A Novel Approach to the Pulmonary Delivery of Liposomes in Dry Powder Form to Eliminate the Deleterious Effects of Milling

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Received 9 May 2001; revised 10 August 2001; accepted 16 August 2001

ABSTRACT: The effect of lyophilization and jet-milling on liposome integrity was investigated as a function of their ability to retain the encapsulated model drug on reconstitution of the dry products. The encapsulation efficiencies of the lyophilized and jet-milled formulations were determined at various concentrations of lactose. Lyophilization resulted in considerable leakage of the model drug at lower concentrations of lactose, and jet-milling further augmented the leakage for all the lyophilized formulations, with optimum retention obtained for formulations containing at least 10:1 molar ratio of lactose/lipid. In an attempt to overcome the deleterious effects of lyophilization and jet-milling, the feasibility of formulating phospholipid-based powders that result in spontaneous formation of liposomes in an aqueous environment has been investigated. Partitioning of three model drugs (viz., ciprofloxacin, CM3 peptide, and salbutamol sulfate) between the aqueous phase and spontaneously formed liposomes was determined in terms of encapsulation efficiency. The effects of several parameters, including lactose concentration, lipid composition, and lipid concentration on the encapsulation efficiency of these model drugs were investigated. The spontaneous formation of liposomes on dispersion of phospholipid-based powder formulations was further evidenced by freeze–fracture scanning electron microscopy. This novel approach for the delivery of liposomes in dry powder form appears promising because lyophilization is not involved and jet-milling of these powder formulations did not impact encapsulation efficiency. Jet-milled phospholipid-based powder formulations showed high encapsulation efficiencies of $96.2 \pm 1.4\%$ for ciprofloxacin, 100% for CM3 peptide, and $45.3 \pm 3.1\%$ for salbutamol sulfate compared with a high amount of leakage ($> 50\%$) observed due to jet-milling of lyophilized liposome formulations encapsulating ciprofloxacin. J Pharm Sci 91:482–491, 2002

Keywords: liposome powders; lyophilized liposomes; jet-milling; freeze–fracture scanning electron microscopy; ciprofloxacin; salbutamol sulfate; cationic peptide

INTRODUCTION

Aerosols are an effective method of delivering therapeutic agents to the respiratory tract tissues and can be delivered by nebulizers, metered dose

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Journal of Pharmaceutical Sciences, Vol. 91, 482–491 (2002)

inhalers, or dry powder inhalers.[1,2] Liposome aerosols are promising vehicles for respiratory delivery of therapeutic drugs and have attracted the attention of many researchers, especially in the area of nebulizers[3,4] and dry powder inhalers.[5,6] However, stability and leakage problems are associated with aqueous dispersions with nebulizers.[7] As an alternative approach, delivery by dry powder has been considered,[5,6,8] mainly based on the fact that liposomes can be more stable when dried by lyophilization.[9,10] With liposome powders as drug carriers for inhalation therapies, the lyophilized precursor should be micronized to particles of ~1–6 μm in diameter for efficient delivery to the lung. This micronization has normally been achieved by jet-milling,[5,8,11] which causes particles to break apart on colliding in a high-velocity air-stream.

Although lyophilization (freezing–drying) is considered a promising means of extending the shelf-life of liposomes, both freezing and drying can lead to structural and functional changes in liposomes.[12,13] In addition, jet-milling is also expected to induce membrane deformation because of high-energy collision during milling, leading to perturbation of the liposome structure and leakage of encapsulated drug on hydration. Mobley[11] investigated the consequences of jet-milling on liposome integrity for formulations prepared with different phospholipids exhibiting different physical properties. He observed that jet-milling led to a significant reduction in retention of the model drug, thus compromising liposome integrity. In this study, we have examined the effect of the concentration of cryoprotectant (lactose) on leakage of a model drug after lyophilization and jet-milling. Ciprofloxacin, a potent antimicrobial agent, was selected as a model drug for liposome encapsulation because it demonstrates increased protection compared with free ciprofloxacin in animal models.[14]

As a measure of circumventing the potentially negative effects of lyophilization and jet-milling, advantage might be made of the fact that phospholipids are known to 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, so that it is feasible to postulate that spontaneous liposome formation would occur following pulmonary deposition of microfine phospholipid-based aerosols. Moreover, incorporation of suitable drugs within such packs should result in the creation of a reservoir of liposomally encapsulated drug that is subsequently released at a controlled rate. This concept was first investigated by Farr et al.[15,16] for phospholipid-based aerosols derived from pressurized metered dose inhaler (MDI) formulations. The study investigated chlorofluorocarbon (CFC)-based formulations containing phospholipid and drug that would facilitate liposome formation following impingement of an aerosol in a water-rich environment and was evaluated *in vitro* through use of a multistage liquid impinger (MLI). They explored this concept only for MDIs. In this paper, we have examined the concept relying on spontaneous *in situ* formation of liposomes in an aqueous environment for dry powder phospholipid-based formulations as a novel approach for the pulmonary delivery of liposomes in dry powder form. The ability of a variety of powder formulations to spontaneously partition several drugs in this manner is examined.

