CHAPTER V

Chitosan microparticles for delivery of Japanese Encephalitis antigen: Physicochemical properties, *in vitro* cellular uptake and *in vivo* immune response in mice

Introduction

Traditional vaccines consist of live attenuated pathogens, whole inactivated organisms or inactivated toxins. In many cases, these approaches have been very successful at inducing protective immune response. Additionally, most live vaccines are able to induce cell-mediated immunity against pathogens, which often establish chronic infections, including human immunodeficiency virus, hepatitis C virus, tuberculosis, and malaria. However, some live attenuated vaccines can cause disease in immunosuppressed individuals and some pathogens are difficult or impossible to grow in culture (*e.g.* hepatitis C virus). Meanwhile, non-living vaccines have generally proven ineffective at inducing such type of immunity. Many traditional inactivated vaccines (*e.g. Bordetella pertussis*) also contain components that can cause undesirable effects and safety problems. As a result of these problems, several new approaches to vaccine development have emerged, which may have significant advantages over more traditional approaches. These approaches include: (1) recombinant protein subunit; (2) synthetic peptides; (3) protein-polysaccharide conjugates; and (4) plasmid DNA. While these new approaches may offer important safety advantages, a general problem is that the vaccines alone are often poorly immunogenic. Traditional vaccines contain many components, some of which can elicit additional T cell help or function as adjuvants, for example, bacterial DNA in whole cell vaccines. Unfortunately, these components have been eliminated from many new generation vaccines. Therefore, there is an urgent need for the development of potent and safe adjuvants and/or delivery systems, that can be used with newer generation vaccines, including DNA vaccines (Goldsby, Kindt and Osborne, 2000; O'Hagan, MacKichan and Singh, 2001; Storni *et al.*, 2005).

A number of studies have reported so far on the application of biodegradable micro-/nanoparticles as vaccine adjuvants or vaccine delivery systems (O'Hagan,

Singh and Gupta, 1998; Singh and O'Hagan, 2002), in particular, those made of poly(lactic acid) and poly(lactic-co-glycolic acid) (Hampl *et al.*, 1997; Johansen *et al.*, 2000; Singh *et al.*, 1997). They were utilized for delivering a variety of antigens, including tetanus (Alonso *et al.*, 1994; Johansen *et al.*, 2001; Thomasin *et al.*, 1996) human immunodeficiency virus (Moore *et al.*, 1995) malaria (Men *et al.*, 1996; Thomasin *et al.*, 1996), influenza (Lemoine *et al.*, 1998), etc. To meet desired properties, the microparticles could be controlled or modified in terms of their biodegradation and/or antigen release rate, making them very promising as efficient vaccine delivery (Thomasin *et al.*, 1996).

Despite the advent of synthetic biodegradable polymers, natural polymers remain much interest due to their availability, inexpensiveness, and capability of a multitude of chemical modifications. Chitosan has been recognized as a promising material for delivery of drugs and labile macromolecular compounds, attributed to its excellent physicochemical and biological properties. It is regarded as a biocompatible and biodegradable polymer of low toxicity (Illum, 1998; Mi *et al.*, 2002). Furthermore, it is extremely important to note that chitosan is soluble in aqueous mild acidic solution, which is an obvious advantage over other biodegradable polymers. Therefore, potential application of chitosan microparticles as a vaccine delivery is promising.

This study was thus aimed at investigating the application of chitosan microparticles as vaccine delivery systems, in comparison with PLGA microparticles. They were investigated both *in vitro* cellular uptake and *in vivo* immunization. If possible, such *in vitro-in vivo* relationship would be evaluated.

Materials and methods

Materials

Chitosan at molecular weight of 37 kDa with 94% degree of deacetylation (LCS) and 100 kDa with 95% degree of deacetylation (HCS) were obtained from Seafresh Chitosan (Bangkok, Thailand). Gelatin (GEL) was supplied from Fluka Chemie (Buchs, Switzerland). Poloxamer 407 (Lutrol F 127) (POL) was obtained as gift from BASF (Florham Park NJ, USA). Poly(lactic-co-glycolic acid), 50:50 with

inherent viscosity of 0.37 dl/g in hexafluoro isopropanol (PLGA) was purchased from Absorable Polymers International (Pelham AL, USA). Japanese Encephalitis (JE) antigen, Beijing strain, as a concentrate solution in TCM199-HBSS media, was kindly supplied by the Government Pharmaceutical Organization (Bangkok, Thailand). All other chemicals were of analytical grade and used as received.

Methods

Preparation of chitosan microparticles

Chitosan and/or the excipient were dissolved in 0.5% acetic acid solution and mixed with JE concentrate solution, in a manner that the final concentration of both components was controlled. The mixture was then spray dried in a bench-top spray dryer (Büchi model 190, Büchi Labortechnik, Flawil, Switzerland). The liquid feed was pumped peristaltically and fed through a two-fluid nozzle (0.5 mm internal diameter) where it was atomized into fine droplets. The standard processing parameters comprised an atomizing air volumetric flow rate of 750 l/hr and the aspirator vacuum of 25 mbar. The inlet drying air temperature and the liquid feed rate was controlled at 120 ^{o}C and 3 ml/min, respectively. The preparation process was performed in an aseptic manner.

