CHAPTER III

MATERIALS AND METHODS

Materials

1. Acrylamide (Batch no. 0109539, Fisher Scientific, UK)
2. Ammonium hydroxide (Batch no. C02043, J.T. Baker, USA)
3. Ammonium molybdate (Batch no. 3420X12465, Mallinckrodt, USA)
4. Aquagent Composite 5, free from pyridine (Batch no. 87958, Scharlau, Spain)
5. Chloroform (Batch no. 07 08 1241, Labscan, Ireland)
6. Cholesterol from lanolin (26740, Batch no. 423504/1, Fluka, Switzerland)
7. Coomassie Brilliant blue R250 (Batch no. K37824253 738, Merck, Germany)
8. Dialysis membrane molecular weight cutoff (MWCO) 100,000 Dalton (Batch no. 131414, Spectra/Por® Biotech CE, Spectrum Laboratories, Inc, USA)
9. D-Mannitol (Batch no. K91617082 709, Merck, Germany)
10. D(+)-Trehalose dihydrate from corn starch (Batch no. 114K7064, Sigma, USA)
11. Ethanol (Batch no. K34041783 450, Merck, Germany)
12. Fiske-Subbarrow reducer (Batch no. 064K7028, Sigma, USA)
13. Glycine (Batch no. AF507086, Ajax Finechem, Australia)
14. HEPES (H3375, Batch no. 066K5422, Sigma, USA)
15. Hydrochloric acid (Batch no. K27100452 944, BDH, UK)
16. Hydrogen peroxide 30 % (Batch no. K32656587 345, Merck, Germany)
17. Hydrogenated soybean phosphatidylcholine (Batch no. 61260, PHOSPHOLIPON® 90H, Nattermann Phospholipid GmbH, Germany)
18. Lactose monohydrate (Granulac® 200, Batch no. 1855, Molkerel, MEGGLE Wasserburg GmbH & Co., Germany)
19. Lysozyme from chicken egg white, lyophilized powder, protein ~95 %, ~50,000 units/mg protein (L6876, Batch no. 056K1690, Sigma, USA)
20. 2-Mercaptoethanol (Batch no. 004508, Fisher Scientific, UK)

21. Methanol, dried (Karl Fischer reagent grade, Batch no. 85772, Scharlau, Spain)
22. Methyl alcohol, anhydrous (Batch no. Y37B03, Mallinckrodt, USA)
23. *Micrococcus lysodeikticus* (Batch no. 015K8704, Sigma, USA)
24. Molybdenum blue spray reagent, 1.3 % (M1942, Batch no. 085K6140, Sigma, USA)
25. Polycarbonate membrane 0.2, 0.4 (Batch no. 4281005, 6104020, Nuclepore®, Whatman, USA) and 1.2 μm (Batch no. R2CN45086, Isopore®, Millipore, Ireland)
26. Polyethylene drain disc (Batch no. 310031, Whatman, USA)
27. Potassium dihydrogen phosphate (Batch no. A531473 425, Merck, Germany)
28. Potassium hydroxide (Batch no. B742533 049, Merck, Germany)
29. 2-Propanol (Batch no. K32632434 346, Merck, Germany)
30. QuantiPro™ BCA assay kit (QPBCA, Batch no. 046K6880, Sigma, USA)
31. SDS-PAGE standards (Batch no. LS1610317, Bio-Rad, Netherlands)
32. Sephadex G-75 (Batch no. 307356, GE Health Care, USA)
33. Sodium chloride (Batch no. K28315204 042, Merck, Germany)
34. Sodium dodecyl sulfate (Batch no. V351612J, BDH, UK)
35. Sodium hydroxide (Batch no. B131198 214, Merck, Germany)
36. Sucrose (Batch no. F3D103, Ajax Finechem, Australia)
37. Sulfuric acid (Batch no. K32417131 337, Merck, Germany)
38. TLC aluminium sheets, Silica gel 60 F_{254} (Batch no. 0B195144, Merck, Germany)
39. TrisGly SDS running buffer (10X) (Novex®, Lot no. 1194870, Invitrogen™, USA)
40. Tris-Glycine SDS sample buffer (2X) (Novex®, Lot no. 1184833, Invitrogen™, USA)
41. Triton X-100 (Batch no. 065K0122, Sigma, USA)
42. Ultrapure® water (Elga®, England)

All chemicals were of analytical or pharmaceutical grades and were used as received.

Equipment

1. Analytical balance (Model AG285, Mettler Toledo, Switzerland)
2. Bath sonicator (Transsonic Digitals, Elma®, Germany)
3. Cooling bath Neslab RTE7 (Thermo Electron Corporation, USA)
4. Differential scanning calorimeter (DSC 822^e, STAR^e system, Mettler Toledo, Switzerland)
5. Fourier Transform Raman Spectrometer (FT-Raman, Spectrum GX, Perkin Elmer, USA)
6. Freeze dryer (Dura-Dry microprocessor, FTS System, USA)
7. Helium pycnometer (Ultrapycnometer 1000, Quantachrome instruments, USA)
8. High pressure homogenizer (Emulsiflex® C-5, Avestin, Canada)
9. Hot air oven (Memmert, Germany)
10. Karl Fischer Titrator (720 KFS Titrino, Metrohm, Switzerland)
11. Liplex® Extruder (Northern Lipids, Canada)
12. Magnetic stirrer (Ika® Werke, Germany)
13. Mastersizer 2000 (Malvern Instruments Ltd., UK)
14. Mastersizer S (Malvern Instruments Ltd., UK)
15. Microplate reader (Anthos Labtec, UK)
16. Optical microscope (Model IX51, Olympus, Japan)
17. pH meter (model 420A, ORION, USA)
18. Rotary evaporator (R-215) with vacuum controller (V-850) (Buchi, Switzerland)
19. Scanning Electron Microscope (Model JSM-5800LV, JOEL®, Japan)
20. Spectropolarimeter (Jassco J-715, Jasco Corp., Japan)
21. Spray drier (Model B-290, Buchi, Switzerland)
22. Transmission Electron Microscope (Model JEM-200CX, JOEL®, Japan)
23. Ultracentrifuge (Model L-80 with a 90Ti rotor, Beckman Coulter, USA)
24. UV-visible Spectrophotometer (Model UV-1601, Shimadzu, Japan)
25. Vortex mixer (Vortex-Genie® 2, Scientific Industries, USA)
26. Water bath (Grant Instruments, England)

27. X-ray diffractometer (Model D8 discover, Bruker AXS, USA)
28. Zetaplus and Bi-mas (Brookhaven, USA)

METHODS

1. Preparation of Liposomes

In this study, liposome formulations were composed of hydrogenated soybean phosphatidylcholine (HPC) and HPC/cholesterol (Chol) in the molar ratios of 9:1, 8:2 and 7:3. HPC only liposomes was prepared by one-step method using high pressure homogenizer (Emulsiflex® C-5) (Brandl et al., 1990, 1993). HPC was dispersed under mechanical stirring in Ultrapure® water with the total lipid concentration of 10 %w/w at 65 °C (the temperature above its T_m) for 2 hours. After that, the lipid dispersion was homogenized by Emulsiflex® C-5 pre-heated to 65 °C using thermostat for 4 cycles at 100 MPa. In this process, solid lipid particles are broken up and undergo forced hydration and self-aggregation into homogeneous dispersions of liposomes without using organic solvents (Brandl et al., 1993). Finally, the liposome dispersion was extruded through two-stacked polycarbonate (PC) membrane filters of 0.2 μm pore size under nitrogen pressure for 10 cycles at 65 °C in an Liplex® Extruder to obtain homogeneous unilamellar vesicles. The extruded liposome dispersion was kept at 4 °C before spray drying process.

Liposomes composed of HPC and Chol were prepared by thin film hydration method (New, 1989). The total lipid mixture weight of 2 g was dissolved in 40 mL of a chloroform/methanol (1:3 v/v) mixture in a 2 L round bottomed flask. The solvent mixture was slowly removed at reduced pressure on a rotary evaporator at 45 °C, 100 rpm for 60 min until a thin film of dry lipids was formed on the wall of the flask. The formed thin film layer was flushed under a stream of nitrogen for 1 min. The lipid film was hydrated using 18 mL Ultrapure® water by swirling in a shaking water bath at 80 °C, 190 rpm for 30 min. After that, the liposomal dispersion was passed through high pressure homogenizer for 4 cycles at 65 °C and pressure of 100 MPa for size

reduction and then was extruded through two-stacked 0.2 μm PC filters for 10 cycles at 65 °C to obtain a more uniform unilamellar liposome dispersion. The liposome dispersion sample was kept at 4 °C prior to spray drying process.

