerase gene under the control of lacUV5 promoter found in this strain.

2.2 Method

2.2.1 Construction of plasmids for inducible ClyA-GFP-6XHistidine tag

Amplification of ClyA-GFP-6XHistidine tag gene by Polymerase chain reaction (PCR)

The pET15b plasmids containing ClyA-GFP-6XHistidine tag was amplified by PCR approach as shown in Table 2.2. Primers for amplification of this insert were 5′-CAGGAATTCATGACCGAGATTGTGGCGGATAAG-3′ (Forward primer) and 5′-CAGAAGCTTTTAGTGGTGGTGGTGGTGGTGGC-3′ (Reverse primer).

Table 2.2 Master mix reaction of polymerase chain reaction (PCR)

Q5 PCR recipes	Final conc.	12.5uL (1x)
DNA template	<1000 ng	1
5x Q5 buffer	1x	2.5
10mM dNTPs	200 µM	0.25
10mM Forward primer	0.5 µM	0.625
10mM Reverse primer	0.5 µM	0.625
Q5	0.02 U	0.125
UP H2O	up to 12.5uL	7.375
Total volume		12.5

DNA fragment analysis and verification by agarose gel electrophoresis

Agarose gel electrophoresis was used to separate genetic material based on the differences of size of DNA fragment. Agarose gel preparation at concentration of 1.2% w/v was performed in 1X TAE Buffer (40mM Tris, 20 mM Acetate and 1 mM EDTA, pH 8.6). When gel was polymerized and formed as a solid phase, PCR product in volume of 12.5 µL was mixed with 6X loading dye (2.5 µL). Later, agarose gel was set in the horizontal electrophoresis (Mupid®-One) filled with 1X TAE Buffer. Then, 1kb HyperLadder™ and PCR product (with 6X loading dye) were loaded into each lane of 1.2% agarose gel. Ladder and samples were basically loaded in the volume of 5 and 15 µL, respectively. Size separation was performed by 100-volt running for 40 min. Finally, the DNA bands of each samples were detected by LED light.

Gel extraction

DNA purification of ClyA-GFP-6XHistidine tag from the agarose gel was performed using QIAquick Gel Extraction Kit. In brief, expected DNA bands were cut and removed to microcentrifuge tubes. Buffer QG was added into microcentrifuge tubes containing DNA fragments of an expected ClyA-GFP-6XHistidine tag at volume ratio of 3 to 1 (300 µl buffer QG to 100 mg of gel fragment). Thereafter, all microcentrifuge tubes were heated at 50 °C for 10 min. Of note, the color of solution inside the tubes should be yellow. Furthermore, isopropanol was added into each tube at a ratio of 1:1 by volume. Hence, all solution in the tubes containing a mixture of DNA fragments was removed to QIAquick column for the further purification process. Next, QIAquick column tubes containing DNA fragment were centrifuged at a speed of 13,000 rpm at room temperature for 1 min. Filtered solution beneath the column was discard and column was washed by 750 µL of Buffer PE and centrifuged at 13,000 rpm for 2 min in room temperature. Filtered Buffer PE beneath the column was completely discard. Of note, it is necessary to centrifuge the empty column to

entirely remove remaining buffer for 5 min. Column was put on top of the new sterile/DNA-free microcentrifuge tubes. Therefore, 20 µL Buffer EB was added into each column and incubated for 1 minute and was therefore further centrifuged at room temperature at 13,000 rpm for 2 min to isolate ClyA-GFP-6XHistidine tag from the column.

Measurement of DNA concentration by NanoDrop™

Briefly, 1 µL of each sample was used to measure the concentration of DNA, which is able to predict the concentration of plasmid DNA based on UV absorbance at 260 nm by NanoDrop™ Microvolume UV-Vis Spectrophotometer. In addition, quality and purify of DNA can be determined by the ratio of 260 to 280 nm (A260/A280) in which ratio of approximately 1.7–2.0 of A260/A280 was accepted as high quality and purity without any contamination. In addition, ratio of A260/A230 that indicates salt mixture should be higher than 1.5 as accepted value.

Restriction digestion of DNA inserts

A mixture of restriction buffer for specific DNA digestion was shown in Table 2.3. Briefly, specific restriction enzymes including EcoRI-HF and HindIII-HF were used. It is noticed that all the solution should be spin down before mixing with ClyAGFP-6XHistidine tag fragments (1 µg) in a volume of 10. A mixture was heated in heat block that was set at 37 °C for 15 min and was moved to 50 °C for 20 min to stop the restriction reaction.

Table 2.3 Mixture of restriction enzyme and DNA insert

Reagent	**Volume (µL)**
Nuclease-free water	6
CutSmart Buffer 10X Concentrate	2
EcoRI-HF	1
HindIII-HF	1
DNA insert	10
Total volume	20

Purification of DNA fragment

After restriction reaction, ClyA-GFP-6XHistidine tag fragment was purified by QIAquick PCR & Gel Cleanup Kit. The ClyA-GFP-6XHistidine tag fragment was added into DNA-free microcentrifuge tubes filled with Buffer QG (volume ratio of 3:1) and was incubated at 50 °C for 10 min by which color of solution should be yellow. Isopropanol was added into a mixture in a ratio of 1:1. Then, all mixture

solution was removed to the QIAquick column and centrifuged at a speed of 13,000 rpm for 1 min. Remaining filtered solution was discarded and column was washed by 750 μL of Buffer PE at room temperature with centrifuge speed of 13,000 rpm for 2 min. Filtered solution beneath the column was discarded and the empty column was further centrifuged to remove all remaining buffer. In addition, 20 μL Buffer EB was added into the column and incubated for 1 min at room temperature before centrifuge at 13,000 rpm for 2 min to isolate ClyA-GFP-6XHistidine tag (Insert) from column. NanoDrop™ was used again to measure DNA concentration of ClyA-GFP-6XHistidine tag (Insert) before ligation process.

DNA ligation

According to protocol from the kit, 1 μL of 10X T4 DNA Ligase Buffer was mixed with 0.5 μL of T4 DNA Ligase and 1 μL of nuclease-free water to make ligation reaction buffer. In addition, the ClyA-GFP-6XHistidine tag (DNA insert) was mixed into the plasmid pMS119 (Vector DNA) in an insert to vector ratio of 2:1, incorporated with ligation reaction buffer. This reaction was incubated in room temperature for 10 min and heated to inactivate ligation process at 65 °C for 10 min.

Table 2.4 A mixture of ligation reaction of plasmid

Reagent	**Volume (μL)**
Nuclease-free water	1
T4 DNA Ligase Buffer 10X concentrate	1
Vector DNA (4 kb)	3.4
Insert DNA (1 kb)	4.1
T4 DNA Ligase	0.5
Total volume	10

Transformation of newly construct plasmid into competent cells

The newly construct plasmid containing ClyA-GFP-6XHistidine tag that use the pMS119 plasmid as a template was transformed into *E. coli* Mach1. For transformation, heat shock approach was used to generate the competent cells (50 μL) in microcentrifuge tube and put it on ice. The pMS119 plasmid containing ClyA-GFP-6XHistidine tag (5 μL) was added into tube containing competent cells (50 μL) and transferred to 42 °C for 30 second and put it on ice again for 2 min. Thereafter, SOC outgrowth media (500 μL) was added into tube containing a mixture of competent cells and plasmid and was incubated in 37 °C on the shaker with a speed of 200 rpm for 1 hour. Furthermore, 100 μL of SOC outgrowth media containing competent cells and plasmid was plated onto LB agar treated with ampicillin and incubated at 37 °C overnight.