Preparation of poly(lactic-co-glycolic acid) microparticles

One part of the concentrate antigen solution and four parts of 2.5% w/v PLGA solution in dichloromethane were mixed together by means of sonication with a 6-mm-tip diameter standard probe at output control of 100 (Vibra cell model VC 130 PB, Sonics and Materials, Newtown CT, USA) for about 15 seconds. The mixture was then dispersed into 20 parts of 2% w/v polyvinyl alcohol (PVA) solution by means of sonication, as described above, and subsequently transferred to 25 parts of magnetically stirred 0.2% w/v PVA solution. The resultant mixture was kept stirring for 4-6 hours. The particles were separated by centrifugation (Eppendorf model 5810, Eppendorf AG, Hamburg, Germany) at 3000 rpm for 15 minutes, washed three times with pH 7.4 phosphate buffered saline (PBS) and eventually re-dispersed with pH 7.4 PBS to the original volume of dichloromethane. The preparation process was performed in an aseptic manner.

Particle size measurement

Particle size and size distribution of the microparticles were measured by laser light-scattering method (Mastersizer 2000, Malvern Instrument, Malvern, UK). Small amount of chitosan microparticles was first dispersed in a few milliliters of absolute ethanol and sonicated with a 3-mm-tip diameter standard probe at output control of 60 for about 30 seconds, in order to deaggregate the microparticles. The dispersion was loaded into a stirred sample cell, containing water as a measuring medium. Calculation of particle size was made from the intensity of light scattered at different angles, based on Mie's theory (Zimmerman, 1997). The particle size is presented in the volume-weighted mode and the 50% undersize diameter $d(v, 0.5)$ is referred to as the particle diameter.

Zeta potential measurement

Chitosan microparticles were dispersed in 1 mM NaCl solution with sonication, as described under the 'particle size measurement' section, while PLGA microparticles were dispersed without sonication. The measurement was carried out on the Zetasizer NanoZS (Malvern Instrument, Malvern, UK). At least 20 sub-runs were performed at room temperature. The results were reported as zeta potential and zeta deviation.

Particle morphology

Chitosan microparticles were mounted onto double-faced adhesive tape, which was attached on a sample stub. PLGA microparticles were filtered and vacuum-dried before being mounted onto the sample stub. The samples were sputtered with gold and viewed under a scanning electron microscope (Jeol model JSM-5410 LV, Tokyo, Japan) at a voltage of 15.0 kV. The photomicrographs were then taken at a magnification of 7500.

Protein content determination

Chitosan and PLGA microparticles were separately dissolved in 0.5% acetic acid solution and the mixture of 1:1 dimethyl sulfoxide and 0.5% dodecyl sulfate in 0.05 N NaOH, respectively. JE content was determined by using bicinchoninic acid kit for protein determination (Sigma-Aldrich, Saint Louis MO, USA). The reaction

was run at room temperature for 2 hours. Optical density of the sample solution was read at 562 nm on a spectrophotometer (Jasco model V-530, Jasco, Tokyo, Japan). Actual antigen loading and entrapment efficiency were then calculated. The experiment was performed in triplicate.

In vitro cellular uptake

Mouse dendritic cells (DC) (ATCC CRL-11904) and mouse splenic macrophages (Mϕ) (ATCC CRL-2471) at concentration of $4x10^6$ cells in 2 ml complete culture medium were seeded in 6-well plate and incubated at 37 °C with 5% CO_2 in air atmosphere until the cell confluency was reached. JE antigen and the chitosan microparticles were labeled with fluorescein-5-isothiocyanate, isomer I (FITC) (Invitrogen, Calsbad CA, USA), according to the manufacturer's information. For PLGA microparticles, JE antigen was first labeled by the same procedure and subsequently incorporated into the microparticles. The FITC-labeled antigen and microparticles were co-incubated with cells for another 4 hours at the same condition. The particle uptake was analyzed with a fluorescence-activated cell sorter (FACS) (BD model FACSCalibur, BD Biosciences, San Jose CA, USA), equipped with argon laser. The fluorescence signal was detected with G1 detector for green fluorescence signal at the excitation wavelength of 485 nm and the emission wavelength of 525 nm. The number of fluorescence event, which correlated to the particles taken up within the cells, was counted. The gated cells with fluorescence signal were defined as the positive cells for cellular uptake. The percentage of positive cells relative to the control experiment (cells co-incubated with FITC solution) was then determined. The uptake event was also observed under a confocal laser scanning electron microscope (Olympus model FV 1000, Olympus, Germany). Glass slips were pre-coated overnight with 0.01% poly-L-lysine solution (Sigma-Aldrich, Saint Louis MO, USA) and placed in the well plate prior to cell seeding. After incubation with the antigen and/or microparticles, cells were fixed with 3.7% paraformaldehyde in pH 7.4 PBS and kept in pH 7.4 PBS at 4 °C. The slip was mounted upside-down on a glass slide with glycerin and observed under the microscope with fluorescence detector at the excitation wavelength of 488 nm and the emission wavelength of 520 nm. The photomicrographs were taken at a magnification of 600.

In vivo immunization in mice

The *in vivo* immunization of JE-loaded microparticles was studied in female BALB/c mice. The experiment was approved by the Ethical Committee of Chulalongkorn University, Thailand. Mice of 3-4 weeks old were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The animals were housed in a 12-hour light/dark cycle at temperature of 25 °C for 1 week prior to the study and allowed access to food and water *ad libitum*. The JE-loaded chitosan microparticles, pre-dispersed in pH 7.4 PBS, and PLGA microparticles were subcutaneously administered into mice at a dose equivalent to 0.2 antigen units. Three doses were given at week 0, 1 and 4 in each group of eight mice, except one group, which was immunized with triple doses of POL LCS microparticles only at week 0. The commercial JE vaccine was also given as the reference. Blood samples were taken from tail vein at week 0, 1, 4, 8 and 12. The serum was separated and kept at –20 °C until the analysis.

