AAPS PharmSciTech, Vol. 11, No. 2, June 2010 (© 2010)
DOI: 10.1208/s12249-010-9442-8

Research Article

Effect of Cholesterol on the Properties of Spray-Dried Lysozyme-Loaded Liposomal Powders

Dusadee Charnvanich,[1] Nontima Vardhanabhuti,[1] and Poj Kulvanich[1,2]

Received 5 February 2010; accepted 20 April 2010; published online 13 May 2010

Abstract. The influence of cholesterol (Chol) in the liposomal bilayer on the properties of inhalable protein-loaded liposomal powders prepared by spray-drying technique was investigated. Lysozyme (LSZ) was used as a model protein. Feed solution for spray drying was prepared by direct mixing of aqueous solution of LSZ with mannitol solution and empty liposome dispersions composed of hydrogenated phosphatidylcholine and Chol at various molar ratios. The spray-dried powders were characterized with respect to morphology, thermal property, and crystallinity using scanning electron microscopy, differential scanning calorimetry, and X-ray diffraction, respectively. Most formulations gave slightly aggregated, spherical particles, and percentage yields of the spray-dried powders decreased with increasing Chol content. Degree of particle aggregation depended on the powder composition. The powders spontaneously formed liposomes which efficiently entrapped LSZ after reconstitution with HEPES buffered saline (HBS) at 37°C. Lysozyme entrapment efficiency and size distribution of the reconstituted liposomes were evaluated after the powders were reconstituted with HBS. Increasing Chol content resulted in a decrease in size of the reconstituted liposomes and an increase in entrapment efficiency of LSZ. These results correlated with thermal behaviors of the reconstituted liposomes. Biological activity of LSZ was not affected by the spray-drying process. It was also demonstrated that LSZ-loaded liposomal powders could be produced without the need to preload the LSZ into liposomes prior to spray-drying process.

KEY WORDS: cholesterol; liposomes; lysozyme; spray-dried powders.

INTRODUCTION

Liposomes have been studied in pulmonary drug delivery for years because they are prepared with phospholipids endogenous to the lung surfactants and can significantly alter the pharmacokinetics of entrapped drugs (1). Some protein/peptide drugs have been incorporated into liposomes to enhance their pulmonary delivery (2,3). Nebulizers have been studied extensively for the delivery of liquid-based liposomes (4,5). However, stability and drug leakage are problems associated with delivery of liposomes by nebulization (6). Dry powders have been considered a promising dosage form because of the drug stability and the propellant-free design (7). When applied to liposomes, dried liposomes would also render liposome stability upon storage (8). In addition, liposomal dry powders have many advantages for pulmonary administration with respect to protection of drug integrity, controlled delivery, increased potency, prevention of local irritation, reduced toxicity, and immunological adjuvant activity (9–11).

Spray-drying technique has been considered a modern one-step process for the production of small particles (<5 μm) for pulmonary administration (12). Spray drying is a very simple and industrially applicable method for bulk preparation of lipid mixture for secondary liposome production (13). Moreover, the spray-drying technique was used to dry drug-loaded liposomes in order to retain their contents during storage, to protect the drug integrity, and to control the release of peptides/proteins or other drugs (9,14–16).

The feasibility of preparing liposomal powders that spontaneously form liposomes in an aqueous environment, thereby creating reservoirs for the encapsulation of drugs, has been investigated (13,17,18). Phospholipids can orient into a liposomal configuration through a spontaneous, entropic process in a water-rich environment. Such conditions exist in the airways of the respiratory tract. Therefore, it is feasible that spontaneous liposome formation may occur following lung deposition of liposomal dry powders (19). Furthermore, the presence of drugs within particles should result in the creation of a reservoir that might improve pulmonary delivery of the drug in the liposomal form.

[1] Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, 10330, Thailand.
[2] To whom correspondence should be addressed. (e-mail: kpoj@chula.ac.th)

ABBREVIATIONS: Chol, Cholesterol; DPPC, Dipalmitoylphosphatidylcholine; DRV, Dehydrated-rehydrated vesicles; DSC, Differential scanning calorimetry; EE, Entrapment efficiency; HBS, HEPES buffered saline; HPC, Hydrogenated soybean phosphatidylcholine; LSZ, Lysozyme; M, Mannitol; MMD, Mass median diameter; PC, Phosphatidylcholine; QPBCA, QuantiPro bicinchoninic acid; SEM, Scanning electron microscopy; T_d, Denaturation melting temperature; T_m, Phase transition temperature; XRPD, X-ray powder diffraction.

Cholesterol has been incorporated in many liposomal formulations because of its effect on the physical properties of bilayers. This additive regulates fluidity and permeability of lipid bilayers (20). Incorporation of Chol into phosphatidylcholine (PC) bilayers in fully hydrated state results in a broadening or elimination of the gel-to-liquid crystalline phase transition peak by an increase (or a decrease) in the order of the phospholipid hydrocarbon chains above (or below) the phase transition temperature (21). However, the effect of Chol on the physicochemical properties of dehydrated liposomes has been scarcely reported, especially liposomes dehydrated by spray-drying process (22,23).

In this research, therefore, the influence of Chol on the physicochemical properties of the spray-dried liposomal powders was investigated. The spray-drying technique was studied to dry preformed empty liposomes simultaneously with protein and additive solutions into powders. The powders spontaneously formed liposomes and entrapped the protein into liposomal structure after reconstitution with aqueous buffer solution. The properties of the reconstituted liposomes were also investigated. Lysozyme was chosen as a model protein because it is commercially available and well characterized (24). Mannitol was used as an additive because it could be prepared into fine powders by spray-drying technique. In addition, mannitol is reported to be the best candidate for dry powder inhalation formulations (25). The results could be useful in the development of spray-dried liposomal powders for entrapment and delivery of protein/peptide drugs to the alveolar region of the lung.

MATERIALS AND METHODS

Materials

Hydrogenated soybean phosphatidylcholine (PHOSPHOLIPON® 90H) was a kind gift from Nattermann Phospholipid GmbH (Cologne, Germany). Chicken egg white lysozyme in lyophilized form (95% purity), cholesterol, and QuantiPro bicinchoninic acid (QPBCA) reagents were purchased from Sigma-Aldrich Co. (USA). D-Mannitol was obtained from Merck (Germany). All chemicals were of an analytical grade and used as received.

Preparation of Empty Liposomes

For the empty liposomes composed of HPC and Chol, the film hydration method (26) was used to achieve a homogeneous distribution of HPC and Chol over the bilayer. HPC and Chol at the molar ratios of 9:1, 8:2, and 7:3 were dissolved in a mixture of chloroform and methanol (1:3). The mixture was dried to form a thin lipid film using a rotary evaporator (Buchi R-215, Switzerland) under controlled vacuum at 45°C. The lipid film was hydrated with Ultrapure® water at 80°C for 1 h. The liposomal dispersion was passed through a high-pressure homogenizer (EmulsiFlex−C5®, Avestin Inc., Canada) preheated at 65°C for four cycles at 100 MPa for size reduction. Then, the dispersion was extruded through 0.2-μm polycarbonate filters under nitrogen pressure for ten cycles at 65°C in a Liplex® Extruder (Northern Lipids, Canada) to obtain homogeneous vesicles. The extruded liposomes were kept at 4°C before spray drying.