2. Study of Feasibility of Liposomal Powder Preparation by Spray Drying Technique

The extruded HPC liposome dispersion was used to study feasibility of preparing liposomal powders using spray drying technique. The liposomal powders may comprise an additive added to improve particle rigidity and production yield (Weers et al., 2005). Therefore, carbohydrates such as sucrose, trehalose, lactose and mannitol were evaluated as an additive for formulation of the spray-dried liposomal powders because they are available in USP-NF pharmaceutical grades and widely applied in aerosolization (Bosquillon et al., 2001).

The extruded HPC liposome dispersion (prepared according to the procedures described in Section 1) was mixed with aqueous solution of an additive to obtain a HPC/additive weight ratio of 1:1 and total solid content of 10 %w/w. The resultant mixture was stirred for 30 min and then spray-dried into powders using a B-290 Buchi mini spray-dryer (Figure 14). The mixture was maintained under continuous stirring at a moderate speed while fed into the spray-dryer. The feed mixture was pumped into the drying chamber and pneumatically atomized through two-fluid external mixing 0.7 mm nozzle. The processing condition is listed in Table 4. Cooling water was circulated through a jacket around the nozzle. The resultant powder was blown through the cyclone separator and collected in a receiving vessel. Exhausted air was extracted out of the cyclone by a vacuum pump and filtered by a fiber filter. The spray-dried product was stored in bottles in a silica gel desiccator at 4 °C until assay. The schematic of the preparation method of the spray-dried liposomal powders is illustrated in Figure 15.

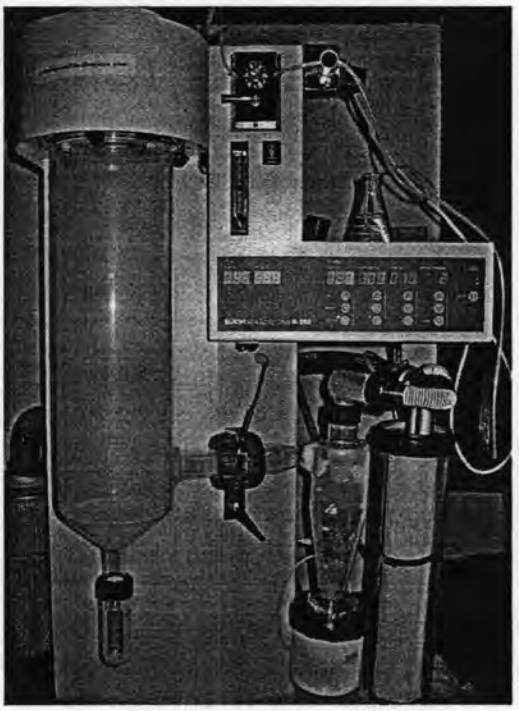

Figure 14 B-290 Buchi spray-dryer for preparing the spray-dried liposomal powders

Table 4 Spray-drying process condition of B-290 Buchi spray-dryer

Condition	Parameter
Inlet (outlet) temperature	120 (74-80) °C
Pump speed	10 % (2.3 mL/min)
Aspirator setting	100 %
Atomizing air volumetric flow rate	30 mm (357 Normlitre/hour)
Total solid content	10 %w/w

In the preliminary study, coarse lipid dispersion was also used to prepare spray-dried liposomal powders to compare with the extruded liposomes. Coarse lipid dispersion was prepared by dispersing HPC in Ultrapure® water at 65 °C for 2 hours and homogenization by high speed homogenizer at 65 °C for 5 min, and then mixed with mannitol solution and spray-dried into powders using the same spray drying condition. Morphology of the powders and the reconstituted liposomes from the spray-dried powders were observed.

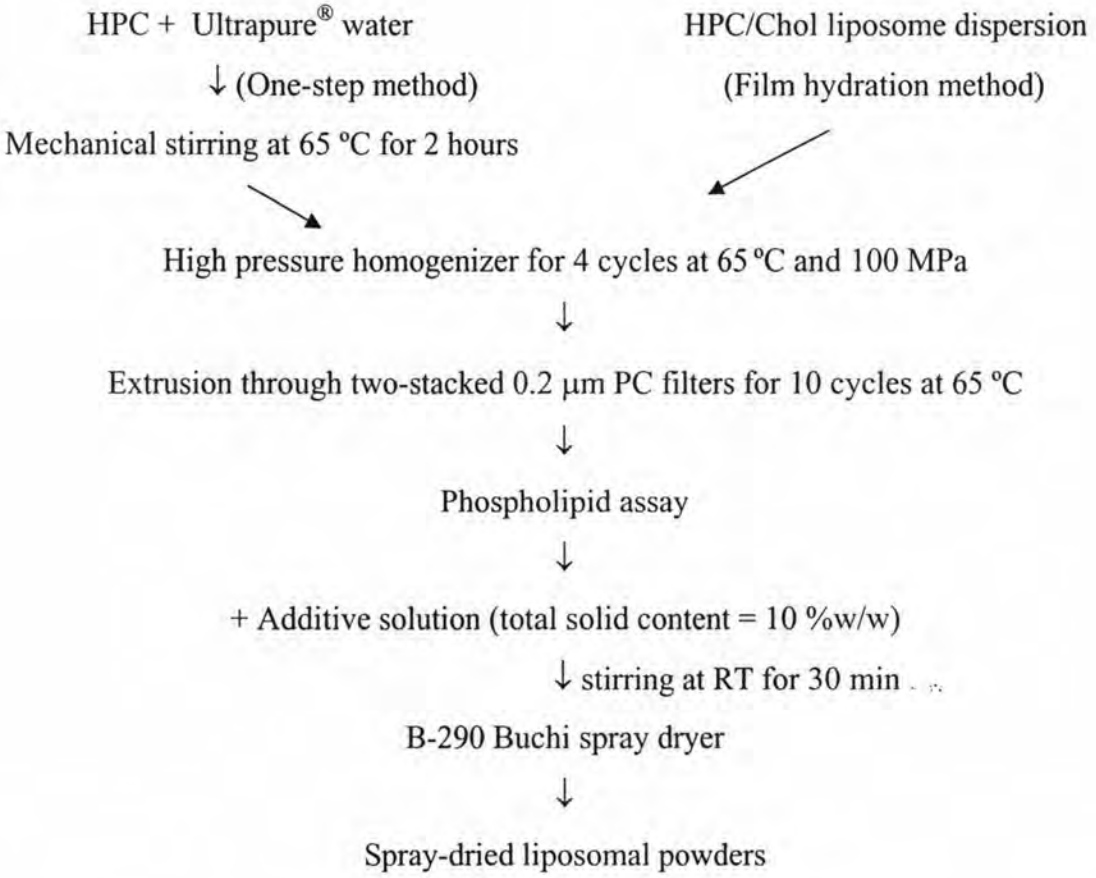

Figure 15 Typical steps of preparation of liposomes and spray-dried liposomal powders composed of HPC or HPC/Chol

3. Chemical Analysis of Phospholipid

3.1 Quantitative Determination of Phospholipids (New, 1989)

Phospholipid concentrations were determined by the Bartlett assay (Bartlett, 1959). The principle of the Bartlett assay is based on the colorimetric determination of inorganic phosphate. The phospholipid content of liposomes can be determined after destruction of the phospholipid with sulfuric acid to inorganic phosphate. This is converted to phospho-molybdic acid by the addition of ammonium molybdate, which is reduced to a blue-coloured complex by 4-amino-2-naphthyl-4-sulfonic acid during heating. This compound can be determined colorimetrically at 800 nm, and is

compared with calibration standards to give phosphorus and hence phospholipid content. The phospholipids contain one mole of phosphorus per mole of phospholipids.

