CHAPTER III

EXPERIMENTAL

Materials

1. Japanese Encephalitis Antigen (GPO, Thailand)
2. DL-Polylactide (Lot 108H 1284, Sigma, USA)
3. Poly (lactide-co-glycolide) 85:15 (Lot 98H 1008, Sigma, USA)
4. Poly (lactide-co-glycolide) 75:25 (Lot 19H 0422, Sigma, USA)
5. Poly (lactide-co-glycolide) 50:50 (Lot 108H 1285, Sigma, USA)
6. Low molecular weight chitosan (Seacure® 143, Norway)
7. Medium molecular weight chitosan (Seacure® 243, Norway)
8. High molecular weight chitosan (Seacure® 343, Norway)
9. Soy bean oil (Lot 99H 0081, Sigma, USA)
10. Dichloromethane (Lot 4881 N18753, Mallinckrodt, USA)
11. Acrylamide (Lot 1610100, Bio-Rad, USA)
12. N, N $''$– methylene bisacrylamide (Lot 89K 0943, Gibco BRL Life Technologies, USA)
13. Glycine (Lot 1610717, Bio-Rad, USA)
14. Sodium tripolyphosphate (Sigma, USA)
15. n-Hexane (J.T. Baker, USA)
16. Trizma base (Lot 74H 5731, Sigma, USA)
17. Ammonium persulfate (Lot 1610700, Bio-Rad, USA)
18. TEMED (Promega, USA)
19. 2-Mercaptoethanol (Lot 110H-060815, Sigma, USA)
20. Sodium chloride (Lot K26811304 952, Merck, Germany)
21. Sodium dodecyl sulfate (Lot 1610301, Bio-Rad, USA)
22. Bicinchoninic acid kit (Lot 119H9800, Sigma, USA)
23. SDS-PAGE standards broad range (Lot 82442, Bio-Rad, USA)

24. Methanol (J.T. Baker, USA)
25. Tetrahydrofuran (Lot 2K040107, Lab-Scan, Ireland)
26. o-Phenylenediamine (Lot 00861626, Zymed Laboratories, USA)
27. Horseradish peroxidase-goat anti-rabbit IgG (H+L) (Lot 00560520, Zymed Laboratories, USA)

Instruments

1. Analytical balance (Mettler Toledo, USA)
2. Autoclave (Hirayama, Tokyo, Japan)
3. Freeze dryer (FTSSYSTEMS, USA)
4. Homogenizer (Ultra-Turrax T 25, Germany)
5. Incubator (Memmert, Germany)
6. Laser particle size analyser (Mastersizer, Malvern Instruments, USA)
7. Laminar air flow (Issco Laminar Flow Model VS124, DWYER Instruments, USA)
8. Magnetic stirrer (Heidolph MR 3001, Germany)
9. Micropipet (Socorex, Switzerland)
10. Multichannel pipet (Socorex, Switzerland)
11. Optical microscope (Olympus, USA)
12. Microplate reader (Bio-Rad, USA)
13. Vortex mixer (Vortex-Genie, Scientific Industries, Thailand)
14. pH meter (Beckman, USA)
15. Scanning electron microscope (JSM-5410 LV, JEOL, Japan)
16. Refrigerated centrifuge (Sigma, USA)
17. Gel permeation chromatograph (Waters 150-CV, Millipore, USA)
18. Gel permeation chromatograph (PL-110, USA)
19. Gas chromatograph with 5 % SE-30 column (Schimidzu 7-AG, Japan)
20. Electrophoresis cell (Mini-PROTEAN ® II, Bio-Rad, USA)

21. Sonicating probe (Soniprep 150, Sanyo, Japan)
22. Shaking water bath (PolyScience, USA)
23. UV-VIS Spectrophotometer (Schimidzu, Japan)