The control empty liposomes with only HPC were produced by the one-step method (27). HPC was dispersed in Ultrapure® water under mechanical stirring at 65°C for 2 h. Then, the lipid dispersion was passed through a high-pressure homogenizer preheated at 65°C for four cycles at 100 MPa. The solid lipid particles are broken up and undergo forced hydration and self-aggregation into homogeneous dispersions of liposomes without using organic solvents (27). Finally, the liposomal dispersion was extruded through 0.2-μm polycarbonate filters similar to the preparation of liposomes with Chol.

Preparation of Spray-Dried Powders

The empty liposomal dispersion was mixed with an aqueous solution of mannitol (M) and LSZ at the lipid/M/LSZ weight ratio of 1:1:0.1. The total amount of lipid and mannitol in the mixture was 10% (*w*/*w*). The mixture (100 g) was spray-dried using a B-290 Buchi spray-dryer (Switzerland). The feed mixture was pumped into the drying chamber and pneumatically atomized through a 0.7-mm nozzle. The spray-drying conditions were set as follows: pump speed of 10% (2.3 mL/min), inlet temperature of 120°C (outlet temperature of 74°C to 80°C), airflow rate of 357 Normlitre/h, and aspirator setting of 100%. The spray-dried product was stored in a desiccator containing silica gel at room temperature prior to characterization.

Characterization of Spray-Dried Powders

Yield

The yield was calculated by comparing the resultant powder amount (in the collector and in the cyclone) after spray-drying process with the total amount of initial solids as in Eq. 1.

$$\%\ \text{Yield} = \frac{\text{Weight of spray} - \text{dried powders}}{\text{Total weight of solids added initially}} \times 100\% \quad (1)$$

Residual Moisture Content

The residual moisture content of the spray-dried liposomal powders was determined using a Karl Fischer titrator (720 KFS Titrino, Metrohm Ltd., Switzerland).

Surface Morphology

The surface morphology of the spray-dried liposomal powders was examined by scanning electron microscopy (JSM-5800LV, JEOL, Japan). Samples were fixed to stubs using double-sided tape and then viewed using an accelerating voltage of 10 kV.

Particle Size and Size Distribution

The particle size of the spray-dried powders was determined by laser diffraction method (Mastersizer® S, Malvern Instruments Inc., UK). The samples were dispersed in 2-propranol saturated with the same additives as those of the samples. Particle size distributions were expressed in terms of mass median diameter (MMD, $D_{0.5}$) and span. 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 diameters at 90%, 50%, and 10% cumulative volumes, respectively. The measurements were recorded in triplicate.

Thermal Property

Thermal properties of the spray-dried powders were analyzed by differential scanning calorimetry (DSC 822e, Mettler Toledo, Switzerland). The sample (4–7 mg) was placed in an aluminum pan with vented lids and heated from 25°C to 250°C at the scan rate of 10°C/min to determine the peak transition temperature (T_m) and from 25°C to 80°C at 1°C/min to determine the transition enthalpy of lipid. An empty aluminum pan was used as a reference.

X-Ray Powder Diffraction

The crystallinity properties of the starting materials and the spray-dried powders were determined by X-ray diffractometer (model D8 discover, Bruker AXS, USA), 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°.

Reconstitution of Spray-Dried Liposomal Powders

The spray-dried liposomal powders were reconstituted to obtain liposomal dispersion with a lipid concentration of 5% (*w*/*w*) with HBS, pH 7.4, at 37°C by vortex mixing. The reconstituted liposomes were then allowed to hydrate by equilibrating the dispersion at 37°C for 15 min without shaking.

Characterization of Reconstituted Liposomes

Photomicrography

The morphology characteristics of the reconstituted liposomes from the spray-dried formulations were investigated by optical microscopy under plain and polarizing filters using Olympus (model IX51, Olympus, Japan) and Nikon optical microscopes (model E200, Nikon, Japan), respectively.

Size of Liposomes

The size distribution of empty liposomes before spray drying was determined using dynamic light scattering method (Zetaplus®, Brookhaven Instrument Co., USA). The effective diameter and polydispersity index were recorded for the initial empty liposomes. For the reconstituted liposomes, laser diffraction method (Mastersizer® 2000, Malvern Instruments Inc.) was used to measure their size distribution. The liposomal samples were dispersed in distilled water and sonicated to reduce aggregation of vesicles. The size distribution of the reconstituted liposomes was described by volume mean diameter ($D_{[4,3]}$) and span.

Entrapment Efficiency of LSZ in the Reconstituted Liposomes

Lysozyme entrapment in the reconstituted liposomes was determined by dialysis and ultracentrifugation techniques. For the dialysis technique, the reconstituted liposomes were loaded into dialysis tubing with a molecular weight cutoff of 100 kDa (Spectrum Laboratories Inc., USA). The dialysis tubing was then placed into cold HBS which was stirred with a magnetic stirrer at 4°C for 24 h. The dialyzed liposomal dispersion was analyzed for the amounts of LSZ and phospholipid by a QPBCA protein assay kit and the Bartlett method (28), respectively. Lysozyme entrapment in liposomes was calculated as the amount of LSZ (g) per the amount of lipid (g). Entrapment efficiency (EE) was defined as the percentage of the theoretical LSZ entrapment. The theoretical value was 0.1 g LSZ/g lipid. All measurements were performed in quadruplicate.

For the ultracentrifugation method, an aliquot of the reconstituted liposomes was diluted fivefold with HBS and then centrifuged at 36,000 rpm for 30 min at 4°C using an

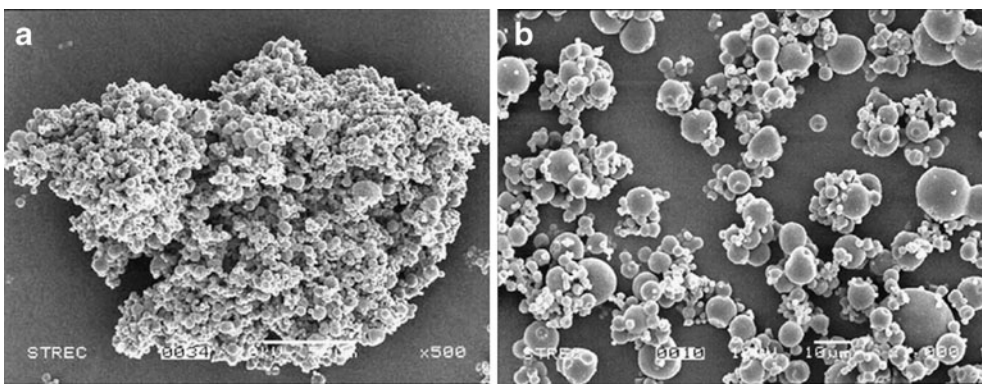

Fig. 1. SEM images of the spray-dried liposomal powders **a** without mannitol (magnification ×500; *scale bar*, 50 μm) and **b** with mannitol in the HPC/M ratio of 1:1 (magnification ×1,000; *scale bar* 10 μm)

Table I. Characteristics of the Spray-Dried LSZ Powders Prepared with Different Formulations

Formulation	Yield (%)	Moisture content (%, mean ± SD)	Physical appearances	$D_{0.5}$ (μm, mean ± span)
Spray-dried LSZ	65.31	13.90±1.41	Aggregates	ND
M/LSZ	74.96	0.90±0.01	Fine powders	ND
HPC/M/LSZ	68.85	3.85±0.06	Fine powders	7.90±1.13
HPC/Chol9:1/M/LSZ	75.10	3.78±0.31	Fine powders	6.59±2.60
HPC/Chol8:2/M/LSZ	60.28	4.07±0.16	Fine powders	6.36±1.67
HPC/Chol7:3/M/LSZ	52.01	2.91±0.18	Aggregates	ND

Chol cholesterol, *HPC* hydrogenated soybean phosphatidylcholine, *LSZ* lysozyme, *M* mannitol, *ND* not determined

L-80 Beckman® ultracentrifuge with a 90Ti rotor (Beckman Coulter, Palo Alto, CA, USA). The pellets were redispersed with HBS. The dispersion was analyzed for the amounts of LSZ and phospholipid as previously described.