Determination of serum IgG and IgG isotype titer

The IgG, IgG_1 and IgG_{2a} serum titers were determined by ELISA. Ninety six-well ELISA microtiter plate was coated with 100-µl of 1:49 JE concentrate in pH 9.6 coating buffer overnight at 4 °C. It was thoroughly washed three times with pH 7.4 PBS-Tween 20 (PBS-T) and allowed to dry. One hundred microliters of 3% gelatin in pH 7.4 PBS-T, as a blocking solution, was added to each well and incubated for 1 hour at 37 °C. The plate was thoroughly washed three times with pH 7.4 PBS-T and allowed to dry. The serum samples were 2-fold serially diluted with diluent (1% w/v gelatin in pH 7.4 PBS-T) and added into each well at 100 µl. The sample plate was incubated at 37 °C for 1 hour, subsequently washed with pH 7.4 PBS-T three times and allowed to dry. The 100-µl goat anti-mouse IgG (H+L)-HRP, goat anti-mouse IgG_1-HRP, and goat anti-mouse IgG_{2a}-HRP were separately added into each sample well and incubated for 1 hour at 37 °C. The plate was washed with pH 7.4 PBS-T three times and allowed to dry. One hundred microliters of substrate, *o*-phenylene diamine, at a concentration of 1 mg per 12-ml pH 5.0 citrate buffer plus 12-µl of 30% hydrogen peroxide solution, were added to each well. The sample plate was incubated for 30 minutes at room temperature with light shield for color development.

Fifty microliters of 4 N sulfuric acid were added to stop the reaction. The optical density was read at 490 nm on a microplate reader (VICTOR^3, Perkin Elmer, USA). The antibody titer was the dilution that yielded the optical density two times higher than blank. The average of natural logarithm of antibody titer was plotted against time.

Results

Physicochemical properties and morphology of microparticles

The JE loaded chitosan and chitosan composite microparticles were prepared by spray drying process. Their physicochemical properties were compared with those of PLGA microparticles, prepared by double-emulsion solvent-evaporation technique. The results are shown in Table 5.1.

Table 5.1 Physicochemical properties of JE-loaded microparticles

Microparticles	JE loading (% w/w)	Entrapment efficiency (%)	Particle size* (μm)	Zeta potential (mV)
LCS	1.99 (0.89)**	84.98	5.968 (0.472)***	15.17 (9.698)**
GEL LCS	2.26 (0.74)	100.42	5.752 (0.450)	22.57 (5.617)
POL LCS	2.86 (1.29)	124.63	4.196 (0.585)	9.26 (13.50)
HCS	1.75 (0.73)	74.76	9.550 (0.538)	11.56 (15.22)
PLGA	2.21 (0.21)	84.31	1.409 (0.833)	-1.064 (7.427)

* Volume-weighted median diameter, $d(v, 0.5)$

** Standard deviation

*** Absolute deviation from the median (Uniformity)

All JE-loaded microparticles contained about 2% w/w of JE antigen, with entrapment efficiency ranged between 74.76-124.63%. The chitosan and chitosan composite microparticles had the comparable size of about 5 μm, with narrow size distribution, except the HCS microparticles, which were relatively larger than the

others. The PLGA microparticles were much smaller than the chitosan microparticles. According to surface charge, chitosan microparticles were obviously cationic. Co-spray drying with the excipients apparently affected the zeta potential of microparticles. While the incorporation of GEL, a cationic polypeptide, clearly increased the zeta potential, that of POL, a non-ionic surfactant, slightly reduced the particle surface charge. Molecular weight of chitosan did not have significant effect on the exposed surface charge of microparticles. In contrast to the chitosan microparticles, the PLGA microparticles were relatively neutral, with slightly negative zeta potential.

As illustrated in Figure 5.1, the antigen-loaded chitosan microparticles had dented surface, but smooth texture. They tended to fuse together at the particle surface, leading to the agglomerate formation. Unlike the chitosan microparticles, the PLGA microparticles were discrete and spherical, with very smooth surface.

***In vitro* cellular uptake**

The FITC-labeled JE antigen and FITC-labeled microparticles at non-toxic loading (Kusonwiriyawong et al., manuscript in preparation) were incubated with DC and Mϕ, which were supposedly target of the particles. The cellular uptake of microparticles was monitored by FACS analysis. The results are illustrated in Figure 5.2.

A comparable amount ranged between 21.65-28.64% of DC was able to take up the soluble JE antigen and all the LCS microparticles, while only 12.93 and 2.99% of DC were found positive for the uptake of HCS and PLGA microparticles, respectively. Mϕ was more active than DC in taking up the LCS and the composite microparticles. About 82% of Mϕ was detected positive for the endocytosis of POL LCS microparticles. A comparable amount about 65 and 66% of Mϕ were able to take up the LCS and the GEL LCS microparticles, respectively. However, Mϕ was evidently less active than DC in taking up the HCS microparticles. Furthermore, a supposedly negligible amount of Mϕ was detected as positive for the endocytosis of PLGA microparticles and soluble JE antigen.

