# CHAPTER III

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

### Introduction

A great deal of attempt to replace the traditional vaccine administered by parenteral route with non-parenteral route has been widely intentional. One approach advocated for non-parenteral vaccine is mucosal immunization and one of an attractive route for mucosal immunization is the nasal route (Zeng, Xu and Pichichero, 2007; Brayden and Baird, 2001; Nugent, Po and Scott, 1998). Nasal route offers a number of important advantages over parenteral route, including easier administration, requiring neither sterile needle nor trained personal. (Chadwick, Kriegel and Amiji, 2010; Atmar et al., 2007; Clark, Jepson and Hirst, 2001). Moreover, unlike oral route, nasal route could circumvent hepatic first-pass elimination (Merkus and Verhoef, 1997). However, various physiological barriers on nasal surface prevent efficient absorption of vaccine into the underlying tissue. One important barrier is the mucociliary clearance causing low uptake efficiency through nasal mucosa. Therefore, an effective vaccine carrier in accordingly needed to ensure the overcoming of this physiological barrier (Brandtzaeg, 2007; Chen, 2000).

Poly (D, L-lactic-co-glycolic acid) polymer (PLGA) has been investigated most extensively in mucosal vaccine delivery. Studies in various animals have demonstrated the potential of PLGA particles as mucosal vaccine carriers. Immunized animals also showed high immunological titer and some studies also demonstrated the protection against live antigen challenges (Azevedo et al., 2006; Van der Lubben et al., 2003; Raghuvanshi et al., 2002; Esperaza and Kissel, 1992). However, in the circumstance of mucosal delivery, the major limitations of PLGA particles are primarily the negative charge of PLGA and the residual PVA coated on PLGA surface during the preparation process, which limit the adhesion of PLGA to nasal surface, in addition to the poor uptake characteristic through nasal mucosa (Chen et al., 2009; Basarkar et al., 2007; Nafee et al., 2007). Thus, one strategy with potential implication for mucoadhesive vaccine delivery is the use of particulate delivery system that intrinsically incorporates a mucoadhesive agent in the

formulation process or by subsequent conjugation to particulate delivery system (Chadwick, Kriegel and Amiji, 2010; Alpar et al., 2004).

The double emulsion-solvent evaporation is one of the most popular methods used to prepare PLGA particles (Mundargi et al., 2008; Wei et al., 2004) and polyvinyl alcohol (PVA) is an emulsifier most commonly used to stabilize the emulsion since it could help to form particles of narrow size distribution (Sahoo et al., 2002). In addition, the carbon chain of PVA could form an interconnection with PLGA surface resulting into a core-shell structure as shown in Fig 3.1A. The hydroxyl groups of PVA with free valence electron on oxygen atom, on the other hand, allow propagation of cross-linking reaction with other substances or the fixation of exogenous molecules to PLGA surface (Jia et al., 2007; Nafee et al., 2007; Fischer et al., 2006; Lu et al., 2006; Zambaux et al., 2000). Moreover, repeated washing in the preparation process does not significantly change the amount of PVA associated to PLGA surface (Gupta et al., 2006).

Chitosan (CS) is a polycationic polymer with mucoadhesive properties and has ability to act as an adsorption enhancer. Biodegradability and capability to open the tight junction to allow paracellular transport of large compounds are also recognized (Lemarchand, Gref and Couvreur, 2004; Vila et al., 2002; Van der Lubben et al., 2001; Kotze' et al., 1997). The C2 position of CS is amino group which could be ionized under acidic or neutral pH condition (Jia et al., 2007) while PVA obtains the surface hydroxyl group with the free valence electron at oxygen atom. Therefore, interaction of CS with surface hydroxyl group of PVA would occur through a strong intermolecular hydrogen bonding which probably be caused by an electrostatic interaction between PVA and CS (Gaumet et al., 2009; Guan et al., 2008; Nafee et al., 2007; Jia et al., 2007; Fischer et al., 2006; Lu et al., 2006; Sandoval et al., 2005; Messai and Delair, 2005) as shown in Fig 3.1B. Accordingly, using this surface hydroxyl group is an uncomplicated method to conjugate PLGA particle with various cationic substances.

Aluminium in the form of aluminium hydroxide, $Al(OH)_3$, is commonly used as an adjuvant in vaccine. The significant advantages of using aluminium adjuvant are the more rapid development of high titer and long lasting antibody response after primary immunization (Lindblad, 2004; Baylor, Egan and Richman, 2002; Matheis,

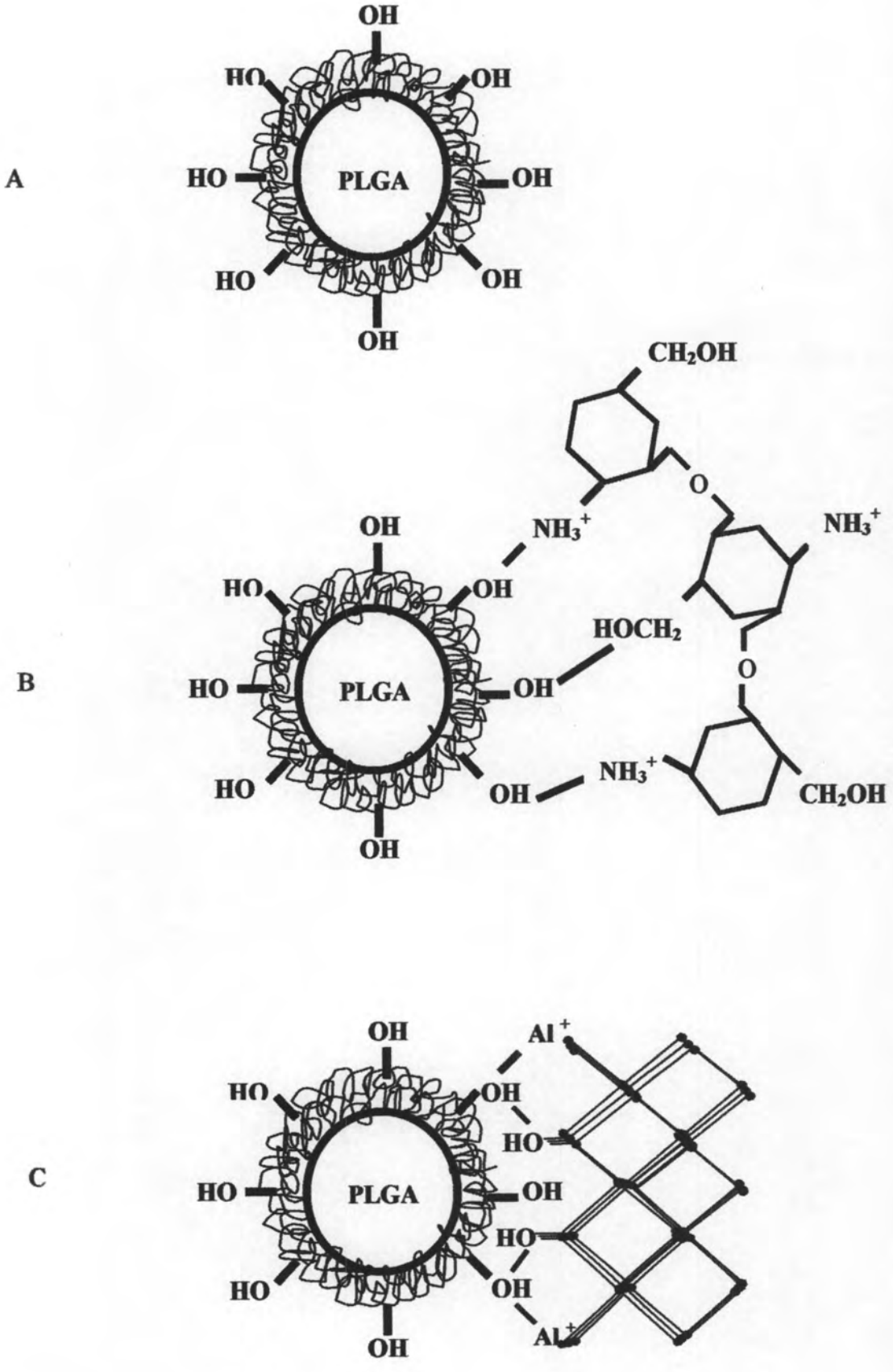


**Figure 3.1** Schematic representation of PLGA, (A), CS conjugated PLGA, (B) and $Al(OH)_3$ conjugated PLGA, (C) particles

Zott and Schwaing, 2002; Gupta, 1995). The results of previous studies indicated that $Al(OH)_3$ gel had strong positive charge at pH range 6.0-7.4 with a great adsorption ability (Sripongsan and Ritthidej, 2007, data not published; Linblad, 2004; Gupta, 1995). This positively charged adjuvant can be potentially attractive to employ as mucoadhesive substances for mucosal vaccine delivery. Moreover, study from Katare Muthukumaran and Panda (2005) indicated that serum antibody titers from single point intramuscular immunization of admixture of PLA particles and alum was comparable with immunization by two divided doses of alum adsorbed TT. Thus, $Al(OH)_3$ is one of an attractive option. $Al(OH)_3$ conjugated with PLGA particles could be depicted as in Fig 3.1C. All in all, the use of $Al(OH)_3$ and CS conjugated on PLGA surface is attempted to be thoroughly examined as effective nasal vaccine carrier.

