The Effect of Jet-Milling on Lyophilized Liposomes

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INTRODUCTION

Because of their capacity to target drugs to cells such as macrophages and to alter drug pharmacokinetics, liposomes are promising vehicles for pulmonary drug delivery (1,2). And among the available pulmonary dosage forms, for liposomes, dry powders are worth examining (3). Liposomes can be more stable when dried, for example, by lyophilization (4,5); and the technology of dry powder delivery is advancing (6). Thus, it should be of value to determine if stable liposome powders can be produced from lyophilized precursors.

For powder particles to be respirable (i.e., to reach the lower airways), the lyophilized precursor should ideally be micronized to particles of approximately 1 to 6 μm in diameter: if smaller, they may be exhaled; and if larger, inertial impaction may prevent them from reaching the lower airways. Jet-milling, which causes particles to break apart upon colliding in a high velocity airstream, can micronize adequately, and it has been used to produce liposome powders (3). However, the consequences of jet-milling to liposome integrity are unclear.

The main purpose of this study was to examine the effect of jet-milling on liposome integrity. Liposomes of different formulations were prepared with an encapsulated marker (calcein), then lyophilized and jet-milled. The liposome formulations were based on DPPC, which has a high transition temperature (42°C) and is a natural component of pulmonary surfactant, and POPC which has a low transition temperature (−3.5°C). DPPC-based preparations were also modified with DPPG to add charge to the membrane. Cholesterol was added to DPPC liposomes to confer fluidity to the membrane (below its transition temperature), and to POPC to strengthen the membrane and prevent a membrane phase transition (7). Sucrose and lactose were included in the formulations, owing to their ability to protect liposomes during lyophilization.

The ability of the liposomes to maintain their integrity upon lyophilization and jet-milling was measured as a function of their ability to retain encapsulated calcein upon reconstitution of the dry products.

MATERIALS AND METHODS

Materials

The following materials were used as purchased: dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG [sodium salt]), and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) from Avanti Polar Lipids (Alabaster, AL, USA); cholesterol (CH) (Sigma grade), sucrose (Sigma Ultra), and Sephadex-G-50-80 from Sigma Chemical Co. (St. Louis, MO, USA); D(+)-lactose monohydrate (Ph.Eur.) from Fluka (Ronkonkoma, NY, USA); and calcein (high purity) from Molecular Probes, Inc. (Eugene, OR, USA).

Liposome Preparation

Liposomes of the following lipid compositions were prepared: DPPC; DPPC/CH, 7:3 molar ratio; DPPC/DPPG, 7:3; POPC/CH, 7:3. Dry lipids (lyophilized from *t*-butanol) were hydrated to a 27.2 mM lipid concentration with 292 mM sucrose and lactose solutions in 25 mM phosphate buffer (Na_2HPO_4/KH_2PO_4, pH = 7.4), containing 3.2 mM of calcein. The multilamellar vesicles, so formed, were extruded through two 100 nm pore-size polycarbonate membranes (Poretics; Livermore, CA, USA) using Liposofast Large (Avestin, Ottawa, ON, Canada). For preparations containing DPPC, the extruder was heated to greater than 42°C.

Extraliposomal calcein was removed from the extruded liposomes by Sephadex gel chromatography using a variation of the dry mini-column method of Fry (8). Sephadex G-50-80 gels, hydrated with phosphate buffered sugar solutions, were loaded into 5 ml disposable plastic syringes, and dried by centrifugation. Extruded liposomes were added to the dry columns, then centrifuged and collected in the eluate.

Liposome Lyophilization

Sephadex-treated liposomes were lyophilized in a Virtis Unitop 400SL Freeze Dryer, equipped with a Sentry Datalog. They were shelf-frozen to between −36 to −38°C (sample temperature), then lyophilized for 24 hours of primary drying at −35°C, followed by 8 hours of secondary drying at 25°C.