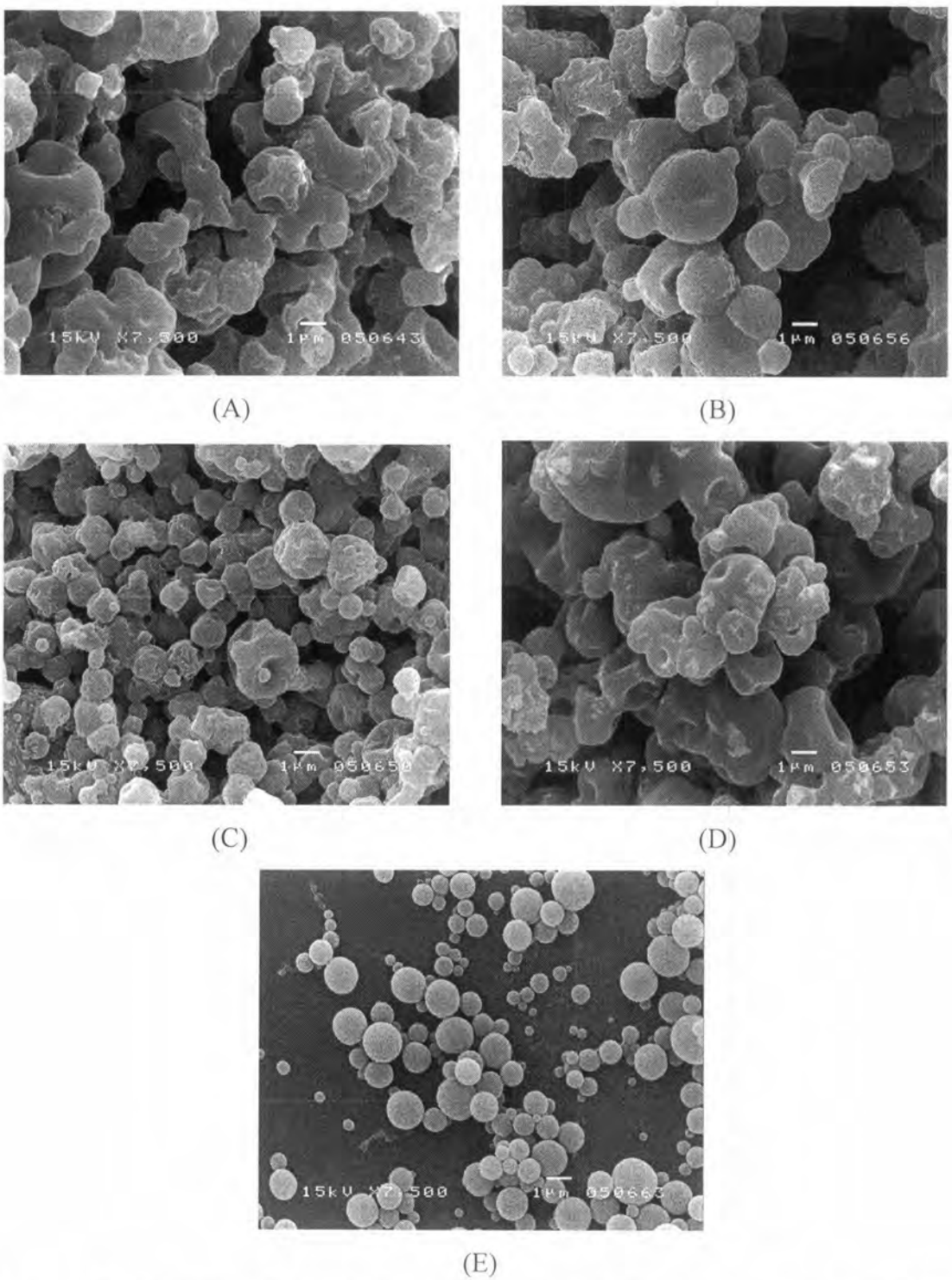


Figure 5.1 Scanning electron photomicrographs of JE-loaded (A) LCS, (B) GEL LCS, (C) POL LCS, (D) HCS, and (E) PLGA microparticles

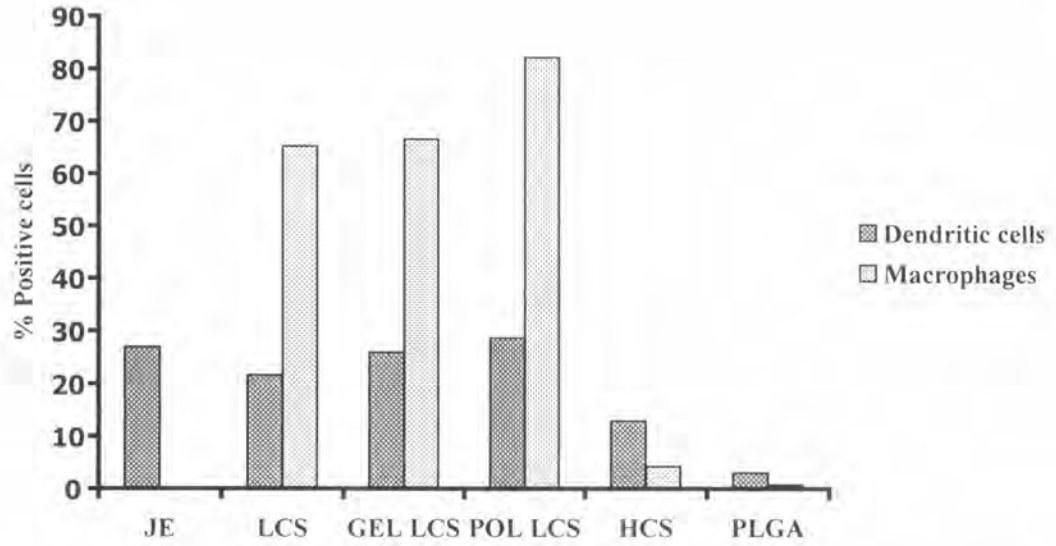


Figure 5.2 *In vitro* cellular uptake of microparticles, as percentage of cells taking up the particles

The cellular uptake of JE antigen and microparticles was further confirmed by CLSM. The results are depicted in Figures 5.3 and 5.4 for DC and Mϕ, respectively.