Plasmid extraction from transformed cells

Bacterial colonies that are resistant to ampicillin were picked up using sterile loop for collection and transferred to 5 ml LB broth containing ampicillin. Tube containing bacterial colony that express the gene of interest was incubated in 37 ℃ on the shaker with a speed of 200 rpm overnight. After overnight incubation, constructed plasmid from transformed cells was isolated using Presto™ Mini Plasmid Kit Quick. In brief, 5 mL of transformed cells was centrifuged at 4 °C with a speed of 16,000xg for 1 min. All the colorless fragment was removed. Then, 200 μL of PD1 buffer was added into and mixed in the tube containing transformed cells. PD2 buffer (200 μL) was added and mixed to break the cells but without destroying plasmid DNA and let it in room temperature for 2 minutes. PD3 buffer (300 μL) was added into the tube and gently mixed as well as further performing a centrifugation at 16,000xg for 3 min. Later, all solution was transferred to the PDH column and centrifuged at 13,000 rpm for 30 seconds. The PDH column was washed by wash buffer (600 μL) and further centrifuged again at 16,000xg for 3 min. The column was transferred to new sterile tube for the next isolation step. Elution buffer (50 μL) was added into the column and perform centrifugation (16,000xg) step for 2 min to elude the plasmid construct from the filter of the column. Plasmid DNA samples can be kept in -20 ℃. Measurement of DNA samples can also be performed using Nanodrop. After plasmid was extracted from transformed cells, the DNA sequence of plasmid was by sanger sequencing using pMSseq primer.

2.2.2 Production of OMVs containing inducible ClyA-GFP-6XHistidine tag

Protein expression of ClyA-GFP-6XHistidine tag in BZB1107 and BL21-DE3 strains of E. coli

The pMS119, which is used as a cloning vector was genetically modified to insert ClyAGFP-6XHistidine tag and ampicillin resistance to and transformed to *E. coli* BZB1107. In addition, pET15b that contains ClyA-GFP-6xHistidine tag was directly transformed to BL21DE3 strain of *E. coli.*

Production of OMVs expressing ClyA-GFP-6XHistidine tag*(Thoma, Manioglu et al. 2018)*

The pMS119 and pET15b plasmids encoding ClyA-GFP-6xHistidine tag were transformed into *E. coli* competent cells including BZB1107 and BL21-DE3 strains, respectively. Inoculation of these two strains in 300 mL of TB broth treated with ampicillin (100μg/ml) was performed and incubated at 37 °C with 250 rpm shaking until OD_{600} of bacterial culture was 0.6-0.8. Isopropyl B-D-1-thiogalactopyranoside (IPTG) 0.5 mM used as an inducer of protein expression was added into bacterial

culture flask. Bacteria was cultured with IPTG at 37 °C with shaking at 250 rpm for 8 – 14 hours.

Collection of OMVs having ClyA-GFP-6XHistidine tag*(Thoma, Manioglu et al. 2018)*

After induction, transformed cells were centrifuged at 8,000 xg for 30 min at 4 °C. As OMVs were secreted into media, supernatant was collected using ultrafiltration membrane that can remove particles with size smaller than 100 kDa. Supernatant remaining on filter membrane was centrifuged at 150,000 g for 3 h at 4 °C. Supernatants was removed and pellets were dissolved in 1 ml PBS (137 mM NaCl, 2.68 mM KCl, 10.1 mM Na2HPO4 and 1.76 mM KH2PO4, pH 7.4). OMV samples in PBS were further characterized.

2.2.3 Characterization of OMV and identification of ClyA-GFP-6XHistidine tag expression in OMV samples

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide (10 %) that includes stacking and separating gels was prepared. Of note, ammonium persulfate and TEMED should be mixed as the last things. Precision plus protein™ standard ladder (4 μL) was used to make the size reference of protein. Boiled samples (20 μL) were loaded into each lane of the Polyacrylamide gel. To separate the protein by size, 80-volt, 120-volt and 150-volt running were performed for 30 min each. Staining buffer was used to fill the gel in the box container with shaker (20 rpm) in room temperature overnight. A day later, staining buffer was removed and replaced with destaining buffer with shaker (20 rpm) until the bands were appeared.

Dynamic light scattering (DLS)

To determine size of the OMVs, measurement of size distribution by DLS experiment using of the newly synthesized OMVs as samples was performed. Indeed, isolated OMVs were 1:10 diluted in PBS. Moreover, diluted OMVs were moved to a disposable cuvette. For determination, a disposable cuvette that contained OMV was put onto the Malvern Zetasizer nano Z590TM at temperature of 25 °C to analyze quality of OMVs**.** This measurement was repeatedly performed in triplicate.

Transmission Electron microscope (TEM)

To ensure that the size and morphology of the newly synthesized OMVs are relevant to standard, transmission electron microscope (TEM) was used to observe these criteria of OMVs. The TEM-Hitachi HT77000 was used to achieve this goal. Indeed, OMVs were sonicated in water bath sonicator for 30 min. OMVs was placed onto the surface of a carbon-coated copper grid. In addition, OMVs were negatively stained with 2% uranyl acetate for 5 min at room temperature to allow evaporation of the remaining solution and TEM figure was captured.

Western blot with SDS-PAGE

SDS-PAGE and western blot analysis was performed to investigate expression of ClyA-GFP-6xHistidine tag, which is a representative antigen used for testing this vaccine delivery system platform. For detection of ClyA-GFP-6xHistidine tag, samples were blocked with 5% non-fat dry milk and stained by mouse anti-6x-His tag monoclonal antibody (Invitrogen) in dilution of 1:2,500 in blocking buffer (5% skim milk). Samples were washed three times using washing buffer. For detection, samples were stained with goat anti-mouse IgG secondary antibody (ThermoFisher Scientific) in dilution of 1:2,500 and incubated with AP substrate and development, and bands were detected.

Stabilization of OMVs

For stability test, OMVs isolated from both BL21-DE3 and BZB1107 were 10-fold diluted with PBS and performed at varying conditions using with or without 5 mM of EDTA on the various temperatures of 25°C, 37 °C and 50°C for 1 h. DLS was also used for this aim.

Enzyme-linked immunosorbent assay (ELISA)

For establishing ELISA for observing the localization of GFP on the surface of OMVs, an indirect ELISA assay was used. First, 96-well plate was coated by OMVs-BL21-DE3, OMVs-BZB1107, RBD-His, BSA and PBS at 4°C overnight. Then, block non-specific with 5% w/v skim milk binding at 37°C for 2 h. Mouse anti 6x-His Tag antibody at the ratio 1:2500 were added on to the plate at RT for 2 h. Secondary antibodies HRP Goat anti-mouse IgG (1:2500) in RT° for 1h. Then signals were developed by 3,3',5,5';-tetramethybenzidine (TMB), stop reaction with H_2SO_4 50 µl and then the absorbance was measured at the optical density of 450 nm.

CHAPTER 3
RESULTS

3.1 Construction of plasmids encoding ClyA-GFP-6xHistidine tag

There are two plasmids including pMS119 and pET15b that were initially used as parental plasmids for making the newly synthesized plasmids. The pMS119 plasmid contains OmpF whereas pET15b contains gene of interest, which is ClyA-GFP-6xHistidine tag. There are two strains of bacteria (*E. coli*) that are used in this study including BZB1107 and BL21-DE3. These appropriate strains were based on expression profiles. BZB1107, a derivative strain of *E. coli* BE containing a Tn5 insertion at the beginning of the OmpF gene, which is resistant to kanamycin and streptomycin. There was no endogenous expression of OMP (Rosenbusch 1974, Ghosh, Steiert et al. 1998). On the other hand, BL21-DE3 strain is a widely used for T7 expression in *E. coli* culture. This strain ideally has protease-deficient B strain and endogenously expresses OMP (Meuskens, Michalik et al. 2017). Newly synthesized pMS119 containing ClyA-GFP-6xHistidine tag construction is needed because BZB1107 strain is unable to express protein from pET15b plasmid that contains T7 promoter.

Therefore, in this work, BZB1107 and BL21-DE3 were used as ideal platforms to produce OMVs that have no or endogenously express OMP, respectively. Accordingly, this design can provide information concerning the roles of OMP on vaccine/OMV platform properties. There are many points related to OMV properties including size of OMV, stabilization, and immunogenic effects. In this study, experimental study was designed for only characterization of the newly synthesized OMVs including stability and size of OMVs that can be comparable to reference OMVs, but for immunogenic effects need to be further investigated.