Thermal Property of the Reconstituted Liposomes

Thermal properties of the reconstituted liposomes were analyzed by DSC. The spray-dried powders were reconsti-

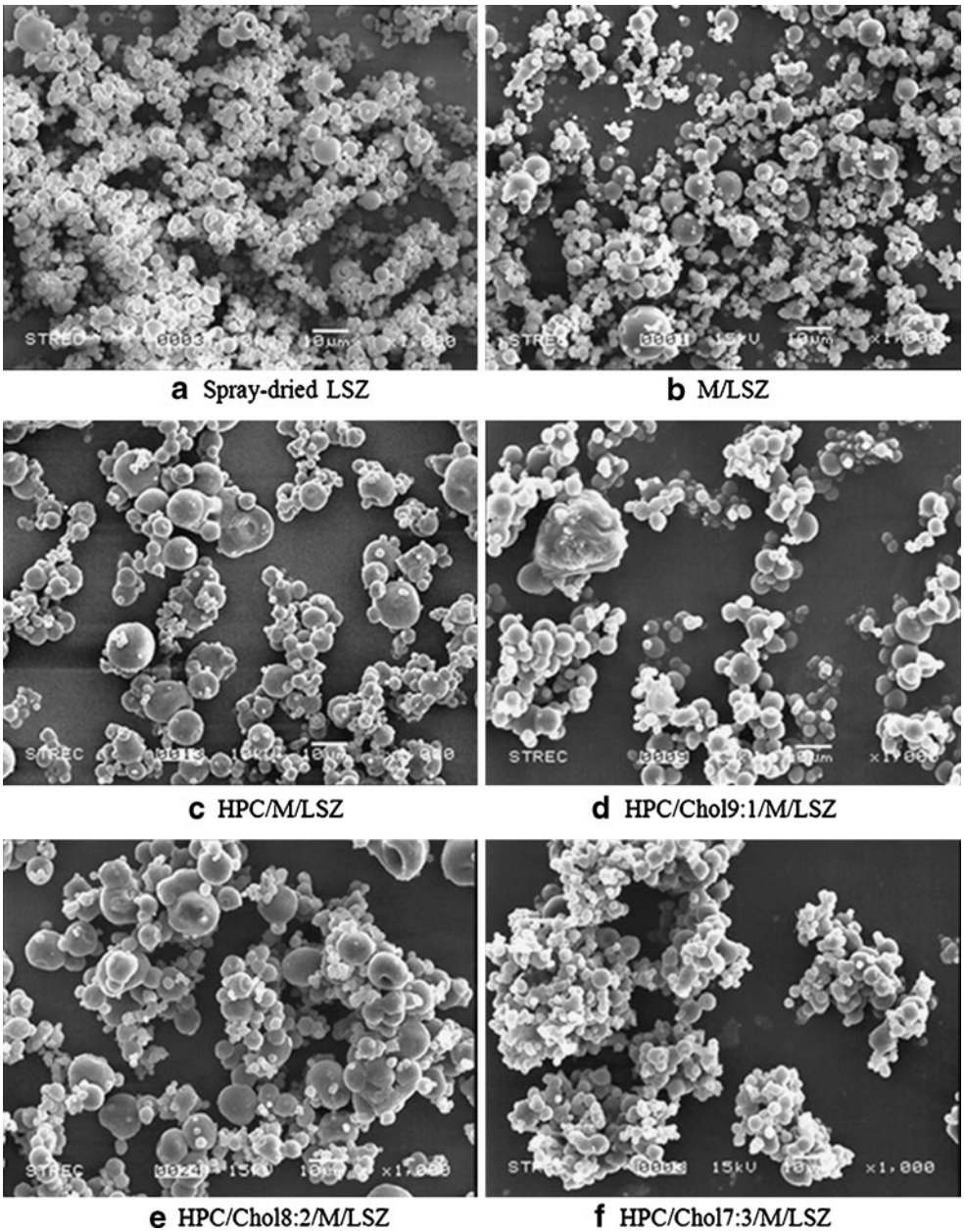

Fig. 2. SEM images of the spray-dried LSZ powders prepared with different formulations (magnification ×1,000; *scale bar* 10 μm)

tuted with HBS to obtain a dispersion with 10% (*w*/*w*) lipid at 37°C for 15 min. The dispersion was accurately weighed (about 10 mg) and loaded into hermetically sealed aluminum pans, and HBS was used as a reference. DSC runs were conducted from 25°C to 60°C at a rate of 5°C/min. Thermodynamic data were analyzed with Mettler-Toledo STAR^e Software 8.10 to determine the T_m. Transition enthalpies were normalized with lipid weight in the sample.

Biological Activity of Lysozyme

LSZ activities in the spray-dried formulations were measured by monitoring the hydrolysis of a bacterial suspension of *Micrococcus lysodeikticus* in phosphate buffer (66 mM, pH 6.24) at 25°C (29). The spray-dried liposomal powders were reconstituted in Ultrapure® water. The LSZ activity was determined after liposomes were lysed with Triton X-100 and diluted with Ultrapure® water to obtain about 10 μg/mL of LSZ. The remaining activity of LSZ was calculated from the percentage of LSZ activity remaining after spray-drying process relative to activity of the starting material. Four replicates of each sample were analyzed.

Statistical Analysis

All data are expressed as mean ± SD. The reconstituted liposome size and LSZ entrapment efficiency were compared using analysis of variance and Student's *t* test. Differences were considered to be significant at a level of $P<0.05$.

RESULTS AND DISCUSSION

Preparation of Spray-Dried Powders

In this study, HPC was chosen for developing the spray-dried liposomal powders because it has high phase transition temperature, displays good stability, and gives high entrapment of peptides (30). The initial empty liposomes before spray drying had the effective diameter of 130–190 nm and polydispersity value of 0.15–0.19 nm, independent of the lipid composition. The nanometer size range of liposomes resulted in a homogenous distribution of a liposomal dispersion with other additives during spray-drying process.

The preliminary study showed that the spray-drying condition used had no statistically significant destructive effect on the chemical stability to both hydrolysis and oxidation of the major structural phospholipid when compared to the HPC starting material ($P>0.05$). This finding agrees with a previous study by Goldbach *et al.* (31).

The spray-dried liposomal powders without mannitol as an additive were large aggregates composed of partially fused spherical particles with a few microns in size and irregular surface (Fig. 1a). This may be attributed to the phase transition temperature (T_m) of lipid in the spray-dried form which was not sufficient to avoid softening of the powders during the spray-drying process and consequently did not prevent aggregation of particles. Addition of mannitol reduced the tendency of the particles to aggregate and decreased the amount of powders adhering to the cyclone wall of the spray dryer. Moreover, mannitol may cause looser packing of HPC hydrocarbon chains in the spray-dried form, leading to lower T_m of the lipid from about 84°C to 72°C (32). From the preliminary study, the best HPC/M weight ratio providing the suitable liposomal powders was 1:1. This ratio gave slightly aggregated spherical particles (Fig. 1b). In the present study, fine liposomal powders were not able to be prepared using sucrose, lactose, and trehalose as an additive by spray-drying technique because of their hygroscopic properties.