Glassware and others

1. Beakers (Pyrex, USA)
2. Centrifuge tubes (Nalgene ®, Nalge Nunc International, USA)
3. Cylinders (Pyrex, USA)
4. Test tubes (Pyrex, USA)
5. Transfering pipettes (HBG, Western Germany)
6. Measuring pipettes (HBG, Western Germany)
7. Microtiter plate (Nunc-Immuno plate, Nalge Nunc International, USA)
8. Micropipet tips (Gilson, USA)
9. Pasteur pipettes (Volac, John poulten, England)
10. Membrane filter (Millipore, USA)
11. Microtube plastic (Treff Lab, Switzerland)
12. Disposable syringes and needles
13. Disposable gloves

Methods

1. Calibration curve of standard protein (BCA assay)

The bicinchoninic acid assay is a colorimetric assay for protein. It is based on chemical principle similar to those of the biuret and Lowry assays. Standard working reagent (S-WR) is prepared by mixing 100 volume of Reagent A, consisting of an aqueous solution of 1% BCA-Na_2, 2% $Na_2CO_3 . H_2O$, 0.16% Na_2 tartrate, 0.4% NaOH and 0.95 % $NaHCO_3$, with 2 volume of Reagent B, consisting of 4% $CuSO_4 . 5 H_2O$ in deionized water. The color of S-WR is apple green (Smith, 1985). A standard solution containing an accurate

amount of bovine serum albumin (BSA) 1 mg/ml was diluted to 6 dilutions with final concentration of 20, 40, 60, 80, 100 and 120 μg/ml.

The standard assay procedure consisted of mixing 1 volume of standard sample with 20 volume of S-WR. Color development proceeded within 2 hours at room temperature. The absorbance was measured at the wavelength of 562 nm versus a reagent blank. The concentration versus average absorbance from triplicate assays was plotted. The relationship between absorbance and concentration was calculated. The concentration of unknown could then be determined from the plot of concentration and absorbance obtained for standard protein.

2. Preparation of unloaded microparticles

2.1 Polyester polymers

The unloaded microparticles were obtained by emulsion solvent evaporation technique (Jeffery, 1993 and Prieto, 1994). The primary w/o emulsion was prepared by adding the 5% w/v PVA aqueous solution into different concentrations ranging from 1.5 to 5% w/v of PLGA polymers in methylene chloride. The mixture was then sonicated for 1 minute at three various intensities by setting the sonicator at output 9, 12, and 15. The resulting emulsion was then added into 100 ml of 1% w/v PVA aqueous solution and homogenized at 9,500 rpm for 10 minutes. The obtained w/o/w emulsion was stirred for up to 8 hours at room temperature to allow solvent evaporation. The microparticles were collected by centrifugation at 12,000 rpm for 10 minutes, washed three times with 100 ml of distilled water and freeze-dried. The formulations of polyester microparticles are shown in Tables 13-16 for DL-PLA, PLGA 85:15, PLGA 75:25 and PLGA 50:50, respectively. The antigen loaded microparticles of polyester were prepared using the same technique as unloaded microparticles. The Japanese

Table 13 Formulation of JE antigen loaded DL-PLA microparticles

Formulation	Polymer	Concentration (%)	Polymer (mg)	DCM (ml)	Antigen (μg)	Sonicate (output)
P1	DL-PLA	1.5	151.2	10	250	9
P2			150.3			12
P3			151.1			15
P4			151.3		500	9
P5			150.5			12
P6			150.6			15
P7			151.3		750	9
P8			150.9			12
P9			149.7			15
P10		3	301.8	10.	250	9
P11			301.2			12
P12			301.3			15
P13			299.2		500	9
P14			300.1			12
P15			301.4			15
P16			302.6		750	9
P17			302.3			12
P18			301.3			15
P19		5	499.5	10	250	9
P20			499.1			12
P21			499.3			15
P22			498.2		500	9
P23			501.4			12
P24			499.8			15
P25			500.1		750	9
P26			501.9			12
P27			500.8			15

Table 14 Formulation of JE antigen loaded PLGA 85:15 microparticles

Formulation	Polymer	Concentration (%)	Polymer (mg)	DCM (ml)	Antigen (μg)	Sonicate (output)
P28	PLGA 85:15	1.5	150.1	10	250	9
P29			150.0			12
P30			150.3			15
P31			149.5		500	9
P32			149.8			12
P33			150.3			15
P34			151.9		750	9
P35			152.8			12
P36			151.1			15
P37		3	299.2	10.	250	9
P38			299.6			12
P39			300.8			15
P40			300.2		500	9
P41			301.1			12
P42			299.7			15
P43			301.8		750	9
P44			302.4			12
P45			302.6			15
P46		5	500.4	10	250	9
P47			501.3			12
P48			500.0			15
P49			501.1		500	9
P50			500.5			12
P51			502.2			15
P52			499.5		750	9
P53			500.7			12
P54			501.2			15