The aim of this experiment was to generate an engineered pMS119 containing ClyA-GFP-6xHistidine tag. At the sites of OmpF and ClyA-GFP-6xHistidine tag in both plasmids, there are similar two restriction sites including EcoR I and Hind III. In brief, ClyA-GFP-6xHistidine tag from pET15b was used as insert and amplified by PCR using forward primer (5′-CAGGAATTCATGACCGAGATTGTGGCGGATAAG-3′) and reverse primer (5′-CAGAAGCTTTTAGTGGTGGTGGTGGTGGTGGC-3′). PCR product of ClyA-GFP-6xHistidine tag was tested in agarose gel and fragment of ClyA-GFP-6xHistidine tag was purified (Fig.3.1A). On the other hand, pMS119 was cut at EcoR I and Hind III to remove OmpF and to linearize this plasmid to make vector template (Fig.3.1B). The molecular size of ClyA-GFP-6xHistidine tag and vector were 1686 bp and 4000 bp, respectively. Ligation of vector and insert was performed. Moreover,

purification of newly synthesized pMS119 plasmid containing ClyA-GFP-6xHistidine tag was performed. In addition, engineered sequencing of pMS119 containing ClyA-GFP-6xHistidine tag was shown below. The PCR products using forward primer (5′-CAGGAATTCATGACCGAGATTGTGGCGGATAAG-3′) and reverse primer (5′-CAGAAGCTTTTAGTGGTGGTGGTGGTGGC-3′) of 20 colonies of bacteria containing ClyA-GFP-6xHistidine tag were analyzed by agarose gel to identify the positive colonies (Fig.3.2). The colony that was proved to have ClyA-GFP-6xHistidine tag was further analyzed by Sanger sequencing (Fig.3.3). Therefore, sequencing data of these constructed plasmids was compared to ClyA-GFP-6xHistidine tag sequence (Fig.3.4), it was found that both plasmids contained the correct sequences of ClyA-GFP-6xHistidine tag sequence

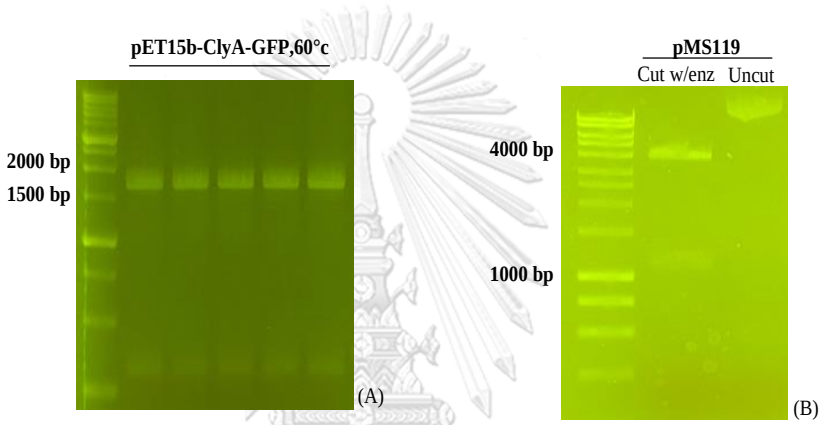

Figure 3.1 Agarose gel electrophoresis of ClyA-GFP-6xHistidine tag fragment (A) and vector (B)

(A) Lane 1: 1 kb DNA Ladder
Lane 2-6: PCR product of ClyA-GFP-6xHistidine tag

(B) Lane 1: 1 kb DNA Ladder
Lane 2: Plasmid pMS119 with EcoRI and HindIII restriction enzyme digestion
Lane 3: Plasmid pMS119 without restriction enzyme digestion

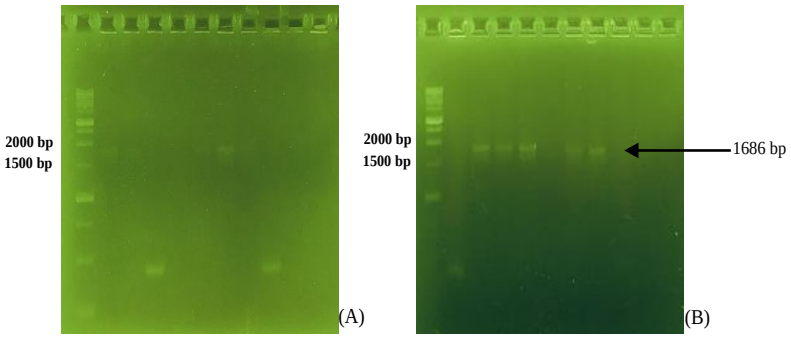

Figure 3.2 Agarose gel electrophosis of PCR products obtained from the screening for positive clone containing pMS119 plasmids with ClyA-GFP-6xHis gene by colony PCR.

(A) Lane 1: 1 kb DNA Ladder
Lane 2-11: PCR product of ClyA-GFP-6xHistidine tag from plasmid pMS119 containing ClyA-GFP-6xHistidine tag clone.1-10

(B) Lane 1: 1 kb DNA Ladder
Lane 2-11: PCR product of ClyA-GFP-6xHistidine tag from plasmid pMS119 containing ClyA-GFP-6xHistidine tag clone.11-20

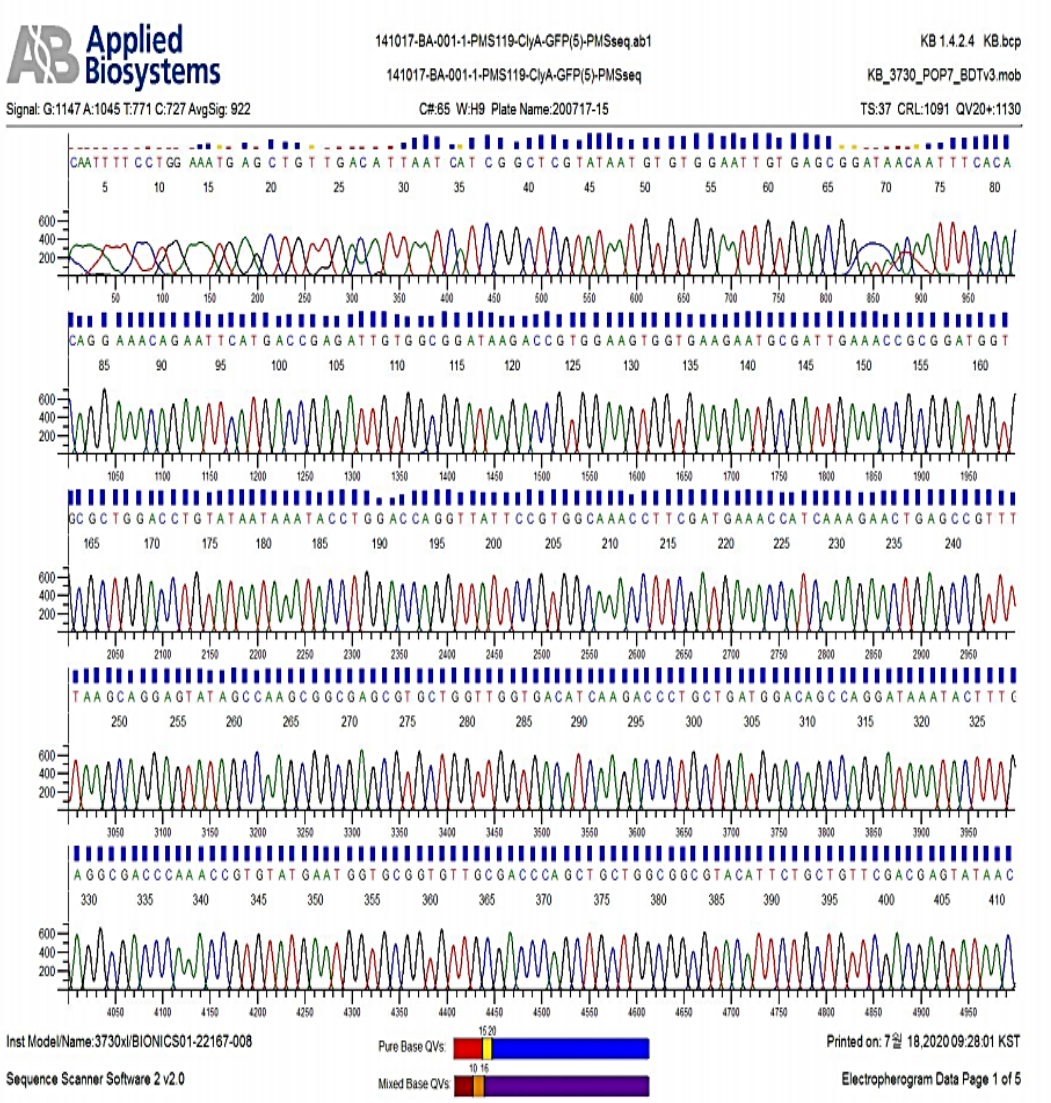

Figure 3.3 Sanger sequencing analyses of our synthesized plasmid pMS119 encoding ClyA-GFP-6xHistidine tag