The liposome dispersion was diluted with Ultrapure® water to give a concentration of approximately 0.1 mg/mL of phospholipid. Five hundred microliters of the diluted sample or the standard solutions was added into separate 10 mL tubes, together with a blank (0.5 mL of Ultrapure® water). Each aliquot of the samples and the standard solutions was acidified with 0.4 mL of 5 M sulfuric acid and then incubated at 180-200 °C for an hour in a hot air oven. After the tubes were cooled down, 0.1 mL of 10 %v/v hydrogen peroxide was added and the mixture was incubated at 180-200 °C for another 30 min. The clear solutions were cooled down to room temperature. Ammonium molybdate-sulfuric acid solution (4.6 mL) was added to each tube and the contents were mixed by vortex. An aliquot (0.2 mL) of Fiske-Subbarrow reducer was added to reduce the solutions. The tubes were then covered and placed in a boiling water bath for 7 min. After the tubes were cooled down, the absorbances of the blue-colored solutions, including the blank, were measured at 800 nm against water. The molar concentration of phosphorus content was equivalent to the molar concentration of phospholipid content in liposome dispersion.

3.2 Chemical Analysis of Degradation of Phospholipids

The chemical stability of HPC during liposome preparation using high pressure homogenizer and during dehydration by spray drying was investigated for both hydrolysis and oxidation with the HPC starting material (Phospholipon 90H®) as a reference. The measurement was performed in quadruplicate.

3.2.1 Hydrolysis

In an aqueous liposome dispersion, phospholipids (PC) can be hydrolyzed to free fatty acids (FFA) and 2-acyl- and 1-acyl-lysophospholipids (LPC). The LPCs hydrolyze further to glycerol phospho compounds (GPC) (Torchilin and

Weissig, 2003). An accurate method to measure hydrolysis is to monitor the PC and LPC content by Thin-layer Chromatography (TLC) in the chloroform phase and GPC by total phosphate analysis of the supernatant (methanol/water phase) after extracting the lipids according to Bligh and Dyer (Bligh and Dyer, 1959 cited in Torchilin and Weissig, 2003). The degree of hydrolysis is expressed as:

$$\% \text{ hydrolysis} = 100\ \% - \frac{[PC] \times 100\%}{[PC] + [LPC] + [GPC]} \quad (1)$$

3.2.1.1 Bligh and Dyer-extraction

The Bligh and Dyer-extraction enables one to separate between various components of the formulation. The lipid dispersion (200 μL) was added into a 10 mL tube. Chloroform (250 μL) and methanol (500 μL) were added to the tube and the mixture was mixed by vortex until a clear solution was obtained. Then 250 μL of 0.1 M HCl and 250 μL of chloroform were added to the tube and the content was mixed and centrifuged at 5000 rpm for 15 min to obtain two clear phases: the upper phase consisted of methanol and water and the lower phase consisted of practically 100 % chloroform.

3.2.1.2 Determination of PC and LPC Contents by Thin-layer Chromatography (TLC)

The HPC starting material or the samples were dispersed and diluted with Ultrapure® water to obtain lipid concentration of 50 mg/mL and then the phospholipid was extracted using acidified Bligh-Dyer procedure. Ten microliters of each sample (the lower chloroform phase obtained from Bligh and Dyer-extraction) was spotted in separate lanes on a TLC plate. The plate was developed with the solvent system of chloroform:methanol:water:ammonium hydroxide (65:35:2.5:2.5 v/v). After the plate was air-dried for 15 min in fume hood, it was sprayed to visualize the lipids with molybdenum blue. The phospholipid would stain blue within 5 min. The plate was air-dried for 30 min in fume hood. The PC and LPC spots were

scrapped off into separate 10 mL tubes and assayed for phospholipid content using the Bartlett method. The tubes were centrifuged at 5000 rpm for 5 min before the absorbance of the supernatant was measured at 800 nm.

3.2.2 Oxidation

Oxidation of phospholipids was estimated by spectrophotometry (Torchilin and Weissig, 2003). The method is based upon the absorbance of conjugated dienes and trienes at 233 and 270 nm, respectively. Phospholipids contain only double bonds which are non-conjugated, and thus have an UV absorbance peak at a very short wavelength (200-205 nm). The initial step in oxidation of acyl chains is supposed to involve a free radical chain mechanism which leads to bond migration and diene conjugation. Since conjugated dienes absorb at 233 nm, the occurrence of oxidative reactions can be monitored by measuring of the change in absorbance at this wavelength.

Fifty microliters of the chloroform phase from Bligh and Dyer-extraction of each sample (from Section 3.2.1.2) was added into separate 10 mL tubes and evaporated to a dried residue. The lipid residue was dissolved in 2 mL of ethanol. The UV absorbance spectrum of each sample was scanned over the range 200-300 nm using ethanol as a blank. The absorbances at 215 nm, 233 nm and 300 nm were measured. The oxidative index was calculated using equation 2.

$$\text{Oxidation index} = \frac{\text{absorbance at 233} - \text{absorbance at 300 nm}}{\text{absorbance at 215} - \text{absorbance at 300 nm}} \qquad (2)$$

4. Effect of HPC/mannitol Ratio on the Preparation of Spray-dried Liposomal Powders

From the preliminary study in Section 2, mannitol was chosen as the best suitable additive for preparing the spray-dried liposomal powders. The liposomes with only HPC were used to study effect of mannitol amount on the preparation of the liposomal powders by spray drying technique. Aqueous solution of mannitol was prepared by dissolution of mannitol in Ultrapure® water to obtain a concentration of 10 %w/w. The extruded liposome dispersion with 10 %w/w HPC was mixed with the mannitol aqueous solution to achieve HPC/mannitol at theoretical weight ratios of 10:0, 9:1, 7:3 5:5, 3:7, 1:9 and 0:10. The total concentration of lipid and mannitol was 10 %w/w. A portion of the resultant mixture (100 gram) was spray-dried using the condition shown in Table 4. The spray-dried powders obtained were characterized as described in Sections 8.1-8.6 and reconstituted as in Section 9.1. The reconstituted liposomes were investigated as in Sections 9.2, 9.4 and 9.5. The highest HPC/mannitol weight ratio providing fine liposomal powders was selected to further study in Sections 5, 6 and 7.

5. Effect of Glycine on the Preparation of Spray-dried HPC Liposomal Powders

Chougule et al. (2006) reported using glycine as an anti-adherent for formulation of liposomal dry powder inhaler. In this study, therefore, the effect of glycine on the properties of the spray-dried HPC liposomal powders was investigated. Glycine was dissolved in Ultrapure® water and then mixed with the mixture of liposome dispersion and mannitol prior to spray drying process. The amounts of glycine studied were 0.5, 1.0, 5.0 and 10.0 %w/w based on the total weight of HPC and mannitol. The resultant spray-dried powders were evaluated as described in Sections 8.1-8.3, 8.5-8.6, and reconstituted as in Section 9.1. The reconstituted liposomes were investigated as in Sections 9.2 and 9.4.

6. Effect of HPC/Chol Molar Ratio on the Preparation of Lysozyme-loaded Liposomal Powders by Spray Drying Technique

The extruded liposomes with 10 %w/w lipid composed of HPC/Chol at the molar ratios of 10:0, 9:1, 8:2 and 7:3 were prepared as described in Section 1. Aqueous solution of lysozyme was mixed with the liposomal dispersion and mannitol solution prior to spray drying. The HPC/mannitol ratio used was obtained from Section 4 and the total amount of lipid and mannitol was 10 %w/w. The mixture (100 gram) was spray-dried into the lysozyme-loaded liposomal powders using the condition present in Table 4. The spray-dried powders produced were evaluated as described in Sections 8.1-8.6 and reconstituted as in Section 9.1. The reconstituted liposomes were investigated as described in Sections 9.2, 9.4-9.7. The spray-dried powders of only lysozyme and lysozyme with mannitol were prepared from 0.5 %w/w of lysozyme solution and solution of 0.5% w/w lysozyme with 5 %w/w mannitol, respectively, to compare with the spray-dried lysozyme-loaded liposomal powders. The HPC/Chol molar ratio providing fine liposomal powders and high entrapment of lysozyme in liposomes was selected for further study in Sections 7 and 10.