Table 15 Formulation of JE antigen loaded PLGA 75:25 microparticles

Formulation	Polymer	Concentration (%)	Polymer (mg)	DCM (ml)	Antigen (μg)	Sonicate (output)
P55	PLGA 75:15	1.5	150.0	10	250	9
P56			151.2			12
P57			150.4			15
P58			150.2		500	9
P59			150.0			12
P60			150.7			15
P61			150.3		750	9
P62			150.3			12
P63			150.3			15
P64		3	300.6	10.	250	9
P65			300.3			12
P66			301.9			15
P67			300.2		500	9
P68			300.1			12
P69			300.5			15
P70			301.2		750	9
P71			301.8			12
P72			301.7			15
P73		5	501.2	10	250	9
P74			501.4			12
P75			500.8			15
P76			500.3		500	9
P77			499.3			12
P78			501.7			15
P79			501.5		750	9
P80			501.8			12
P81			500.1			15

Table 16 Formulation of JE antigen loaded PLGA 50:50 microparticles

Formulation	Polymer	Concentration (%)	Polymer (mg)	DCM (ml)	Antigen (μg)	Sonicate (output)
P82	PLGA 50:50	1.5	149.0	10	250	9
P83			152.9			12
P84			153.0			15
P85			150.8		500	9
P86			149.9			12
P87			151.5			15
P88			150.6		750	9
P89			150.4			12
P90			149.9			15
P91		3	302.8	10.	250	9
P92			300.0			12
P93			299.6			15
P94			300.4		500	9
P95			300.7			12
P96			301.0			15
P97			301.4		750	9
P98			300.5			12
P99			301.9			15
P100		5	501.2	10	250	9
P101			499.0			12
P102			501.3			15
P103			502.3		500	9
P104			501.5			12
P105			499.8			15
P106			499.9		750	9
P107			499.0			12
P108			503.7			15

Encephalitis antigen was added into the aqueous phase of PVA. The formulations of antigen-loaded PLGA microparticles were the same as unloaded PLGA microparticles.

2.2 Chitosan polymers

The unloaded chitosan microparticles were prepared by emulsification and ionotropic gelation method (Lim, 1997). The dispersed phase consisted of different concentration ranging from 1 to 3% w/v of chitosan in 2% v/v acetic acid. The continuous phase consisted of soy bean oil containing 2% w/v Span 80 as emulsifier. The dispersed phase was added into the continuous phase under homogenization at 13,500 rpm for 10 minutes to form a water-in-oil emulsion. After a specific emulsification time, 15 ml of 10% w/v sodium tripolyphosphate solution was gradually dropped into the emulsion under agitation. Stirring was continued for a specific time period of 3 hours. The microparticles were collected by centrifugation at 12,000 rpm for 10 minutes, washed 5 times with 30 ml of n-hexane followed by 3 times with 100 ml of distilled water and freeze-dried. The formulations of chitosan microparticles are shown in Tables 17-19 for LMW, MMW and HMW CS, respectively. The antigen loaded microparticles of chitosan were prepared. The Japanese Encephalitis antigen was added into the aqueous phase of chitosan. The formulations of JE- loaded chitosan microparticles were the same as unloaded chitosan microparticles.

3. Characterization of microparticles

3.1 Polyester microparticles

3.1.1 Shape and surface morphology

Morphological evaluation of microparticles was determined by scanning electron microscopy (SEM, JEOL, JSM-5410LV). The microparticles were attached to the specimen holder with a double-coated adheisve tape and coated with a layer of gold. The scanning electron images were examined and photographed.