The formulation with the lipid/M weight ratio of 1:1 was used to investigate the influence of Chol incorporation into HPC liposomal bilayer on the properties of LSZ-loaded liposomal powders. The powders were prepared by mixing LSZ solution with liposomal dispersion and mannitol solution before spray drying to avoid possible instability of LSZ during liposomal process including high temperature. Spray drying of the dispersion containing empty liposomes, protein, and mannitol resulted in the formation of stacked lipid bilayers with intercalated solutes. Solute molecules were incorporated into resealed vesicles upon rehydration of the powders. The spray-dried LSZ without and with mannitol were also prepared to compare the properties of the spray-dried powders.

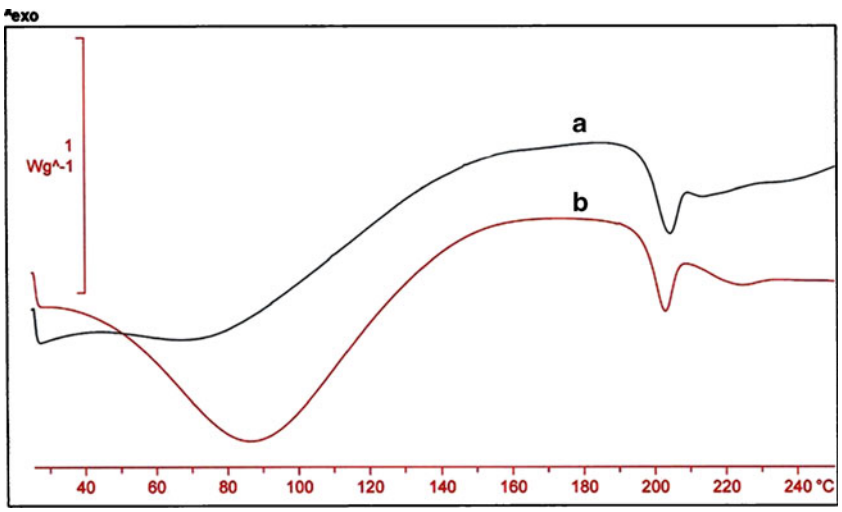

Fig. 3. DSC thermograms of **a** the LSZ starting material and **b** the spray-dried LSZ

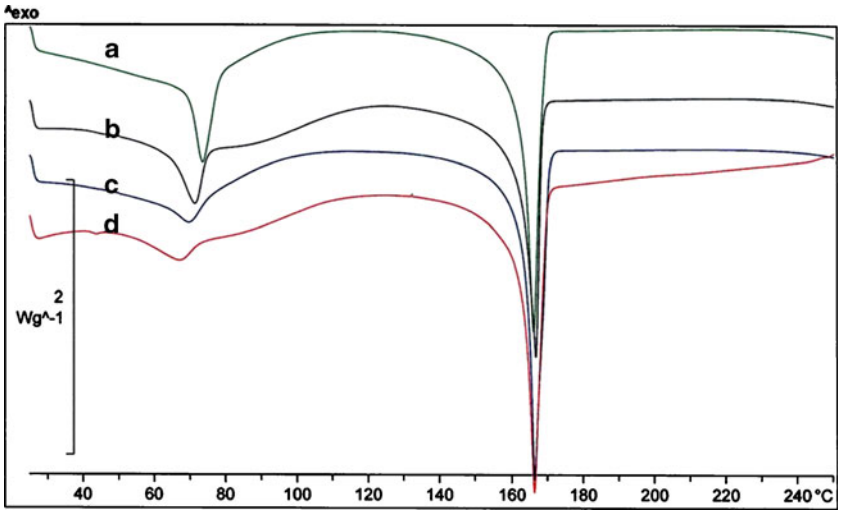

Fig. 4. DSC thermograms of the spray-dried LSZ-loaded liposomal powders with different HPC/Chol molar ratios: **a** 10:0; **b** 9:1; **c** 8:2; **d** 7:3

Characterization of the Spray-Dried Formulations

The characteristics of the spray-dried LSZ with/without mannitol and the spray-dried LSZ-loaded liposomal powders with various amounts of Chol are summarized in Table I. The spray-drying process yield decreased with higher Chol content. The moisture content of the spray-dried liposomal powders with various HPC/Chol ratios was not significantly different. The spray-dried pure LSZ powders had the highest moisture content. Addition of mannitol decreased the moisture content of the spray-dried LSZ powders. The $D_{0.5}$ of the spray-dried liposomal powders with HPC/Chol ratios of 10:0 to 8:2 ranged between 6.36 and 7.90 μm.

Scanning electron microscopy (SEM) images in Fig. 2 reveal that the morphology of the spray-dried powders was influenced by the formulation compositions. The spray-dried LSZ without and with mannitol were spherical in shape and had a few dimples on their surface (Fig. 2a, b). The spray drying of LSZ without mannitol gave loosely agglomerated powders, which might be due to high surface electrostatic force. The morphology of the liposomal powders with various HPC/Chol molar ratios was not significantly different, except the ratio of 7:3 which apparently gave the smaller size, but more aggregate, of the spray-dried powders. The increase in powder aggregation might be attributed to the more fluid state of the dehydrated membrane with high Chol content (22). Increasing mannitol proportion may be required to reduce aggregation of the spray-dried liposomal powders containing high Chol content. The role of mannitol in reducing liposomal powder aggregation was clearly evident in the formulation with only HPC (see Fig. 1).

Representative DSC curves of the LSZ starting material, the spray-dried LSZ, and the spray-dried LSZ-loaded liposomal powders with different HPC/Chol molar ratios are illustrated in Figs. 3 and 4, respectively. A summary of their thermal properties is presented in Table II. Thermal behavior of the spray-dried LSZ was similar to that of the starting material. The curves were characterized by two endotherms, one being very broad at about 55–100°C and a second endotherm at about 202°C. The broad endotherm was due to water loss. The endotherm at higher temperatures was thought to represent the denaturation transition, and the peak was considered the denaturation melting temperature (T_d). T_d is analogous to the melting of a crystal when the native and unfolded states are in equilibrium (33). The T_d value of the LSZ starting material (203.15°C) was close to that of the spray-dried LSZ (201.91°C). This result indicated the thermal stability of LSZ during the spray-drying process. This finding is not consistent with the data reported by Elkordy *et al.* (34) who found that the T_d of spray-dried LSZ was significantly higher than that of the unprocessed form. The spray-dried alone LSZ powders were in amorphous state

Table II. Thermal Properties of the LSZ Starting Material and the Spray-Dried LSZ Powders with Different Formulations (Mean ± SD, n=3)