7. Effect of Presence of Lysozyme with and without Mannitol in Liposome Structure prior to Spray Drying

Lysozyme-loaded liposomes were prepared by the dehydration-rehydration method described by Gregoriadis et al. (1999). The liposomes composed of only HPC and HPC/Chol (in the molar ratio obtained from Section 6) were prepared by the method described in Section 1. After passing through the high pressure homogenizer, the liposome dispersion was mixed with aqueous solution of lysozyme at the weight ratio of 10:1. The resultant mixture was frozen (-20 °C) overnight and then freeze-dried for 2 days. The freeze-dried powders were stored at -20 °C until use. For rehydration, Ultrapure® water (0.1 mL per 32 μmol of phospholipid) prewarmed at 60 °C was added to the freeze-dried material. The mixture was vortexed and incubated for 30 min at 60 °C. The rehydration process was repeated twice with Ultrapure®

water to obtain the final lipid concentration of 10 %w/w. Multilamellar dehydrated-rehydrated vesicles (DRV) were generated and extruded at 60 °C through PC membrane filters with pore sizes of 1.2 μm 3 times, 0.4 μm 3 times and finally 0.2 μm 10 times, respectively. The vesicles were further stabilized by hydration for 2 h at room temperature. Entrapment efficiency of lysozyme in liposomes was investigated after gel filtration chromatography as described in Section 9.6.

To study the effect of osmotic pressure and/or the initial mannitol concentration gradient across the liposomal membrane on the rupture of liposome bilayer during spray drying process, mannitol solution was introduced either only on the outside or on both sides of the bilayer. For mannitol introduced only on the outside of the vesicles, the extruded DRV liposomes with free lysozyme were mixed with mannitol solution and then spray dried into the powders using the condition shown in Table 4. The spray-dried powders obtained from this method were called "DRV HPC or DRV HPC/Chol8:2" depending on the lipid composition used. In the case where mannitol was present on both sides, mannitol solution was used for rehydration of the freeze-dried liposomes instead of Ultrapure® water and these formulations were called "DRV HPC in M or DRV HPC/Chol8:2 in M". The amount of mannitol used depended on the HPC/mannitol ratio obtained from Section 4. The total amount of lipid and mannitol was 10 %w/w. The spray-dried powders obtained were characterized as described in Sections 8.1-8.3, 8.7 and reconstituted as in Section 9.1. The reconstituted liposomes were investigated as described in Sections 9.2, 9.4 and 9.6-9.7. The schematic of the dehydration-rehydration method and preparation of the spray-dried powders is illustrated in Figure 16.

Summary of the different formulations of the spray-dried powders is shown in Table 5. The amount of each compound was expressed as gram per 100 grams of dispersion before spray drying process.

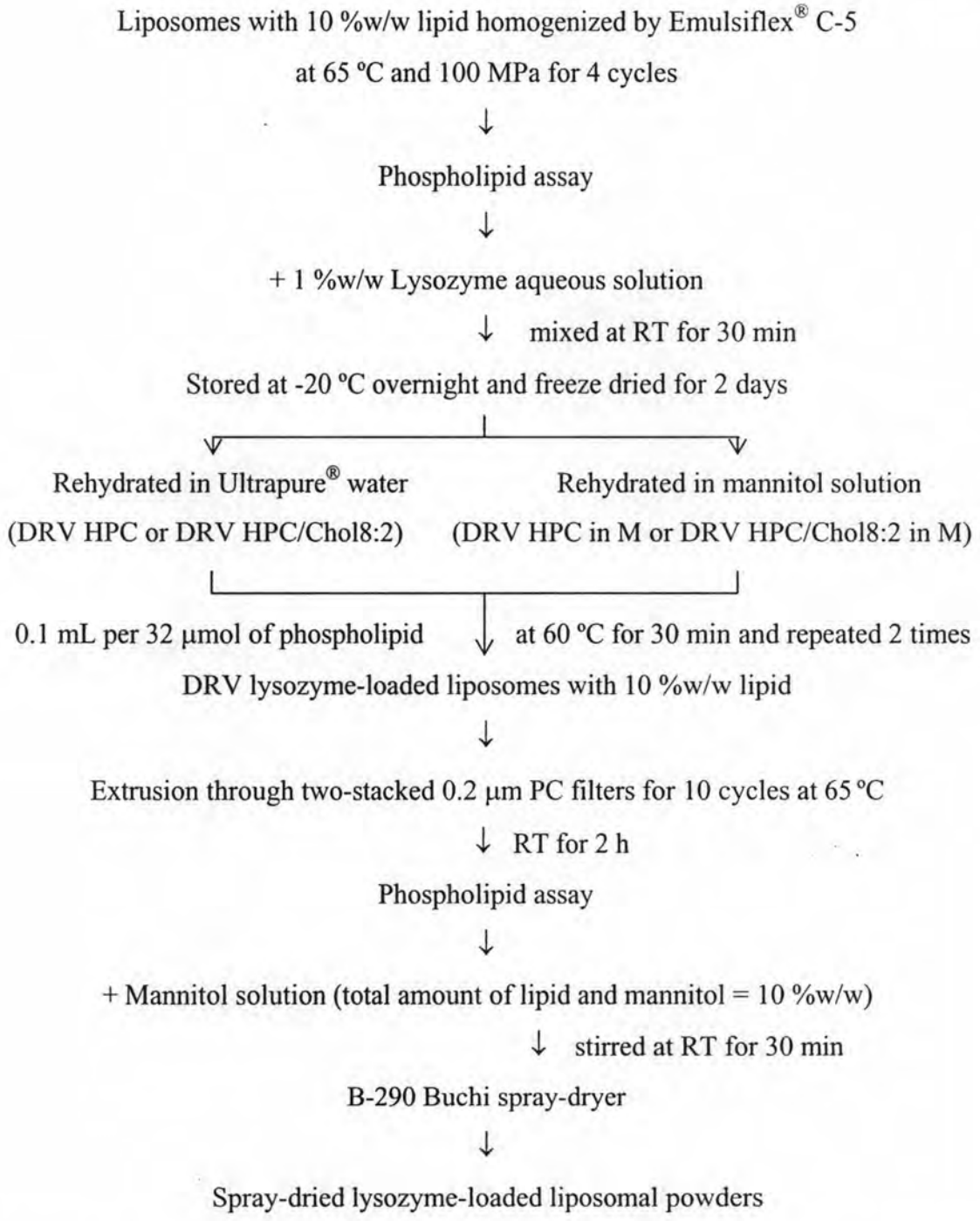

Figure 16 Typical steps of the preparation of DRV liposomes and spray-dried liposomal powders

Table 5 Formulation code of the spray-dried powders and composition of dispersion prior to spray drying process

Formulation code	Composition (%w/w in dispersion)				
	HPC	Chol	Mannitol	Glycine	Lysozyme
HPC/M 10:0	10	-	-	-	-
HPC/M 9:1	9	-	1	-	-
HPC/M 7:3	7	-	3	-	-
HPC/M 5:5	5	-	5	-	-
HPC/M 3:7	3	-	7	-	-
HPC/M 1:9	1	-	9	-	-
HPC/M 0:10	-	-	10	-	-
HPC/M/G0.5	5	-	5	0.05	-
HPC/M/G1	5	-	5	0.1	-
HPC/M/G5	5	-	5	0.5	-
HPC/M/G10	5	-	5	1	-
Spray-dried LSZ	-	-	-	-	0.5
M/L	-	-	5	-	0.5
HPC/M/L	5	-	5	-	0.5
HPC/Chol9:1/M/L	4.75	0.26	5	-	0.5
HPC/Chol8:2/M/L	4.448	0.552	5	-	0.5
HPC/Chol7:3/M/L	4.125	0.875	5	-	0.5
DRV HPC	5	-	5	-	0.5
DRV HPC in M	5	-	5	-	0.5
DRV HPC/Chol8:2	4.448	0.552	5	-	0.5
DRV HPC/Chol8:2 in M	4.448	0.552	5	-	0.5

HPC: Hydrogenated soybean phosphatidylcholine

Chol: Cholesterol

M: Mannitol

G: Glycine

LSZ, L: Lysozyme

DRV: Lysozyme-loaded liposomes prepared by dehydration-rehydration method

8. Characterization of Spray-dried Formulations

8.1 Yield

The spray-drying process yield was calculated as a percentage by dividing the mass of the powders collected from the collector and the cyclone of spray dryer by the initial mass of substances added (lipid, mannitol, lysozyme and other additives) as follows:

$$\% \text{ Yield} = \frac{\text{Weight of spray-dried powders}}{\text{Total weight of solids added initially}} \times 100\ \% \quad (3)$$

8.2 Residual Moisture Content

The residual moisture content of the spray-dried powders was measured by Karl-Fischer Moisture Titrator. The samples (50 mg) were titrated in triplicate after calibration with a standard solution of water in methanol.