Table 17 Formulation of JE antigen loaded LMW CS microparticles

Formulation	Polymer	Concentration (%)	Ratio Aqueous:oil phase	Polymer (mg)	Antigen (μg)
CS 1	LMW CS (Seacure® 143)	1	1:5	200	250
CS 2					500
CS 3					750
CS 4			2:5	400	250
CS 5					500
CS 6					750
CS 7		2	1:5	400	250
CS 8					500
CS 9					750
CS 10			2:5	800	250
CS 11					500
CS 12					750
CS 13		3	1:5	600	250
CS 14					500
CS 15					750
CS 16			2:5	1,200	250
CS 17					500
CS 18					750

Table 18 Formulation of JE antigen loaded MMW CS microparticles

Formulation	Polymer	Concentration (%)	Ratio Aqueous:oil phase	Polymer (mg)	Antigen (μg)
CS 19	MMW CS (Seacure® 243)	1	1:5	200	250
CS 20					500
CS 21					750
CS 22			2:5	400	250
CS 23					500
CS 24					750
CS 25		2	1:5	400	250
CS 26					500
CS 27					750
CS 28			2:5	800	250
CS 29					500
CS 30					750
CS 31		3	1:5	600	250
CS 32					500
CS 33					750
CS 34			2:5	1,200	250
CS 35					500
CS 36					750

Table 19 Formulation of JE antigen loaded HMW CS microparticles

Formulation	Polymer	Concentration (%)	Ratio Aqueous:oil phase	Polymer (mg)	Antigen (μg)
CS 37	HMW CS (Seacure ® 343)	1	1:5	200	250
CS 38					500
CS 39					750
CS 40			2:5	400	250
CS 41					500
CS 42					750
CS 43		2	1:5	400	250
CS 44					500
CS 45					750
CS 46			2:5	800	250
CS 47					500
CS 48					750
CS 49		3	1:5	600	250
CS 50					500
CS 51					750
CS 52			2:5	1,200	250
CS 53					500
CS 54					750

3.1.2 Particle size analysis

The microparticles were suspended in 1% Tween 80 solution and bath sonicated to deaggregate. The volume-based particle size and size distribution were determined by a laser particle size analyser (Mastersizer, Malvern Instruments). The results reported the percentile sizes for 10, 50 and 90% which expressed as D(v,0.1), D (v,0.5) and D(v,0.9), respectively and the width of the distribution, which was the measure of the absolute deviations from the median expressed as uniformity.

3.1.3 Antigen content and entrapment efficiency

About 100 mg of microparticles were accurately weighed and dissolved in 1 ml of DMSO. The dispersion was incubated for 1 hour with occasionally shaking. A 5 ml of 0.05 N NaOH solution containing 0.5% w/v SDS was added and gently mixed. The mixture was shaken at room temperature for 12 hours. The sample was aliquoted and the amount of antigen was measured by bicinchoninic acid (BCA) protein assay (Sah, 1997). The percentage entrapment efficiency and the antigen content were calculated (Kawashima, 1998).

$$\underset{(\mu g\ Ag\ /100\ mg\ microparticles)}{\text{Antigen content}} = \frac{\text{amount of antigen in harvested microparticles}(\mu g)}{\text{amount of harvested microparticles}(mg)} \times 100$$

$$\%\ \text{Entrapment efficiency} = \frac{\text{amount of antigen in harvested microparticles}}{\text{amount of antigen loaded in system}} \times 100$$

3.1.4 In vitro release of antigen

About 100 mg of freeze-dried microparticles were accurately weighed and suspended in 5 ml of phosphate buffer saline (PBS) pH 7.4 containing 0.02% sodium azide. The samples were retained in a shaking water bath at 37°C and 150 rpm. The supernatant was withdrawn at appropriate time interval and centrifuged at 12,000 rpm for 5 minutes. The antigen in the supernatant was analysed by BCA protein assay.

3.1.5 Antigen integrity (Cohen, 1991)

The integrity of JE antigen was analysed by SDS-polyacrylamide gel electrophoresis (PAGE). The sample of antigen from microparticles, pure JE antigen and a molecular weight marker were heated at 95 ° C with sample buffer (Appendix I) for 1 hour and then loaded onto a 12% w/w slab gel and subjected to electrophoresis at 80 V. The gel was stained with coomassie brilliant blue in water/methanol/acetic acid (45:45:10) for 1 hour and destained for many times until the bands of protein were visualized.