MATERIALS AND METHODS

Materials

Dipalmitoyl phosphatidylcholine (DPPC; >99%) and dimyristoyl phosphatidylglycerol (DMPG, sodium salt; > 99%) were purchased from Genzyme Pharmaceuticals (Cambridge, MA). Egg phosphatidylcholine (EPC; > 99%) was purchased from Avanti Polar Lipids (Alabaster, AL). Inhalation-grade lactose, Pharmatose 325M (mean particle size of ~65 μm), was a generous gift from DMV International (Veghel, The Netherlands). Cholesterol (CH), sodium chloride, ammonium sulfate, and salbutamol sulfate were purchased from Sigma Chemicals Company (St. Louise, MO). Ciprofloxacin hydrochloride (> 99%) was purchased from Serologicals Corporation (Kankakee, IL). CM3 peptide was synthesized by Fmoc (9-fluorenylmethoxycarbonyl) chemistry at the Nucleic Acid/Protein Service Laboratory, University of British Columbia, Vancouver, BC, Canada. The amino acid sequence of CM3 is KWKKFIKSLTKSAAKTVVKTAKKPLIV, as described in Scott et al.[17] All the materials were used as received.

Methods

Liposome Preparation

Liposome-encapsulated ciprofloxacin used in this study was prepared using the remote loading

procedure. These liposomes were prepared using a lipid composition of EPC/CH in a 5.5:4.5 molar ratio. The lipids were dissolved in 4 mL of chloroform, and the organic solvent was removed by rotaevaporation under reduced pressure to form a thin lipid film. Dry lipid film was then rehydrated to 0.1 M lipid concentration with 0.6 M ammonium sulfate containing desired amount of Pharmatose 325 M. Six different formulations containing 1:0, 1:1, 1:3, 1:5, 1:10, and 1:20 molar ratios of lipid/Pharmatose 325 M were prepared. The aqueous dispersions were frozen in liquid nitrogen and then thawed at 45°C five times. The multilamellar vesicles thus formed were extruded 10 times through 0.2-μm polycarbonate filters. The dispersion was placed in dialysis buffer (0.9% sodium chloride) for 18 h, with buffer change after every 2 h. For drug loading, ciprofloxacin was then added at a dosage of 22.5 mg/mL, and the ciprofloxacin–liposome mixture was then incubated at 45°C for 2 h. The encapsulation efficiency of ciprofloxacin was $97 \pm 1\%$ before lyophilization. If lower encapsulation efficiency was obtained, then extraliposomal ciprofloxacin was removed by centrifuging the dispersion at 50,000 rpm and 4°C for 30 min. The supernatant was then removed, and the dispersion was reconstituted to its original volume to achieve $97 \pm 1\%$ encapsulation efficiency. The encapsulation efficiency was measured by determining the amount of ciprofloxacin in supernatant and residue by ultraviolet (UV) absorption at 274 nm. The liposomes were then immediately frozen in liquid nitrogen.

Liposome Lyophilization

Liposomes encapsulating ciprofloxacin were lyophilized for 24 h at -47°C and 67 mbar using a Labconco freeze–dry system (St. Louis, MO).

Jet Milling

The lyophilized liposome cakes were divided into two portions before the reconstitution study; that is a nonmilled portion and another portion for jet-milling. Micronized powders were generated by the principle of opposing jets and cyclone separation. The lyophilized formulations were first ground to fine powder with pestle and mortar and then milled with a Trost Impact Pulverizer (Garlock Inc., Plastomer Products, Newton, MA), at a grinding nozzle pressure and pusher nozzle pressure of 90 psi. The jet-milled powders were collected from the collection vessel for further reconstitution studies.

Reconstitution of Lyophilized Liposome and Determination of Encapsulation Efficiency

Dried liposomes were reconstituted at their original concentration with 0.9% saline at room temperature (24 ± 1°C). Extraliposomal ciprofloxacin was then separated from the reconstituted liposomes by centrifuging the dispersion at 15,300 rpm at 4°C (Beckman Coulter, CA), and separating the supernatant and pellet by decantation. After dilution with methanol, the amount of ciprofloxacin was separately determined in supernatant and residue by UV spectroscopy ($\lambda = 274$ nm). The percentage of drug encapsulated was calculated as the ratio of the drug in the pellet to the sum of the drug in the pellet and the drug in the supernatant. All experiments of lyophilization and jet-milling were performed for three separate batches of each formulation.

Mixing Powders and Determination of Encapsulation Efficiency

To demonstrate the concept of spontaneous formation of liposomes, various formulations containing phospholipid(s), model drug, and Pharmatose 325M were dispersed in saline, and subsequently encapsulation efficiency of these various formulations was determined. To study the effect of lactose concentration on the encapsulation efficiency, phospholipids, drug, and Pharmatose 325M were mixed in such a way that when powders were dispersed in 0.9% saline, then lipid/lactose molar ratios of 1:1, 1:5, and 1:10 were achieved. To study the effect of lipid concentration on encapsulation of different model drugs, different lipid concentrations were adjusted depending on the nature of the drug to be encapsulated and the nature of the lipid. Ciprofloxacin was added at a dosage of 7 mg/mL, salbutamol sulfate at a dose level of 2 mg/mL, and CM3 peptide at a dose level of 1 mg/mL. The encapsulation efficiencies of different drugs were determined by dispersing the powder formulations before and after jet-milling to study the effect of jet-milling on encapsulation of various drugs. To determine the encapsulation efficiency, appropriately weighed formulations (to achieve the desired concentration) were dispersed in 0.9% saline by gentle vortex mixing for 1 min at room temperature (24 ± 1°C). Thephospholipids were then allowed to hydrate by equilibrating the dispersions for 15 min at room temperature before centrifugation. Unencapsulated drug was then separated from the hydrated liposomes by centrifuging the dispersions at 15,300 rpm and 4°C