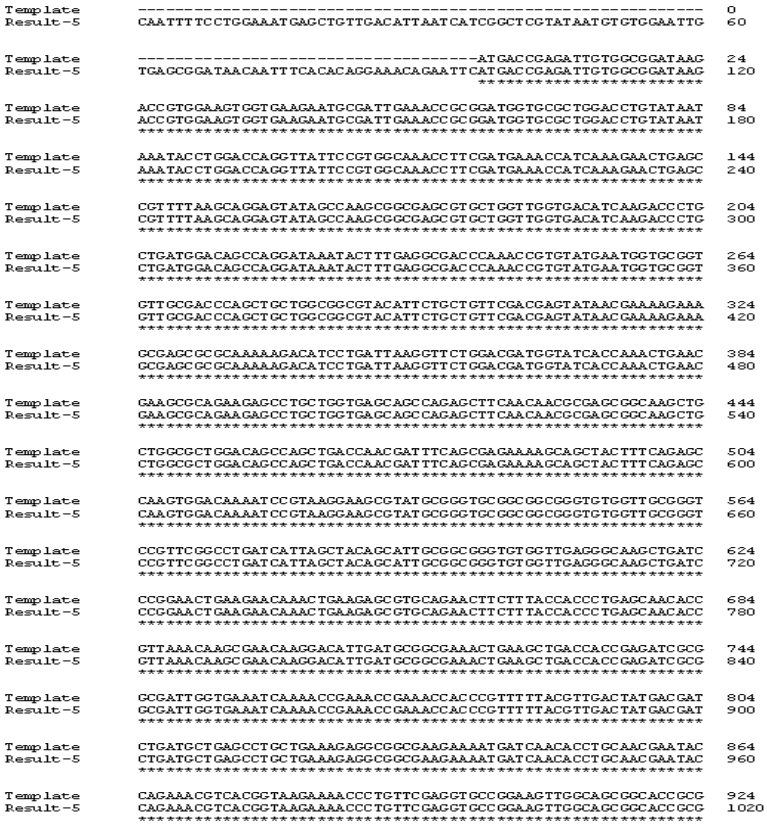

Figure 3.4 Sequence alignment between plasmid pMS119 containing ClyA-GFP-6xHistidine tag and template sequences (ClyA-GFP-6xHistidine tag)

3.2 Evaluation of ClyA-GFP-6xHis expression in outer membrane vesicles

The OMP proteins have been considered as one of the key mediators that may affect efficiency and stability of OMVs. Therefore, it is necessary to definitely define the exact role of OMP in determining functional OMV system. In fact, bacteria with and without expression of OMP is required for this experiment. In this experimental setting, plasmids containing ClyA-GFP-6x Histidine tag was transformed into host competent bacterial cells to produce these recombinant proteins. Experimentally, GFP was used as a representative antigen whereas ClyA, an anchor protein, was used to bring GFP to membrane. In addition, 6x Histidine tag was used for detection. To investigate whether ClyA-GFP-6x Histidine tag are expressed on OMVs derived from *E. coli* BZB1107 (without OMP expression) and BL21DE3 (having OMP expression) strains, the western blot and SDS-PAGE were performed and indicated that ClyA-GFP-6xHistidine tag expression could be detected in BZB1107 and BL21DE3 cell

pellets with inducer (IPTG; 0.5 mM). ClyA-GFP-6xHistidine tag band was found at ~ 62 kDa, which was consistent with the correspondence to expected molecular size. On the other hand, as shown in Fig. 3.5A – 3.5B, in both strains without inducer (IPTG; 0.5 mM), expression of ClyA-GFP-6xHistidine tag could be detected in neither BZB1107 nor BL21DE3 cell pellets. Of particular importance, in purified OMV samples from both strains, protein band corresponding to expected molecular size and ClyA-GFP-6xHistidine tag bands were detected by SDS-PAGE and western blot in Fig. 3.6A-3.6B, respectively. ClyA-GFP-6xHistidine tag bands still detected at ~ 62 kDa which were comparable to the size of ClyA-GFP-6xHistidine indicating that development of OMV containing antigen platform was successful.

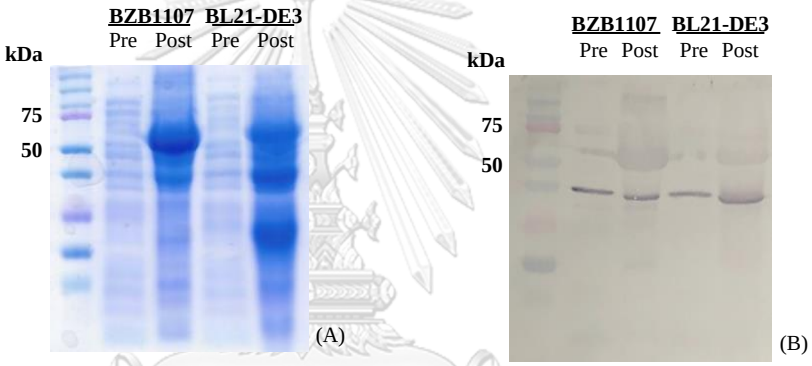

Figure 3.5 *E. coli* expression of ClyA-GFP-6xHis from Bacterial cell pellets in the presence and absence of IPTG. Analyzed by SDS-PAGE (A) and western blot using mouse anti-histag antibody (B)

Lane 1: Precision Plus Protein^{TM} Standards

Lane 2(Pre): BZB1107 cell pellets without inducer

Lane 3(Post): BZB1107 cell pellets with inducer

Lane 4(Pre): BL21-DE3 cell pellets without inducer

Lane 5(Post): BL21-DE3 cell pellets with inducer

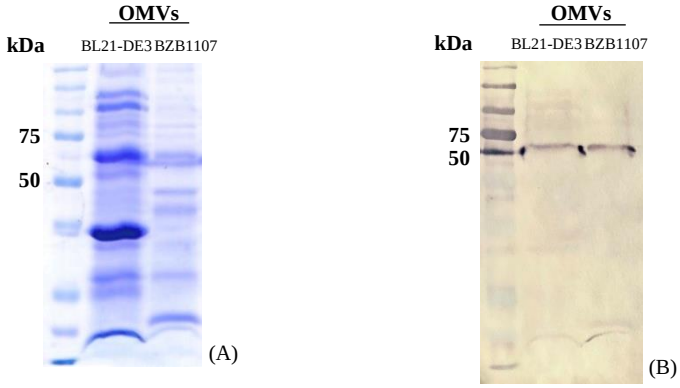

Figure 3.6 Identification of ClyA-GFP-6xHis that inserted into outer membrane vesicles (OMVs) isolated from *E. coli* BL21-DE3 and BZB1107 by SDS-PAGE(A) and western blot (B)

Lane 1: Precision Plus Protein™ Standards

Lane 2: OMVs of BL21-DE3

Lane 3: OMVs of BZB1107

3.3 Size distribution of OMVs by DLS

Dynamic light scattering or DLS was used to measure the size distribution of OMVs. It was found that DLS revealed the size distribution of OMVs from BL21-DE3 and BZB1107 with diameters of 43 to 300 nm in diameter (Fig. 3.7A) and 40 to 200 nm in diameter (Fig. 3.7B), respectively. These sizes of both newly synthesized OMVs can be comparable to previously reported size of OMV (from 20 – 200 nm in diameter) (Bachmann and Jennings 2010, Ellis and Kuehn 2010, Klimentova and Stulik 2015, van der Pol, Stork et al. 2015, Collins, Nice et al. 2021). Moreover, the average size of OMVs from BL21DE3 and BZB1107 strains were 103.94 ± 14.7 nm and 86.77 ± 3.39 nm, respectively. In addition, from the data as shown in Table 3.1, it represents the mean number percent of each size of OMVs from BL21DE3 and BZB1107strains.

