CHAPTER VI
DISCUSSION

The production of VLPs in mammalian cell based systems tends to be increasingly used in VLPs based vaccine development. DENVs VLP is one of the DENVs vaccine candidates because it is safe, but triggers a strong immune response. VLPs mimick the morphology of natural virions and VLPs vaccine can apply in many applications, for example, targeted drug delivery, gene therapy and immune therapy in vaccination. There are several commercial VLPs vaccine such as GlaxoSmithKline (GSK)'s Engerix®, GenHevac B®, Sci-B-Vac® and Cervarix® which are commercial VLPs vaccine of hepatitis B virus and human papillomavirus, respectively (62). In case of DENVs, there is evidence that co-expression of *Flavivirus* prM and E proteins with or without a nucleocapsid, could produce VLPs. Interestingly VLPs could induce neutralizing antibodies and virus-specific cytotoxic T lymphocytes response in host cells. DENVs nucleocapsid-like particles were constructed by cloning of C protein gene (297 bp) of DENV-2 strain Jamaica 1409 into the pQE30 vector and expressed in *E. coli* (XL1-blue strain). After immunization in mice, DENVs antibodies could not detect in mice sera but it possibly induces cellular immune response (63). DNA sequence encoding prM and E structural proteins was frequently used in DENVs VLPs construction and the expression systems reported for prM-E VLPs were *E. coli*, yeast, baculovirus and mammalian cells. Zhou and colleagues constructed DENVs serotype 1-4 VLPs. While DENV-3 and 4 VLPs constructed with only signal peptide of prM, DENV-1 and 2 were added with alpha-factor. This sequence is an N-terminal secretion signal from *Saccharomyces cerevisiae* alpha-factor. Then, four recombinant plasmids were electroporated into the *Pichia pastoris (P. pastoris)*. Yeast cells were then harvested and VLPs were purified by sucrose density gradient. After 25 µg of monovalent VLPs and a combination of 25 µg of each serotype VLPs (tetravalent VLPs) were intraperitoneally inoculated in mice, the result showed better protective efficacy in tetravalent vaccine than monovalent but the compatibility

adjustment still needs improvement (54). Another publication from Zhang and colleagues suggested that the JEV signal sequence (JESS) at the 5' terminus of the prM-E gene could increase the production of DENVs VLPs by detection of expression of E protein in supernatant. Chimeric constructs, containing full length of prM and E protein in which the 20% of 3' terminus was replaced with the corresponding sequence JEV, could solve the DENVs ER retention signal problem. The most effective constructs to secrete E protein into supernatant contained both of JESS and the 3' terminus of JEV corresponding gene (64). In this study, all of DENV 2 VLPs contained an E protein ER retention signal. This might be the main reason for inefficient E protein secretion because the prM-E proteins with ER retention signal might accumulate in ER and were unable to travel to Golgi apparatus for further processing prior to secretion.

In this study, DENVs VLPs with or without YPTI motif and C signal sequence namely pC2-D2prM-E, pC2-D2YPTI-prM-E, pC2-D2-C-prM-E and pC2-D2-C-YPTI-prM-E were constructed to compare the production of VLPs in mammalian cells. Preliminarily, Western blot analysis showed that only pC2-D2-C-prM-E and pC2-D2-C-YPTI-prM-E could express E protein in the transfected cells since day 1 and day 2 post transfection, respectively and substantially decreased over time. But no detectable E protein band in supernatant could be detected from all constructs in all 3 days post transfection. Interestingly, only the constructs containing a signal sequence (19 amino acid sequence at 3' terminus of capsid protein (C)) could express the VLPs production in cell lysate. This signal sequence might facilitate the expression of E protein inside the transfected cell but it was not sufficient to enhance the secretion of VLPs out of cells.

Because of ineffective VLPs production in the transfected cell, the presence of DENV-2 VLPs constructs in the cell was observed by PCR. The 2 constructs containing C sequence, which E protein could be detected by Western blot analysis, VLPs plasmid could not be detected inside transfected cells by PCR amplification. The result was contrary in the pC2-D2prM-E and pC2-D2YPTI-prM-E transfected cells which VLPs plasmids could be detected but there was no protein expression in the cell lysate. This phenomenon might be resulted from the toxicity of the expressed VLPs to the cells resulting in cells dying after E protein expression, or

the cells tried to eject the constructed plasmid for cell survival. Thereby, the trend of E protein expression in pC2-D2C-prM-E and pC2-D2YPTI-C-prM-E transfection showed substantially decreasing overtime which might be result from the loss of VLPs expressing cell death.

Many attempts had been made to monitor VLPs expression in the supernatant of the VLPs transfected cells. Supernatant from pC2-D2C-prM-E and pC2-D2YPTI-C-prM-E transfected cell were concentrated by centrifugal evaporator or protein precipitated using $(NH_4)_2SO_4$ precipitation prior to applying on SDS-PAGE or directly dot blot on the nitrocellulose membrane. There was also no detectable band of E protein secretion in supernatant.

