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Liposomal Dry Powders as Aerosols for Pulmonary Delivery of Proteins

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ABSTRACT

The purpose of this research was to develop liposomal dry powder aerosols for protein delivery. The delivery of stable protein formulations is essential for protein subunit vaccine delivery, which requires local delivery to macrophages in the lungs. β-Glucuronidase (GUS) was used as a model protein to evaluate dry powder liposomes as inhaled delivery vehicles. Dimyristoyl phosphatylcholine:cholesterol (7:3) was selected as the liposome composition. The lyophilization of liposomes, micronization of the powders, aerosolization using a dry powder inhaler (DPI), and in vitro aerodynamic fine particle fraction upon collection in a twin-stage liquid impinger were evaluated. After lyophilization and jet-milling, the total amount of GUS and its activity, representing encapsulation efficiency and stability, were evaluated. The GUS amount and activity were measured and compared with freshly-prepared liposomes in the presence of mannitol, 43% of initial GUS amount, 29% of GUS activity after lyophilization and 36% of GUS amount, 22% of activity after micronization were obtained. Emitted doses from dry powder inhaler were 53%, 58%, 66%, and 73% for liposome powder:mannitol carrier ratios of 1:0, 1:4, 1:9, and 1:19. Fifteen percent of the liposome particles were less than 6.4 μm in aerodynamic diameter. The results demonstrate that milled liposome powders containing protein molecules can be aerosolized effectively at a fixed flow rate. Influences of different cryoprotectants on lyophilization of protein liposome formulations are reported. The feasibility of using liposomal dry powder aerosols for protein delivery has been demonstrated but further optimization is required in the context of specific therapeutic proteins.

KEYWORDS: protein, liposome, lyophilization, dry powders, aerosol, pulmonary delivery.

INTRODUCTION

Drug delivery via the lungs received considerable attention throughout the 1990s.[1] Difficulties associated with the delivery of peptides and proteins, coupled with the likelihood of acceptable pulmonary bioavailability led to interest in their presentation for absorption via the lungs.

Liposomes are promising vehicles for pulmonary drug delivery owing to their capacity to target drug to cells, such as macrophages, and to alter pharmacokinetics of drugs.[2,3] They also provide sustained release, prevent local irritation, increase drug potency, reduce toxicity, and uniformly deposit active drugs locally.[4,5]

Some proteins and enzymes, such as glutathione,[6] superoxide dismutase,[7] and catalase,[8] have been encapsulated by liposomes to improve their pulmonary delivery, and most of them were administered intratracheally into the respiratory tract in liquid-based liposomes.[2] Nebulizers have been used extensively for the delivery of liposomes.[9,10] However, delivery of liposomes by nebulization may be hampered by long-term instability problems that lead liposome dispersions to undergo physiochemical changes resulting in leakage of the encapsulated drug.[11] Among formulations employed for drug delivery to the lungs, dry powders stand out because of the stability of drugs and formulations. Dry powder delivery is an important inhalation technology. Dry powder products may consist of drug alone or blended with excipient that acts as a carrier for delivery to the lungs.[12] Freeze-dried liposomes have been prepared for aerosol delivery to improve liposome stability.[13] Another method of preparing dry powder liposomes is by spray-drying.[14] Some small molecules, such as Budsonide[15] and Ketotifen,[16] have been prepared in freeze-dried liposomes to form a dry powder dispersion for inhalation. These investigations demonstrated the possibility of delivering liposomally entrapped small molecules to terminal bronchioles in therapeutic doses and offered the exciting possibility of aerosol delivery as dry powder formulations.

Few studies have been performed to evaluate whether therapeutic proteins in liposomes can be aerosolized into the respiratory tract in dry powder formulations. Superoxide dismutase was encapsulated in the spray-dried liposomes to evaluate respirable properties.[14] In our present studies, a model protein was used to evaluate both the feasibility of delivering dry powder liposome formulations for protein and, more importantly, whether the process of lyophilization could be used to prepare dry powders suitable for pulmonary delivery.

The majority of studies describing pulmonary delivery of proteins and peptides have focused on systemic delivery of drugs.[17-21] The present studies are concerned with the

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delivery of proteins for local activity, specifically the delivery of subunit vaccines to elicit cell-mediated responses for tuberculosis prevention.[22] Many of these proteins are in limited supply and a formulation strategy requires an approach validated using model proteins. The enzyme, β-glucuronidase (GUS), was evaluated as a model protein in preliminary studies of liposome formulation, lyophilization, micronization, and aerodynamic size distributions. The specific aims of the study are to (1) prepare liposomes containing the model protein, (2) adopt methods for lyophilization and milling that result in fine particles suitable for inhalation, and (3) to characterize the powder in terms of protein structure or activity before and after the various processes and aerosolization.