Formulation	T_m of lipid (°C)[a]	Enthalpy (ΔH, J/g)[b]	Melting peak (°C)
LSZ starting material	–	–	203.15±0.64
Spray-dried LSZ	–	–	201.91±0.72
M/LSZ	–	–	166.22±0.30
HPC/M/LSZ	72.91±0.55	38.29±0.32	165.87±0.53
HPC/Chol9:1/M/LSZ	71.60±0.43	30.56±0.31	166.37±0.34
HPC/Chol8:2/M/LSZ	68.90±0.61	21.57±0.36	166.22±0.24
HPC/Chol7:3/M/LSZ	66.48±0.25	18.80±0.61	166.05±0.28

Chol cholesterol, *HPC* hydrogenated soybean phosphatidylcholine, *LSZ* lysozyme, *M* mannitol
[a] Scan rate of 10°C/min
[b] Scan rate of 1°C/min

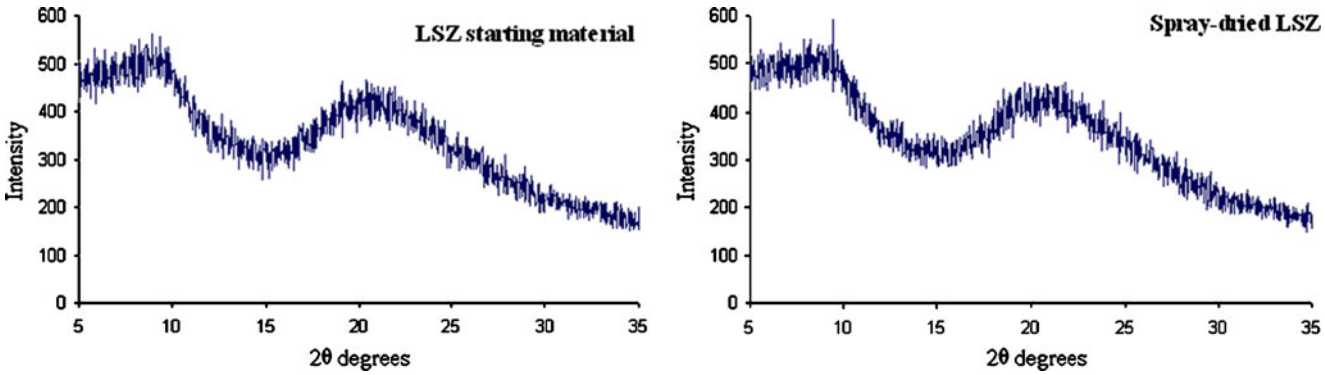

Fig. 5. X-ray diffraction patterns of the LSZ starting material and the spray-dried LSZ

similar to the starting material, as proved by X-ray powder diffraction (XRPD; Fig. 5).

DSC thermograms of the spray-dried LSZ-loaded liposomal powders do not show the endotherm of LSZ (Fig. 4). However, the thermogram of a physical mixture of the empty liposomal powders and LSZ at a weight ratio of 2:0.1 displays a very faint endothermic peak of LSZ at 200.91°C. This finding is consistent with previous studies by Chougule *et al.* (35) and Nirale *et al.* (36). The absence of peaks specific to LSZ might be attributed to dispersion of LSZ in molecular level in the spray-dried formulation and/or encapsulation of LSZ in the formulation. Therefore, this part of the study was specifically purposed to investigate the thermal properties of the lipid and mannitol additive.

A comparison of DSC thermograms of the spray-dried formulations with different HPC/Chol molar ratios indicated that interactions took place between the lipid compositions under the experimental conditions. The endothermic peak of lipid mixture shifted to a lower temperature from 72.91°C to 66.48°C when Chol content increased (Table II and Fig. 4). The enthalpy of transition decreased from 38.29 to 18.80 J/g of lipid with increasing Chol content. The effect of Chol on the thermal behavior of the spray-dried HPC liposomes is consistent with previous studies on the freeze-dried dipalmitoylphosphatidylcholine (DPPC) liposomes (22) and the air-dried 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphatidylcholine liposomes (23). The intercalation of Chol into the lipid bilayers destabilizes the phospholipid packing in the gel state, resulting in a decrease in the T_m and the enthalpy of the dehydrated liposomes (22). This finding indicated that the dehydrated liposomal membrane was in more fluid state in the presence of Chol, leading to a reduction in the spray-drying process yield. Chol incorporation did not affect the melting point (about 166°C) of mannitol in the spray-dried liposomal powders. The XRPD patterns in Fig. 6 show that the HPC/Chol molar ratios studied did not affect the crystallinity of the spray-dried liposomal powders.

Characterization of the Reconstituted Liposomes

After the reconstitution of the spray-dried liposomal powders with various HPC/Chol ratios in HBS at 37°C, the liposomes formed spontaneously with different sizes (Table III and Figs. 7 and 8). The polarizing microphotograph of the reconstituted liposomes with HPC/Chol ratio of 8:2 confirmed the formation of spherical liposomes with birefringent lamellar structure (Fig. 9) (37). The reconstituted liposomes with only HPC and HPC/Chol ratio of 9:1 showed no birefringence and appeared dark under polarized microscope. This might imply that the liposomal powders without Chol or with low Chol content formed a non-bilayer structure after reconstitution under this condition.

The volume mean diameter ($D_{[4,3]}$) of the reconstituted liposomes decreased significantly ($P<0.05$) when Chol content increased (Table III and Fig. 8). This result was related to enhanced fluidity of the HPC liposomal bilayer by inclusion of Chol. In a similar way, Lee *et al.* (38) reported that the sizes of soybean PC vesicles ($T_m<0$°C) prepared by dehydration–rehydration method (DRV) increased with increasing Chol content due to enhanced rigidity of the membrane. The increased fluidity of HPC liposomal bilayer with Chol was supported by the thermal properties of the reconstituted liposomes as shown in Table IV. The effect of Chol on liposomes is pointed out in the two regions as a function of the temperature (20). At the temperature below T_m of hydrated HPC (about 52–53°C), increasing Chol content leads to an increase in the membrane fluidity. The opposite effect is observed at the temperature above T_m where the membrane is condensed with Chol. This can be due to the formation of an intermediate gel state caused by a hydrophobic interaction of Chol with the fatty acyl chains of saturated PC (39). In this study, the temperature for reconstitution was 37°C, and inclusion of Chol into the HPC bilayers resulted in less order and thus rigidity of the bilayer. This could reduce fusion of the vesicles during rehydration and thus result in the smaller size of the reconstituted

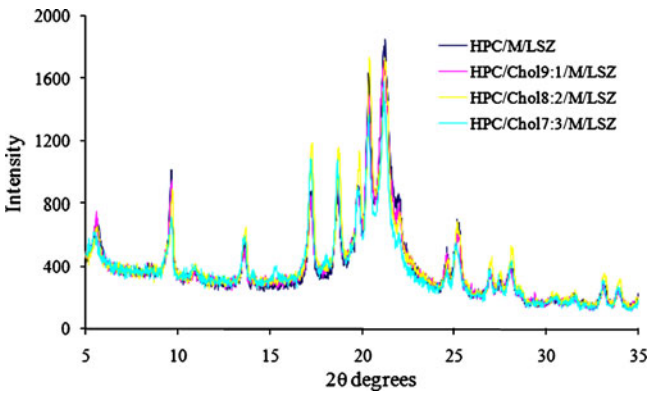

Fig. 6. X-ray diffraction patterns of the spray-dried LSZ-loaded liposomal powders with different HPC/Chol molar ratios

Table III. Sizes of the Initial Empty Liposomes and the Reconstituted Liposomes with Various HPC/Chol Molar Ratios (Mean ± SD, n=3)

Formulation	Before spray drying		After spray drying	
	Effective diameter (nm)	Polydispersity index	$D_{[4,3]}$ (μm)	Span
HPC/M/LSZ	138.2±0.3	0.189±0.013	14.34±0.05	1.28±0.002
HPC/Chol9:1/M/LSZ	186.4±0.7	0.168±0.082	9.43±0.002	1.05±0.001
HPC/Chol8:2/M/LSZ	184.6±3.8	0.153±0.023	7.39±0.002	1.27±0.003
HPC/Chol7:3/M/LSZ	190.6±4.3	0.189±0.022	5.15±0.02	1.21±0.01

Chol cholesterol, *HPC* hydrogenated soybean phosphatidylcholine, *LSZ* lysozyme, *M* mannitol

liposomes. Apparently, the enhanced fluidity of the lipid bilayers made them less sensitive to the thermal stress-induced lysis during the spray-drying process. This result is in agreement with a previous study in that the presence of Chol increases the resistance of DPPC liposomal bilayers to freeze-drying stress (40).

