CHAPTER VI
CONCLUSIONS

The results of this study were concluded as followed:

1. Preparation and characterization of $Al(OH)_3$ and chitosan conjugated PLGA microparticles as nasal vaccine carriers

1.1 Particles sizes and uniformity of PLGA prepared by double emulsion solvent evaporation technique were increased when primary w/o ratio and secondary (w/o)/w ratio were raised from 1:10 to 1:2.5 and from 1:4 to 1:2, respectively, while particles sizes and uniformity were conversely decreased as the elevation of PVA concentration from 1% to 4%.

1.2 The influence of formulation parameters in 1.1 on particles sizes and uniformity were ranked as followed: primary w/o ratio > PVA concentration > secondary (w/o)/w ratio, respectively.

1.3 Probe sonicator produced the particles of smaller size with less uniformity compared to bath sonicator. Though, the PVA concentration obtained more effect on uniformity than sonication output in only bath sonicator.

1.4 Particles sizes and uniformity of selected formulations of 1, 5 and 15μm particles were slightly increased after conjugation with 0.2% CS, especially 1μm particles that uniformity was considerably elevated from 0.27 to 7.72. The particles sizes and uniformity of 1, 5 and 15μm particles after conjugation with 0.75% and 1.5% $Al(OH)_3$ were elevated slightly, except 1μm particles conjugated with 1.5% $Al(OH)_3$ that the particle size was noticeably raised from 0.92 to 3.44. Surface charges of all $Al(OH)_3$ and CS formulations were considerably positive.

1.5 SEM and AFM revealed the spherical shape with smooth surface of 1, 5 and 15μm particles. After conjugation with CS, transparent gel appeared to cover the surface of particles while white-opaque layer occurred to cover the surface of particles when conjugation with $Al(OH)_3$.

1.6 FTIR spectra revealed that the residue PVA on surface of PLGA particles could interact with $Al(OH)_3$ by surface-adsorbed interaction and interact with CS by intermolecular hydrogen bonding.

1.7 The release was ranked as followed : 1 μm > 5 μm > 15 μm, respectively. After conjugation with CS and $Al(OH)_3$, burst releases were decreased. However, the early stage of release from conjugated particles was slower and slightly increased until comparable to un-conjugated particles on approximately day 60^{th}.

1.8 CD spectra revealed that the total conformation of purified JE vaccine was β-sheet and still remained as β-sheet after sonication but changed to α-helix after contacting with dichloromethane. SDS-PAGE showed that the surface proteins of JE as well as the epitope E protein at 53kDa was well retained after exposure to all vigorous conditions of preparation conditions.

2. *Ex vivo evaluation of $Al(OH)_3$ and chitosan conjugated PLGA microparticles as nasal vaccine carriers in porcine nasal mucosa*

2.1 Uptake study by CLSM of 1, 5 and 15μm particles in porcine nasal mucosa expressed that 1μm particles were taken up with more extent than 5 and 15μm at both 20 and 90 minutes while a very rare of 5 and 15μm particles of precisely size could be observed inside tissue at both points of time, not as similar as 1μm particles. The characteristic of 1μm particles taken up by nasal tissues was grouped in an irregular shape scattering inside the tissues.

2.2 Uptake study by CLSM of 1C and 1A particles in porcine nasal mucosa also revealed that the percentage of 1C taken up at both 20 and 90 minutes was unexpectedly lower compared to 1μm particles while the percentage of 1A particles taken up at 20 minutes was relatively equal to 1μm particles and noticeably more than 1μm particles at 90 minutes. The morphology of 1C inside tissues was fairly similar to 1μm particles while 1A particles occurred to be grouped collectively inside tissues and also adhered on tissue surface.

2.3 Adhesion property of 15μm particles was more pronounced than those of 5 μm and 1μm particles on tissue surface, respectively. After conjugation with CS and $Al(OH)_3$, the adhesion property of 1μm particles was improved to which it was quite comparable to the adhesion property of 15μm particles for 1A and even better for 1C. These results were corresponded to the results of SEM that the particles of large size of 15μm or particles of small size with positively surfaced charge of 1C and 1A could be clearly observed on tissue surface.

2.4 Cytotoxicity of 15μm particles was more stated than 5μm and 1μm particles, respectively according to the same dose. After conjugation with CS and $Al(OH)_3$, the decrease in percentage of cell's viability appeared in both formulations, especially 1C particles by mean of the acidic effect of 1C formulation.

2.5 Permeated contents of particles via nasal mucosa of 1, 5 and 15μm particles were almost equal in which the 1μm particles were considered to be the least of all contents of permeation. After conjugation with CS and $Al(OH)_3$, permeation was considerably increased for both formulations, especially 1C formulation. The permeation of release JE was very small of less than 7% totally.

3. In vivo evaluation of $Al(OH)_3$ and chitosan conjugated PLGA microparticles as nasal Japanese encephalitis vaccine carrier

3.1 After purification process, JE virus remained in spherical shape of 50nm as revealed by TEM and the integrity shown by SDS-PAGE illustrated that the surface

proteins of NS3, NS1, prM including epitope protein of JE (E protein) were well retained after purification. CD spectra revealed that the total conformation of JE vaccine was α-helix and progressively changed to β-sheet after the purification process.

3.2 Dose-response relationship was observed as an increasing of JE dose from 10μg to 80μg resulted in a raise of IgG antibody titer, however, antibody titer of 80μg was declined at week 6 as a results of too high dose of new administered vaccine that eliminate the latest generated antibody. Though, dose-response relationship was in the range between 10-40μg and could not reach 80μg for JE vaccine. This relationship could not be investigated with IgA as the stimulated titer of IgA was relatively low.

3.3 Size-response relationship was examined. Selected sizes PLGA particles encapsulated JE vaccines for intranasal immunization were 1, 5 and 15μm. The results revealed that the level of IgG antibody titer could be ranked as 1 μm >5 μm >15 μm, respectively in which the elicited titers of all sizes tended to be higher than non-particulate immunization, especially at week 8 but the booster dose still required. The particulate vaccine stimulated only small level of IgA and acquired more time to raise the titer compared to non-particulate vaccine. Though, size-response relationship could not be observed with IgA antibody response.

3.4 Surface-response relationship was also examined in this study. Particles of 1μm size were conjugated with CS and $Al(OH)_3$ and immunize in experimental animal. The results revealed that the antibody titer, IgG of both 1C and 1A groups were relatively high, even higher than 1μm particles and could persist for long period of time without any booster dose as well as IgA antibody titer that the generated titer was higher than 1μm particles and persisted without any booster dose. Surface-response relationship was detected in both IgG and IgA which represent systemic and mucosal immune response, respectively.

3.5 Stability of particles in term of size, uniformity and morphology of 1μm, 1C and 1A particles were determined. The 1C tended to be the most degradable formulation compared to 1μm and 1A particles, respectively. Integrity of released JE from 1, 5 and 15μm retained the surface protein of JE with α-helix rich structure revealed by CD spectra while these methods could not apply to investigate the JE released from 1C and 1A particles as JE was interconnected observably to $Al(OH)_3$ and CS.