8.3 Surface Morphology

The surface morphology of the spray-dried powders was examined by scanning electron microscopy (SEM). The samples from the collector of the spray-drier were attached to sample stubs using double sided tape, then coated with gold, and viewed using an accelerating voltage of 10 kilovolt (kV) at the magnification of 500x to 5000x. The physical appearance of the spray-dried powders was visually observed.

8.4 Particle Size and Size Distribution

The particle size of the spray-dried powders was determined by a Mastersizer S laser diffraction analyzer. The spray-dried formulations were dispersed in 2-propranol saturated with the same additives as the samples. The suspension was

sonicated for 5 min so as to disperse any possible agglomerates before being added to a stirred sample cell. Particle size distributions were expressed in terms of mass median diameter (MMD) and span. The MMD is the diameter at the 50% point of the entire volume distribution. The span is defined as $[D_{0.9} - D_{0.1}] / D_{0.5}$, where $D_{0.9}$, $D_{0.5}$ and $D_{0.1}$ are the respective diameters at 90%, 50% and 10% cumulative volumes. The average particle size distribution was measured from three replicates of each sample.

8.5 Thermal Properties

Thermal properties of the starting materials and the spray-dried powders were investigated using DSC. An empty aluminum pan with vented lid was used as the reference for all measurements. Accurately weighed solid samples (3-6 mg) were placed in 40 μL perforated aluminum pans and sealed. Heat runs for each sample were set from 25 to 250 °C at the scan rate of 10 °C/min, using nitrogen as the blanket gas. The instrument was calibrated with indium. The phase transition temperature (T_m) was described as peak temperature of the transition curve. The transition enthalpy of the spray-dried liposomal powders with various HPC/Chol molar ratios was determined using the scan rate of 1 °C/min from 25 to 90 °C. The slower scan rate is preferable for improved resolution (Torchilin and Weissig, 2003).

8.6 Crystallinity

The crystallinity properties of the starting materials and the spray-dried powders were determined by X-ray powder diffraction (XRPD), with a copper line as the source of radiation. The measurements were done at room temperature using a 40 kV voltage, a 40 mA current and a scanning rate of 0.02 °/min over a 2θ range of 5-35 °.

8.7 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

The spray-dried lysozyme was dissolved in Ultrapure® water to obtain a concentration of about 5 μg/μL. The lysozyme starting material was used as the

control. The protein solution was mixed with sample buffer and reducing agent (β-mercaptoethanol). The mixture was then heated at 100 °C for 10 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in TrisGly SDS running buffer at 25 mA for 1 h at room temperature. The gel consisted of a separating gel containing 15 % acrylamide in 0.375 M Tris-Cl, pH 8.8 and 0.1% SDS, and a stacking gel containing 4% acrylamide in 0.125 M Tris-Cl, pH 6.8 and 0.1% SDS with 0.75 mm thick. The amount of sample loaded on the gel was adjusted to 5 μg of protein per well. Protein bands were stained with staining solution (0.025% Coomassie Brilliant blue R250, 40% methanol and 7% acetic acid) and then destained with solution of 30% methanol and 10% acetic acid. The gel image was scanned on a scanner. A broad range molecular weight standard was used as the molecular weight marker.

8.8 Remaining Activity of Lysozyme

Stability of lysozyme in various formulations was determined by an enzymatic activity assay after reconstitution in comparison to that of the lysozyme starting material. The remaining activity of lysozyme in the DRV liposomes before and after spray drying, the spray-dried lysozyme, and the spray-dried liposomal powders studied in experimental design was investigated. Each of the spray-dried liposomal powders (approximately 10 mg) was reconstituted with Ultrapure® water in separate 10-mL tubes. The liposomal dispersion was lysed with Triton X-100 and diluted with Ultrapure® water to obtain about 10 µg/mL of lysozyme. The biological activity and the amount of lysozyme were determined. The effect of lipids and Triton X-100 on the determination of lysozyme activity was compensated by assaying blank and lysozyme standards in the presence of equivalent amount of blank liposomes and Triton X-100 solution. The remaining activity of lysozyme in percentage was calculated from the percentage of lysozyme activity remaining after the DRV process or the spray drying process relative to that of the lysozyme starting material. Four assay replicates per sample were performed. In addition, lysozyme solution heated at 60 °C for 90 min (the condition for liposome preparation by the DRV method) was

also evaluated for biological activity. Conformation stability by circular dichroism analysis was also investigated to confirm stability of lysozyme during this process.

Lysozyme is an enzyme that digests bacteria cell walls. To take advantage of this property, the bacteria *Micrococcus lysodeikticus* was used as the substrate in the standard activity assay for lysozyme (Shugar, 1952). The digestion of bacterial membrane causes a decrease in the absorbance at 450 nm. Briefly, a 0.015 %w/v suspension of *M. lsyodeikticus* was prepared in potassium phosphate buffer (66 mM; pH 6.24) at 25 °C. An aliquot (0.1 mL) of appropriately diluted lysozyme solution was added to a cuvette cell containing 2.5 mL of the bacteria suspension and the mixture was immediately mixed by inversion. The absorbance at 450 nm (A_{450}) was recorded every 10 sec during a total incubation period of 120 sec using a UV-Visible spectrophotometer. The change in absorbance at 450 nm per minute was obtained during 10-70 sec, which gave maximum linear rate for both the test and the blank. The blank was Ultrapure® water containing the equivalent concentration of lipid and Triton X-100. A decrease of 0.001 A_{450}/min was defined as 1 unit of enzyme activity. The specific enzyme activity was calculated using the following equation:

$$\text{Units/mg lysozyme} = \frac{(\Delta A_{450nm}/\text{min Test} - \Delta A_{450nm}/\text{min Blank})}{(0.001)\ (0.1)\ (\text{mg/ml lysozyme})} \qquad (4)$$

where 0.001 is the change in A_{450} per min according to the unit definition; and 0.1 is the volume (mL) of the sample/standard used. The concentration of lysozyme was determined by QuantiPro bicinchoninic acid (QPBCA) assay kit as described in Section 9.7.

Biological activity assay of lysozyme in Ultrapure® water containing 0.01 % HPC/Chol (8:2) and 0.2 % Triton X-100 was validated for accuracy and precision. In addition, aqueous solution of lysozyme heated at 80 °C for 60 min was assayed for the remaining activity of lysozyme to evaluate reliability of the assay method. Conformation stability using circular dichroism analysis was also investigated to confirm whether the native structure of lysozyme was required for its activity.

8.8.1 Accuracy

The evaluation of accuracy of lysozyme biological activity assay was performed by determining the analytical recovery of the six determinations (from six stock solutions) of 10 μg/mL lysozyme solution. The analytical recovery (in percent) of each determination was calculated by dividing the specific enzyme activity of the sample by that of the lysozyme standard. The mean of specific enzyme activity obtained from the precision assay was used as the activity of lysozyme standard. The mean, standard deviation and percent coefficient of variation (% CV) were determined. The mean value should be within 15% of the actual value (ICH Topic Q 6 B, 1999; USFDA, 2001).

8.8.2 Precision

The precision was evaluated by analyzing the specific enzyme activity of six determinations (from the same stock solution) of 10 μg/mL lysozyme solution measured within the same day. The mean, standard deviation and percent coefficient of variation (% CV) were determined. The coefficient of variation should not exceed 15% (ICH Topic Q 6 B, 1999; USFDA, 2001).