Milling

The lyophilized liposomes were divided into two portions: a non-milled portion for reconstitution studies and the other portion for jet-milling. The latter were first ground to a fine powder by vortex-mixing the lyophilisates with a Teflon-coated magnetic stir-bar in lyophilization vials. The resulting powders were fed from a vibratory trough into the jet-mill (Model 00 Jet-O-Mizer: Fluid Energy Aljet, Plumsteadville, PA), and milled with dry nitrogen gas at a grinding nozzle pressure of 100 p.s.i. and a pusher nozzle pressure of 90 p.s.i. The jet-milled powders were collected in a small volume filter-collector attached to the mill. The powders were later retrieved off the mill filter and stored in microcentrifuge tubes in a desiccator at room temperature (21–25°C) until reconstitution. The relative humidity during milling ranged from 22 to 35%.

Characterization of the Liposome Powders

Liposome powder samples were gold-coated, then examined with a Hitachi S4000 Field Emission scanning electron microscope. Sample aerodynamic particle sizes were measured by laser light scattering, using an Aerosizer particle size analyzer (Amherst Process Instruments, Hadley, MA) (9), equipped

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with an Aero-Disperser and run with Version 7.02 software, with the density set at 1.46.

Dry Liposome Reconstitution and Filtration

Dry liposomes were reconstituted to one-third their original concentration with phosphate buffered 0.9% saline (PBS) solution at between 34 and 36°C. Extraliposomal calcein was removed from the reconstituted liposomes by centrifuge-filtration (Ultrafree-MC Filter Unit, 30,000 NMWL: Millipore Corporation; Bedford, MA).

Determination of Calcein Retention

The fraction of calcein retained within reconstituted liposomes was calculated as the lipid-normalized calcein fluorescence in the centrifuge-filtered liposomes divided by that in non-filtered liposomes. Calcein contents were determined in Triton-X 100 solubilized aliquots, with fluorescence read at an excitation wavelength of 490 nm and emission wavelength of 510 nm. The phospholipid contents of separate liposome aliquots were determined by a modification of an assay by Stewart (10), employing an ammonium ferrothiocyanate dye. The absorbances of the phospholipid-dye complexes in chloroform were measured at 475 nm.

To normalize calcein fluorescence to phospholipid content, calcein fluorescence was divided by phospholipid-dye absorbance. The fraction of calcein retained was calculated from these values, and retention is reported here as the mean of three sample replicates: three separate liposome preparations, lyophilized separately, and milled on the same day. Analysis of variance was applied to compare the means.

RESULTS AND DISCUSSION

Characterization of Liposome Powders

Scanning electron micrographs of DPPC-lactose powders are shown in Figure 1. The powder fed into the mill (Fig. 1-A and B) appears as pieces of the lyophilisate with liposomes still embedded within the glassy sugar matrix. The jet-milled powder (Fig. 1-C and D) has smaller powder particles, and there is evidence of liposome aggregation and release of liposomes from their sugar matrix.

Aerodynamic particle size distributions of the DPPC-lactose powders (Fig. 2) illustrate that jet-milling produced powder