The major goal of this study has been to develop $Al(OH)_3$ conjugated PLGA and CS conjugated PLGA particles as nasal vaccine carriers using Japanese encephalitis (JE) vaccine as a model antigen. The effect of formulation parameters on size and uniformity of PLGA particles was first evaluated. Then, the PLGA particles with appropriate size were selected to prepare $Al(OH)_3$ and CS conjugated PLGA particles. The effect of each conjugating material was also evaluated. In addition, the physicochemical characteristics of both conjugated and un-conjugated PLGA particles were also determined.

## Materials and Methods

### *Materials*

Poly (D,L-lactic co-glycolic acid), (PLGA) with lactide : glycolide 50 : 50 and bicinchoninic acid kit (BCA kit) were purchased from Sigma-Aldrich (Saint Louise MO, USA). Polyvinyl alcohol, PVA, (MW 67,000) was obtained from Fluka Chemical (Switzerland). CS at molecular weight of 37kDA with 94% degree of deacetylation was procured from Seafresh® (Bangkok, Thailand). Aluminium hydroxide, $Al(OH)_3$, was a gift from Bureau of Veterinary Biologic (Bangkok, Thailand). Japanese encephalitis vaccine (JE) was kindly provided by Government Pharmaceutical Organization (Bangkok, Thailand). JE was purified and concentrated

before use by membrane centrifuged tube. The other chemicals were of analytical grade and used as received.

*Preparation of particles*

Various sizes of PLGA particles were prepared by double emulsion solvent evaporation technique (Prieto et al., 1994) with a range of formulation parameters. In brief, JE antigen was added into 8 ml of 5%PLGA in dichloromethane (DCM) with the ratio of 1:2.5, 1:5 and 1:10, respectively. The mixture was immediately sonicated by 3-mm diameter standard probe sonicator at output control of 20 for 10 seconds to form primary w/o emulsion. Then, 1%, 2% and 4% PVA solutions were added to the primary emulsions with the ratio of primary emulsion : PVA of 1:2 and 1:4, respectively, to obtain secondary emulsions by sonication with either bath sonicator or probe sonicator. The double emulsions were diluted in 100 ml of 1% PVA and the solvent was eliminated by stirring up to 3 hours at 500 rpm. The resulting particles were washed and collected by centrifugation at 10,000 g for 5 minutes. The CS conjugated formulation was prepared by either adding 0.2% CS into the concave of centrifuged particles or blending 0.2% CS with 1%PVA at the final process of dilution. The volume of 0.2% CS added to the concave of centrifuged particles was excess in order to obtain the complete conjugation. The particulate formulation containing CS were then vortexed for 15 minutes and shaken for 2 hours subsequently. After shaking, the mixture was left overnight and the excess of un-conjugated CS was removed by centrifugation at 10,000g for 5 minutes. $Al(OH)_3$ conjugated formulation was obtained by adding either 0.75% or 1.5% $Al(OH)_3$ onto the concave of the centrifuged particles following the regulation of US code of federal regulations (610.15(a)) that the amount of aluminium is limited to $\leq$ 0.85mg for a single human dose of vaccine.

*Characterization of particles*

*Size and size distribution*

The size and size distribution of samples were observed under a laser diffractometer (Mastersizer 2000, Malvern, UK). The samples were suspended in 1% v/v tween 80 solution and bath sonicated to deaggregrate before measurement. The percentile sizes for 10, 50 and 90% which were expressed as D(v,0.1), D(v,0.5) and D(v,0.9), respectively, were reported. The width of the distributions which was the measurement of the absolute deviation from the median was expressed as uniformity.

*Morphology*

Morphology of samples was determined by scanning electron microscopy (SEM, JEOL, JSM-5410LV, Jeol, Japan). The samples were attached to the specimen holder with a double coated adhesive tape and coated with a layer of gold. The scanning electron images were examined and photographed.

*Surface morphology*

Surface morphology was determined by atomic force microscopy (SPA 400-DFM, Seiko Instruments Inc., Japan). The samples were pasted as thin film on a clear and smooth platform. The measurement was operated by laser spot reflected from the top of cantilever into an array of photodiodes. Three-dimensional surface profile was presented as the topography of the sample.

*Surface charge*

Surface charge was determined by a photon correlation spectrophotometer (Zetasizer nanoseries, Nano-ZS, Malvern, UK). The samples were diluted into 2 ml and the zeta potential was measured at 25°C. The results were presented as a mean surface charge.

*Entrapment efficiency*

The selected samples were separated from non-encapsulated material by centrifugation. The supernatants were used indirectly to estimate the percentage of JE vaccine entrapped in particles by BCA protein assay. The unloaded particles were employed as blank in order to avoid the interference of PVA on BCA assay. The percentage of entrapment efficiency was calculated using the following equation :

$$\text{Percentage of entrapment} = \frac{(Ja-Js) \times 100}{Ja} \quad \ldots\ldots(1)$$

Where Ja is the total amount of Japanese encephalitis added

Js is the amount of Japanese encephalitis in supernatant

*Organic solvent residue*

Dichloromethane residue in selected PLGA particles was determined by gas chromatography (Schimudzu GC-7AG, Japan). Samples of 3 ml were transferred to vials and sealed. These vials were then heated at 50°C to vaporize the dichloromethane. The vapor of dichloromethane was then injected to 5% SE-30 column (3.3mm.x 2mm.) under the following conditions : column temperature 50°C, injecting temperature 180°C, nitrogen gas flow rate 20ml/min. The standard curve of various concentrations was investigated by the same method.

*Polymeric interaction*

The interaction between PVA, and CS or PVA and $Al(OH)_3$, and the spectrum of each component were investigated by Fourier transform spectroscopy, a high sensitivity of IR (FTIR 1760X, Perkin Elmer, USA). The interaction of functional groups among each polymer investigated by FT-IR was acquired by mulling method with the ratio of PVA : CS, 1:2 and PVA : $Al(OH)_3$, 5:1, respectively. The samples

were dried overnight by desicator before evaluation. The wave number used was 750-4000$cm^{-1}$.

*In vitro release of antigen*

The 5 ml of samples were retained in a shaking bath at 37°C, 150 rpm. At appropriate time intervals, samples were centrifuged at 10,000 g for 5 minutes and the supernatants were then collected. The antigen in the supernatant was analyzed by BCA kit for quantitative determination. The unloaded particles were used as blank in order to avoid the interference of PVA, CS and $Al(OH)_3$ on BCA assay.

*Integrity of entrapped material*

The entrapped material in this study was JE vaccine. SDS-PAGE and circular dichroism (CD) were attributed to investigate the integrity and the conformation of epitope proteins of JE virus. The JE virus was exposed to vigorous conditions as in the preparation processes which were the organic solvent and sonication force before an evaluation by SDS-PAGE and CD. According to SDS-PAGE, samples were mixed with reducing buffer and boiled at 95°C for 2 minutes. The mixtures of samples were loaded into 12% polyacrylamide gel and the electricity at 80V was applied to the gel for 2 hours. The gels were stained by simply blue solution and destained by distilled water. Regarding to CD, the ellipticity of samples was recorded between 200-290 nm on spectropolarimeter (Jasco model J-715, Jasco, Tokyo, Japan). The CD spectra was obtained by plotting molar ellipticity against wavelength.

## Results and discussions

*Factors affecting size and uniformity of PLGA particles*

*Formulation parameters*

Particle sizes of 0.68-7.88μm and uniformity of 0.27-5.46 were obtained from PLGA particles prepared by probe sonication during both primary emulsion and secondary emulsion process as shown in Fig 3.2.