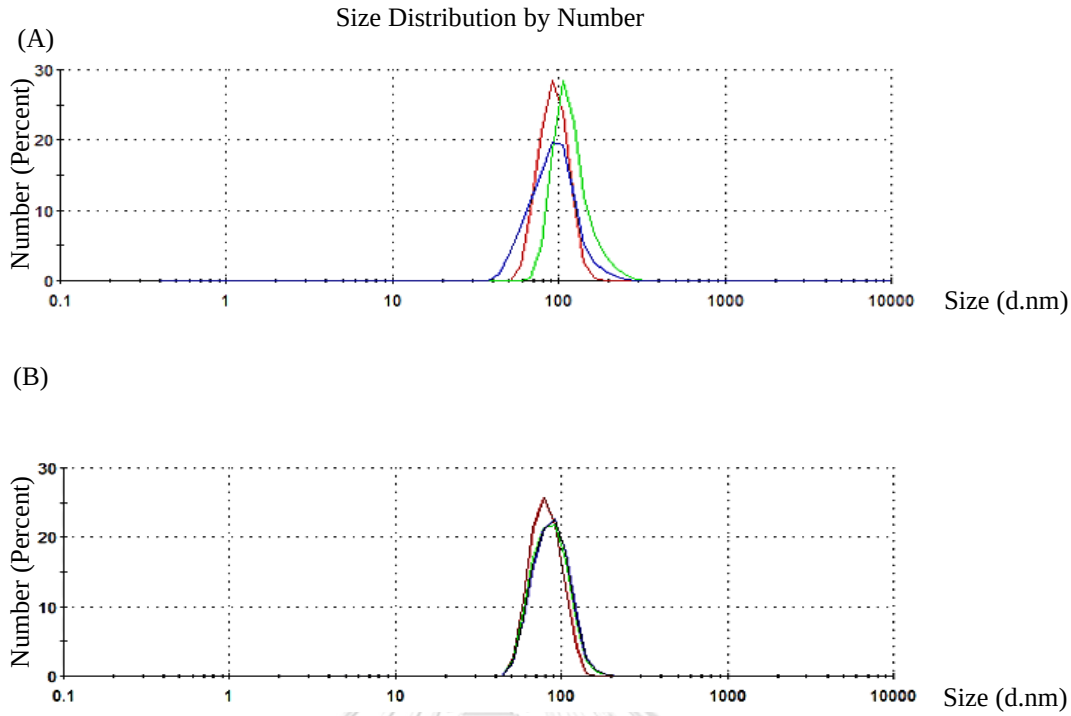

Figure 3.7 Size distribution of OMVs from BL21-DE3(A) and BZB1107(B) strains determined by DLS

Table 3.1 Mean number percent of OMV particles

BL21-DE3		**BZB1107**	
Size (d.nm)	**Mean number percent**	**Size (d.nm)**	**Mean number percent**
40 - 100	48.30	40 – 100	74.80
101 - 150	45.50	101 – 150	24.40
151 - 190	4.80	151 – 190	0.50
> 190	1.20	> 190	0.30

3.4 Ultrastructure of OMVs derived from BL21DE3 and BZB1107 strains

To visualize the ultrastructure of OMVs, transmission electron microscope or TEM was performed. It was found that size and morphology of OMVs were shown in Fig 8, which consisted of a structure that contain two layers of phospholipid in size of approximately 20 - 100 nm. Interestingly, size of OMVs from BL21DE3 were larger than those from BZB1107, which is in consistent with DLS result. Average size of OMVs isolated from BL21DE3 and BZB1107 strains (Fig. 3.8) were measured by

ImageJ software and statistical analysis was further performed by Graphpad prism. Average size of each OMPs was calculated by three particles and sixty-three particles of OMVs from BL21-DE3 and BZB1107 strains in TEM (Fig. 3.9).

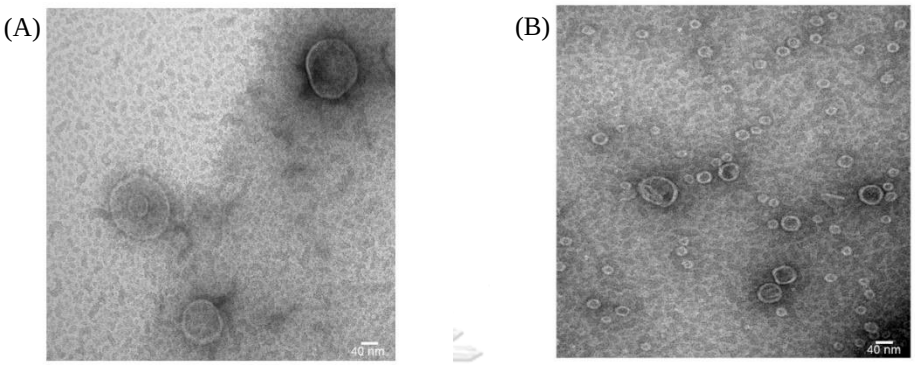

Figure 3.8 Ultrastructure of OMVs from BL21-DE3 (A) and BZB1107(B) strains in TEM

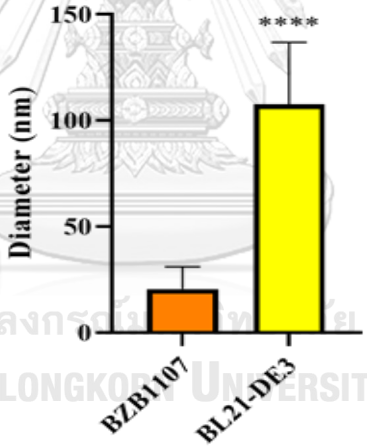

Figure 3.9 Comparison of the size of OMVs expressing ClyA-GFP-6xHistidine tag isolated from BZB1107 and BL21-DE3 strain. Results were statistically analyzed from diameter OMVs and expressed as a mean of OMV size ± S.E.M. in nanometer scale of each types of OMVs (n = 3). ****, $p < 0.0001$ compared between OMVs from BZB1107 and BL21-DE3 strains (One-way ANOVA).

3.5 ClyA-GFP-6xHistidine tag expression on the surface of OMVs

To identify the localization of ClyA-GFP-6xHistidine tag which is a representative antigen for this study, we speculate that GFP should present on the surface of OMV. After generating OMVs containing ClyA-GFP-6xHistidine tag from two platforms including those from BL21-DE3 and BZB1107 strains, indirect ELISA for detecting

6xHistidine tag was used. Basically, if protein antigen (ClyA-GFP-6xHistidine tag) expresses on surface of OMVs, ELISA will be able to detect the signals. In contrast, if protein antigen is in OMV lumens, ELISA will not detect the signal. After coating OMVs on the plate, it was found that OMVs isolated from both BL21-DE3 and BZB1107 strains could be detected as seen in a concentration-dependent manner. Importantly, in negative control groups (PBS-treated and BSA-treated groups), (Fig.3.10) ELISA could not detect fluorescent signals as expected. These results strongly support that ClyA-GFP-6xHistidine tag (representative antigen) expressed on the membrane surface of OMVs isolated from both strains, indicating the successful establishment of OMV platform for vaccine delivery system.