The endocytosed JE antigen was observed as many small spots of FITC scattered throughout the cytosol of DC (Figure 5.3A), while the up-taken microparticles were noticed as larger spots located within the cellular compartment (Figure 5.3B-5.3F). It was noticed that some cells were able to take up more than one particle. The photomicrographs of Mϕ well confirmed the endocytic activity of the cells. Multiple particles of the chitosan and the chitosan composite microparticles were observed within the cells, while only a few particles were observed for the PLGA microparticles. In contrast, only small amount of the JE antigen could be taken up, evident by a few spots of FITC within the cytosol.

***In vivo* immune response**

The vaccine formulations were immunized subcutaneously in mice. At predetermined time, the blood samples were collected and analyzed for various types of antigen-specific IgG by ELISA. The results are illustrated in Figure 5.5.

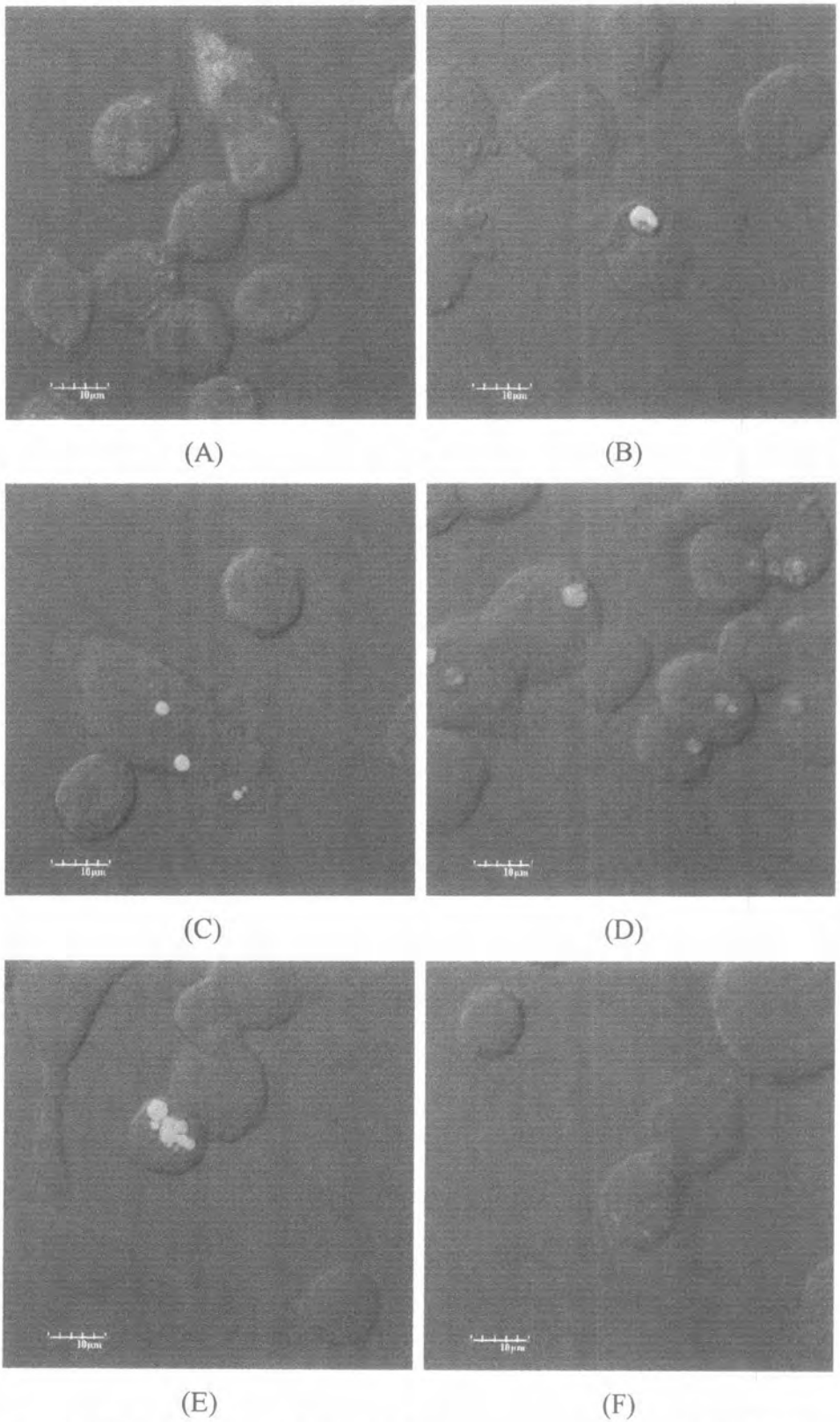
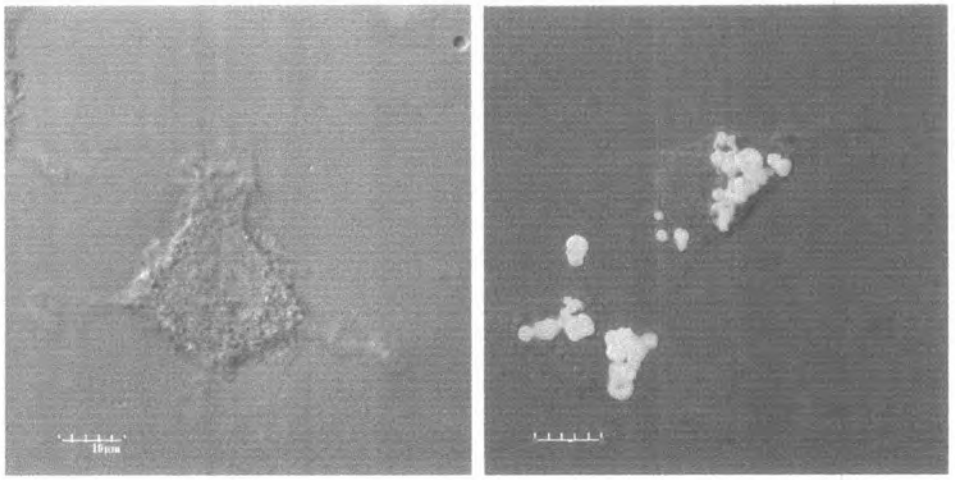
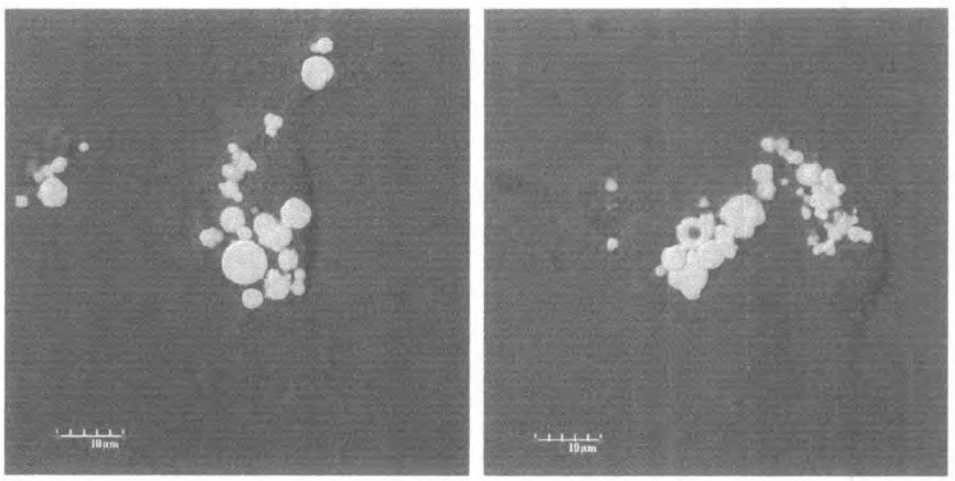