for 60 min, and separating the supernatant and residue. After dilution with methanol, the amount of drug was determined separately in supernatant and residue by UV spectroscopy. The percentage of drug encapsulated was then calculated as the ratio of the drug in the pellet to the sum of the drug in the pellet and the drug in the supernatant. The reported values of encapsulation efficiencies indicate the mean of three sample replicates.

Determination of Liposome Size Distribution

Particle size distributions of liposome dispersions were determined by dynamic light scattering analysis, using a Brookhaven B190 submicron particle analyzer (Brookhaven Instruments, Holtsville, NY).

Freeze–Fracture Scanning Electron Microscopy

Freeze–fracture scanning electron microscopy (SEM) was used to study the morphology of spontaneously formed liposomes. The spontaneously formed liposome samples were immediately taken for SEM study after hydrating the formulations in saline, and equilibrating for 15 min at room temperature. The specimens were fast frozen by plunging in liquid nitrogen at -150°C in a cryostage (Emitech K1250) and transferred cold into a high-vacuum freeze–fracture system. Specimens were then fractured in liquid nitrogen with a Balzers complementary replica device. The fractured surfaces were then coated by gold sputtering for $\sim$30 s. The coated samples were viewed under a Jeol JSM-6301 FXV scanning electron microscope.

RESULTS AND DISCUSSION

Effect of the Concentration of Lactose on Ciprofloxacin Retention Before and After Jet-Milling

The percent encapsulation efficiency of ciprofloxacin before and after jet-milling, as a function of concentration of lactose, is shown in Figure 1. There is a clear trend of increase in the encapsulation efficiency with increasing concentration of lactose for nonmilled as well as jet-milled formulations. For nonmilled lyophilized liposomes, the maximum retention of ciprofloxacin was observed at a liposome/lactose molar ratio of 1:10. Further increases in lactose concentration did not show any increase in the ciprofloxacin retention. Similarly, in the case of jet-milled powders, the formulation with liposome/lactose molar ratios of $\geq$ 1:10 showed highest retention of ciprofloxacin. Also, lyophilization leads to considerable leakage of drug at the lower concentrations of lactose and jet-milling further augmented this leakage in the case of all the lyophilized liposomes, with the highest retention obtained for the 1:10 ($38.7 \pm 2.5\%$) and 1:20 ($40.1 \pm 2.7\%$) formulations.

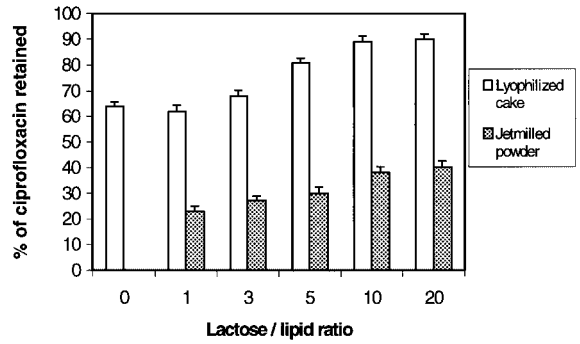


Figure 1. Histogram representation of ciprofloxacin retention (%) of reconstituted liposomes as a function of lactose concentration ($n = 3$; error bars indicate SD).

For nonmilled lyophilized liposomes, the leakage can be mainly attributed to stress-induced lysis that may have occurred due to the lyophilization process (freezing–dehydration), which leads to shrinkage of the membrane, thereby causing membrane stress.[9] The relatively greater retention in the 1:10 and 1:20 formulations reflects a greater resistance of these liposome membranes to stress-induced lysis. Another cause of leakage could be liposome aggregation and fusion,[10] as evidenced by the effect of lactose concentration on the liposome particle size (see Table 1). The results in Table 1 indicate that

Table 1. Mean Particle Size of Lyophilized/Rehydrated Liposomes as a Function of Lactose/Lipid Molar Ratio ($n = 3$, $\pm$SD)

Lipid/Lactose Molar Ratio	Lyophilized Cake (μm)	Jet-Milled Powder (μm)
1:0	6.74 ± 0.22	—
1:1	2.10 ± 0.12	1.69 ± 0.14
1:3	1.83 ± 0.14	1.48 ± 0.12
1:5	1.61 ± 0.11	1.44 ± 0.16
1:10	1.30 ± 0.13	1.33 ± 0.11
1:20	1.23 ± 0.11	1.30 ± 0.13

incorporation of lactose in lyophilized liposomes drastically decreased the particle size of lyophilized/rehydrated liposomes, possibly indicating limited aggregation and fusion of liposomes. Further augmentation of liposomal ciprofloxacin loss was caused by jet-milling, which may be mainly attributed to the rupture of liposome membranes during jet-milling. Particle size analyses of the milled powder did not show any appreciable difference in the vesicle size compared with the respective nonmilled formulation, indicating that aggregation behavior of liposomes on rehydration in nonmilled and jet-milled formulations is the same. However, the vesicle size decreased with increasing lactose concentration and remained nearly constant after 1:10 molar ratio for nonmilled as well as jet-milled formulations.