To improve the VLPs production, stable transfection was performed to accumulate cells harboring DENVs VLPs. Normally, stable transfection is the introduction of target gene into the host genome and maintained selected cells in conditioned media (67). Retroviral vector is frequently used to delivery target gene into genome of host cell. In a publication by Wong and colleagues, retroviral vector was used for delivery of the codon optimized prME gene into HeLa cells. Co-transfection of the optimized prM-E gene in retroviral vector, pcDNA-VSV-G and p8.71 (modified HIV provirus coding for gag and polymerase) into 293T cells was performed. Because pol proteins are responsible for integration into host DNA, infectious VSV-G-pseudotyped retroviral particles containing prME gene was secreted to supernatant. The supernatant from the transfected cells was collected at day 2 post transfection and infected to HeLa cells. Hela-prME cells were cultured and maintained in medium containing 500 mg/ml of hygromycin for two weeks and observed the level of E protein expression by flow cytometry (61). In an easier way, Crabb and colleagues transfected DHFR-TS gene from *Toxoplasnma gondii* with pyrimethamine resistance vector into *Plasmodium falciparum* and they used only pyrimethamine to select the positive cells and selected cells were maintained for a further 2 weeks (67). In this study pC2-D2-C-prM-E and pC2-D2-C-YPTI-prM-E were moved to transfected in HEK 293 instead of HEK 293T cells. HEK 293T are a derivative of 293 cells, but stably express the SV40 large T antigen which can bind to SV40 enhancers of expression vectors to increase protein production. In SV 40 large T antigen cassette contains neomycin resistance gene. It makes HEK 293T resistance to

gentamicin/neomycin whereas HEK 293 cells are sensitive. G418, an aminoglycoside antibiotic similar in structure to gentamycin B1, was used for the selection and maintenance of DENV-2 VLPs transfected cell in this study. Amount of VLPs harboring cell was validated by IFA. The percentage of pC2-D2-C-prM-E, pC2-D2-C-YPTI-prM-E and pcDNA-D2opt.prM-E harboring cells in 1 and 2 months stable transfection were approximately 20-30% whereas 10-20% transfected cells showed in day 1 and day 2 post transfection. It seemed that the VLPs harboring cells could not expand well while the untransfected cells could not be eliminated by 1500 µg/ml of G418. This situation might be explained in the same phenomenon as shown by PCR detection of the absence of plasmid in the cells. DENV-2 VLP constructs, which could express E protein, were toxic and death or plasmid was ejected from the cells while the remaining transfected cells in population could not expand as much. On the other hand, the rate of untransfected cells expansion might be higher than rate of transfected cells. Thus, it gave the low percentage of the VLPs harboring cells in stable transfection.

As above mentioned, codon optimization was used to increase protein expression. The more favored codons correspond to more abundant tRNAs. Moreover, differentiation of codons usage generally happens among genes in each individual organism, thus codon optimization can improve rate and accuracy of translation in different cell culture systems. There are many codon optimized software such as Codon optimizer, Gene Designer, Java Codon Adaptation Tool or OPTIMIZER. They are mainly focused on the synonymous codons that are frequently used in each expression system to improve protein expression (65). In term of vaccine development, Baud and colleagues constructed HPV16 VLPs and they expressed in *Salmonella enterica* serovar Typhimurium. Codon optimization of HPV16 L1 capsid gene with the most frequently used codons in Salmonella organism resulted in a shorter time to reach mid-log growth phase after inoculation to LB broth with a single colony when compared with native L1 gene. The native L1-encoding plasmid was rapidly lost in salmonella without antibiotics. In contrast, the optimized L1 gene was completely stable for at least 2 weeks. Optimized L1 gene created high titers of neutralizing antibodies in mice immunization with live bacteria through either the nasal or oral route (66). Codon optimization of DENVs E gene was reported by Wang

and colleagues. This optimized prM-E gene significantly improved E protein expression in HeLa cells when analyzed by flow cytometry. Percentage of positive cells increased by almost 3 folds comparing to native prM-E construct (from 37.02% to 94.87%) (59). Hence, pcDNA-D2opt.prM-E was constructed in this study to solve the ineffective VLPs production problem. This construct contained both optimized D2-prM-E gene with a preferred set of codon that frequently use in mammalian cell and a VSV-G signal sequence was used instead of dengue native signal sequence.

Although the VLPs construct with codon optimization and VSV-G signal sequence improved the expression in cell lysate, small amount of VLPs could be observed in the supernatant. In detection step by Western blot analysis, supernatants from VLPs transfected cells were analyzed using both reducing and non-reducing dye as loading buffer for SDS-PAGE. Precipitated supernatants with reducing dye had no signal when they were detected by antibody against dengue viral protein. In contrast, precipitated supernatants with non-reducing dye (without DTT) could show the band of E protein secretion with higher intensity. One interesting point might be the conformation of the expressed protein which exposed the antibody binding sites differently in native or denatured forms. It might be involved in antibody binding step that was interrupted by DTT or antibody used to detect DENV-2 E protein was recognized in region that exposed only in native structure of E protein. DTT is used to reduce the disulfide bonds of proteins and peptides prior to analysis by Western blot. DTT reduces a disulfide bond by two thiol-disulfide exchange reactions resulting in DTT becoming a six-member ring structure. β-mercaptoethanol is commonly used to break disulfide bond the same as DTT. Both of them overcome some forms of tertiary protein folding, and break up quaternary protein. The analysis of the conformational changes of protein and the effect to antibody docking was performed by Vakser and colleagues who showed that conformational changes in hemagglutinin had affect to their side chain and resulted in penetrations area of antibody. It created a tolerance or discrepancies of antibody docking (68).