3.1.6 Determination of PLGA microparticle degradation (Hausberger, 1995)

About 100 mg of unloaded microparticles were accurately weighed and dispersed in PBS pH 7.4. The dispersion was incubated in a shaking water bath at 37°C and 150 rpm. The samples were collected at different time intervals such as 2, 4, 6, 8, 12 and 16 weeks. The microparticles were dried and dissolved in tetrahydrofuran (THF). The solution was filtered through 0.45 μm membrane filter. The molecular weight of polymer was measured by gel permeation chromatogaph using Waters 150 – CV. The test conditions were as followed ; tetrahydrofuran as eluent , column temperature of 30° C, injection volume of 100 μl and flow rate of 1.0 ml/min. All molecular weight data and polydispersity were reported with respect to polystyrene standard. The polydispersity of the polymer represented the breadth of the molecular weight distribution.

3.1.7 Levels of residual solvent

The residual dichloromethane in polyester microparticles was determined by gas chromatography (Schimidzu 7-AG). About 500 mg of microparticles were accurately weighed and transferred to 5-ml vial sealed with rubber stopper and aluminum cap. The vials were heated to 50 ° C and the vapor of DCM was detected

using gas chromatograph with 5% SE-30 column. The operating conditions were : column temperature of 50°C, injection temperature of 180°C , detector temperature of 180°C , nitrogen flow rate at 25 ml/ minute and injection volume of 1 ml. The data were reported with respect to standard curve of DCM in hexane at various concentrations.

3.2 Chitosan microparticles

3.2.1 Shape and surface morphology

Shape and surface morphology of microparticles were evaluated by Cryo-scanning electron microscopy. The microparticles were dispersed in Tween 80 and sonicated to deaggregate. The samples were placed on the specimen holder and freezed with liquid nitrogen. The specimen were coated with gold. Then SEM micrographs were examined and photographed.

3.2.2 Particle size analysis

The particle size and size distribution were determined by a laser particle size analyser as described in 3.1.2.

3.2.3 Antigen content and % entrapment efficiency

About 100 mg of loaded chitosan microparticles were accurately weighed and dissolved in 0.1 N HCl containing 1% w/v sodium dodecyl sulfate(SDS). The sample was shaken at room temperature for 12 hours. An aliquot was neutralized and the amount of antigen was determined by BCA protein assay. The percentage and antigen content were calculated as described in 3.1.3.

3.2.4 In vitro release of antigen

About 100 mg of loaded chitosan microparticles were accurately

weighed and suspended in 5 ml of PBS pH 7.4 containing 0.02% sodium azide. The samples were agitated in a bath incubator shaker at 37° C and 150 rpm. At appropriate time intervals, the medium was collected and centrifuged at 12,000 rpm for 5 minutes. The amount of antigen in supernatant was analysed by BCA protein assay.

3.2.5 Antigen integrity

The integrity of JE antigen encapsulated in microparticles was detected by PAGE as described in 3.1.5.

3.2.6 Determination of chitosan microparticle degradation

About 100 mg of unloaded chitosan microparticles were accurately weighed and dispersed in PBS pH 7.4. The dispersion was incubated in a shaking water bath at 37°C and 150 rpm. The samples were collected at different time intervals of 4, 8, 12 and 16 weeks. The microparticles were dried and dissolved in 0.5 M acetic acid and 0.5 M sodium acetate in water (1:1 volume ratio). The solution was filtered through a 0.45 μm membrane filter. The molecular weight was measured using a gel permeation chromatograph, PL-110 with ultralinear hydrogel column MW resolving range 1,000-20,000,000. The operational conditions were as followed ; 0.5 M acetic acid and 0.5 M sodium acetate in water (1:1 volume ratio) as eluent, column temperature of 30°C , injection volume of 20 μl and flow rate of 0.6 ml/min. Pullulans(MW 5,900-788,000) were used as polymer standard. All molecular weight values and polydispersity were calculated relative to pullulan standard.