8.9 Circular Dichroism (CD) Analysis of Lysozyme

The spray-dried lysozyme was dissolved in Ultrapure® water to obtain concentration of 0.1 mg/mL and its structural integrity was evaluated using circular dichroism in comparison with the lysozyme starting material. Ultrapure® water was used as blank. Far-UV CD spectra providing an estimation of the secondary structure of protein were measured from 190 nm to 260 nm at room temperature using a cell with a path length of 0.5 cm. Near-UV CD spectra indicating tertiary structure of protein were measured from 250-320 nm using a cell with a path length of 1 cm. Other parameters were set as follows: resolution = 0.5 nm, bandwidth = 2.0 nm, sensitivity = 50 mdeg, response = 2 sec, and scan speed = 50 nm/min. Four scans were averaged to obtain one spectrum. The measurement was performed in triplicate

for each sample. The CD absorbance was expressed as ellipticity (mdeg). It was calculated as molar ellipticity [θ] using equation 5 (Morrow et al., 2000).

$$[\theta] = \theta\,(MRW) / (10.l.c) \qquad (5)$$

$[\theta]$ = Molar ellipticity ($deg.cm^2/dmol$)

θ = Measured ellipticity (deg)

MRW = Mean residual weight

$$= \frac{\text{Molecular mass of polypeptide chain (Da)}}{(\text{number of amino acids} - 1)}$$

l = Path length of cell (cm)

c = Protein concentration (g/mL)

The molecular ellipticity at 222 nm ($[\theta]_{222}$) is a standard measure of helical content of a protein and has been used to estimate the secondary structural change of the protein. The decrease in the intensity of the negative band at 222 nm represents the decrease in the content of α–helix and changes in the secondary structure of lysozyme. The α–helix content of lysozyme was calculated from the [θ] value at 222 nm using equation 6:

$$\%\ \alpha\text{-helix} = \frac{(-[\theta]_{222} + 3000)}{39{,}000} \times 100 \qquad (6)$$

as described by Morrow et al. (2000). The relative α–helical content (RHC) was calculated according to equation 7:

$$\%\ RHC = \frac{\%\ \alpha\text{–helix after spray-drying}}{\%\ \alpha\text{–helix of the starting material}} \times 100 \qquad (7)$$

8.10 Fourier Transform Raman Spectroscopy

The secondary structure of a protein can be determined by analysis of the amide band shape and position in Fourier Transform Raman (FT-Raman) spectra. The most sensitive modes to conformation in the FT-Raman are the amide I which arises from C=O stretching and amide III that mainly arises from the N-H bending (δN–H). Proteins with α–helical structures show a strong amide I band at about 1645-1660 cm^{-1}, whilst proteins with β–structure show an intense amide I band at about 1660-1670 cm^{-1} (Carey, 1982). The amide III band is at about 1250-1350 cm^{-1}. The FT-Raman spectroscopy was employed to investigate the secondary structure of the starting material and the spray-dried lysozyme in both solid state and aqueous solution (7.5 %w/v). The FT-Raman spectra were collected using a Spectrum GX spectrometer with FRA 106 FT-Raman module equipped with a Nd^{3+}: YAG laser emitting at a wavelength of 1064 nm. Solids was analyzed in stainless steel sample cups and solutions were assessed in a 1 cm^3 quartz cuvette with a mirrored rear surface, to enhance the intensity of the FT-Raman signal by multiple reflection of the FT-Raman radiation. The laser power was 450 mW for the solid samples and 2 W for solutions. Spectra were the average of 1000 scans for solids and 4000 scans for solutions at 4 cm^{-1} resolution over the range 3500-500 cm^{-1} at 25±1 °C.

9. Characterization of Liposomes

9.1 Reconstitution of Spray-dried Liposomal Powders

The spray-dried liposomal powders with and without lysozyme were reconstituted to obtain 5% lipid concentration with 10 mM HEPES and 140 mM NaCl, pH 7.4 (HBS) at 37 °C by vortex mixing for 15 sec twice. The liposomal dispersion was then allowed to hydrate by equilibrating the dispersion at 37 °C for 15 min before further characterization. In addition, the spray-dried lysozyme-loaded liposomal powders with HPC only and HPC/Chol in the ratio chosen from Section 6

were also reconstituted with HBS at 60 °C for 15 min to study the effect of temperature above T_m on the properties of the reconstituted liposomes.

9.2 Optical Microscopy

All liposome formulations before spray-drying and the reconstituted liposomes from the spray-dried liposomal powders were diluted to 1 %w/w of lipid with HBS and then investigated for the morphology characteristics by optical microscopy viewed under plain and polarizing filters by Olympus optical microscope (x400 magnification) and Nikon optical microscope (x1000 magnification), respectively.

9.3 Transmission Electron Microscopy

The morphological examination of some extruded liposomes was conducted by transmission electron microscopy (TEM). A drop of liposome suspension was applied to a copper grid covered with a thick Formvar® film. After leaving for 5 min to allow adsorption of liposomes to the grid, the excess was removed by filter paper. The samples were negative stained with 1 %w/v phosphotungstic acid (PTA). Then the grid was air-dried for approximately 10 min and examined under a transmission electron microscope.

9.4 Size and Size Distribution of Liposomes

The size distribution of liposomes prior to spray drying was determined using dynamic light scattering with Brookhaven Zetaplus. The liposomal vesicles were diluted in Ultrapure® water. The effective diameter and polydispersity were recorded for liposome size before spray-drying. Laser diffraction with Mastersizer 2000 was used to measure the size distribution of the reconstituted liposomes from the spray-dried powders. The samples were sonicated to reduce aggregation of vesicles before measuring during 2 min and 5 min for the formulations with HPC and HPC/Chol,

respectively. The vesicle size was described by the volume mean diameter ($D_{[4,3]}$). The polydispersity of the vesicles was expressed by the span.

9.5 Thermal Properties of Liposomes

The gel-to-liquid crystalline phase transition temperatures (T_m) of the initial liposomes and the reconstituted liposomes from the spray-dried liposomal powders with the different HPC/Chol molar ratios were determined by differential scanning calorimetry (DSC). About 10 µL of the initial liposomes with 10 %w/w lipid was accurately weighed into 40 µL hermetically sealed aluminium pans and analyzed. A reference pan was prepared with 10 µL of Ultrapure® water. For the rehydrated liposomes, the spray-dried powders were reconstituted with HBS at 37 °C for 15 min to obtain 10 %w/w lipid. About 10 mg accurately weighed samples were loaded into aluminium pans and HBS was used as the reference. DSC runs were conducted from 25 to 60 °C at a rate of 5 °C/min. The scan rate used for these thermograms was chosen as a compromise between signal-to-noise ratio and resolution. High scan rates give high signal-to-noise ratios, but they also broaden peaks. Low scan rates are used for resolving narrow peaks, but one suffers decreased peak height (Koyama et al., 2000). Thermodynamic data were analyzed with Mettler-Toledo STAR^e Software 8.10 to determine the peak temperature (T_m). Enthalpies were normalized with respect to lipid weight in the sample. The instrument was calibrated with indium (T_{onset} = 156.67 °C; enthalpy = 28.66 J/g). All measurements were performed in triplicate. The mean and standard deviation of the peak temperature (°C) and enthalpy change ΔH (J/g) were reported.

9.6 Entrapment Efficiency

For determination of lysozyme entrapment in liposomes prepared by the DRV method, the liposomes with entrapped lysozyme were separated from unencapsulated lysozyme by gel filtration chromatography. A column of Sephadex G-75 was prepared in a burette (25 cm x 1 cm) and equilibrated with Ultrapure® water. The column was pre-saturated with empty liposomes composed of the same lipid

composition as the sample (about 20 mg of lipid in the form of the extruded liposomes per 10 g of gel (New, 1989)). The extruded DRV liposome dispersion was diluted to 1 % lipid with Ultrapure® water and then 1 mL of the diluted dispersion was loaded on top of the column. Liposomes were eluted from the column with Ultrapure® water at 0.8 mL/min flow rate. Fractions were collected for 12 grams. Liposomal dispersion with entrapped lysozyme was eluted at gram of 6-8, then diluted to suitable concentration and assayed for the amounts of lysozyme and phospholipid by QuantiPro bicinchoninic acid (QPBCA) protein assay kit and Bartlett method, respectively.

For the reconstituted liposomes from the spray-dried liposomal powders, lysozyme entrapment was investigated by dialysis technique because of the large liposome size which was not able to use gel filtration. The spray-dried lysozyme-loaded liposomal powders were reconstituted with HBS at 37 °C to obtain lipid concentration of 5% as in Section 8.1 and then diluted to 1% lipid with HBS prior to dialysis through cellulose ester membrane (MWCO 100K Da). Dialysis membrane packaged wet in 0.1 % sodium azide was soaked in a large volume of DI water for 30 min to remove sodium azide and then kept into HBS for 30 min at room temperature. After that, 2.4 mL of the reconstituted liposome dispersion was loaded into dialysis tubing which was then placed into 900 mL of a cold HBS. The medium was stirred with a magnetic stirrer at 4 °C for 24 hours. The dialyzed liposome dispersion was analyzed for the amounts of lysozyme and phospholipid by QPBCA assay and the Bartlett method, respectively.