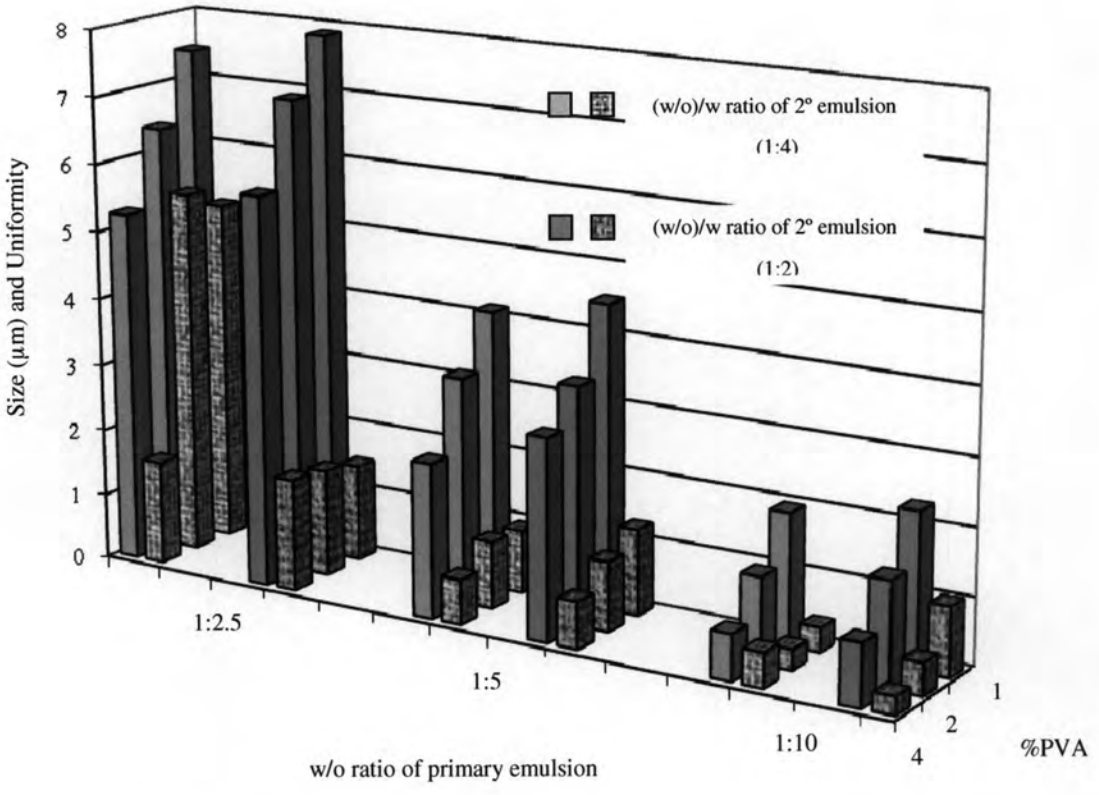


**Figure 3.2** Effect of w/o ratio of primary emulsion, (w/o)/w ratio of secondary emulsion and % PVA of secondary emulsion on size and uniformity (plain bar and strip bar of each pair bar) of PLGA particles prepared by probe sonication during secondary emulsion process

*The effect of w/o ratio of primary emulsion*

The particles sizes were clearly decreased when the primary w/o ratio was reduced from 1:2.5 to 1:10. This tendency was observed at all formulations of different % of PVA and different ratios of secondary w/o. A decrease in the uniformity value was slightly noticed. Moreover, formulations prepared with primary w/o ratio of 1:2.5, secondary (w/o)/w ratio of 1:4 and PVA of 1 and 2% comprised dramatically high uniformity values as the formulations were prepared under vigorous conditions.

Under the same input force from probe sonicator, the larger droplet size of primary emulsion prepared with higher primary w/o ratio was observed since larger volume of internal phase probably required higher force to disperse to be the small and uniform droplets. Moreover, dissolving JE would result in an increase in the viscosity of water phase which rendered the dispersion of water, in turn, large particles with high size distribution were formed (Nafee et al., 2007; Mainardes and Evangelista, 2005). The effect of the internal phase volume of the primary emulsion on the particle size of PLGA had also been previously reported that an increase in the volume of the internal phase led to an increase in particle size (Parikh et al., 2003; Jeffery, Davis and O'Hagan, 1993).

*The effect of (w/o)/w ratio of secondary emulsion*

The particle sizes were slightly increased when secondary (w/o)/w ratio was ascended from 1:4 to 1:2. However, a shift of particle size influenced by secondary (w/o)/w ratio was minute when compared to the influence from primary w/o ratio. The uniformity values of all formulations were small of less than 2 and tended to be stable or slightly decreased when decreased the secondary (w/o)/w ratio from 1:2 to 1:4 except the two formulations mentioned previously.

After the secondary emulsion was formed, the solvent was then evaporated and the circular layer of organic phase was shrunk to cover the internal water phase and solidified (Bakan, 2000; Kissel and Koneberg, 1996). The factor affecting the coalescence of organic phase was the distance of separation which correlated to the

volume of continuous phase and disperse phase in accordance with the theory of phase emulsion stability. The droplets in this study were stabilized by stabilizer PVA in which the applied theory could be the DLVO theory of electrostatic stabilization and steric stabilization which mainly relevant to non-ionic surfactant and polymers. Potential energy of interaction was in reference to the distance of separation, charge and steric effect. The emulsion system needed to overcome both of the primary minimum force of attraction between liquid droplets and the energy barrier of repulsion to reach the most stable stage of secondary minimum in which the droplets were stable and not coalesced at this stage (Mondal et al., 2007; Mao et al., 2007; Kumar et al., 2004; Parikh et al., 2003 and Eccleston, 1997). The 1:4 ratio of secondary (w/o)/w provided the system a better possibility to reach the stable stage of secondary minimum and to avoid the collision of droplets by increasing the distance of separation and by the more amount of PVA compared to ratio 1:2 in which by these reasons, the droplets could be protected from aggregation. Thus, the size and consistency of particles prepared with secondary (w/o)/w 1:4 were slightly smaller and more uniform compared to secondary (w/o)/w of 1:2 except the formulations prepared with PVA 1 and 2% and primary w/o ratio of 1:2.5. The uniformity values of these formulations were considerably high even though these formulations were prepared with secondary (w/o)/w of 1:4 which could be concluded that the critical effect of primary w/o ratio was more pronounce than the effect of secondary (w/o)/w ratio on the uniformity value.

The effect of primary w/o ratio on droplets size was considerably more important than the effect of secondary (w/o)/w ratio since the small and uniform size of primary emulsion droplets would provide a greater opportunity for the organic phase to form small and uniform circular layer and consequently to obtain small and uniform size of secondary emulsion droplets. Thus, the huge and inconsistency of secondary emulsion droplets notably replied to the size and uniformity of primary emulsion droplets (Mainardes and Evangelista, 2005).

*The effect of % PVA in secondary emulsion*

The particles sizes were gradually decreased when the percentage of PVA was increased from 1% to 4% in all formulations with different primary w/o and

secondary (w/o)/w ratios. Consequently, amount of PVA was more pronounced to influence the particles size than secondary (w/o)/w ratio but not as critical as primary w/o ratio as the shift of particles size was much in lesser extent than the shift of particles size influenced by primary w/o ratio. This was due to the primary emulsion droplets supposed to be small and uniform before they were stabilized by PVA in the step of secondary emulsion forming according to an equal input force. The uniformity value was stable or slightly decreased when increased the percentage of PVA especially, formulation prepared with primary w/o ratio of 1:2.5, PVA of 4% and secondary (w/o)/w of 1:4 that the uniformity was considerably decreased when the amount of PVA was increased from 2% to 4%. Compared to the change of uniformity value influenced by different parameter, the effect of PVA on uniformity value was quite noticeable, but not as essential as primary w/o ratio.

The size and uniformity of particles relied upon the size and stability of emulsion droplets which partly depended on the availability of sufficient amounts of PVA in the system to completely cover the droplets surface and to stabilize the small emulsion droplets in order to create the smaller sizes of particles. (Astete, Kumar and Sabliov, 2007; Mainardes and Evangelista, 2005; Sahoo et al., 2002; Edan and Celebi, 1996). The stabilization of stabilizer was from the reduction of the free energy (*Gf*) of emulsion system by both of steric and electrostatic effects from PVA that prevent the coalescence of droplets. (Jiao and Burgess, 2003; Garti and Aserin, 2000; Bakan, 2000; Nakagaki et al., 1999 and Eccleston, 1996). PLGA particles had been previously prepared by using 1-5% PVA as stabilizer and found that the particles size was significantly decreased as the amount of PVA was increased (Nafee. et al., 2007 and Krishnamachari, Madan, and Lin., 2007). Similar result was also observed in this study.

*Process parameter : Sonication equipment*

According to the sonication equipments, probe sonicator could produce particles of smaller size than bath sonicator in all percentages of PVA as could be seen in Fig 3.3. The overall uniformity values of particles prepared by probe sonicator were correspondingly less than bath sonicator. Moreover, the uniformity

values of particles prepared by bath sonicator were increased with the increasing amount of PVA while the uniformity values of particles prepared by probe sonicator showed the opposite effect.