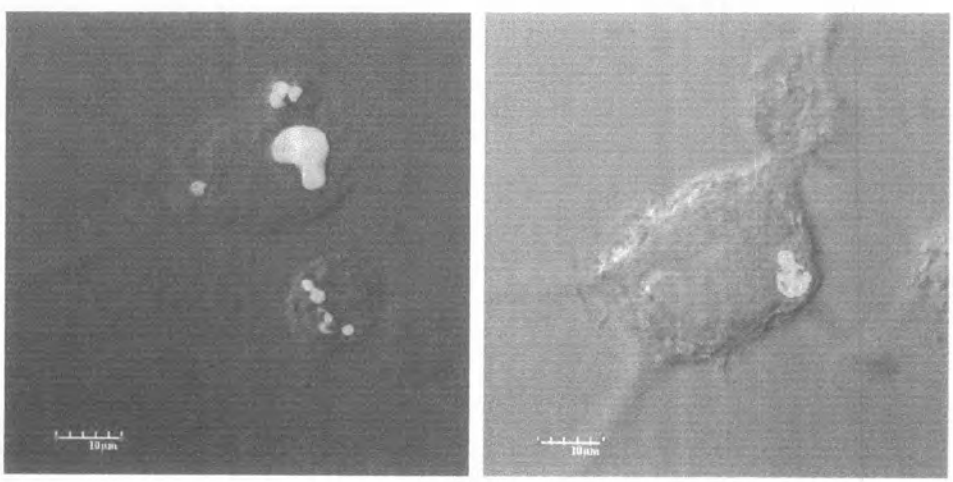
Figure 5.3 Confocal laser-scanning photomicrographs of dendritic cells taking up (A) JE antigen, JE-loaded (B) LCS, (C) GEL LCS, (D) POL LCS, (E) HCS, and (F) PLGA microparticles



(A) (B)



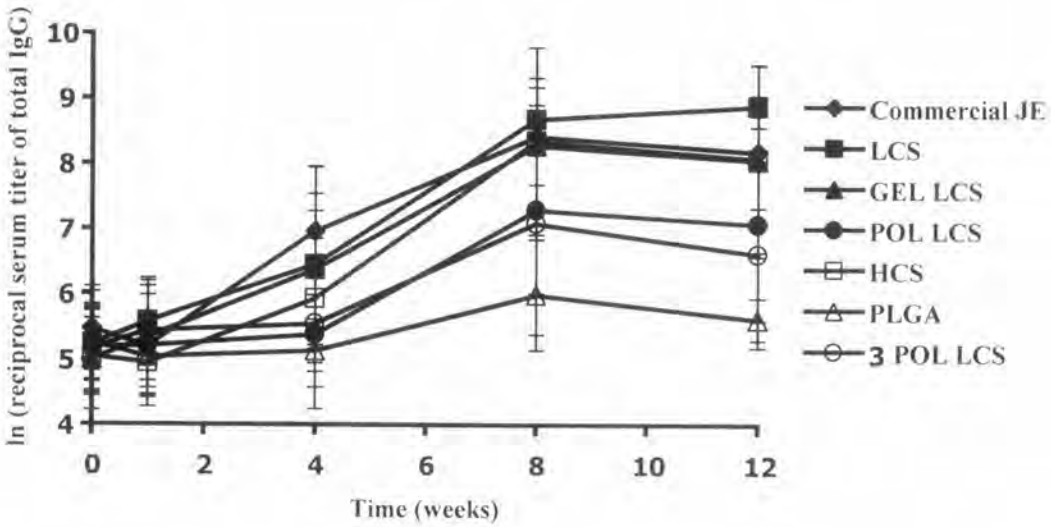
(C) (D)