Liposome dispersion with entrapped lysozyme was treated with Triton X-100 solution at 65 °C for 5-15 min to disrupt the liposome vesicles and diluted to 1 mL with Ultrapure® water or HBS for protein assay. The amounts of Triton X-100 used for liposomes containing 0.1 gram of HPC and HPC/Chol were about 0.05 g and 2.5-4 g, respectively. The amount of lysozyme was determined by a colorimetric assay using a QPBCA protein assay kit. All measurements were performed in quadruplicate. Entrapment efficiency of lysozyme in liposomes was calculated as the amount of lysozyme (μg) per the amount of lipid (mg).

9.7 Quantitative Analysis of Lysozyme by QPBCA (Product information of Sigma)

The principle of the bicinchoninic acid (BCA) assay relies on the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+}. The extent of reduction is proportional to the protein present. It has been shown that cysteine, cystine, tryptophan, tyrosine, and the peptide bond are able to reduce Cu^{2+} to Cu^{1+}. BCA forms a purple-blue complex with Cu^{1+} in alkaline environments, thus providing a basis to monitor the reduction of alkaline Cu^{2+} by proteins. The QPBCA assay kit is based on the same principle as the BCA kit, except that the QPBCA kit gives a linear response from 0.5 to 30 μg/mL of protein while the BCA kit gives a linear response at higher concentrations of protein (200-1000 μg/mL). Reaction schematic of BCA-containing protein assay is illustrated in Figure 17.

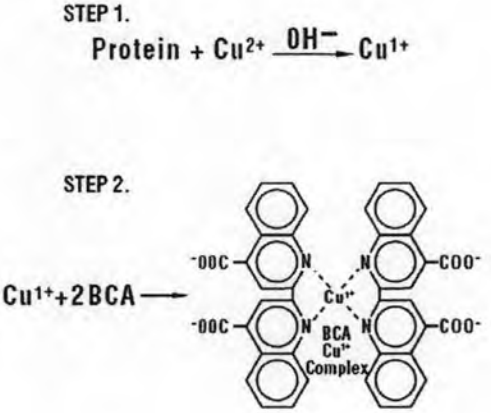

Figure 17 Reaction schematic for the BCA-containing protein assay

The amount of lysozyme was determined by QPBCA assay kit. Standard curves were generated using known concentrations of lysozyme (2-25 μg/ml). Lysozyme (5 mg) was weighed into a 5-mL volumetric flask, then dissolved and diluted to volume with Ultrapure® water. For working standard solutions, 10, 25, 50, 75, 100 and 125 μL of the stock lysozyme solution were transferred into separate 5-

mL volumetric flasks, and diluted to 5 mL with Ultrapure® water. For determination of lysozyme entrapment, HBS was used as the medium instead of Ultrapure® water. The effect of lipid and Triton X-100 on the quantification of lysozyme was compensated by performing blank subtraction and by using lysozyme standards dissolved in the corresponding lipid and Triton X-100 solution. Table 6 summarizes the final concentrations of lipid and Triton X-100 used in standard solutions for lysozyme assay in the study of remaining biological activity and entrapment efficiency. The liposomal samples treated with Triton X-100 were diluted to appropriate concentration of lysozyme. Aliquots (150 μL) of the blank, the lysozyme standards and the samples were pipetted into a 96-well plate in triplicate. The QPBCA working reagent (150 μL) made up according to manufacturer's instructions was added into each well. After incubation at room temperature for about 16 hours, the absorbance was measured with a microplate reader at measurement filter 550 nm without a reference filter. The net absorbance at 550 nm (Net A_{550}) is found by subtracting the absorbance (A_{550}) of the blank from the recorded A_{550} values for the standards and samples. The lysozyme concentration was determined by comparison of the net A_{550} of the unknown samples to the standard curve prepared using the lysozyme standards. The total amount of lysozyme present in the unknown samples was calculated by using equation 8.

$$\mu g \text{ of lysozyme} = (\mu g/mL \text{ of sample}) \times (mL \text{ of sample}) \times (\text{dilution factor}) \quad (8)$$

Table 6 Summary of the final concentrations of lipid and Triton X-100 in standard solutions for lysozyme assay in the study of remaining biological activity and entrapment efficiency

Composition	Final concentration (%w/v)			
	Biological activity		Entrapment efficiency	
	HPC	HPC/Chol	HPC	HPC/Chol
Lipid	0.01	0.01	0.2	0.05
Triton X-100	0.005	0.2	0.1	2

Because Triton X-100 and HBS had a high impact on QPBCA protein assay, validation of QPBCA protein assay was performed in the HBS with maximum amounts of lipid and detergent used for entrapment efficiency study. HBS containing 0.05 %w/v of HPC/Chol (8:2) and 2 %w/v of Triton X-100 was used as the vehicle for preparing standard lysozyme solutions as well as the blank. Lysozyme (5 mg) was accurately weighed into a 5-mL volumetric flask, then dissolved and diluted with HBS. Six dilutions were made with HBS composed of the lipid and Triton x-100 in six 5-mL volumetric flasks to obtain standard solutions with concentrations ranging from 2 to 25 μg/mL. The dispersion was warmed at 65°C for 15 min to obtain clear solution. The validation was performed under the following conditions (ICH Topic Q 6 B, 1999; USFDA, 2001).

9.7.1 Linearity

The linearity was determined by plotting the standard curve between the net A_{550} and the concentration of lysozyme (μg/mL). The standard curve was fitted using linear regression analysis. The coefficient of determination (R^2) and the equation for the line were calculated.

9.7.2 Accuracy

The evaluation of accuracy of lysozyme assayed by QPBCA assay was done by analyzing percent recoveries of the five determinations (from five stock solutions) per concentration of 5, 15, and 25 μg/mL lysozyme solution. Analytical recovery in percent of each determination was calculated by dividing the concentration fitted from a calibration curve by the known concentration. The mean, standard deviation and percent coefficient of variation (% CV) were determined. The mean value should be within 15 % of the actual value.

9.7.3 Precision

9.7.3.1 Within-run precision

The within-run precision was evaluated by analyzing the fitted concentration of lysozyme of five determinations per concentration of 5, 15, and 25 μg/mL lysozyme solution measured within the same day. The mean, standard deviation and percent coefficient of variation (% CV) of each concentration were determined. The precision determined at each concentration level should not exceed 15 % of the coefficient of variation.

9.7.3.2 Between-run precision

The between-run precision was evaluated by analyzing the fitted concentration of lysozyme of five sets of 5, 15, and 25 μg/mL lysozyme solution measured on different days. The mean, standard deviation and percent coefficient of variation (% CV) of each concentration were determined. The precision determined at each concentration level should not exceed 15 % of the coefficient of variation.

10. Experimental Design and Optimization of Spray Drying Conditions for Lysozyme-loaded Liposomal Powder Formulation

The formulation of the spray-dried lysozyme-loaded liposomal powders obtained from Section 6 was selected for studying the effects and relationships among the spray drying conditions on the properties of the powders using factorial design and response surface methodology.

A 2^3 full factorial design was built to evaluate main effects and interactions of the three spray drying condition factors (Table 7) on the four responses, namely process yield, moisture content, particle size and entrapment efficiency. First order models were employed for the factorial design. The model presented was evaluated in term

of statistical significance using analysis of variance (ANOVA). Three center points were added to the design in order to allow a better estimate of the experimental error and to evaluate possible response curvature. Eleven batches, one preparation per factorial point and three replicates of center point, of the spray-dried lysozyme-loaded liposomal powders were prepared. The complete set up of the 2^3 factorial design and center point is reported in Table 8. The curvature is measured by difference between the average of the center points and the average of the factorial points. In the ANOVA table for factorial model, if the quadratic curvature was statistically significant at $P < 0.05$, the quadratic response surface methodology would be used for further optimization of the spray drying condition factors.