However, the sizes of the reconstituted liposomes with various HPC/Chol ratios increased when compared to those of the extruded liposomes before spray drying (Table III). The increased sizes could be mainly attributed to thermal stress-induced breaking of lipid bilayer during the spray-drying process. Aggregation and/or fusion of liposomes are a mechanism to dissipate the excess surface energy originating from the distorted molecular packing to obtain a greater stability state (41). The result indicated that mannitol was not effective in preserving the HPC liposome integrity during spray-drying process.

For studying the entrapment of LSZ in the reconstituted liposomes, dialysis and ultracentrifugation techniques were used for the separation of free LSZ. Dialysis technique was found to be an appropriate method because it is a gentle and efficient method, but is time-consuming. The entrapment efficiency of LSZ in the reconstituted liposomes with various Chol contents is presented in Fig. 8. The EE of HPC/M/LSZ formulation by dialysis was 6.74±0.25%. The ultracentrifugation technique gave higher EE of 9.73±0.62%, which might be due to the amount of LSZ associated with liposomal surface. However, ultracentrifugation may result in damage of liposomal bilayer due to high centrifuge force and pellet washing process which gave the entrapment underestimate. The dialysis study using a mixture of the reconstituted empty HPC/Chol(8:2) liposomes and LSZ solution confirmed that the LSZ amount assayed in the liposomal dispersion after dialysis process was LSZ entrapped into liposomes, not attributed to adsorption phenomena.

The LSZ entrapment may occur from the re-encapsulation of LSZ into the liposomes which were spontaneously

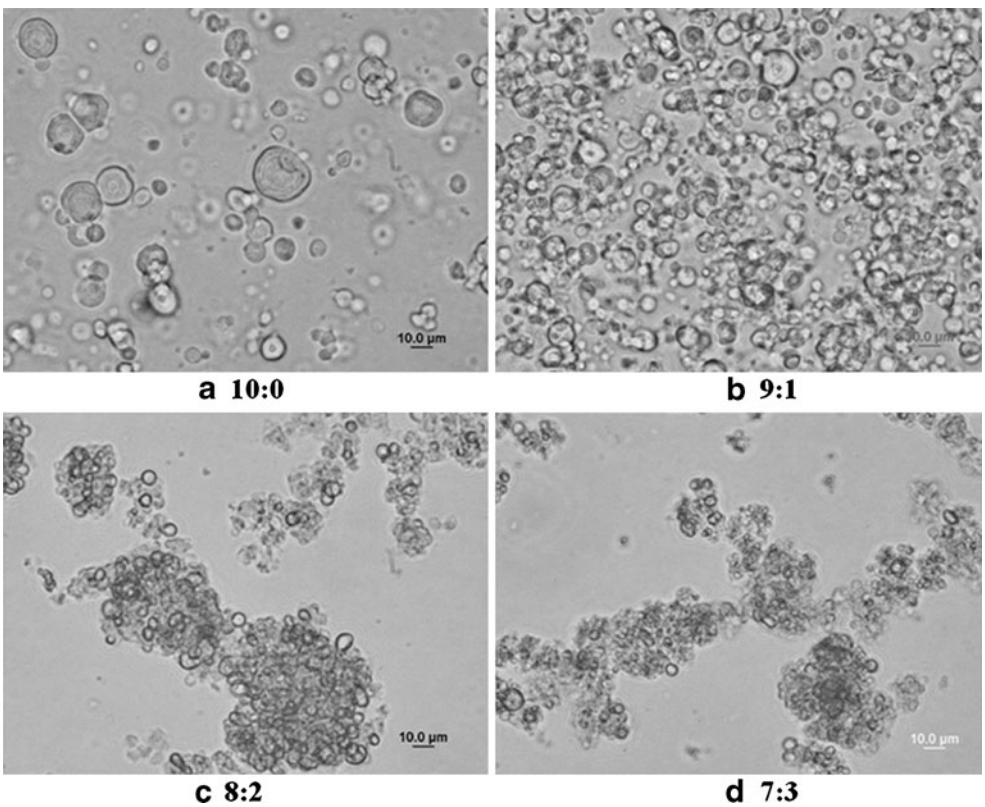

Fig. 7. Optical microphotographs of the reconstituted liposomes from the spray-dried LSZ-loaded liposomal powders with various HPC/Chol molar ratios in HBS at 37°C (magnification ×400; *scale bar* 10 μm)

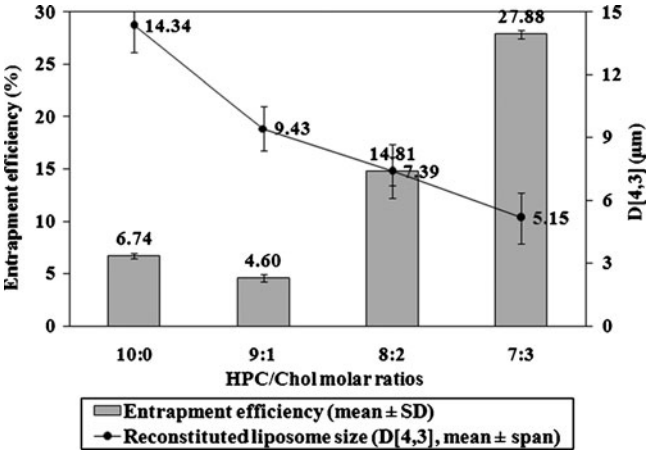

Fig. 8. Entrapment efficiency and volume mean diameter of the reconstituted liposomes from the spray-dried LSZ-loaded liposomal powders with various HPC/Chol molar ratios in HBS at 37°C

formed after reconstitution of the powders with HBS at 37°C. Lysozyme may be incorporated by entrapment into aqueous compartment or by hydrophobic interaction between the protein and the lipid bilayer (42). The EE of LSZ in the reconstituted liposomes increased significantly ($P<0.05$) with increasing Chol content from 10 to 30 mol%. A higher proportion of Chol resulted in less order and thus rigidity of HPC bilayer at a temperature below its T_m. It might enhance hydrophobic interactions between the enzyme and the lipid membrane, hence an increase in the re-encapsulated LSZ amount (43). The formulations without Chol or with HPC/Chol ratio of 9:1 gave low LSZ entrapment which might be a consequence of either improper formation of the liposomal structure or high rigidity of the lipid bilayer. On the contrary, from our preliminary data, the LSZ entrapment decreased with increasing Chol contents when the liposomal powders were reconstituted at 60°C (temperature above T_m of hydrated lipids). The presence of Chol in the lipid bilayers reduced the affinity of LSZ for the lipid membrane because of enhanced rigidity of bilayers (43). The results disagree with