Considering to probe sonication, high frequency vibrations were produced by probe which gave an intense microscopic vacuum bubbles imploded at very high rate directly at the point of collapse. This phenomenon was known as caviation. The point of collapse for emulsion was the interphase of water and oil, and the caviation nuclei was the suspended entrapped materials (Figiel, 2007; Reich, 1998). Conversely for bath sonication, the high frequency of electrical energy was converted into ultrasound waves. These high frequency waves were created in the liquid countless inside the tank to form the microscopic vacuum bubbles which rapidly expanded and collapsed. This phenomenon was termed cavitation. The wave in bath sonicator, however, was required to exceed through the glassware thickness prior to attain the water-oil mixture.

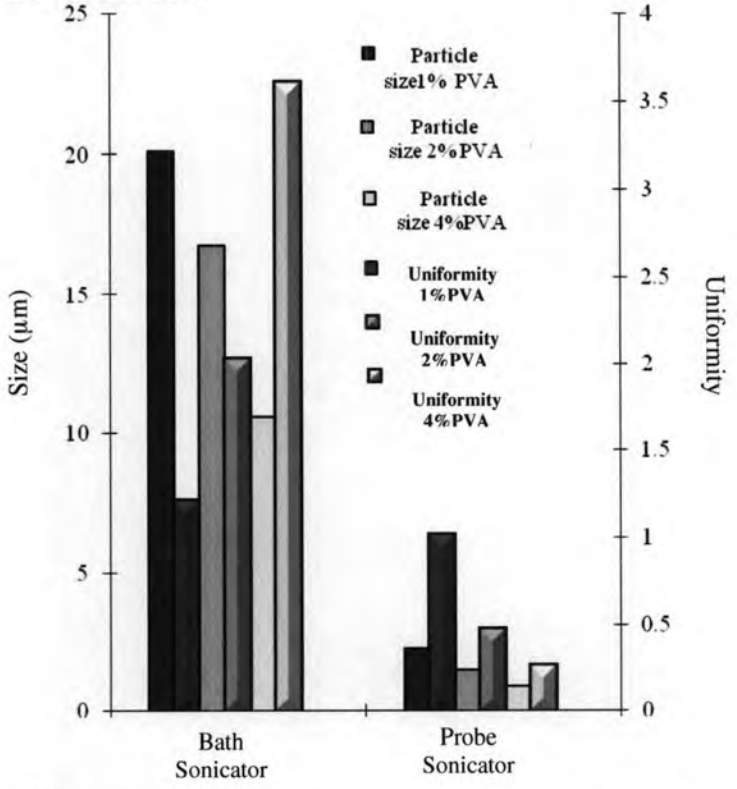


**Figure 3.3** Effect of sonication equipment in the process of secondary emulsion forming on size and uniformity of PLGA particles prepared with primary w/o and secondary (w/o)/w of 1:10 and 1:2, respectively.

In this study, the sizes of particles prepared by bath sonicator were about ten times larger than particles prepared by probe sonicator since the efficiency of the expanded waves in bath sonicator was not as effective as intense-collapsed waves from probe sonicator according to an equal intensity. Moreover, the uniformity of particles prepared by bath sonicator obviously depended on the viscosity of the external water phase conversely to the effect by probe sonicator. This phenomenon was occurred since the origin of vibration of probe sonicator was immense directly into the external water phase while the bath sonicator only transmitted the ultrasound wave to produce the bubbles which the viscosity could be considered as an effectual factor.

*Effect of mucoadhesive substances on physicochemical properties of size-selected PLGA particles*

*Characteristics of size-selected PLGA particles*

PLGA particles were prepared by double emulsion solvent evaporation technique with selected formulation parameters in order to prepare particles of required sizes as shown in Table 3.1. These formulations were obtained from previous results that yielded the closest particle sizes of 1, 5 and 15 μm with good characteristics of in close proximity to the target size, small uniformity value with smooth particulate surface and high entrapment efficiency. These particles were chosen since the micron sizes of particles were reported to stimulate higher immune response via mucosal route compared to nano-sizes particles (Gutierro et al., 2002). Additionally, high and long lasting antibody titer were observed upon IM immunization with 2–8 μm size particles and immunization with either small particles (<2 μm) or with intermediate size range particles (10–70 μm) both elicited the comparable antibody response according to the same single point immunization but lower in comparison to that achieved with immunizing by 2–8 μm size particles (Katare, Mathukumaran and Panda, 2005). Thus, the 1, 5 and 15 μm sizes were selected in this study in order to compare the effect of particles in different size range

of small, intermediate and large. The physicochemical properties and morphology of resulted particles are shown in Table 3.1 and Fig 3.4A, respectively.

Although the size and uniformity of these selected formulations were satisfactorily met the requirement, the entrapment efficiency of 15μm particles was moderate, at 62.24%, since the low viscosity of PVA applied in 15μm particles led to the submission to the outward diffusion of entrapped materials from internal aqueous phase to external aqueous phase compared to high viscosity PVA (Sahoo et al., 2002; Coombes et al., 1998) while the entrapment efficiency of 1 and 5μm particles were quite satisfied, at 89.39 and 94.17%, respectively. Additionally, the smaller surface area and less internal volume of large particles would provide poor opportunity for the internal water phase to be entrapped inside during the preparation process. According to organic solvent residue, within an equal evaporation time, the organic solvent residue left in 15μm particles was higher than 1 and 5 μm particles as shown in Table 3.1 due to the slower evaporation rate from particles of the less surface area. Still, level of residue of all formulations was in the restriction of the United State Pharmacopeia of 75 ppm (USP23).

The charge of PLGA without any PVA in neutral buffer was normally at about -45mv (Sahoo et. al., 2002; Stolnik et al., 1995). However, the PVA residues at particles surface which were the result from the interpenetration of PVA into the organic phase could shield the surface charge of PLGA and move the shear plane outward. Thus, the charge of PLGA particles prepared by using PVA as stabilizer was increased to be slightly negative of about -6.5 to -15mv at pH 7 and -5 to -10mv at pH 6 (Sahoo et. al., 2002). The result in this study was corresponding to the charge observed in previous report.

The morphology of 1, 5 and 15μm PLGA particles determined by scanning electron microscope (SEM) are shown in Fig 3.4, A1-A3. It was found that the shapes of all formulations were spherical without any pore. The particles surfaces of all formulations were smooth. Surface morphology investigated by atomic force microscopy (AFM) was corresponded to SEM pictures that the surface of PLGA particles was smooth as shown in Fig 3.5A1.

**Table 3.1** Formulation parameters, characteristics of particles and effect of CS and $Al(OH)_3$ on size, uniformity and surface charge of size selected PLGA formulations.

| Size selected formulations (μm) | Input force[1] | w/o ratio [2] | (w/o)/w ratio [3] | %PVA[4] (w/v) | Chitosan (%w/v) | $Al(OH)_3$ (%w/v) | Median size (μm) , uniformity n=3 | Zeta potential (mv±SD) n=3 | Entrapment efficiency (%±SD) n=3 | Solvent residue (ppm±SD) n=3 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Probe sonicator | 1:10 | 1:2 | 4% | - | - | 0.92, 0.27 | -5.38±0.13 | 89.39±3.46x$10^{-4}$ | 10.98±0.24 |
| | | | | | 0.2 | - | 1.22, 7.72 | +22.17±2.67 | | |
| | | | | | | 0.75 | 1.62, 1.59 | +15.36±1.27 | | |
| | | | | | | 1.5 | 3.44, 2.22 | +16.90±0.12 | | |
| 5 | Probe sonicator | 1:2.5 | 1:4 | 4% | - | - | 5.26, 1.57 | -7.42±0.31 | 94.17±6.24x$10^{-4}$ | 11.52±0.19 |
| | | | | | 0.2 | - | 7.59, 2.52 | +23.23±2.93 | | |
| | | | | | | 0.75 | 6.87, 2.34 | +16.40±0.38 | | |
| | | | | | | 1.5 | 6.89, 2.96 | +19.71±1.40 | | |
| 15 | Bath sonicator | 1:10 | 1:2 | 2% | - | - | 16.76, 2.03 | -6.90±0.86 | 62.24±4.19x$10^{-4}$ | 21.32±0.68 |
| | | | | | 0.2 | - | 17.18, 2.77 | +25.62±4.85 | | |
| | | | | | | 0.75 | 16.85, 1.98 | +22.45±2.66 | | |
| | | | | | | 1.5 | 16.95, 1.83 | +19.52±3.95 | | |