It is concluded from this study that lyophilization–rehydration causes considerable leakage of drug from the lyophilized liposome matrix and the leakage is a function of the concentration of lactose. A lipid/lactose molar ratio of 1:10 appears to be the optimum ratio to achieve maximum retention of the drug in nonmilled as well as milled formulations. The rupture of liposome membrane during jet-milling further augmented the leakage considerably. As observed in this study as well as by Mobley,[11] jet-milling leads to high amounts of leakage of the encapsulated drug. These observations raise concerns about the feasibility of jet-milling as a practical method of producing liposome powders. Further investigation is required to eliminate the sources of the detrimental effect of jet-milling on liposome integrity.

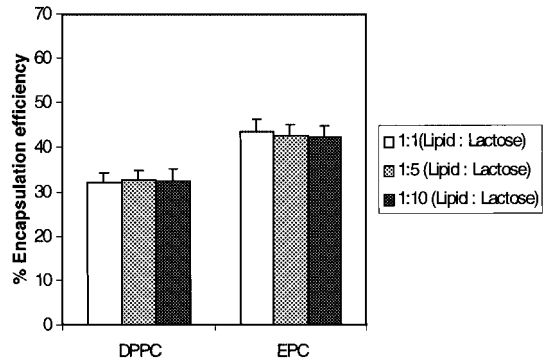


Figure 2. Histogram representation of percent encapsulation efficiency of ciprofloxacin (in spontaneously formed liposomes) on dispersion of DPPC- and EPC-based formulations at various concentrations of lactose (lipid concentration, 0.1 M; $n = 3$; error bars indicate SD).

Determination of Encapsulation Efficiency in Phospholipid-Based Formulations

In an attempt to overcome the problem associated with the leakage of drug due to the detrimental effect of jet-milling, we examined the spontaneous formation concept for phospholipid-based dry powder formulations. The effects of lactose concentration on the encapsulation efficiency of ciprofloxacin in DPPC- and EPC-based powder formulations are shown in Figure 2. The lipid weights were adjusted in such a way that the lipid concentration of 0.1M was achieved on dispersion in saline. It is interesting to note here that EPC-based powder formulations showed encapsulation of $43.6 \pm 2.3\%$, whereas DPPC-based formulation showed encapsulation of $32.7 \pm 2.1\%$, both by merely dispersing phospholipid-, lactose-, and ciprofloxacin-based powders in saline. This result indicates the spontaneous formation of liposomes on hydration and subsequent partitioning of ciprofloxacin from the aqueous phase into the vesicular structure. It can also be seen that unlike lyophilized liposomes, the encapsulation efficiency for mixed powder formulations is independent of the lactose concentration, which is expected because lactose does not act as a protectant and no cryoprocess is involved here. Because the concentration of lactose has no significant impact on the encapsulation efficiency, a molar ratio of 1:5 (lipid/lactose) was selected as representative for the remainder of the study. It should be noted here that the main purpose of adding lactose in the powder formulations was to increase the total bulk of the mixed powders to achieve better yield upon jet-milling because jet-milling results in substantial loss of powder if lower quantities are used for milling.

Ciprofloxacin

The effect of lipid concentrations and the nature of lipid on the encapsulation efficiency of ciprofloxacin are shown in Figure 3. As the concentration of lipid was increased from 0.05 to 0.1 M, the maximum encapsulation of DPPC- and EPC-based formulations (32.7 ± 2.1 and $43.6 \pm 2.3\%$ respectively) were achieved. Further increase in the lipid concentration did not increase the encapsulation further. The results in Figure 3

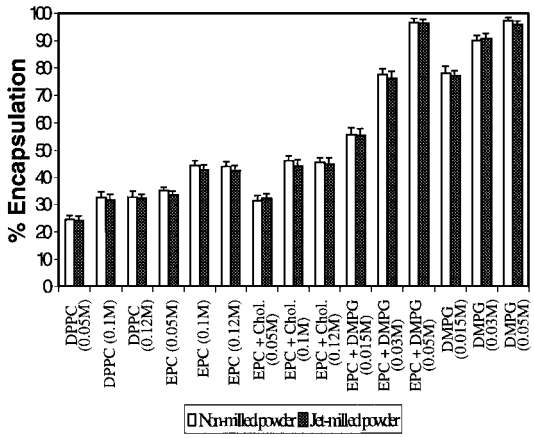


Figure 3. Histogram representation of percent encapsulation efficiency of ciprofloxacin (in spontaneously formed liposomes) on dispersion of various formulations consisting of different lipids at various concentrations (lipid/lactose molar ratio was kept constant at 1:5; $n = 3$; error bars indicate SD).