(E) (F)

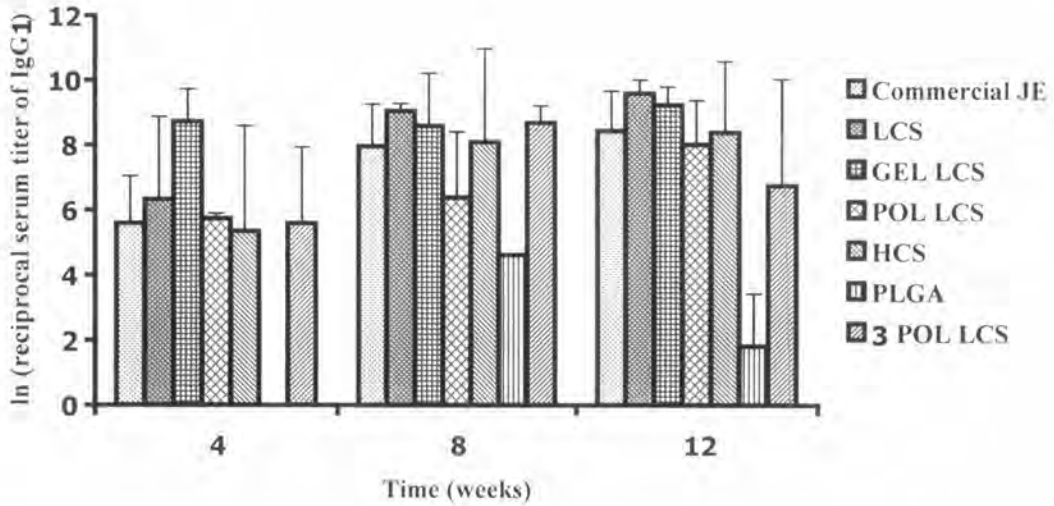
Figure 5.4 Confocal laser-scanning photomicrographs of macrophages taking up (A) JE antigen, JE-loaded (B) LCS, (C) GEL LCS, (D) POL LCS, (E) HCS, and (F) PLGA microparticles

During week 0-8 after the first immunization, the LCS, GEL LCS and HCS formulations yielded the comparable serum titer of total IgG to the commercial JE preparation (Figure 5.5A). At week 12, the total IgG serum titer induced by the LCS microparticles was slightly increasing, while that of the others tended to decrease. The effect seemed not to be significantly different though. The incorporation of POL into LCS microparticles apparently resulted in lowering the serum titer. Surprisingly, a single immunization of the POL LCS microparticles at triple doses only yielded a comparable profile of total IgG serum titer to the triple immunizations at a single dose of the same formulation. The PLGA microparticles gave the lowest serum titer of JE-specific IgG.

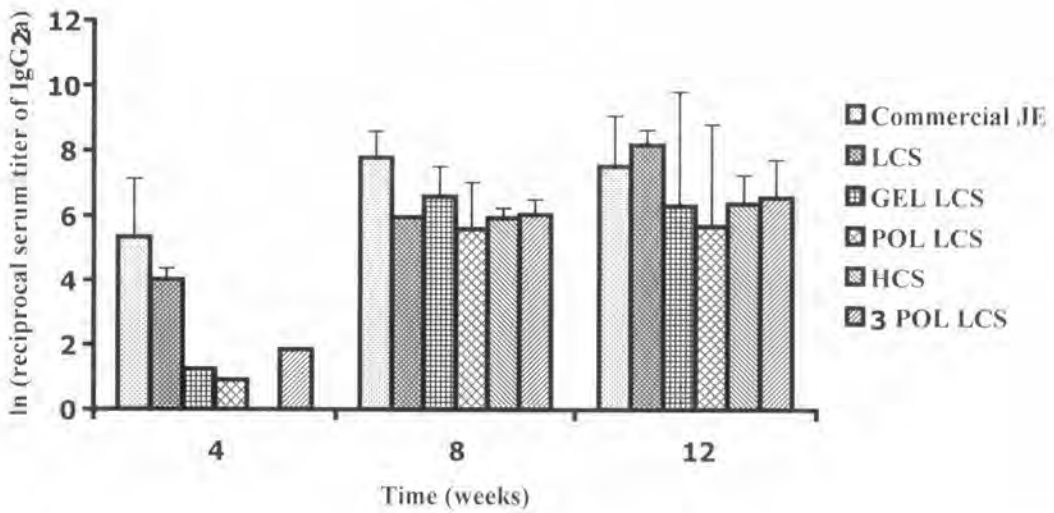


(A)

Figure 5.5 Serum titer of (A) total IgG, (B) IgG_1 isotype, and (C) IgG_{2a} isotype induced by the commercial JE vaccine and JE-loaded microparticles, as plots of natural logarithm of reciprocal serum titer against time (n=8)



(B)



(C)

Figure 5.5 Serum titer of (A) total IgG, (B) IgG_1 isotype, and (C) IgG_{2a} isotype induced by the commercial JE vaccine and JE-loaded microparticles, as plots of natural logarithm of reciprocal serum titer against time (n=8) (continued)

Two IgG subtypes were also analyzed by the same method. The serum titer of IgG_1 induced by all investigated formulations was first detectable at week 4 after the first immunization (Figure 5.5B). While the serum titer of total IgG seemed to be stable or started to decrease at week 12 (Figure 5.5A), that of the IgG_1 still kept increasing for most formulations, except for PLGA and triple-dose POL LCS microparticles. Interestingly, the LCS and GEL LCS microparticles were able to

initiate the production of antigen-specific IgG_1 higher than the commercial vaccine product at all time points, while the other chitosan microparticles could induce the comparable or slightly lower production of the titer. In case of the PLGA microparticles, the IgG_1 serum level at week 4 was below the detection limit of the quantification method. Although it was detectable at the later time point, the serum titer was still low, when compared with other formulations.