[1] Input force of secondary emulsion

[2] Volume ratio of primary w/o emulsion

[3] Volume ratio of secondary (w/o)/w emulsion

[4] %PVA of secondary emulsion

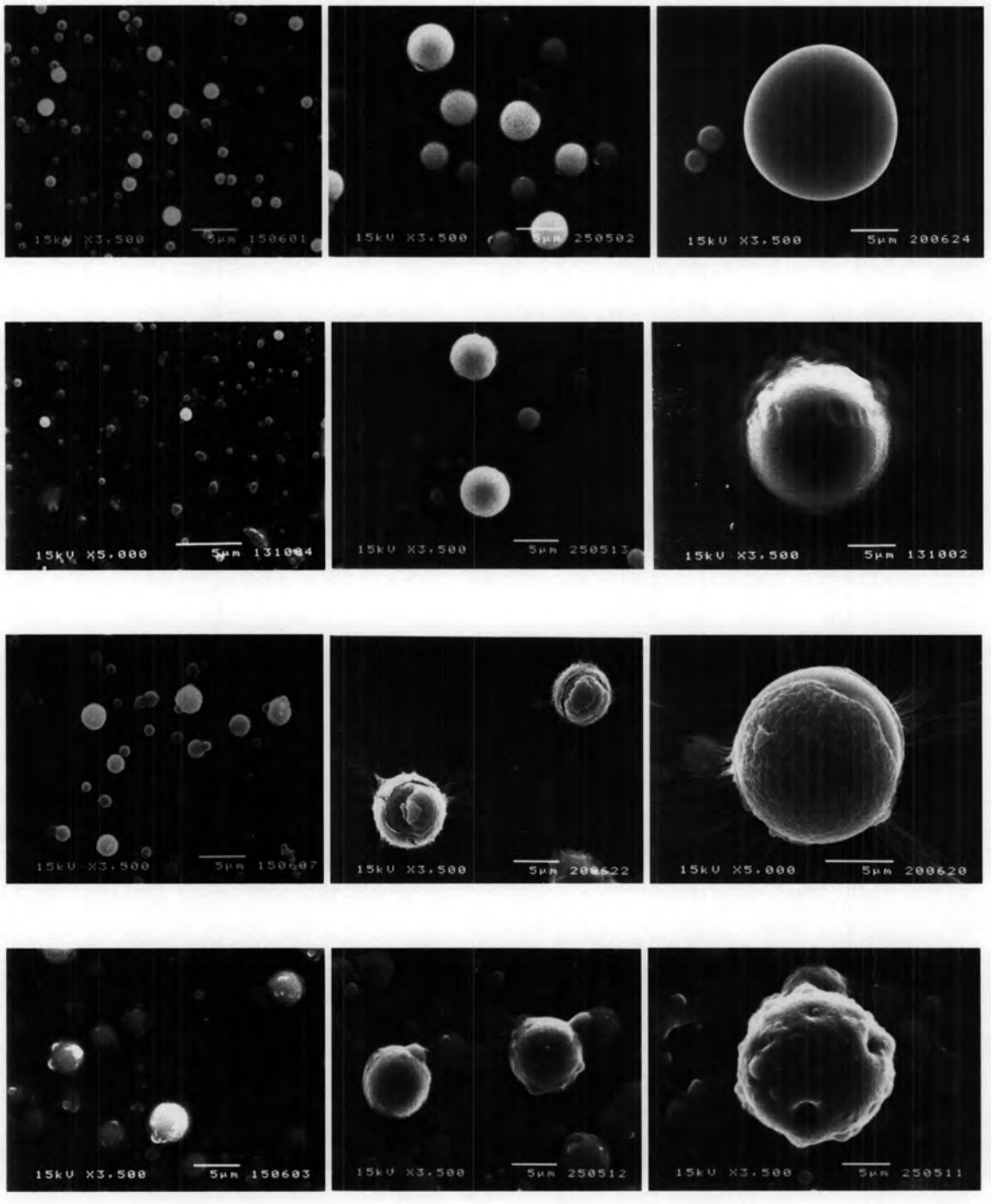


**Figure 3.4** SEM photomicrograph of PLGA, CS conjugated PLGA, 0.75% $Al(OH)_3$ conjugated PLGA and 1.5% $Al(OH)_3$ conjugated PLGA particles (A → D, respectively) with different particles size of 1, 5 and 15μm (1,2,3, respectively).

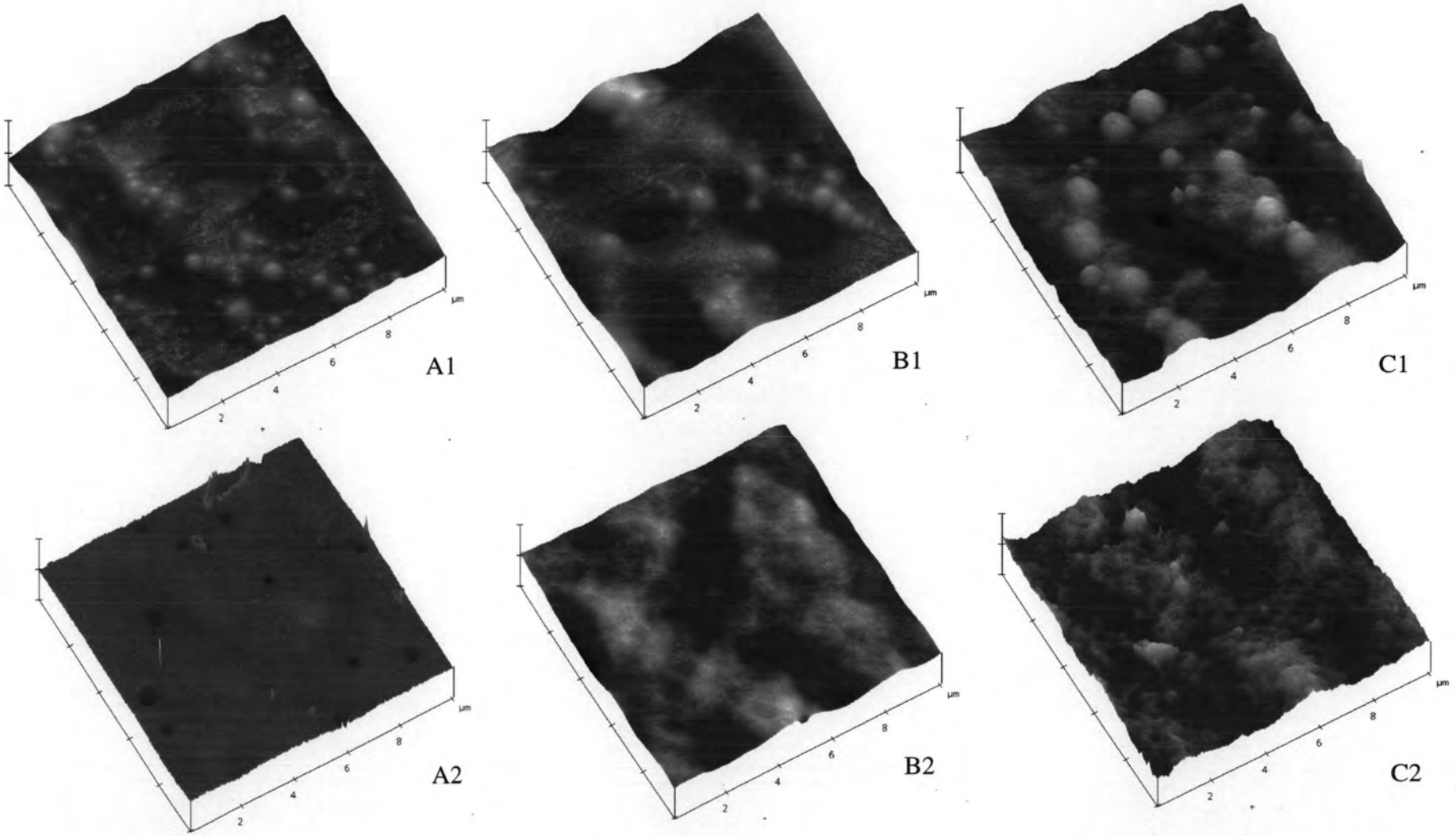


**Figure 3.5** Surface morphology of PLGA (A1), PVA (A2), CS conjugated PLGA (B1), CS (B2), 0.75% $Al(OH)_3$ conjugated PLGA (C1) and $Al(OH)_3$ (C2) using AFM technique

*Effect of CS conjugation*

An increase in particle size upon conjugation of particles by 0.2% CS was considerably insignificant as shown in Table 3.1. The uniformity value of 1μm particles was obviously increased while the uniformity values of 5 and 15μm particles were moderately elevated. The surface charges of all CS conjugated formulations were obviously increased from mildly negative to moderately positive of about +22 toward +25mv as the concentration of CS added had been a saturation of surface charge density and the put in of CS in this study was excess.