Table 2. Mean Particle Size of Liposomes Derived After Hydration of Various Lipid Formulations Encapsulating Ciprofloxacin[a]

Formulation	Non milled Powder (μm)	Jet-milled Powder (μm)
DPPC (0.05 M)	1.36 ± 0.25	0.56 ± 0.13
DPPC (0.1 M)	1.52 ± 0.21	0.71 ± 0.16
DPPC (0.12 M)	1.59 ± 0.20	0.69 ± 0.15
EPC (0.05 M)	1.23 ± 0.18	0.49 ± 0.11
EPC (0.1 M)	1.32 ± 0.21	0.56 ± 0.13
EPC (0.12 M)	1.43 ± 0.23	0.67 ± 0.15
EPC + Chol. (0.05 M)	1.89 ± 0.16	0.91 ± 0.09
EPC + Chol. (0.1 M)	2.13 ± 0.21	1.02 ± 0.12
EPC + Chol. (0.12 M)	2.06 ± 0.23	0.99 ± 0.12
EPC + DMPG (0.015 M)	1.67 ± 0.13	0.59 ± 0.09
EPC + DMPG (0.03 M)	1.87 ± 0.16	0.65 ± 0.08
EPC + DMPG (0.05 M)	1.93 ± 0.16	0.62 ± 0.12
DMPG (0.015 M)	1.25 ± 0.11	0.99 ± 0.14
DMPG (0.03 M)	1.57 ± 0.15	1.02 ± 0.12
DMPG (0.05 M)	1.61 ± 0.13	1.06 ± 0.15

[a] $n = 3$; ± SD; lipid/lactose molar ratio was kept constant at 1:5 for all the formulations.

also indicate that incorporation of cholesterol in EPC-based formulations at a molar ratio of 1:1 had no appreciable effect on the encapsulation efficiency compared with only EPC-based formulations. The lack of effect of cholesterol on ciprofloxacin partitioning suggests that ciprofloxacin may be residing in the aqueous channels of liposomes.[16]

It is also interesting to note that the incorporation of negatively charged lipid, DMPG, in the EPC-based formulation dramatically increased the encapsulation of ciprofloxacin from 43.6 ± 2.3 to $96.2 \pm 1.4\%$. It is known that the inclusion of a charged lipid into the phospholipid bilayers causes electrostatic separation of the bilayers and is a method by which uptake of drugs associated with aqueous volume may be improved.[18,19] It is also interesting to note that such a high encapsulation was achieved even at a lower lipid concentration of 0.05 M. The DMPG-based formulations showed encapsulation at 0.03 M of $90.5 \pm 2.2\%$, increasing to $95.8 \pm 1.4\%$ at 0.05 M concentrations. The increased entrapment of ciprofloxacin in the presence of negatively charged lipid may also be attributed to the formation of a lipophilic ion pair between the positive center of ciprofloxacin and negatively charged moiety of DMPG. Higher entrapment in EPC-based liposomes compared with DPPC-based liposomes may be attributed to the difference in the phase transition temperatures of the two lipids. Because the lipids were hydrated at room temperature ($24 \pm 1^\circ$C), and because of the higher phase transition temperature of DPPC (43°C), most of the lipid molecules may remain in the gel phase rather than liquid crystalline phase, thereby yielding lower entrapment. In contrast, because of the lower phase transition temperature of EPC (-3°C), most of the lipid molecules are in the fluid phase on hydration, thereby exhibiting higher encapsulation. Similarly, because of a lower phase transition temperature of DMPG (23°C) than the hydration temperature, higher encapsulation efficiency was observed.

It is also interesting to note that jet-milling of the powder formulations did not show any appreciable impact on the encapsulation efficiency. The particle size distribution of liposomes derived from various nonmilled and jet-milled formulations encapsulating ciprofloxacin is shown in Table 2.

Cationic Peptide CM3

These encouraging results further motivated us to study the partitioning of other drugs, which are being considered for pulmonary treatment. Cationic peptides are being developed as a new class of antibiotics.[20] They are effective against bacteria, *P. aeruginosa*, which commonly infect cystic

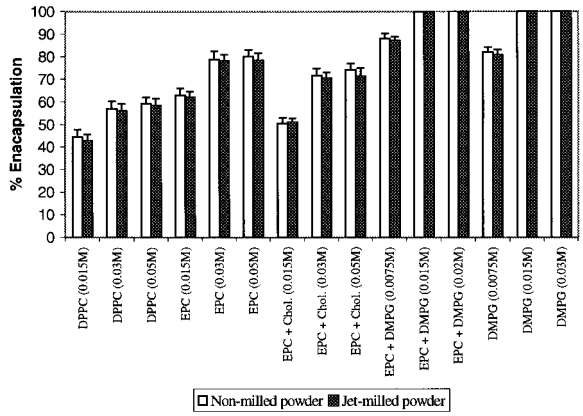


Figure 4. Histogram representation of percent encapsulation efficiency of CM3 peptide (in spontaneously formed liposomes) on dispersion of various formulations consisting of different lipids at various concentrations (lipid/lactose molar ratio was kept constant at 1:5; $n = 3$; error bars indicate SD).