4. Stability study

The antigen-loaded microparticles of polyester and chitosan were incubated at

elevated temperature of 40°C for 1 month (Kreuter, 1981). The stability assessment was undertaken including particle shape and surface morphology, particle size, antigen content, in vitro release of antigen and antigen integrity.

5. Immunization study

5.1 Immunization protocol

Approximately two-month-old female white New Zealand rabbits weighing about 1.5-2 kg were housed for 1-2 weeks prior to any experiments. Any group of five rabbits were immunized by subcutaneous route with the following formulations

1. aqueous solution of pure JE antigen (dose 50 μg/ml)
2. antigen- loaded on 1.5% PLGA 50:50 microparticles with a dose equivalent to 50 μg/ml of JE antigen
3. antigen-loaded on 5% PLGA 50:50 microparticles with a dose equivalent to 150 μg/ml of JE antigen
4. antigen-loaded on 1.5% PLGA 50:50 microparticles with a dose equivalent to 150 μg/ml of JE antigen
5. antigen-loaded on 2% medium molecular weight chitosan microparticles with a dose equivalent to 150 μg/ml of JE antigen
6. Freeze-dried supernatant of microparticle preparation with a dose equivalent to 50 μg/ml of JE antigen

The group 1 of rabbits received a single dose of 1 ml of an aqueous solution of pure JE antigen at week 0 whereas the group 2 received three doses of an aqueous solution of pure JE antigen. The first two doses of 1 ml each were administered at an interval of 2 weeks. One additional administration of 1 ml dose was injected a month after the last immunization. For microparticle preparations, the microparticles were suspended in 1 ml of an aqueous solution containing 0.2% sodium carboxymethyl-

cellulose and 0.02% Tween 80 (Alonso, 1994 and Sah, 1995). A single dose of 1 ml of formulations # 2, 3, 4, 5 and 6 was administered in each group of the rabbit. The blood samples were collected at appropriate time intervals, every 1-2 weeks for 3-4 months. The serum was separated by centrifugation at 8,000 rpm for 5 minutes and frozen at –20 °C until analysed by enzyme-linked immunosorbent assay (ELISA).

5.2 Enzyme linked immunosorbent assay (ELISA) (Coombes, 1996)

The serum was analysed for antibodies using an ELISA technique. The 96–well ELISA microtiter plate was coated with 100 μl per well of a 100 μg /ml JE antigen in coating buffer pH 9.6 overnight at 4° C. The plate was thoroughly washed three times with phosphate buffer saline with Tween 20 (PBST) pH 7.4 and allowed to dry. The 100 μl of blocking solution, 3% gelatin in PBST, was added to each well of the coated plate and incubated for 1 hour at room temperature. The plate was thoroughly washed three times with PBST and allowed to dry. The serum samples were serially diluted with diluent (1% gelatin in PBST), and 100 μl of each sample was added to each well of the coated plate. The sample plate was incubated at room temperature for 1 hour. The plate was washed with PBST for three times. The 100 μl of a horseradish peroxidase-goat anti-rabbit IgG (H+L), diluted 1 in 3,000 with diluent, was added to each well and incubated for 1 hour at room temperature. The plate was washed again with PBST for three times. The 100 μl of the substrate, o-phenylene diamine, at a concentration of 1 tablet per 12 ml of a citrate buffer plus 12 μl of 30% hydrogen peroxide, was added to each well. The plate was covered and incubated for 30 minutes at room temperature for color development. The 50 μl of 4 N sulfuric acid was added to each well to stop the reaction. The plate was mixed and and the optical density (OD) was read at wavelength 491 nm using a microplate reader. The end-point titres were expressed as the last dilution which gave an optical density at 0.5 at 491. The serum samples from individual animals were studied to determine the titre range

and the mean immune response at each time point. The immune responses which evoked from pure JE antigen, encapsulated antigens with chitosan and PLGA polymers were compared.

5.3 Statistical analysis

The results are expressed as mean ± SD for each group of rabbits. An unpaired student's t-test was used to assess statistical significance of the mean values at various time points. Results were considered statistically significant if $p < 0.05$.