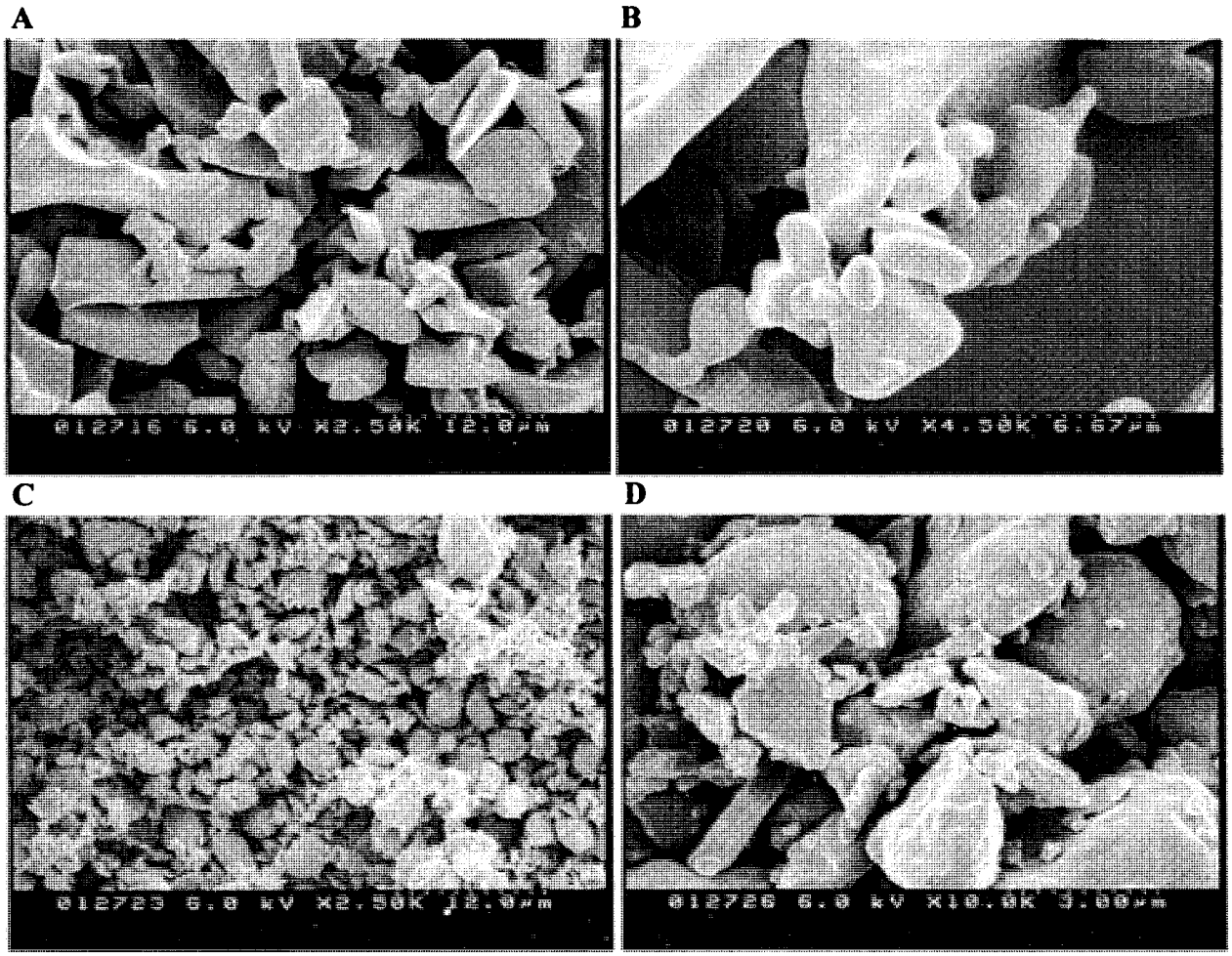

Fig. 1. Scanning electron micrographs (at different magnifications) of DPPC-lactose liposome powders before and after jet-milling: **A and B.** Powder produced by vortexing the lyophilisate with a stir-bar. (This powder was fed into the jet-mill.) **C and D.** Jet-milled liposome powder.