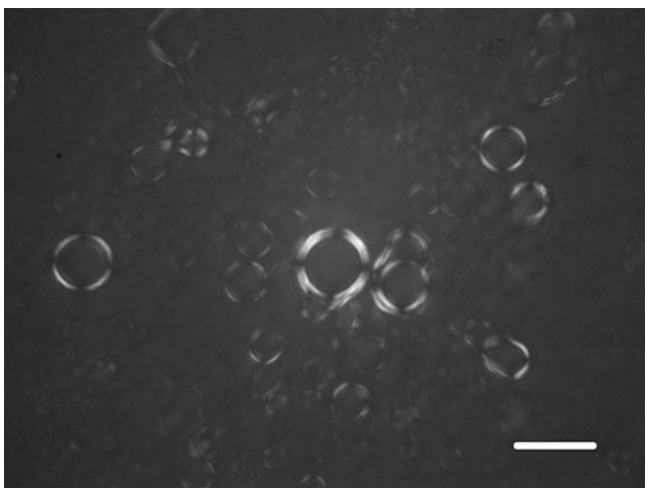

Fig. 9. Polarizing microphotograph of the reconstituted liposomes with HPC/Chol molar ratio of 8:2 in HBS at 37°C (magnification ×1,000; *scale bar* 10 μm)

Table IV. Phase Transition of the Reconstituted Liposomes from the Spray-Dried LSZ-Loaded Liposomal Powders with Various HPC/Chol Molar Ratios (Mean ± SD, $n=3$)

Formulation	T_m (°C)	Enthalpy (ΔH, J/g)
HPC/M/LSZ	52.98±0.34	50.69±1.61
HPC/Chol9:1/M/LSZ	52.29±0.25	51.45±0.75
HPC/Chol8:2/M/LSZ	52.14±0.73	30.83±1.58
HPC/Chol7:3/M/LSZ	53.28±0.37	21.73±0.77

Chol cholesterol, *HPC* hydrogenated soybean phosphatidylcholine, *LSZ* lysozyme, *M* mannitol

a previous report by Rodriguez-Nogales and Lopez (44) in that Chol inclusion into egg PC DRV (T_m, −15°C) increases the EE of β-galactosidase because of improved membrane rigidity and enzyme retention in liposomes. The disagreement could be due to the difference in the dehydration method of liposomes. In the previous study, freeze drying, which is a slow process, was used to dry the mixture of liposomal dispersion and enzyme solution, whereas in the present study, spray-drying process was used. Spray drying is a process using very strong drying conditions that evaporate droplets rapidly, resulting in drug particles embedded in the matrix (45). Nevertheless, the underlying mechanism was not known. At high Chol content, however, a reduction in EE was also seen in the previous study (44), which could be explained by the reduced polypeptide affinity for the membrane due to bilayer-condensing effect (43). Therefore, the entrapment of enzyme into the reconstituted liposomes depended on lipid compositions (type of phospholipid and amount of Chol) and temperature for rehydration relative to the T_m of the hydrated lipid bilayer.

Biological Activity of Lysozyme

The biological enzymatic activity is the method widely used for the investigation of LSZ stability during various processes. For all spray-dried formulations prepared in the present study, the remaining activity of LSZ was not significantly different from the activity of the starting material. It is possible that the spray-drying condition used did not affect the biological activity of LSZ. Nevertheless, Fourier transform Raman spectral change of the spray-dried LSZ powders indicated the occurrence of LSZ aggregation (data not shown). The aggregation of LSZ was reversible upon reconstitution in diluted solution as determined by the enzymatic activity and the circular dichroism analysis (data not shown). Thus, the study of LSZ activity in solution may not reflect its conformational damage, which may occur during the spray-drying process (34). For other protein/peptide drugs, stabilizers should be included to improve the stability of these drugs during spray-drying process.

CONCLUSIONS

Lysozyme-loaded liposomal powders could be successfully prepared by spray drying the mixture of preformed empty liposomes and LSZ solution using mannitol as an additive. Chol incorporated into HPC liposomal bilayer had an essential influence on sizes and LSZ entrapment efficiency

of the reconstituted liposomes. The results may aid in the development of the liposomal powders by spray-drying technique for pulmonary protein delivery.

ACKNOWLEDGMENTS

The authors are thankful to grants from the Graduate School of Chulalongkorn University, Faculty of Pharmaceutical Sciences and from the Commission on Higher Education of Thailand.