Table 3. Mean Particle Size of Liposomes Derived After Hydration of Various Lipid Formulations Encapsulating CM3 Peptide[a]

Formulation	Non milled Powder (μm)	Jet-milled Powder (μm)
DPPC (0.015 M)	3.44 ± 0.23	0.66 ± 0.13
DPPC (0.03 M)	3.68 ± 0.23	0.71 ± 0.14
DPPC (0.05 M)	3.89 ± 0.20	0.73 ± 0.12
EPC (0.015 M)	2.68 ± 0.26	0.53 ± 0.09
EPC (0.03 M)	2.88 ± 0.23	0.62 ± 0.13
EPC (0.05 M)	2.92 ± 0.24	0.59 ± 0.12
EPC + Chol. (0.015 M)	3.13 ± 0.19	0.69 ± 0.12
EPC + Chol. (0.03 M)	3.44 ± 0.24	0.74 ± 0.14
EPC + Chol. (0.05 M)	3.35 ± 0.22	0.71 ± 0.12
EPC + DMPG (0.0075 M)	3.17 ± 0.20	0.48 ± 0.09
EPC + DMPG (0.015 M)	3.31 ± 0.24	0.54 ± 0.11
EPC + DMPG (0.03 M)	3.55 ± 0.27	0.52 ± 0.11
DMPG (0.0075 M)	3.47 ± 0.18	0.69 ± 0.14
DMPG (0.015 M)	3.98 ± 0.23	0.72 ± 0.13
DMPG (0.03 M)	3.88 ± 0.21	0.73 ± 0.12

[a] $n = 3$; $\pm$ SD; lipid/lactose molar ratio was kept constant at 1:5 for all the formulations.

fibrosis patients. Scott et al.[17] carried out extensive *in vitro* studies of several cationic peptides and demonstrated that CM3 had good activity against gram-negative bacteria. CM3 was a candidate for this study because of its potential to be toxic when applied systemically and because liposomal encapsulation may alter its pharmacokinetics after delivery to the lung.

The encapsulation efficiency of CM3 in spontaneously formed liposomes on hydration of different powder formulations prepared with different phospholipids at various concentrations is shown in Figure 4. It is interesting to note that CM3 peptides showed even higher encapsulation compared with ciprofloxacin for the respective lipid. Again, incorporation of cholesterol in EPC-based formulations, at a molar ratio of 1:1, did not show any significant difference in encapsulation efficiency compared with only EPC-based formulations. EPC-based formulations at a concentration of 0.03 M showed higher entrapment than DPPC (0.03 M)-based formulations. Further increases in lipid concentration did not improve the entrapment of CM3 for either lipid. Thus, the concentration of EPC and DPPC required to achieve optimum encapsulation of CM3 is considerably lower than that required to achieve optimum encapsulation of ciprofloxacin. It was also observed that incorporation of negatively charged DMPG in EPC-based formulation at a molar ratio of 1:1 significantly improved encapsulation from 78 ± 3.2 to 100% with no CM3 found in the supernatant after centrifugation of the spontaneously formed liposome dispersions. Similarly, incorporation of CM3 in only DMPG-based liposomes showed nearly complete encapsulation at a concentration as low as 0.015 M. As observed earlier, the results in Figure 4 show that jet milling of the powder formulations did not exhibit any appreciable impact on the encapsulation efficiency. The particle size distribution of various hydrated nonmilled and jet-milled formulations encapsulating the CM3 peptide is shown in Table 3.

Salbutamol Sulfate

To further examine the robustness of the spontaneous formation of liposomes from powders, we also investigated the encapsulation of salbutamol sulfate, a commonly used bronchodilator incorporated in various commercial inhalers. The encapsulation efficiency of salbutamol sulfate in various powder formulations prepared with different phospholipids at various concentrations is shown in Figure 5. Salbutamol sulfate exhibits lower encapsulation efficiency in DPPC ($24.9 \pm 2.2\%$) and EPC ($39.1 \pm 3.1\%$) formulations as compared with ciprofloxacin and CM3 peptide. Even incorporation of negatively charged lipid (DMPG)

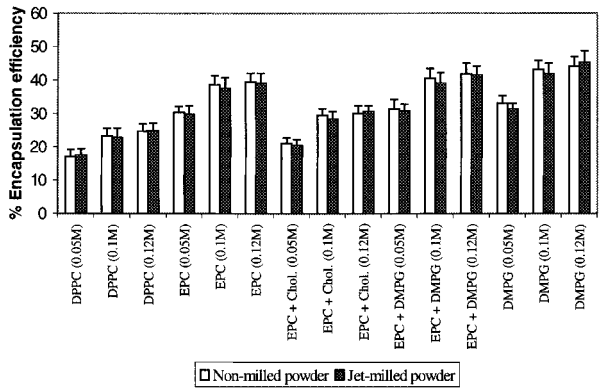


Figure 5. Histogram representation of percent encapsulation efficiency of salbutamol sulfate (in spontaneously formed liposomes) on dispersion of various formulations consisting of different lipids at various concentrations (lipid/lactose molar ratio was kept constant at 1:5; $n = 3$; error bars indicate SD).