After non-reducing loading dye using in SDS-PAGE, clearer detectable E protein band was observed. Western blot result showed that the pcDNA-D2opt.prM-E transfected cells could express E protein and it substantially increased in 3 days post transfection. The E protein with size approximately 55 KDa was also detected in

precipitated supernatant. Beside the 55 KDa band, commonly found for E protein detection in previous study, bigger bands with size approximately 100, 200 and above 250 KDa were detected in both precipitated supernatant from pcDNA-D2opt.prM-E transfected cells and positive controls (precipitated supernatant from DENV-2 infected cells). Because of non-reducing dye retain the native, a secondary or a tertiary structure of protein, the 100, 200 and above 250 KDa protein bands probably are secondary and tertiary structure of DENV-2 E protein and they can be detected in only non-reducing conditions. From the result, VSV-G in the VLP construct showed better result of VLPs secretion than the native signal sequence of DENVs C gene. In addition, 2 factors that promoted the secretion of DENV-2 VLPs into supernatant were optimized including prM-E codon and VSV-G signal sequence. For further analysis, DENV-2 VLPs structure will be observed by electron microscope and the immunogenicity of the VLPs produced by the pcDNA-D2opt.prM-E construct should be investigated by vaccination in mice and determination of neutralize antibody in mouse sera using plaque reduction, ELISA or antibody neutralization assay (64).

Interaction and re-organization of cellular membranes which compose of phospholipids, glycolipids and sterols plays an important role in fusion and genome replication of many viruses. When the virus enters a host cell, cell membrane are re-organizated to generate the endosomal vesicle prior to membrane fusion and uncoating of virus. Mostly lipids in cell membrane diffuse freely in the plasma membrane for clustering into discrete domains, called lipid rafts, and it is important for virus entering process in both of enveloped and non-enveloped virus (69). Alterations of lipid composition in cell membrane can block viral release and entry. For positive-strand RNA viruses such as *Flavivirus*, most of them induce formation of many cytoplasmic vesicles especially in the replication site (70). Furthermore, Dengue virus replication showed that fatty acid synthetase is recruited to replication sites through the viral NS3 protein and create the fatty acid biosynthesis and de novo synthesized lipids in DENV infected cells. Some hypothesis suggested that lipid could control the site of RNA replication process or linked between virus replication and assembly (71). Perera and colleagues showed the up- regulation of phospholipids, sphingolipids in DENV infected cells compared with the UV-inactivated DENV (UV-DENV) which possess only binding, entry and translation abilities but not replication.

In their study, stearic acid was up-regulated in the DENV infected cells while oleic acid was up-regulated in UV-DENV exposed cells. It is suggested that oleic acid plays a role in initial step of viral entry (72). Interestingly, Gutsche and colleagues showed that NS1 hexamer forms a lipoprotein particle composed of protein shell and central channel rich in lipids. Fatty acids that were separated from DENV-1 NS1 triglycerides showed two major peak types those were saturated palmitic acid and unsaturated oleic acid. This study suggests that DENV NS1 could mimick or hijack cellular lipid metabolic pathways (73). In this study, 100 µM oleic acid was used to improve E protein expression of the DENV-2 VLPs transfected cells. Oleic acid was normally used in cell culture to stimulate lipid droplet formation by activating the long-chain fatty acid receptor and stimulated the signaling cascades to increase the number of lipid droplets (74). Free fatty acid BSA was used as a carrier of oleic acid into the cell. However, BSA caused a problem of E protein detection in supernatant because protein precipitation method will also precipitate BSA. It had cross-reaction with E protein antibody and showed large protein band in the same size of E protein in Western blot analysis.

In summary from this study it appears that the factors necessary to improve both intracellular and extracellular DENV E protein expression include the addition of a strong signal sequence at the 5' terminus and codon optimization to a codon usage preferable in mammalian cells. Moreover, elimination of the strong ER retention signal of DENV and replacement with the corresponding sequence in JEV is likely to significantly improve DENV E protein secretion. Combined, these processes are likely to significantly improve DENV VLP construction and secretion and the combination should be explored in further studies.

Nowadays, the development of safer and more effective dengue vaccine is a worldwide priority. Although DENVs vaccines with other platform are under clinical trials, the protection against all 4 serotypes is still problematic. Unfortunately, VLPs vaccine is still expensive to produce and it is unable to produce in a mass-scale. The clinical trial of VLPs vaccine and others effective dengue vaccines should develop to more rapid and robust efficacy antibody response after vaccination to naive recipients and produce an equally level of antibody among 4 serotypes of DENVs. It will lead to accomplished prevention and treatment of DENVs (51, 62, 75).