CS covered the PLGA surface by conjugation with hydroxyl group of PVA which interconnected on particle surface. The amount of PVA on the surface per weight of particles was increased with the high specific surface area of particles as the particles size decreased or the surface PVA density was decreased as the further increase of particle size (Lee et al., 1999). Therefore, the surface PVA density of 1, 5 and 15μm particles in this study could be ranked as 1>5>15μm, respectively. The increase in size was expected and attributed to the conjugation of CS (Kumar et al., 2004). The interaction of PVA and CS could occur by both electrostatic interaction and intermolecular hydrogen bonding. It could be stated that three mechanisms were involved in the PVA-CS interaction which were, the adsorption, the covalent binding and the physical interaction. The uncomplicated manner to form this connection was the polyelectrolyte complex of which it represented the physical relations by mean of the electrostatic interaction leading to intermolecular hydrogen bonding (Guo and Gemeinhart, 2008; Reist, Mayer and Gurny, 2004). Electrostatic interaction occurred between the total negatively charged particles with positively charged CS leading to the formation of the polyelectrolyte complex. The intermolecular hydrogen bonding was as well be formed between amino group of CS and hydroxyl group of PVA (Guan et al., 2008; Nafee et al., 2007; Jia et al., 2007; Lu et al., 2006; Sandoval et al., 2005).

After conjugation, the sizes of all formulations were increased by the coated layer of CS. This finding was corresponded to the study of Chen et al. (2009) that the particle size was increased as the molecular layers of CS coating on the particulate surface increased. However, the particles of small size such as 1μm provided a high

possibility of impaction, thus, causing high agglomeration of particles. The conjugation of 1μm particles would be more frequent and easier when compared to 5 and 15 μm particles within the same volume of formulation resulting in higher uniformity value of 1 μm formulation. It was noticed from SEM photomicrograph in Fig 3.4B that 1μm particles were agglomerated by the attachment of their CS-outer layer as the small particles with high surface area gained a high possibility of collision compared to 5 and 15 μm formulations. The positive surfaced charge of particles was the result of the protonated amino groups of coated CS which completely shield the negatively charged of particles (Yang et al., 2009; Guan et al., 2008). Moreover, the protonated amino group was presumably present on the surface of particles as the surface of PVA/CS blended film investigated by Chaung et al. (1999) was also selectively enriched with nitrogen atom of amino groups of CS on the film surface.

The results from AFM as illustrated in Fig 3.5A1, showed that the surface morphology of PLGA particles was quite smooth while the surface morphology of CS conjugated PLGA particles could not obviously be seen since the surface was covered by the transparent-layer of CS as shown in Fig 3.5B1. AFM could be considered as novel technique for investigating roughness and morphology of the surface of materials more obviously than SEM. The surface morphology of CS conjugated PLGA particles resemble to the blinking-transparent gel of CS as could be observed in Fig 3.5B2.

*Effect of $Al(OH)_3$ conjugation*

Upon conjugation by $Al(OH)_3$, the particles of 1μm would be clearly increase in size and size distribution especially at higher level of 1.5% $Al(OH)_3$. The effect of conjugation on particle size and size distribution on larger particles was respectively decreased as shown in Table 3.1. It would also be seen that the surface charge of all formulations was positive but with lower value than that of CS conjugation.

PLGA particles in this study were dispersed and trapped in $Al(OH)_3$ gel-like structure by forming weak bonding interaction between the layer of PVA residue on PLGA surface and $Al(OH)_3$ (Kanchan, Katare and Panda, 2009). Mitsumata, Hachiya and Nitta (2008) revealed that the structure of $Al(OH)_3$ which appeared as inorganic

particles, were randomly dispersed in the PVA solution like a pre-gel solution with the partial contact of each particles. The macromolecules such as PVA were the linear and flexible molecules which adsorbed non-specifically on the surface of oxides. The interaction of the surface took place through hydrogen bonds between polar functional groups of the polymer chain and the hydroxylated and protonated groups of $Al(OH)_3$ (Contreras et al., 2006). Thus, three primary mechanisms could be proposed to explain the conjugated mechanism of PVA and $Al(OH)_3$ in this study which were the electrostatic attraction, hydrophobic attraction and ligand exchange (Morefield et al., 2005; Rinella, White and Hem, 1995; Shakhshir et al., 1995). $Al(OH)_3$ particles could also be assumed to interact with PVA by the hydrogen bonding between hydroxyl groups of PVA and hydroxyl groups of $Al(OH)_3$ (Beruto, Botter and Converti, 2008; Shah, 2006;) or could presume to interact by the attraction of free valence electron of oxygen in hydroxyl group of PVA and positive charge of aluminium.

The size of particles of 1μm covered by the 1.5% $Al(OH)_3$ were clearly elevated as a consequence of the much amount of $Al(OH)_3$ added which covered the surface of particles and adsorbed the nearby particles as a cluster of particles. Mitsumata Hachiya and Nitta (2008) investigated the microscopic characteristics of PVA/$Al(OH)_3$ gel and found the clear network of gel consisting the partial contact between $Al(OH)_3$ particles causing the heterogeneous structures of composited gel. Thus, the high possibility of particulate impaction of small particles and the partial contact of $Al(OH)_3$ particles could be a reason of huge median size of 1μm conjugated 1.5% $Al(OH)_3$ while the larger size of 5 and 15μm obtained a lower possibility of particulate impaction by the fact that surface area of large particles were less than small particles resulting in a small change of particles size and uniformity.

Morphologies of $Al(OH)_3$ conjugated PLGA particles are shown in Fig 3.4C-3.4D. The particles of all sizes were spherical and were coated by $Al(OH)_3$ as a white-opaque outer layer. It was revealed that 0.75% $Al(OH)_3$ was sufficient to cover the whole surface of 1μm particles, but not, 5 and 15 μm particles considering to one single particles as the surface area of one large single particles was more than one small single particles. However, $Al(OH)_3$ of 1.5% was adequate to cover the surface of 5 and 15μm particles. The amount of $Al(OH)_3$ added was corresponded to the

surface PVA since the surface PVA assumed to interact with $Al(OH)_3$ (Morefield et al., 2005; Rinella, White and Hem, 1995; Shakhshir et al., 1995). The surface PVA was increased with the particles size decreased (Lee et al., 1999) and could be ranked as 1>5>15μm, respectively in this study. Thus, 1μm particles attributed to the high surface PVA could assume to trap the higher amount of $Al(OH)_3$ added to cover the whole surface compared to 5 and 15μm particles. Consequently, 0.75% $Al(OH)_3$ was enough to cover the whole surface of 1μm particles. Surface morphology investigated by AFM was shown in Fig 3.5C1. Surface of $Al(OH)_3$ conjugated particles were relatively rough as a consequence of the $Al(OH)_3$ coated outside. The surface morphology of $Al(OH)_3$ was also shown in Fig 3.5C2 and the characteristics of $Al(OH)_3$ was quite alike to the $Al(OH)_3$ coated on PLGA surface.

*Polymeric interaction*

The interactions between PVA/CS and PVA/$Al(OH)_3$ were investigated by FT-IR as shown in Fig 3.6.

The principal band of PVA spectra was at 3439.68 $cm^{-1}$ which was attributed to –OH stretching vibrations of hydroxyl group. The considered peak of $Al(OH)_3$ was the –OH stretching peak found at 3310.76$cm^{-1}$ (Contreras, 2006; Lindblad et al., 2004; Shirodkar et al., 1990 and Wolska and Szajda, 1983) while CS showed FT-IR absorption feature of –OH and $–NH_2$ stretching vibrations at 3390.34 $cm^{-1}$ (Niu et al., 2009; Jia et al., 2006; Zheng et al., 2001).

Regarding to FT-IR spectra of PVA-$Al(OH)_3$, the peak illustrated at 3437.13 $cm^{1}$ was attributed to –OH stretching peak of PVA. The adsorption band of hydroxyl group of $Al(OH)_3$ associated with both hydrogen bonding and bridged hydrogen bonding were normally appeared around 3375-3525 $cm^{1}$ which could not be seen as these peak would be overlapped by –OH stretching peak of PVA. However, the new peak at 1631 $cm^{1}$ represented the water of hydration or surface-adsorbed molecule of $Al(OH)_3$ corresponding to the bending mode of water was represented (Kutty, Jayaraman and Periaswami, 1996). Thus, the peak of surface-adsorbed molecule of $Al(OH)_3$ apparently appeared which could be concluded that there was a surface-adsorbed interaction between PVA and $Al(OH)_3$.