Table 7 Values of factors and levels for factorial design and central composite design

	Factor Level		
Factor	-1	0	+1
A: Inlet temperature (T, °C)	110	130	150
B: Pump speed (P, %) (mL/min)	5 (1.15)	10 (2.3)	15 (3.45)
C: Total solid content (C, %w/w)	2.975	5.775	8.575

The response surface methodology was performed by central composite design for optimization of the spray-drying condition factors. This design is suitable for exploring quadratic response surfaces and constructing second-order polynomial models. Axial runs are added to allow the quadratic terms to be incorporated into the model. Rotational designs were selected to obtain constant variances of the predicted responses at all points that were equal from the centre of the design (Montgomery, 2005). This design provides equal precision of estimation in all directions. The distance α of the axial runs from the design center for rotatability is 1.682 [α = (no. of point in the factorial design)$^{1/4}$]. The complete design consisted of 17 experimental points that included eight factorial points, three replications at the center point, and six axial points as presented in Tables 8 and 9. The nonlinear quadratic model generated by the design is as follows:

$$Y = \beta_0 + \beta_A A + \beta_B B + \beta_C C + \beta_{AB} A*B + \beta_{AC} A*C + \beta_{BC} B*C + \beta_{AA} A^2 + \beta_{BB} B^2 + \beta_{CC} C^2 \quad (9)$$

where Y is the measured response (dependent variable) associated with each factor-level combination. β_0 is an intercept and β_i are the multiple regression coefficients representing estimates of main effects and interactions. In particular, β_A, β_B, β_C represent main effects, β_{AB}, β_{AC}, β_{BC}, β_{AA}, β_{BB}, and β_{CC} represent two-factor interactions. A, B, and C are the independent factors studied. Values and levels of the independent factors studied are listed in Table 8. Software package, Design-Expert 7.1.4 (Stat-Ease, Inc., Minneapolis, USA) which can be downloaded for free trial for 45 days (in this study during 2008 Mar, 20–2008 May, 3) was used for regression analysis of experimental data and to plot response surface. Analysis of variance (ANOVA) was used to estimate the statistical parameters. The extent of fitting the experimental results to the polynomial model equation was expressed by the coefficient of determination, R^2. F-test was used to estimate the significance of all terms in the polynomial equation within 95% confidence interval.

Contour plots of central composite design describing the variable effects on the response were built to define the proper conditions for improvement of responses. In addition, model prediction efficiency was assessed by preparing the spray-dried liposomal powders with the optimum condition giving high yield, low moisture content, low particle size, and high entrapment efficiency as predicted from the contour plots. The experiment was performed in three batches.

Table 8 Matrix of experiments of the factorial design and the center point

Experiment	Factor		
	T (°C)	Pump speed (%)	Total solid content (%)
1	(-) 110	(-) 5	(-) 2.975
2	(+) 150	(-) 5	(-) 2.975
3	(-) 110	(+) 15	(-) 2.975
4	(+) 150	(+) 15	(-) 2.975
5	(-) 110	(-) 5	(+) 8.575
6	(+) 150	(-) 5	(+) 8.575
7	(-) 110	(+) 15	(+) 8.575
8	(+) 150	(+) 15	(+) 8.575
9	(0) 130	(0) 10	(0) 5.775
10	(0) 130	(0) 10	(0) 5.775
11	(0) 130	(0) 10	(0) 5.775

Experiments 1-8: factorial points

Experiments 9-11: center points

Table 9 Values attributed to the factors for the additional experiments of the central composite design

Experiment	Factor		
	T (°C)	Pump speed (%)	Total solid content (%)
12	(-1.682) 96	(0) 10	(0) 5.775
13	(+1.682) 164	(0) 10	(0) 5.775
14	(0) 130	(-1.682) 2	(0) 5.775
15	(0) 130	(+1.682) 18	(0) 5.775
16	(0) 130	(0) 10	(-1.682) 1.05
17	(0) 130	(0) 10	(+1.682) 10.5

Experiments 12-17: axial points

11. Other Properties of the Lysozyme-loaded Liposomal Powders Prepared from the Optimum Spray Drying Condition

11.1 Lysozyme Content

The amount of lysozyme in the spray-dried lysozyme-loaded liposomal powders was determined by QPBCA protein assay kit. About 10 mg of the powders was accurately weighed into 5-mL volumetric flask, then dispersed and vortexed with 1 mL Ultrapure® water. The liposomal dispersion was lysed with 1 mL of 10 %w/w Triton X-100 and warmed at 65°C for 15 min. The clear solution was adjusted to volume with Ultrapure® water. This solution (0.5 mL) was pipetted and transferred into a 5-mL volumetric flask, then adjusted with Ultrapure® water and assayed by QPBCA assay. The measurement was performed in 6 replicates. Blank and standard solutions were prepared with aqueous solution containing 0.01 % of HPC/Chol (8:2) and 0.2 % of Triton X-100. The amount of lysozyme was determined from the standard curve. The lysozyme content and loading efficiency were calculated using equations 10 and 11, respectively. The mean value and standard deviation of lysozyme content and loading efficiency were reported.

$$\text{Observed content (\%w/w)} = \frac{\text{Assayed amount of lysozyme}}{\text{Amount of the spray-dried powders}} \times 100 \quad (10)$$

$$\text{Loading efficiency (\%)} = \frac{\text{Observed content (\%w/w)}}{4.76\ \text{\%w/w}} \times 100 \quad (11)$$

The figure 4.76 %w/w was the theoretical content of lysozyme in the spray-dried powders composed of lipid, mannitol and lysozyme in the weight ratio of 1:1:0.1.

11.2 Density and Flow Characteristics

The apparent particle density (excluding open pores, but including closed pores) of the spray-dried formulations prepared from the factorial design was determined using a helium pycnometer with 0.3-0.5 g of sample placed in the sample holder. The measurement was performed with 5 purges.

Bulk and tapped densities of the spray-dried liposomal powders prepared with the optimum condition were measured. The apparent volume occupied by about 3 g of powder mass, carefully placed into a 10-mL graduated cylinder, was determined before and after packing. The cylinder was tapped 1000 times using the equipment developed by Department of Industrial Pharmacy in order to obtain the closet packed densities (Council of Europe, 2005). Bulk and tapped density values allow the determination of the Carr's compressibility index by the formula:

$$\text{Carr's index (\%)} = \frac{\text{Tapped} - \text{Bulk}}{\text{Tapped}} \times 100\ \% \qquad (12)$$

11.3 Theoretical Aerodynamic Diameter

An equivalent diameter of particular importance to respiratory deliery is the aerodynamic diameter. Aerodynamic diameter is the diameter of a unit-density sphere that has the same settling velocity as the measured particle or the diameter of water droplet having aerodynamic property equivalent to the measured particle regardless of shape, density and physical size of the measured particle (Hind, 1999). The theoretical aerodynamic diameter (D_{aer}) of the spray-dried lysozyme-loded liposomal powders can be calculated using equation 13:

$$D_{aer} = \frac{D_{0.5}\sqrt{\rho / \rho_0}}{\gamma} \qquad (13)$$

where $D_{0.5}$ is the diameter at the 50% point of the entire volume distribution (MMD), ρ is tapped density, ρ_0 is 1 g/cm^3, and γ is a shape factor (for a spherical particle, γ =1).

11.4 Scanning Electron Microscopy of the Reconstituted Liposomes

The morphology of the reconstituted liposomes obtained from the optimum formulation was explored by a scanning electron microscope (SEM). The reconstituted liposomes were dialyzed through cellulose ester membrane (MWCO 100KDa) to remove free lysozyme and mannitol. A few drops of liposome suspension were smeared on a slide and air-dried for approximately 5 min. The sample was stained with 1 % osmium tetroxide for 1 h. Then the slide was immersed in DI water for 5 min. This step was repeated twice. After that, the sample was dehydrated with absolute ethanol for 5 min for 2 times. The sample was dried using a critical point dryer and finally coated with gold.

12. Statistical Analysis

All data are expressed as mean $\pm$ standard deviation (SD). Statistical analysis of the chemical stability of phospholipid was performed using analysis of variance (ANOVA) and Tukey's multiple range test. *P* values less than 0.05 was considered significantly different from the control. Design-Expert version 7.1.4 software was applied for experimental design to study the effects and relationships among various spray drying factors on the properties of the spray-dried lysozyme-loaded liposomal powders.