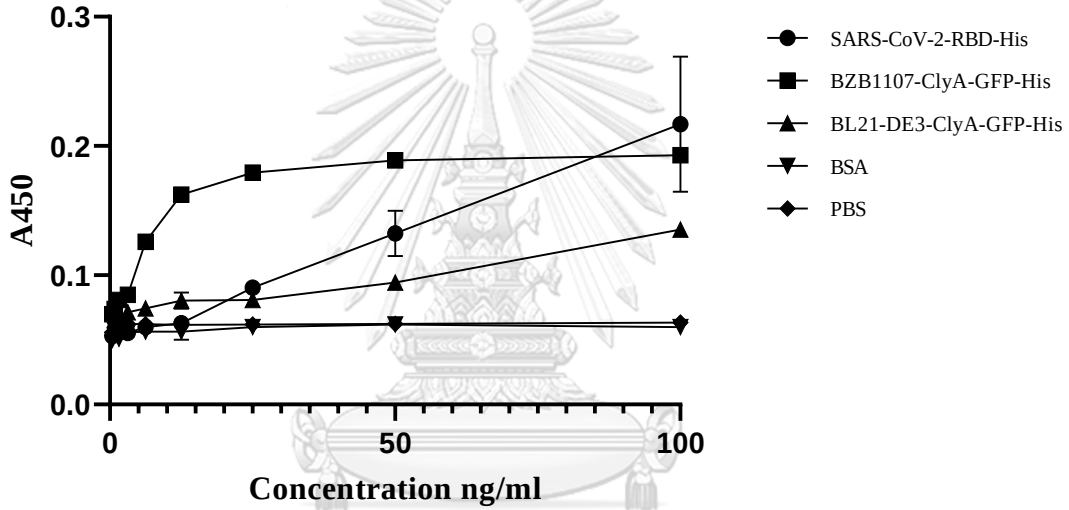

Figure 3.10 Binding properties of ClyA-GFP-6xHistidine tag expression on the surface of OMVs. Indirect ELISA was used for this investigation. BSA and PBS (as negative control) and SARS-CoV-2-RBD-His (as positive control)

3.6 Stabilization of OMVs

Next, to investigate whether our newly synthesized OMVs are stable, DLS was used to observe the size distribution of OMVs from BL21-DE3 and BZB1107 that expose to various challenging condition. Here, heat-induced destabilization of OMVs was performed. In brief, there are three experimental conditions including 25 °C, 37 °C, and 50 °C, incubated for an hour. These challenging temperatures are reported to induce physicochemical alteration of OMVs and protein structure denaturation (Schulz, Karagianni et al. 2020). Of note, BL21-DE3 expresses OMPs and other membrane proteins whereas BZB1107 have no expression of OMPs. DLS results revealed that OMVs isolated from both strains were stable although they were exposed to challenging temperature including 25 °C, 37 °C, and 50 °C. Furthermore,

EDTA was used to destabilize OMVs to investigate roles of OMPs in stabilizing OMVs. Indeed, stabilization of membrane proteins and outer-leaflet integrity of OMVs requires divalent cations (especially Mg^{2+} and Ca^{2+}) that directly interact to the negative charge of the phosphate groups in the inner core (Schneck, Schubert et al. 2010, Clifton, Skoda et al. 2015). In fact, EDTA is a chelator of Mg^{2+} and Ca^{2+} that can be used to permeabilize membrane vesicles for inducing destabilization. Interestingly, OMVs from BL21-DE3 treated with EDTA, number of OMVs (size ~ 20 – 100 nm) were less than those of OMVs from EDTA-untreated, BL21-DE3 group. Surprisingly, EDTA had no effect on stability of OMVs from BZB1107. These data strongly indicated that OMPs may involve in maintaining stabilization of OMVs isolated from BL21-DE3 strain. In contrast, OMVs from BZB1107 strain that have no OMP expression were not sensitized by EDTA. Therefore, it is possible that OMVs from BZB1107 strain is more stabilized than those from BL21-DE3 strain. Details of data are shown in Fig.3.11.

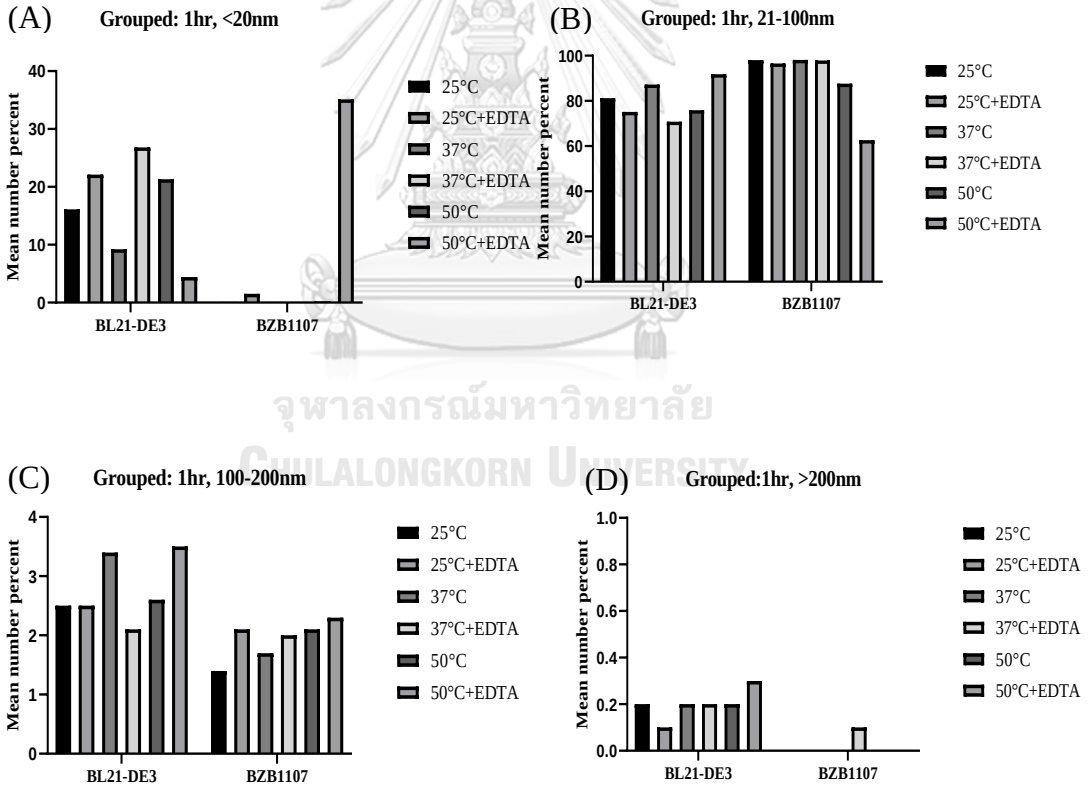

Figure 3.11 Graphs presenting the mean number percent of each ranges of OMV size isolated from BZB1107 and BL21-DE3 strain after 1 h incubation at various temperature with or without EDTA

CHAPTER 4
DISCUSSION

OMV platform for vaccine delivery, current issues, and significances of problem

Vaccine, especially subunit vaccine, is produced from purified antigen with aim at stimulating adaptive immune response and T cell to produce antibody for prevention and attenuating severity of diseases (Baxter 2007). To potentiate vaccine's effectiveness, the delivery system of the vaccine is necessary. In this study, vaccine delivery system was developed from outer membrane vesicles (OMVs) of gram-negative bacteria in which this platform can effectively deliver the vaccine to target immune cells. It is noticed that delivery system of the vaccine may be able to improve or potentiate the effect of vaccine-induced immune boost-up. The OMV is usually a small with diameter of ~ 20 – 200 nm, that is capable of penetrating into lymph vessels and can be recognized and captured by the antigen presenting cells (Kuehn and Kesty 2005, Baxter 2007, Bachmann and Jennings 2010).

Components of OMVs is based on the properties of bacterial membrane that include polysaccharides, phospholipids, and outer membrane protein (OMP) (Kulp and Kuehn 2010). These components can induce both cell-mediated and humeral immune response (Ellis and Kuehn 2010). OMV platform has recently been used commercially for many diseases (Gorringe and Pajon 2012). Because OMVs is derived from bacteria, there are generally some adverse impacts on human body, e.g., LPS-mediated hyperimmune reactivity. Therefore, it is necessary to improve this platform. There are many parts of research questions and characterization that are needed to furnish further studies. In this study, new construct of plasmid containing ClyA-GFP fusion protein was performed in bacterial strains that express or does not express OMP. From this design, it may be able to figure out roles of OMP in this delivery platform, especially the effects of OMV-mediated vaccination and its morphology. Information that is obtained from this study may lead to an improvement of new delivery system for the vaccine development in the future.