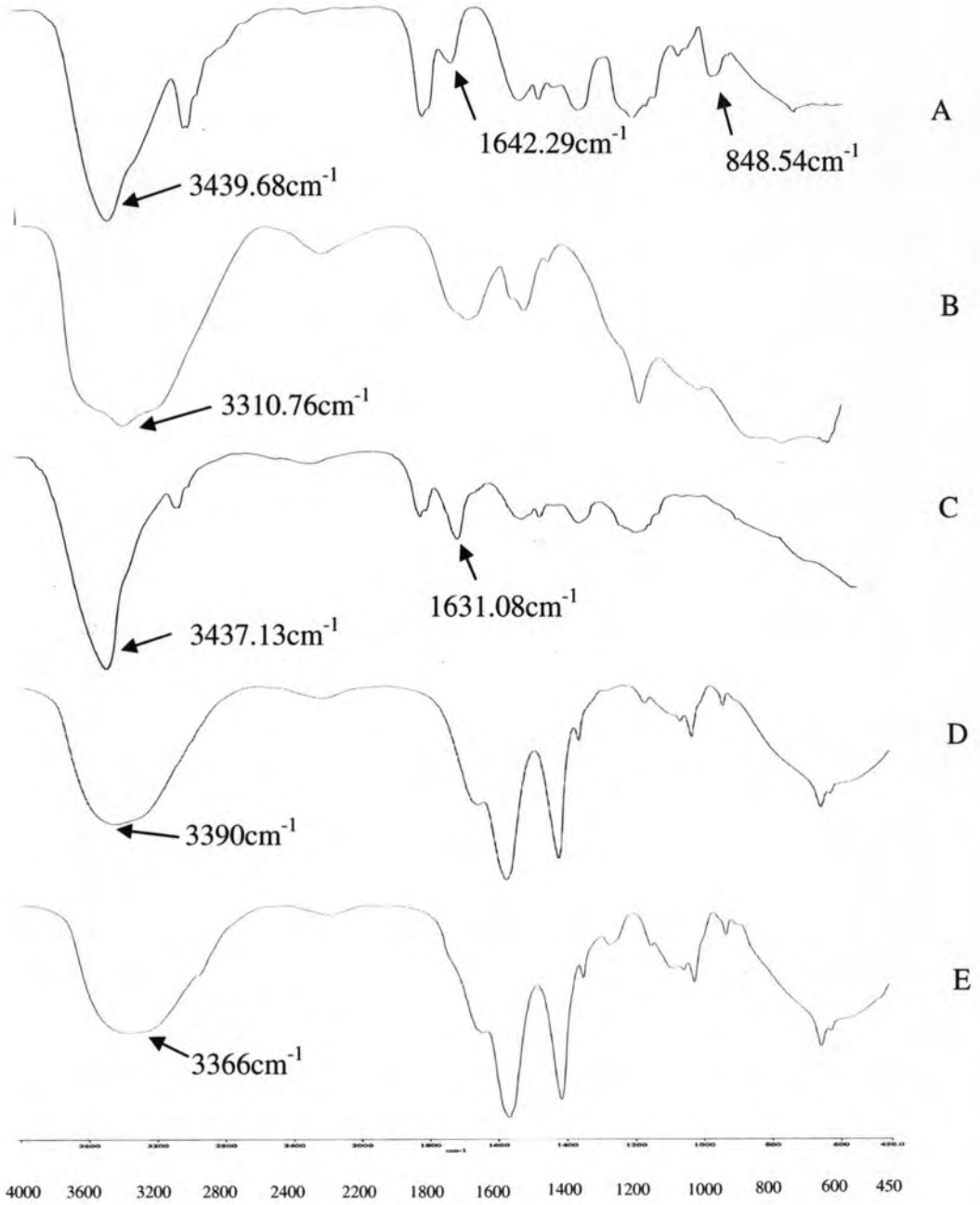


**Figure 3.6** The FT-IR spectra of PVA (A), $Al(OH)_3$ (B), PVA/$Al(OH)_3$ (C), CS (D), and PVA/CS (E)

According to FT-IR spectra of PVA- CS, the spectra showed that the -OH stretching peak was broaden and shifted to the lower number at 3366.09cm$^{-1}$. This result was caused by the dissociation of hydrogen bond of –OH group of PVA to form the intermolecular hydrogen bonding with CS (Lu et al., 2006; Jia et al., 2007; and

Srinivasa et al., 2003). As a result, the intermolecular hydrogen bonding assumed to be the main mechanism of interaction between PVA and CS.

*In vitro release*

The 1, 5 and 15μm formulations produced initially burst release of 15-20% as shown in Fig 3.7. The release rate of 5 and 15μm particles were gradually slower when compared to that of 1μm particles in which the release reached 32.08% at day 60$^{th}$. From day 30$^{th}$, the amount of JE antigen released from 5 μm particles was higher than 15 μm and continuously elevated until end of experiment at day 60$^{th}$. After conjugation of 1μm PLGA particles by CS and 0.75% $Al(OH)_3$, the release of JE antigen, especially $Al(OH)_3$ conjugated formulations, was obviously less than that of un-conjugated one. In addition, the burst releases from both conjugated formulations were merely noted. Although, CS conjugated formulation slowly released JE antigen, the release was comparable to 5 and 15μm un-conjugated formulation at day14$^{th}$ and gradually exceeding and reached 30.29% at day 60$^{th}$. $Al(OH)_3$ conjugated particles initially exhibited the slowed release but was quite comparable to that of 5 μm un-conjugated formulation at day 60$^{th}$.

The high surface particles presumed to liberate more encapsulated materials (Panyam, et al., 2003). Moreover, the initial release rate decreased with an increase in microsphere size and the release profile changed from first order to concave-upward as the microsphere size was increased (Berchan et al., 2007). In this study, the initial burst release was graphically observed at all sizes of 1, 5 and 15μm formulations as the antigen was water-soluble and the amount of antigen that located on the surface of particles could be immediately released. According to the second phase of release, smaller particles had larger surface area for the entrapped material to be diffused out. Thus, the amount of release antigen from different size of particles in this study could be ranked; 1 >5 >15 μm, respectively. As regarding to the conjugated formulations, the initial burst release of conjugated particles was accordingly suppressed as the surface of particles was covered by the layers of CS (Manca et al., 2008) and $Al(OH)_3$ (Kanchan, Katare and Panda, 2009). These conjugated materials considerably retarded the second phase of release by delaying

the diffusion of JE antigen as the conjugated materials acted as a barrier for buffered solution to penetrate into the particles to dissolve JE antigen and for entrapped antigen to penetrate out of particles. The other possible mechanism to retard the release might be the interaction between the released antigen and conjugated material (Yushu and Venkatraman, 2005; Sahoo et al., 2002 and Kreuter, 2002).

This phenomenon could represent the efficacy of conjugated formulations as vaccine carriers, since they had a great ability to act as sustained depot system for the antigen over a period of time, especially $Al(OH)_3$ which was known to be a good vaccine adjuvant by their depot characteristic (Skea and Barber., 1993; and Rosenkrands et al., 2005). Thus, the release rate of $Al(OH)_3$ conjugated particles was slower than CS conjugated particles since $Al(OH)_3$ had a better ability to act as a depot. Besides, the released antigen such as peptide and protein, could be absorbed with $Al(OH)_3$ by means of electrostatic interaction and retard the release rate (Trujillo, Valencia and Calvo, 2006). Tahara et al. (2008) investigated the release of PLGA particles coated with CS and found that CS could reduce the initial burst release and prolonged the drugs releasing at the later stage.