Table 4. Mean Particle Size of Liposomes Derived After Hydration of Various Lipid Formulations Encapsulating Salbutamol Sulfate[a]

Formulation	Non milled Powder (μm)	Jet-milled Powder (μm)
DPPC (0.05 M)	1.92 ± 0.23	0.62 ± 0.13
DPPC (0.1 M)	2.22 ± 0.18	0.71 ± 0.16
DPPC (0.12 M)	2.16 ± 0.20	0.76 ± 0.12
EPC (0.05 M)	1.23 ± 0.21	0.39 ± 0.09
EPC (0.1 M)	1.35 ± 0.17	0.41 ± 0.13
EPC (0.12 M)	1.42 ± 0.20	0.52 ± 0.10
EPC + Chol. (0.05 M)	1.98 ± 0.15	0.64 ± 0.14
EPC + Chol. (0.1 M)	2.13 ± 0.24	0.67 ± 0.13
EPC + Chol. (0.12 M)	2.34 ± 0.20	0.71 ± 0.13
EPC + DMPG (0.05 M)	1.43 ± 0.21	0.54 ± 0.15
EPC + DMPG (0.1 M)	1.65 ± 0.23	0.61 ± 0.11
EPC + DMPG (0.12 M)	1.60 ± 0.28	0.69 ± 0.17
DMPG (0.05 M)	1.63 ± 0.16	0.46 ± 0.10
DMPG (0.1 M)	1.69 ± 0.23	0.51 ± 0.11
DMPG (0.12 M)	1.75 ± 0.28	0.54 ± 0.15

[a] $n = 3$; $\pm$SD; lipid/lactose molar ratio was kept constant at 1:5 for all the formulations.

in EPC-based formulations did not appreciably enhance the encapsulation. It may be assumed that the ion-pair complex formed between salbutamol sulfate and negatively charged lipid may not be as hydrophobic as those formed between CM3 and ciprofloxacin with the charged lipid, thereby exhibiting lower entrapment. In other words, the increased entrapment of ciprofloxacin and CM3 peptide in the presence of DMPG reflects the relatively higher hydrophobicity of the complex in EPC/DMPG-based liposome systems. As observed earlier, incorporation of cholesterol did not affect the encapsulation efficiency. The particle size distribution of various hydrated nonmilled and jet-milled formulations encapsulating salbutamol sulfate is shown in Table 4.

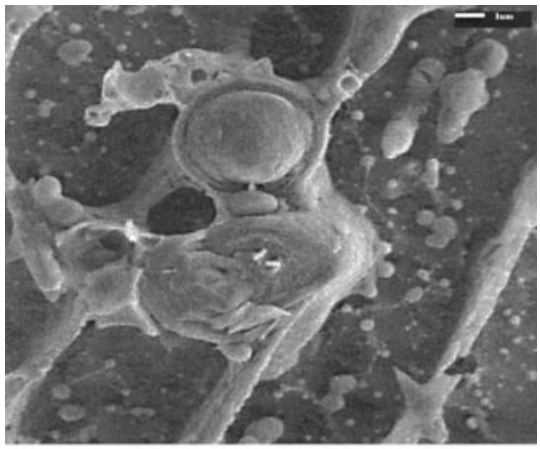
Freeze–Fractured SEM

The spontaneous formation of liposomes on hydration of phospholipids was further authenticated by freeze–fracture SEM. The electron microscopes of freeze–fractured surfaces derived from EPC + DMPG (1:1)-based formulations encapsulating ciprofloxacin, CM3 peptide, and salbutamol sulfate are shown in Figures 6a, b and c respectively. Figures 6d and e show electron microscopy of freeze–fractured surfaces derived from EPC- and DPPC-based formulations, respectively, encapsulating ciprofloxacin. It is evident from these micrographs that multilamellar vesicles (MLVs) are formed on hydration of phospholipids spontaneously, which act as a reservoir for encapsulating drugs.

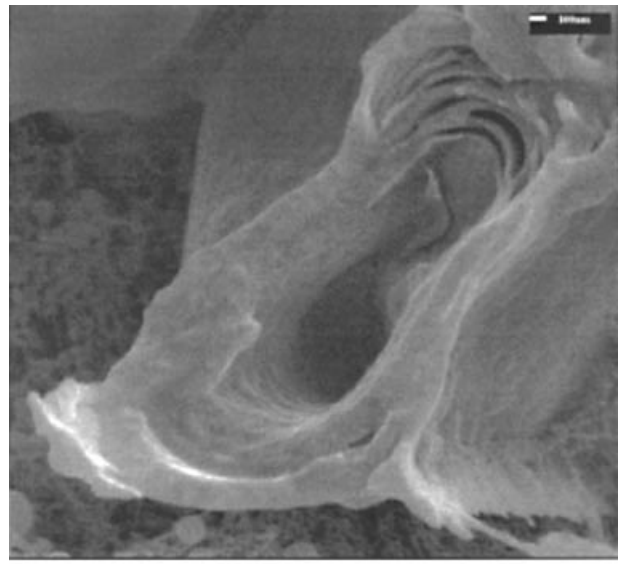
CONCLUSIONS AND PERSPECTIVES

We have demonstrated the spontaneous formation of MLVs on dispersion of various phospholipid-based powder formulations in saline. This novel approach of delivering liposomes in dry powder form was advantageous to avoid the detrimental effects of lyophilization and jet-milling on leakage of the encapsulated drug. The encapsulation efficiency was dependent on the nature of the drug and lipid composition.