Characterization of newly engineered OMVs

According to the DLS data, it was able to provide a conclusion of the successful establishment of OMVs. Of particular importance, size of OMVs can be more than 200 nm in diameter to 300 nm but 200 nm is better in terms of its efficiency (Fantappie, de Santis et al. 2014, van der Pol, Stork et al. 2015, Collins, Nice et al. 2021). Indeed, the reason that 200 nm OMVs is better because, in this size (< 200 nm), OMVs can effectively pass through epithelial barrier into lymph vessels, and it is easier for the uptake of OMV/antigen into antigen presenting cells (Bachmann and Jennings 2010, van der Pol, Stork et al. 2015). Larger sizes of OMVs might be difficult to passively diffuse to lymph vessels. In more detail, most of OMVs purified from BZB1107 strain was approximately 40 – 100 nm in diameter (74.8%).

In contrast, OMVs from BL21-DE3 were about 40 – 100 nm and 101 – 150 nm at the level of equal distribution (45.5 – 48.3%). Size, kinetics, molecular patterns and geometry are considered as key factors in vaccine delivery platform for inducing immune responses that promote adaptive immunity (Bachmann and Jennings 2010). According to the basics of simple diffusion, smaller, uncharged particles can easily transport across polarized epithelia than larger molecules (Venable, Kramer et al. 2019). Theoretically, OMVs from BZB1107 strain that was relatively smaller than those from BL21-DE3 strain may be better in terms of its delivery process. Based on the standard size of OMV in range of 20 – 200 nm (Kuehn and Kesty 2005, Baxter 2007, Bachmann and Jennings 2010), if OMV size is in this range may be able to be used clinically because these sizes can be easily uptake to immune cells. However, based on Fick's law of diffusion, uptake of bigger size of OMV may be more difficult than smaller one. It can conclude that, without OMP, product of OMVs was quite smaller. Nevertheless, there is still a need for the further investigation in both *in vitro* and *in vivo* models, or in human in the future before making a precise conclusion. In addition, it is not clear about the roles of OMP in determining size of OMVs.

Moreover, ultrastructure of these engineered OMVs was demonstrated by TEM. This method was used to further confirm the size and the advantage of this approach can visualize the morphology of the newly synthesized OMVs as well. Indeed, OMVs were stained by 2% uranyl acetate to allow evaporation of the remaining solution and TEM figure was captured. The OMVs from both bacterial strains contain two layers of phospholipid in a similar manner with standard OMV circular morphology. Considering the size of OMVs here, they were approximately 20 - 100 nm. Importantly, size of OMVs from BL21DE3 were larger than those from BZB1107, which is in consistent with DLS result. In fact, 20 nm OMVs were mostly seen in TEM image. However, DLS peak at 20 nm was undetectable. Therefore, it is possible that size evaluation of the light scattering of DLS is an indirect measure that may be less sensitive to small-sized OMVs. Indeed, both OMVs generated from both strains of *E. coli* bacteria are mixtures of different sizes of OMV. Accordingly, DLS detected larger particles usually make more weight that may be resulted in increasing average of light scattering pattern. Therefore, in case of the OMV from BZB1107 strain, it is possible if we were unable to detect the smallest particle of OMV in DLS technique.

Western blot analysis revealed the intense band below an expected size of ClyA-GFP-6xHis tag in bacterial cell pellet but not in the purified OMV sample. This strange finding may be due to three main factors including non-specific action of antibody detection, endogenous enzymatic cleavage of ClyA-GFP-6xHis tag protein or immature ClyA-GFP-6xHis tag protein synthesis. First, since antibody recognize short amino sequence of 6xHis tag, there might be some intracellular *E. coli* protein that is structurally homologue, in part, to some 6xHis tag sequence, leading to detection of non-specific band. Second, compared to purified OMVs, it is highly more complex in live *E. coli*. There are several types of enzymes exist including proteases.

It is possible that there might be some endogenous enzymatic cleavage of ClyA-GFP-6xHis tag protein in *E. coli*, resulting shorter size of protein molecular weight. Finally, translational and post-translational processes of protein synthesis in live *E. coli* may produce both mature and immature protein. Therefore, it may not be extremely surprised if detection of immature protein band of ClyA-GFP-6xHis tag protein that may lack some part. This may be possible cause of detection of shorter sequence of immature protein in lower size of expected molecular weight. Although it is known that C-terminus is involved in activity of Cytolysin A, there was evidence supporting that eliminating the C-terminus of ClyA did not change the capacity of ClyA to bind to the membrane(Sathyanarayana, Desikan et al. 2016). Indeed, cytolysin A is considered as an anchoring protein that always insert to membrane. It is possible that connecting ClyA with GFP-6xHis tag at ClyA's C-terminus may not interfere membrane insertion process of ClyA. Therefore, GPF, a representative antigen that is linked to ClyA, will be actually inserted into the membrane of OMVs. Therefore, our engineered OMV with expressing ClyA-GFP-6xHis tag protein at C-terminus of ClyA may not interfere its membrane insert as well.

Stability of OMV-based vaccine derived from BL21-DE3 and BZB1107 strain

Temperature-dependent OMV destabilization experiment in collaboration with DLS that challenged OMVs with various conditions strongly revealed these types of OMVs were tolerant to extreme temperature. It is not similar to previous study, for example, the case scenario of the storage of OMVs of Neisseria meningitidis group B. Of particular importance, collection of this type of OMVs at high temperatures (37 or 50 °C) promote the morphologic eradication of OMV and PorA denaturation followed by chemical degradation of OMVs of *Neisseria meningitidis* group B (Arigita, Jiskoot et al. 2004). Furthermore, temperature challenging can trigger the gel/liquid crystalline phase transition of LPS molecule that may alter structure and immunogenic function of OMVs. Of note, low temperatures induced gel state of LPS that was strongly associated with rigid chains, and high temperatures promoted liquid-like phase of LPS with highly fluid chains, In addition, immunogenic effect of OMVs of *Neisseria meningitidis* group B was completely lost in extreme high temperature, however, they were not affected by freeze-dried state or in the frozen or (Arigita, Jiskoot et al. 2004). Moreover, EDTA was used to destabilize OMP to investigate roles of OMP in stabilizing OMVs. Interestingly, EDTA decreased the number of OMVs (size ~ 20 – 100 nm) from BL21-DE3. In contrast, OMVs from BZB1107 were resistant to EDTA, OMP may be a determinant factor of OMV stability. It is possible that existing expression of OMP may prefer to interact with many types of harmful inducers that may destabilize OMVs. Generally, divalent cations including Ca2+ and Mg2+ have long been known to stabilize integral proteins located on the surface of and outer-leaflet integrity of OMVs requires (Schneck, Schubert et al. 2010, Clifton, Skoda et al. 2015). EDTA that can chelate divalent cations can destroy the integrity of OMV. However, Stability of OMV-based vaccine derived from both

strains need to be further investigated. Freeze-thaw experiment may be performed and see the stability using DLS. In addition to size of OMVs, stability can be indicated by morphology of OMV using TEM as well. Furthermore, testing antigen expression may be useful as another stability parameter.

Localization of representative protein antigen on the surface of OMVs

It is necessary to know if ClyA-GFP-6xHistidine tag expresses on the surface of OMVs. Ideally, antigen used as immune activator in vaccination should be expressed on the membrane surface of OMVs. In this study, GFP is used as representative antigen. However, GFP alone would preferentially localizes inside the OMVs. Indeed, when GFP is linked with ClyA, an anchor membrane protein, GFP was transferred to membrane. Here, GFP-specific ELISA was performed to investigate whether GFP expresses on membrane surface. Theoretically, ELISA would not be able to recognize GFP. In this study, it was found that ELISA could detect ClyA-GFP-6xHistidine tag from both OMV samples from BL21-DE3 and BZB1107. This result indicated that our OMVs were usable as a similar platform of standard OMVs that have surface expression of protein antigen.

Bacterial strains used as a platform

There are two strains of bacteria (*E. coli*) that are used in this study including BZB1107 and BL21-DE3. These appropriate strains were based on expression profiles. BZB1107, a derivative strain of *E. coli* B^E containing a Tn5 insertion at the beginning of the OmpF gene, which is resistant to kanamycin and streptomycin. There was no endogenous expression of OMP [9840808, 4609976]. On the other hand, BL21-DE3 strain is a widely used for T7 expression in *E. coli* culture. This strain ideally has protease-deficient B strain and endogenously expresses OMP [29164072].