REFERENCES

1. Zeng XM, Martin GP, Marriott C. The controlled drug delivery to the lung. Int J Pharm. 1995;124:149–64. doi:10.1016/0378-5173(95)00104-Q.
2. Huang Y-Y, Wang C-H. Pulmonary delivery of insulin by liposomal carriers. J Control Release. 2006;113:9–14. doi:10.1016/j.jconrel.2006.03.014.
3. Briscoe P, Caniggia I, Graves A, Benson B, Huang L, Tanswell AK *et al*. Delivery of superoxide dismutase to pulmonary epithelium via pH-sensitive liposomes. Am J Physiol. 1995;268:L374–80.
4. Schreier H, Gonazales-Rothi RJ, Stecenko AA. Pulmonary delivery of liposomes. J Control Release. 1992;24:209–23. doi:10.1016/0168-3659(93)90180-D.
5. Zaru M, Mourtas S, Klepetsanis P, Fadda AM, Antimisiaris SG. Liposomes for drug delivery to the lungs by nebulization. Eur J Pharm Biopharm. 2007;67:655–66. doi:10.1016/j.ejpb.2007.04.005.
6. Niven RW, Carvajal MA, Schreier H. Nebulization of liposomes. III. The effects of operating conditions and local environment. Pharm Res. 1992;9:515–20. doi:10.1023/A:1015844430695.
7. Prime D, Atkins PJ, Slater A, Sumby B. Review of dry powder inhalers. Adv Drug Deliv Rev. 1997;26:51–8. doi:10.1016/S0169-409X(97)00510-3.
8. Kim C-K, Chung H-S, Lee M-K, Choi L-N, Kim M-H. Development of dried liposomes containing β-galactosidase for the digestion of lactose in milk. Int J Pharm. 1999;183:185–93. doi:10.1016/S0378-5173(99)00115-5.
9. Lo Y, Tsai J, Kuo J. Liposomes and disaccharides as carriers in spray-dried powder formulations of superoxide dismutase. J Control Release. 2004;94:259–72. doi:10.1016/j.jconrel.2003.09.019.
10. Kellaway IW, Farr SJ. Liposomes as drug delivery systems to the lung. Adv Drug Deliv Rev. 1990;5:149–61. doi:10.1016/0169-409X(90)90012-H.
11. Cryan S-A. Carrier-based strategies for targeting protein and peptide drugs to the lungs. AAPS J. 2005;7(1):E20–40. doi:10.1208/aapsj070104.
12. Shoyele SA, Cawthorne S. Particle engineering techniques for inhaled biopharmaceuticals. Adv Drug Deliv Rev. 2006;58:1009–29. doi:10.1016/j.addr.2006.07.010.
13. Kikuchi H, Yamauchi H, Hirota S. A spray-drying method for mass production of liposomes. Chem Pharm Bull. 1991;39:1522–7.
14. Goldbach P, Brochart H, Stamm A. Spray-drying of liposomes for a pulmonary administration. II. Retention of encapsulated materials. Drug Dev Ind Pharm. 1993;19:2623–36. doi:10.3109/03639049309047205.
15. Grenha A, Remunan-Lopez C, Carvalho ELS, Seijo B. Microspheres containing lipid/chitosan nanoparticles complexes for pulmonary delivery of therapeutic proteins. Eur J Pharm Biopharm. 2008;69:83–93. doi:10.1016/j.ejpb.2007.10.017.
16. Chougule MB, Padhi BK, Misra A. Development of spray dried liposomal dry powder inhaler of dapsone. AAPS PharmSciTech. 2008;9(1):47–53. doi:10.1208/s12249-007-9024-6.
17. Skalko-Basnet N, Pavelic Z, Becirevic-Lacan M. Liposomes containing drug and cyclodextrin prepared by the one-step spray-drying method. Drug Dev Ind Pharm. 2000;26(12):1279–84. doi:10.1081/DDC-100102309.
18. Weers JG, Tarara TE, Tzannis S. Lipid formulations for spontaneous drug encapsulation. US patent application 20050214224, 4 Nov 2004.
19. Desai TR, Wong JP, Hancock REW, Finlay WH. A novel approach to the pulmonary delivery of liposomes in dry powder form to eliminate the deleterious effects of milling. J Pharm Sci. 2002;91:482–91. doi:10.1002/jps.10021.
20. Coderch L, Fonollosa J, Pera MD, Estelrich J, Maza ADL, Parra JL. Influence of cholesterol on liposome fluidity by EPR relationship with percutaneous absorption. J Control Release. 2000;68:85–95. doi:10.1016/S0168-3659(00)00240-6.
21. McMullen TPW, Lewis RNAH, McElhaney RN. Differential scanning calorimetric study of the effect of cholesterol on the thermotropic phase behavior of a homologous series of linear saturated phosphatidylcholines. Biochemistry. 1993;32:516–22. doi:10.1021/bi00053a016.
22. Ohtake S, Schebor C, Palecek SP, de Pablo JJ. Phase behavior of freeze-dried phospholipid–cholesterol mixtures stabilized with trehalose. Biochim Biophys Acta. 2005;1713:57–64. doi:10.1016/j.bbamem.2005.05.001.
23. Popova AV, Hincha DK. Effects of cholesterol on dry bilayers: interactions between phosphatidylcholine unsaturation and glycolipid or free sugar. Biophys J. 2007;93:1204–14. doi:10.1529/biophysj.107.108886.
24. Rosenberger F. Protein crystallization. J Cryst Growth. 1996;166:40–54. doi:10.1016/0022-0248(95)00921-3.
25. Steckel H, Bolzen N. Alternative sugars as potential carriers for dry powder inhalations. Int J Pharm. 2004;270:297–306. doi:10.1016/j.ijpharm.2003.10.039.
26. New RRC. Liposomes a practical approach. New York: Oxford University Press; 1990.
27. Brandl M, Bachmann D, Drechsler M, Bauer KH. Liposome preparation by a new high pressure homogenizer gaulin micron Lab 40. Drug Dev Ind Pharm. 1990;16:2167–91. doi:10.3109/03639049009023648.
28. Bartlett GR. Phosphorus assay in column chromatography. J Biol Chem. 1959;234:466–8.
29. Shugar D. Measurement of lysozyme activity and the ultra violet inactivation of lysozyme. Biochim Biophys Acta. 1952;8:302–9.
30. Weiner AL. Liposomes as carriers for polypeptides. Adv Drug Deliv Rev. 1989;3:307–41. doi:10.1016/0169-409X(89)90026-4.
31. Goldbach P, Brochart H, Stamm A. Spray-drying of liposomes for a pulmonary administration. I. Chemical stability of phospholipids. Drug Dev Ind Pharm. 1993;19(19):2611–22. doi:10.3109/03639049309047204.
32. Crowe JH, Crowe LM, Carpenter JF, Rudolph AS, Wistrom CA, Spargo BJ *et al*. Interactions of sugars with membranes. Biochim Biophys Acta. 1988;947:367–84. doi:10.1016/0304-4157(88)90015-9.
33. Hoffmann H. Analytical methods and stability testing of biopharmaceuticals. In: McNally EJ, editor. Protein formulation and delivery. New York: Marcel Dekker; 2000. p. 71–110.
34. Elkordy AA, Forbes RT, Barry BW. Integrity of crystalline lysozyme exceeds that of a spray-dried form. Int J Pharm. 2002;247:79–90. doi:10.1016/S0378-5173(02)00379-4.
35. Chougule MB, Padhi BK, Misra A. Nano-liposomal dry powder inhaler of amiloride hydrochloride. J Nanosci Nanotech. 2006;6:3001–9. doi:10.1166/jnn.2006.405.
36. Nirale NM, Vidhate RD, Nagarsenker MS. Fluticasone propionate liposomes for pulmonary delivery. Indian J Pharm Sci. 2009;71:709–11.
37. Giulieria F, Krafft MP. Tubular microstructures made from nonchiral single-chain fluorinated amphiphiles: Impact of the structure of the hydrophobic chain on the rolling-up of bilayer membrane. J Colloid Interf Sci. 2003;258(2):335–44. doi:10.1016/S0021-9797(03)00016-X.
38. Lee S-C, Lee K-E, Kim J-J, Lim S-H. The effect of cholesterol in the liposome bilayer on the stabilization of incorporated retinol. J Liposome Res. 2005;15:157–66. doi:10.1080/08982100500364131.
39. Demel RA, Kruyff BD. The function of sterols in membranes. Biochim Biophys Acta. 1976;457:109–32. doi:10.1016/0304-4157(76)90008-3.
40. van Winden ECA, Zhang W, Crommelin DJA. Effect of freezing rate on the stability of liposomes during freeze-drying and rehydration. Pharm Res. 1997;14(9):1151–60. doi:10.1023/A:1012142520912.

41. Lentz B, Carpenter T, Alford DR. Spontaneous fusion of phosphatidylcholine small unilamellar vesicles in the fluid phase. Biochemistry. 1985;26:5389–97. doi:10.1021/bi00391a026.
42. Gorbenko GP, Ioffe VM, Kinnunen PKJ. Binding of lysozyme to phospholipid bilayers: evidence for protein aggregation upon membrane association. Biophys J. 2007;93:140–53. doi:10.1529/biophysj.106.102749.
43. Laridi R, Kheadr EE, Benech R-O, Vuillemard JC, Lacroix C, Fliss I. Liposome encapsulated nisin Z: optimization, stability and release during milk fermentation. Int Dairy J. 2003;13:325–36. doi:10.1016/S0958-6946(02)00194-2.
44. Rodriguez-Nogales JM, Lopez AD. A novel approach to develop β-galactosidase entrapped in liposomes in order to prevent an immediate hydrolysis of lactose in milk. Int Dairy J. 2006;16:354–60. doi:10.1016/j.idairyj.2005.05.007.
45. Sebti T, Amighi K. Preparation and *in vitro* evaluation of lipidic carriers and fillers for inhalation. Eur J Pharm Biopharm. 2006;63:51–8. doi:10.1016/j.ejpb.2005.11.003.