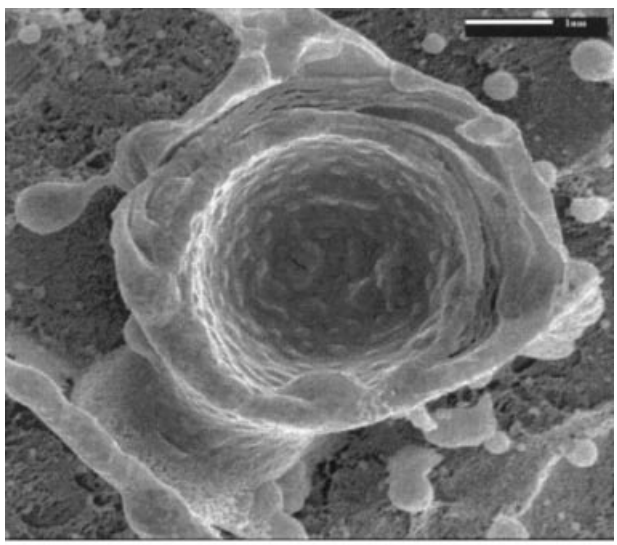
It has been observed previously that aerosol dispersion of micronized powder formulations prepared from lyophilized cakes is difficult because of the adhesive behavior of the lyophilized cakes and aggregation of the micronized powders particles. However, using the present approach, no lyophilized cake is involved in formulating powders and, hence, it can be assumed that these powder formulations will exhibit improved aerodynamic properties. Work to verify this assumption is in progress in our laboratory.



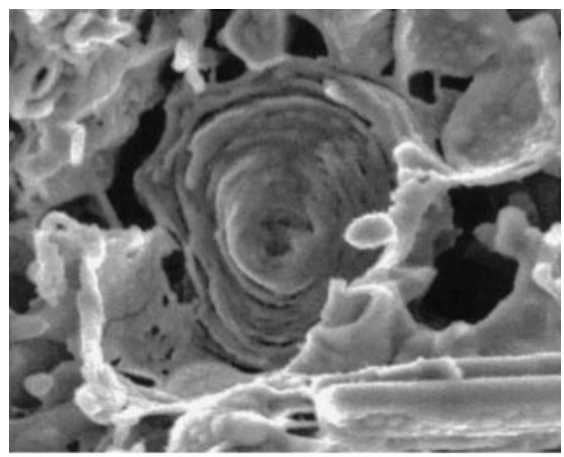
a



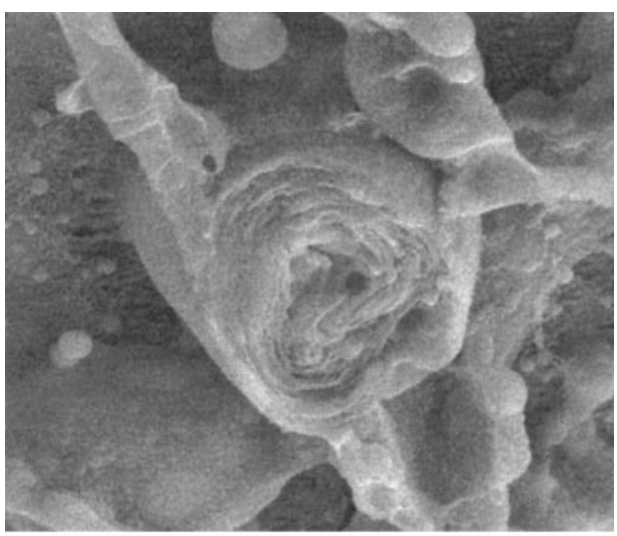
b



c



d



e

Figure 6. (a) Freeze–fractured SEM of EPC + DMPG (molar ratio 1:1)-encapsulating ciprofloxacin (lipid concentration: 0.05 M; lipid/lactose ratio was adjusted to 1:5). (b) Freeze–fractured SEM of EPC + DMPG (molar ratio, 1:1) encapsulating CM3 (lipid concentration, 0.0015 M; lipid/lactose ratio was adjusted to 1:5). (c) Freeze–fractured SEM of EPC + DMPG (molar ratio, 1:1) encapsulating salbutamol sulfate (lipid concentration, 0.1 M; lipid/lactose ratio was adjusted to 1:5). (d) Freeze–fractured SEM of EPC encapsulating ciprofloxacin (lipid concentration, 0.1 M; lipid/lactose ratio was adjusted to 1:5). (e) Freeze–fractured SEM of DPPC encapsulating ciprofloxacin (lipid concentration, 0.1 M; lipid/lactose ratio was adjusted to 1:5).

ACKNOWLEDGMENTS

The authors thank Ms. Karen Blasetti for the excellent laboratory help. Supply of Pharmatose 325M from DMV International, The Netherlands, is gratefully acknowledged. W.F. gratefully acknowledges the support of the Canadian Cystic Fibrosis Foundation in this work. R.H. acknowledges the support of the Canadian Bacterial Diseases Network and Canadian Cystic Fibrosis Foundation's SPARx program and the receipt of Canada Research Chair.

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