Therefore, in this work, BZB1107 and BL21-DE3 were used as ideal platforms to produce OMVs that have no or endogenously express OMP, respectively. Accordingly, this design can provide information concerning the roles of OMP on vaccine/OMV platform properties. There are many points related to OMV properties including size of OMV, stabilization, and immunogenic effects. In this thesis work, experimental study was designed for only characterization of the newly synthesized OMVs including stability and size of OMVs that can be comparable to reference OMVs, but for both *in vitro* and *in vivo* immunogenic effects need to be further investigated.

Of note, using two strains and OMP overexpressing BZB1107 are acceptable in term of experimental data validity. Both have advantage and disadvantage. OMP overexpressing BZB1107 may take longer time and spent more money to employ the experiments to validate and characterize, but this approach may be useful as good control for BZB1107. In our lab setting, we have two strains that have no and have OMP expression endogenously. Although two strains cannot be 100% comparable to

each other, both are *E. coli* and using this approach may reduce the possible artifact from overexpression experiment.

CHAPTER 5
CONCLUSION

The main aims of this study are 1) to produce engineered OMVs containing ClyA-GPF-6xHis-tag isolated from BL21-DE3 and BZB1107 and 2) to characterize these newly synthesized OMVs in term of its size, morphology, antigen expression and localization. Expectation of this study is to establish and improve a vaccine delivery platform that are efficient for inducing immune response in prevention of human diseases. Here, GPF was used as a representative antigen and ClyA, an anchor porin, was used to maintain GFP stabilization on the membrane surface of OMVs. Expression and localization of antigen and its interacting partners were detected using specific antibody against 6xHis-tag. This study is limited on the production and characterization process of OMVs. *In vivo* and *In vitro* efficiencies of vaccine in OMV platform were not yet elucidated in this study. Of particular interest, the main difference of BL21-DE3 and BZB1107 strains of *E. coli* is expression of OMPs. In fact, BL21-DE3 strain has OMP expression whereas there is no OMPs detected in BZB1107 strain. Based on property of these strains, roles of OMPs were defined in the process of OMV production.

Establishment of OMVs containing ClyA-GPF-6xHis-tag

The constructs including pMS119 and pET15b plasmids that contain inducible ClyA-GPF-6xHis-tag sequence were transformed into BZB1107 and BL21-DE3 competent cells, respectively. For induction process, IPTG was used to promote expression of ClyA-GPF-6xHis-tag in host cells. Then, OMVs with or without OMPs were isolated from BL21-DE3 and BZB1107 strains, respectively. Using SDS-PAGE and western blot analysis, expression of ClyA-GPF-6xHis-tag can be detected in IPTG-treated OMVs from both strains. This data strongly suggested the successful establishment of OMVs containing ClyA-GPF-6xHis-tag.

Characterization of OMVs containing ClyA-GPF-6xHis-tag

In this study, the newly synthesized OMVs produced from BL21-DE3 and BZB1107 strains of *E. coli* were characterized. The characteristics that are concerned including OMV size, morphology, antigen localization, and OMV stabilization. Using DLS, it was found that OMVs containing ClyA-GPF-6xHis-tag produced from both BL21-DE3 and BZB1107 strains had size corresponding to OMV size that has been previously reported (20 – 200 nm in diameter). Transmission electron microscope (TEM) revealed that morphologic structure of these types of OMVs composed of bi-layers of phospholipids and size was quite similar to the DLS data. ELISA was used to perform the experiment for investigating ClyA-GPF-6xHis-tag localization. It was found that ClyA-GPF-6xHis-tag localized on the membrane surface of OMVs. In

addition, these OMVs were stable in all extreme temperature, but only OMVs without OMPs from BZB1107 strains were resistant to EDTA-induced OMV destabilization, suggesting that OMPs may be involved in stability of OMVs. Altogether, all characteristics here are consistent with the basic properties of OMVs. Therefore, the successful development of this OMV platform with engineered/modified antigen can be applied for the efficient vaccine delivery system in the future.

Future Perspectives

The OMVs were discovered over past 50 years and the licensed OMV vaccine against *N. meningitidis* for humans is currently available in the clinical practice. This approach is considered as an effective platform of vaccine because OMVs can be both a delivery system and adjuvant mediator (Tan, Li et al. 2018). Similar to the prevention of *N. meningitidis* by vaccine/OMVs platform derived from *E. coli* that was able to effectively stimulate both cell and humoral immunity, especially mediated IFN-γ and IL-17 T-cell dependent responsiveness. Hence, OMV-based vaccine and adjuvants were reported to be relatively superior to aluminum adjuvants only for triggered B cell immunity (Tan, Li et al. 2018). Based on this principle, OMV-based vaccine is fascinating to be improved and developed for the future era of vaccine. Here, OMVs with or without OMP expression were developed. Their characteristics were similar to standard size and morphology of OMVs. In addition, OMP-deficient OMVs may be better than OMP-positive OMVs due to its smaller sizes and more stable structure. Nonetheless, there are still required for the further investigation of these OMV-based vaccine to characterize their properties and effectiveness for clinical usage.

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APPENDIX A

Mean number percent of OMV particles at condition 25 °C, 1 h without EDTA

BL21DE3		BZB1107	
Size (d.nm)	Mean number percent	Size (d.nm)	Mean number percent
<20	16.1	<20	0
21 - 100	81.2	21 - 100	98
101 - 200	2.5	101 - 200	1.9
> 200	0.2	> 200	0

Mean number percent of OMV particles at condition 37 °C, 1 h without EDTA

BL21DE3		BZB1107	
Size (d.nm)	Mean number percent	Size (d.nm)	Mean number percent
<20	9.2	<20	0
21 - 100	87.2	21 - 100	98.1
101 - 200	3.4	101 - 200	1.7
> 200	0.2	> 200	0

Mean number percent of OMV particles at condition 50 °C, 1 h without EDTA

BL21DE3		BZB1107	
Size (d.nm)	Mean number percent	Size (d.nm)	Mean number percent
<20	21.3	<20	0.2
21 - 100	75.9	21 - 100	97.6
101 - 200	2.6	101 - 200	2.1
> 200	0.2	> 200	0

Mean number percent of OMV particles at condition 25 °C, 1 h with EDTA

BL21DE3		BZB1107	
Size (d.nm)	Mean number percent	Size (d.nm)	Mean number percent
<20	22.1	<20	1.5
21 - 100	75.1	21 - 100	96.3
101 - 200	2.6	101 - 200	2.1
> 200	0.1	> 200	0

Mean number percent of OMV particles at condition 37 °C, 1 h with EDTA

BL21DE3		BZB1107	
Size (d.nm)	Mean number percent	Size (d.nm)	Mean number percent
<20	26.8	<20	0
21 - 100	70.8	21 - 100	97.9
101 - 200	2.1	101 - 200	2
> 200	0.2	> 200	0.1

Mean number percent of OMV particles at condition 50 °C, 1 h with EDTA

BL21DE3		BZB1107	
Size (d.nm)	Mean number percent	Size (d.nm)	Mean number percent
<20	4.4	<20	35.1
21 - 100	91.8	21 - 100	62.6
101 - 200	3.5	101 - 200	2.3
> 200	0.3	> 200	0

APPENDIX B

Preparation of the LAB reagents

Ampicillin stock solution 100 mg/ml

- Ampicillin 1 g
- Dissolved in DI water 10 ml

Tris-HCl 1.5 M, pH 8.8 (50ml)

- Tris 9.08 g
- DI water 50 ml
- Adjust the pH to 8.8 with HCL

Tris-HCl 0.5 M, pH 6.8 (50ml)

- Tris 3.03 g
- DI water 50 ml
- Adjust the pH to 6.8 with HCL

SDS-PAGE running buffer (10X) 1L

- Tris-base 30.3 g
- Glycine 144.1 g
- SDS 10 g
- Adjust the volume to 1 L with DI water

Staining Buffer 500ml

- Isopropanol 50 ml
- Acetic acid 50 ml
- DI water 400 ml
- Coomassie Brilliant Blue R250 0.025%

Destaining Buffer 500ml

- Isopropanol 50 ml
- Acetic acid 50 ml
- DI water 400 ml

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