Similar to the serum IgG_1 isotype, the serum IgG_{2a} isotype was first detectable at week 4 after the first immunization (Figure 5.5C). Although the serum titer initiated by the chitosan microparticles was lower than that initiated by the commercial JE preparation at week 4 after the first immunization, it was only slightly lower at week 8 and 12. All formulations induced a comparable titer to one another, except the LCS microparticles, which could induce the production of serum IgG_{2a} titer higher than all other formulations, including the commercial vaccine, at week 12. In contrast, the induction resulted from the PLGA microparticles was under the detection limit of the quantification method at all time point.

Discussion

The chitosan or chitosan composite microparticles and the PLGA microparticles, which were prepared by different techniques, could be produced within the appropriate size range for passive targeting to the antigen presenting cells (Tabata and Ikada, 1988). It could be postulated that the size of microparticles was a major factor, while the surface charge seemed to have less influence, in determining the cellular uptake by DC. For the LCS, GEL LCS and POL LCS microparticles, they were efficiently endocytosed by DC in a comparable extent, probably due to their similar sizes. They exposed different levels of positive zeta potential though. This was further confirmed with the HCS microparticles. Although the particles exposed a similar positive zeta potential to the LCS microparticles, they were slightly larger than the LCS microparticles and hence the lower cellular uptake was obtained. In case of PLGA microparticles, which held the particle size close to the most appropriate size range of 1-2 μm for the uptake by macrophages (Tabata and Ikada, 1988), were less

taken up than the chitosan microparticles. This likely resulted from the exposed negative charge on the particle surface (Thiele *et al.* 2001).

The antigen concentrate was a relatively clear colloidal solution of the inactivated whole-cell JE flavivirus. The virus was morphologically sphere, with approximately 40-50 nm in diameter (Yang *et al.*, 2004). Although size of the antigen was much smaller than the optimal size range for the cellular uptake, they were taken up in a comparable extent to the LCS and the composite microparticles. It was possible that the particles of different sizes were taken up into the cytosol by different mechanisms.

The optimal size range of particles for the cellular uptake by Mϕ seemed to be narrower than that for the uptake by DC. The LCS and the composite microparticles were taken up very efficiently by Mϕ, especially the POL LCS microparticles, although they exposed the surface charge density differently. The HCS, the PLGA microparticles, and the JE antigen likely owned either too large or too small size for being endocytosed by Mϕ.

Since the antigen uptake by the antigen presenting cells is the very first step in initiating the immune response (Singh and O'Hagan, 2003; Zinkernagel *et al.*, 1997), it was thus expected that the vaccine formulations, which could be efficiently taken up by the cells, would likely initiate a high immune response. It was noticed that the microparticulate vaccine formulations, including all chitosan microparticles and the commercial preparation, which were well taken up by DC, could initiate a substantial immune response. The effect was independent of the number of cells found positive for the particle uptake. In other words, the microparticles, which were endocytosed by the cells in a large extent, did not initiate the higher immune response or serum titer than the formulations, which were taken up in a smaller extent. The POL LCS microparticles were the good example in this case, although POL was reported to be capable of enhancing the immune response after the mucosal immunization (Westerink *et al.*, 2002) or even stimulating the Th_1-type response (Spitzer *et al.*, 1999). This led to an assumption that there might be some other important factors,

determining the initiation of immune response, apart from the efficient uptake by the antigen presenting cells. The composition of the vaccine formulation would be another important one.

Mϕ seemed to be less efficient in initiating the immune response than DC, since the extent of immune response did not relate to the extent of particle uptake by the cells.

It was also evident that, for vaccine, a single immunization of multiple doses did not always induce a higher or more prolonged immune response than the multiple immunization of divided doses. However, this effect might be partly resulted from the efficacy of the vaccine formulation as well.

The serum level of IgG isotypes could be used as an indirect indicator for the dominant type of elicited immune response. IgG_1 and IgG_{2a} are the isotypes known to be associated with the stimulation of B-lymphocytes by the T helper cells type 2 (T_H2) and T helper cells type 1 (T_H1) lymphocytes, respectively. The T_H1 type response is responsible for classic cell mediated functions, such as delayed-type hypersensitivity and the activation of cytotoxic T-lymphocytes, while the T_H2 type response functions more effectively as a helper for B-cell activation (Goldsby, Kindt and Osborne, 2000). Although the chitosan microparticles could initiate both types of immune response, they seemed to be biased to the T_H2 type response. In contrast, the commercial vaccine preparation was able to induce both types of immune response more efficiently. It was interesting that LCS microparticles constantly induced the production of investigated IgG isotypes, since both titers seemed to keep increasing within time frame of the investigation. The production of both isotypes was even higher than the commercial vaccine, implying their potential application as a prolonged vaccine delivery.

Conclusion

The chitosan microparticles for vaccine delivery were well developed. The polymer of low molecular weight was efficiently taken up by both DC and Mϕ and, as

a consequence, was able to initiate immune response in a similar extent to that of the commercial vaccine. Although, the chitosan composite and the chitosan microparticles of high molecular weight were taken up by both cells in a substantial extent, the immune response was not higher, or even lower in some cases, than the commercial preparation. Due to low cellular uptake, the low immune response was obtained from PLGA microparticles. It was noticed that, at week 12, the chitosan microparticles of low molecular weight still induced the immune response higher than the commpercial vaccine preparation. The effect seemed to keep increasing. This indicated their potential to be developed as a prolonged vaccine delivery system.