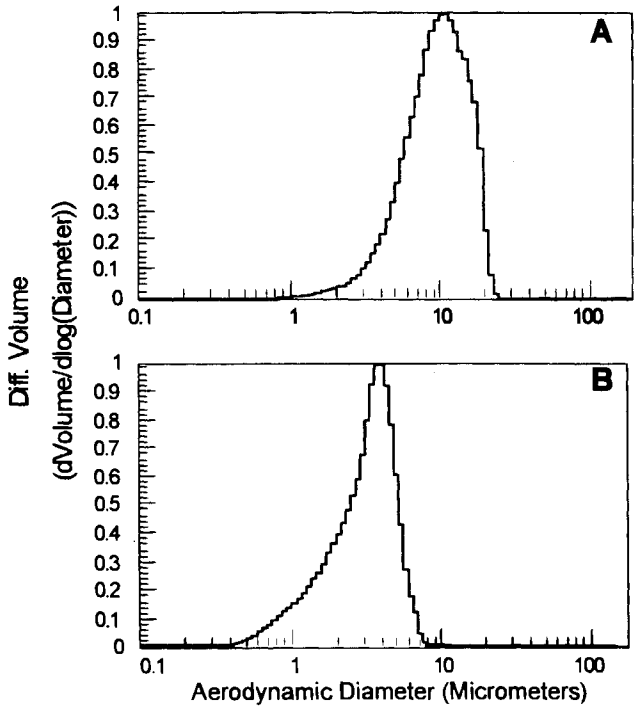

Fig. 2. Particle size distributions of DPPC-lactose liposome powders before and after jet-milling: **A.** Size-distribution for the powder produced by vortexing with a stir-bar (mean diameter = 9.93 μm, s.d. = 1.69). **B.** Jet-milled powder particle size distribution of the same powder shown in Fig. 1-C and D (mean diameter = 3.15 μm, s.d. = 1.73).

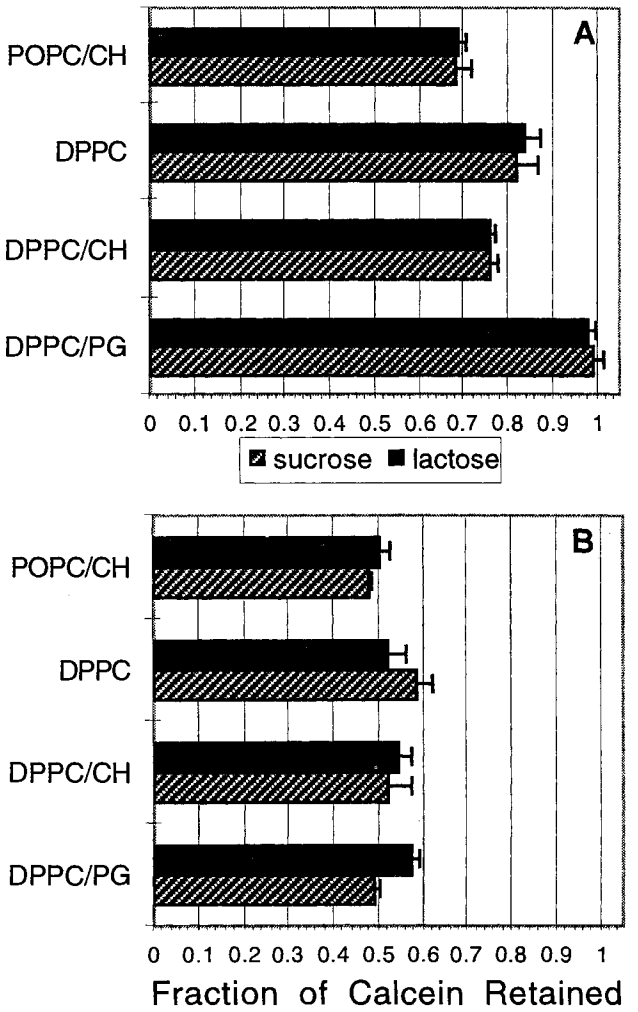

Fig. 3. Calcein retention of reconstituted lyophilized liposomes: **A.** Non-milled liposomes (Non-perturbed lyophilisate). **B.** Jet-milled liposomes. Each of the data points represent the mean ± S.E.M. for three separate batches of each formulation.

particles with a respirable size distribution. While the powders fed into the jet-mill had mean aerodynamic particle diameters of approximately 7 to 10 μm, upon jet-milling, particle size was reduced considerably, with an average mean diameter of 3.16 μm (0.14 μm s.d., n = 4), calculated from the measurements of four different liposome formulations. It is worth noting that particle diameters of 3 μm may be ideal for maximizing pulmonary deposition of dry powders (11).