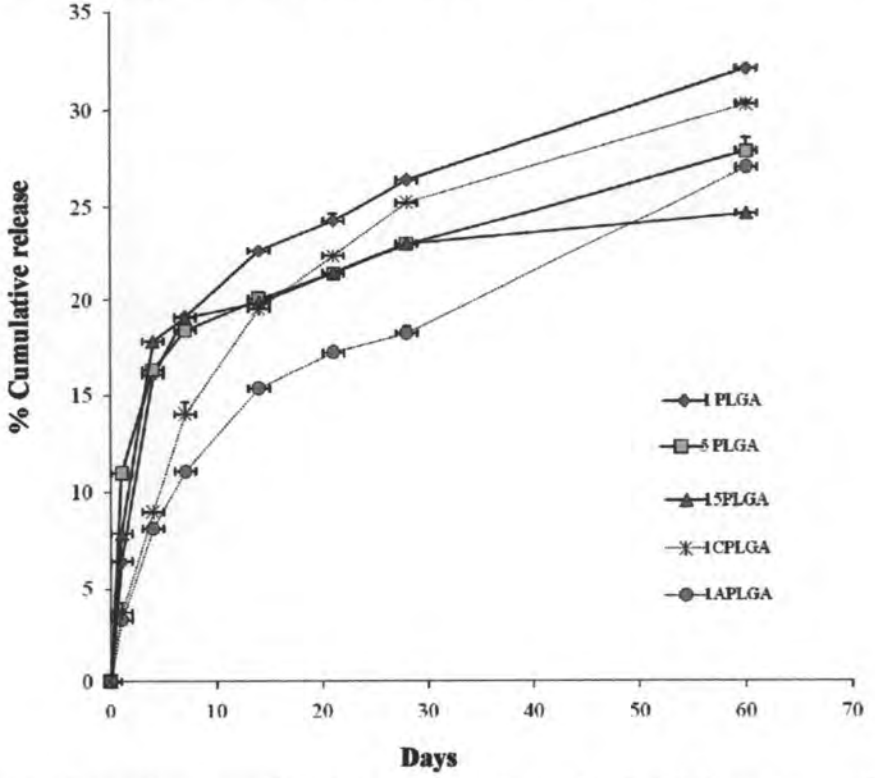


**Figure 3.7** The release profile of 1μm, 5μm, 15μm PLGA, CS conjugated PLGA (1C) and 0.75% $Al(OH)_3$ conjugated PLGA particles (1A).

*Integrity of entrapped material*

*SDS-PAGE*

The structural integrity study by SDS-PAGE revealed the identical protein bands from three samples of JE vaccine with dichloromethane, sonicated JE vaccine and JE vaccine as shown in Fig 3.8 which were NS3 at 68 kDa, Env at 53kDa, NS1 at 42 kDa, prM at 24 kDa, C at 14 kDa and M at 8 kDa, respectively. This result indicating that the structural integrity of JE antigen was not affected by preparation process and organic solvent. The amphiphilic protein E, comprises spikes of virion, is potentially immunogenic as E protein could neutralizes virus infectivity (Halstead and Tsai, 2004; Heinz, 1993 and Volk, 1991). Thus, the structural integrity of E-protein should be retained in order to stimulate the JE immune response. It could be seen that the band of E protein which at molecular weight of 53 kDa could be obviously observed for all three samples.

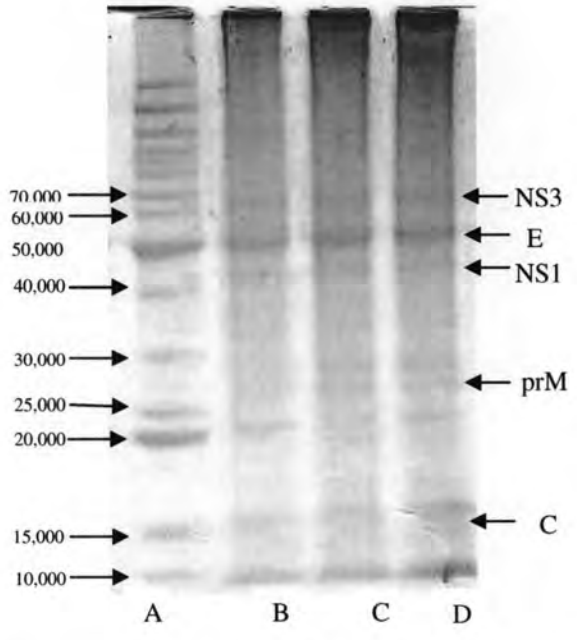


**Figure 3.8** Protein profile of JE vaccine by SDS-PAGE, Lane A: standard molecular weight marker, Lane B: JE vaccine with dichloromethane, Lane C: sonicated JE vaccine and Lane D: JE vaccine

*Circular dichroism*

The conformation of protein obtained from concentrated and purified JE vaccine was predominantly illustrated as ß-sheets as the minima at *ca* exhibited a

single ellipticity at around 220 as shown in Figure 3.9. Following sonication, the conformation and molar ellipticity of concentrated JE were remained express as ß-sheets. However, after contacting with DCM at dilution 1:10, the conformation was mainly changed as the molar ellipticities were found at around 226 and 208 which corresponded to α-helix conformation (Kelly and Price, 1996).

These results indicated that the conformation of JE protein was changed from ß-sheets rich to α-helix rich as a consequence of organic solvent, DCM while the sonication force had no effect on the conformation of JE surface protein. The conformation changes of JE when contacted DCM could possibly be resulted from the entanglement between molecules caused by the stretched conformation of protein after contacting with organic solvent. However, the other possibility might be obtained such as the diffusion of protein molecule to the organic solvent and the aggregation between each molecule (Tanaka et al., 2001; Kelly and Price, 1996). Although the conformation of JE protein was modified by DCM, the antigenic E protein epitope observed by SDS-PAGE still retained. Thus, effectiveness of JE vaccine to stimulate the immune response still remained.

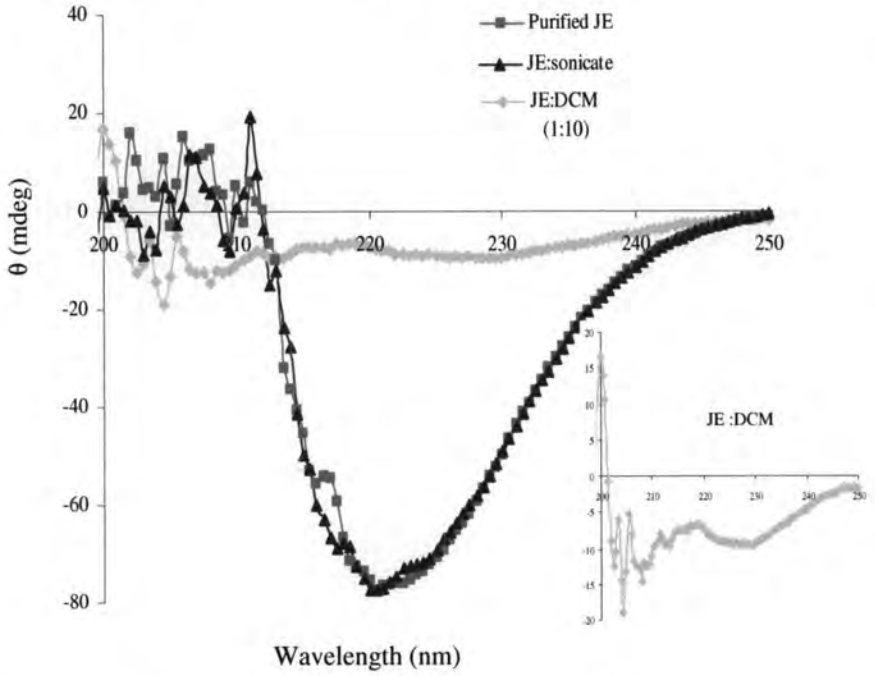


**Figure 3.9** Protein characteristics by CD of purified JE vaccine, sonicated purified JE vaccine and purified JE vaccine blended with DCM, dilution 1:10

**Conclusions**

The PLGA particles of different sizes and $Al(OH)_3$ conjugated PLGA, CS conjugated PLGA particles were successfully prepared using JE as model vaccine for an intension as nasal vaccine carrier. Particle size was considered as an important parameter obtained by different formulation variables such as primary w/o ratio, secondary w/o/w ratio, sonication output, amount and volume of stabilizer PVA, respectively. Evaluation of the effect of formulation variables on particle size and uniformity revealed that an increasing in primary w/o ratio, secondary w/o/w ratio and volume of PVA resulted in a larger size of particles with a high uniformity value while small size of particles could be obtained conversely by raising the percentage of PVA as well as increasing the sonication output, particularly those prepared from probe sonicator. By conjugating with CS and $Al(OH)_3$, the surface charge of all particles were shown positive, indicating that CS and $Al(OH)_3$ were associated with particles that could enhance the binding to negative surface charge of tissue and cell surface. Conjugated particles with appropriate amount of CS and $Al(OH)_3$, not only render the positive surface charge, but also provided particles with uniform size and spherical shape as observed by SEM and AFM. The results of JE integrity before and during the preparation process revealed that surface proteins of JE, especially E protein were well maintained. This investigation provided the useful information for improving intranasal delivery of particular vaccine, Japanese encephalitis, by using particles with suitable size in conjunction with positively charged-mucoadhesive substance without any transformation of epitope protein of Japanese encephalitis. Ex vivo study in porcine nasal mucosa and in vivo study in experimental animal of vaccine entrapped particles are subsequently in progress.