Calcein Retention Results

Jet-milling led to a significant reduction in calcein retention (Fig. 3), thus compromised liposome integrity. Non-milled lyophilized liposomes (Fig. 3A) retained calcein in a lipid-dependent manner, with a rank-order for calcein retention of DPPC/DPPG (98.4%) > DPPC (82.6%), DPPC/CH (75.7%) > POPC/CH (68.4%). These percentages are averages of lactose- and sucrose-containing formulations (n = 6), as the two sugars afforded statistically equal protection. Jet-milling augmented calcein loss from all lyophilized liposomes (Fig. 3B), with a mean calcein retention of 52.7% (DPPC/DPPG [52.9%], DPPC [55.5%], DPPC/CH [53.3%], and POPC/CH [49.0%]), and with no statistically significant difference attributable to either lipid or sugar. Thus, jet-milled liposomes lost approximately half of their aqueous contents upon reconstitution, regardless of composition.

For non-milled lyophilized liposomes, there are several possible causes of leakage during lyophilization including membrane phase transitions, stress-induced liposome lysis, and liposome aggregation and fusion (5,12,13). The glassy sugar matrix should have limited liposome aggregation and fusion (5). Although, for the DPPC-based liposomes, the inclusion of the negatively charged DPPG (to limit aggregation and fusion) improved calcein retention, implying that aggregation and fusion may have played some role in calcein loss for DPPC liposomes without DPPG. This possibility is supported by the fact that DPPC liposomes can aggregate below their transition temperature (14), and thus may be aggregated before the glassy sugar matrix forms during freezing. It is doubtful that membrane phase transitions played a role in calcein loss, as DPPC-based liposomes do not undergo a transition over the temperature range used (−38°C to +36 °C), and the inclusion of cholesterol in POPC liposomes should have eliminated the POPC transition at −3.5 °C (9). Stress-induced liposome lysis may have occurred, as slow freezing (≈ 1 °C/min) in the lyophilizer, can cause liposome dehydration and shrinkage (4), leading to membrane stress. The relatively greater retention in the DPPC-based vs. POPC/CH liposomes may reflect a greater resistance of those liposomes to stress-induced lysis.

The augmentation of liposomal calcein-loss caused by jet-milling may have been due to liposome rupture during jet-milling; and liposome aggregation or fusion, and stress-induced lysis during rehydration. Jet-milled liposomes showed no obvious visible damage (Fig. 1-C and D), although damage from the high energy collisions during jet-milling cannot yet be excluded. It is likely that aggregation and fusion may have contributed to calcein loss in the jet-milled products. Micrographic evidence of liposome aggregation and release from their glassy matrix (Fig. 1.-D), along with the probable interparticulate cohesive forces imparted by jet milling (15), suggests a possible tendency towards particle aggregation. The likelihood of liposome aggregation or fusion was supported by particle-size analyses (data not shown), which showed a size growth of liposomes following reconstitution of jet-milled powders.

The rehydration process may have also contributed to calcein loss from jet-milled liposomes. Whereas reconstitution was practically instantaneous for non-milled lyophilized liposomes, the jet-milled liposomes required several seconds and tapping of the centrifuge tubes to achieve full reconstitution. During this time, several forces may have contributed to calcein loss: There may have been localized osmotic gradients with associated membrane stresses and consequent liposome lysis; and increased chances for membrane apposition with consequent liposomal aggregation and fusion at the temporarily low water contents (5). Perhaps better powder dispersal may improve powder wetting, and minimize these effects.

In conclusion, for jet-milling to be a practical method of producing pulmonary liposome powders, further studies are needed to determine and eliminate the sources of the detrimental effect of jet-milling on liposome integrity.

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