Materials and Methods

Materials

Dipalmitoyl phosphatidylcholine (DPPC), dimyristoyl phosphatidylcholine (DMPC), dioleoyl phosphatidylcholine (DOPC), Dioleoyl trimethylammonium propane (DOTAP), dioleoyl phosphatidylglycerol (DOPG), and cholesterol (CH) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). D-mannitol, sucrose, sodium phosphate monobasic, HEPES, ammoniumferrothiocyanate, phenolphthalein glucuronic acid, and GUS from bovine liver (type B-1) were purchased from Sigma (St Louis, MO). Povidone (polyvinylpyrrolidone [PVP], Mw 40 000) was from Spectrum (Gardena, CA). PAGE gel (Bio-rad ready gels, 7.5% Tris-HCl) and protein quantification kit DC protein assay package were from Bio-Rad (Hercules, CA). All materials were used as received.

Methods

Liposome Preparation

Different components of liposomes were prepared by dehydration-rehydration and then freezing- thawing methods. Different fatty acid chain length (DOPC, DMPC, DPPC) and charged lipids (DOPG and DOTAP) together with cholesterol were screened for encapsulation efficiency. DOPC- CH (7:3), DOPC-CH-DOTAP (7:2:1, 7:1:2), DOPC-CH-DOPG (7:2:1), DMPC-CH (7:3) and DPPC-CH (7:3) were used as lipid components. Lipids were dissolved in chloroform, and the solvent was evaporated by nitrogen. The lipid films were put into vacuum desiccators in the presence of dry silica gel for 2 hours to remove chloroform. The dry lipid films were hydrated in 20 mM sodium phosphate buffer (pH 7.4), containing 25 mM lipids, 250 mM sugar, and 3.75 mg/mL β-glucuronidase. The multilamellar vesicles were freeze-thawed for 20 cycles, −80°C to 37°C, using liquid nitrogen and temperature-controlled water bath. The free proteins were removed by centrifugation at g = 25 000, 10°C for 30 minutes. The liposome pellets were rinsed (4 times) until the ratio of free protein in the supernatant to encapsulated protein in liposomes was less than 2%. The freshly prepared liposomes were subjected to quasi-elastic, dynamic light scattering particle size analysis (NICOMP Submicron Particle Sizer autodilute, model 370, Santa Barbara, CA). A volume of 600 μL 0.1% Triton X-100 was added into 400 μL freshly prepared liposomes to disrupt the liposome vehicles for protein quantification and activity assay.

Liposome Lyophilization

Liposome suspensions were frozen in dry ice-acetone. The liposomes were lyophilized (Labconco Freeze Dry System, Freezone 6, Kansas City, Missouri) at −45°C for 48 hours.

Milling

One gram of the lyophilized liposomes was micronized (Trost GEM-T jet mill, Plastomer Products, Newton, NJ) with dry nitrogen gas (60 and 40 pounds per square inch gauge [psig] of pusher and grinder pressure, respectively). The powders were collected from cyclone and jar. The micronized particles were stored in a desiccator containing silica gel under vacuum at room temperature (20°C-21°C) until required. Powders were stored for a maximum of 2 days before characterization and further assay were performed.

Characterization of Liposomes

Lyophilized liposomes, before and after jet-milling, were characterized by Scanning Electron Microscopy (SEM, model 6300, JEOL, Peabody, NY). The stubs were coated with gold-palladium alloy (150-250Å) using a sputter coater (Polaron 5200, Structure Probe Supplies, West Chester, PA). The coater was operated at 2.2k V, 20 mV, 0.1 torr (argon) for 90 seconds. An accelerating voltage of 15 kV was used.

Dry Liposome Reconstitution

Quantities of lyophilized or jet-milled liposomes were reconstituted in 20 mM phosphate buffer (pH 7.4) to original concentrations in preparation. Samples were vortexed for 2 seconds and centrifuged twice to separate the free drug at g = 25 000, 10°C for 30 minutes. A volume of 1000 μl of 0.1% Triton X-100 was added into the lipid pellets to disrupt lipid vesicles for protein quantification and activity assay.

GUS Quantification and Activity Assay

Lowry protein quantification method (Bio-Rad DC protein assay) was used to determine the total amount of GUS.[23] The standard curve of UV absorbance (λ_{max} = 750 nm) versus GUS concentration was prepared. Absorbance was linear with respect to GUS concentration from 10 μg/mL to

100 μg/mL with R^2 = 0.998. Lipid interference was negligible when lipid concentration was lower than 0.08mM. In all the quantification assays, lipid concentrations were under 0.08mM.

The substrate employed in the enzyme activity assay was phenolphthalein glucuronic acid. The UV absorption versus mass of phenolphthalein curve was established in the phenolphthalein mass range of 0 to 50 μg with R^2= 0.999. The units of enzyme were calculated as follows:

- Units/mL enzyme = (μg phenolphthalein released)(2)(df)/0.1
- 2 = conversion factor from 30 minutes to 1 hour as per the unit definition
- df = dilution factor
- 0.1 = volume (in mL) of enzyme used

A volume of 0.7 mL sodium acetate buffer (pH 5.0), 0.7 mL 1.2 Mm substrate solution, and 100 μL enzyme sample were incubated in a 37°C water bath for 30 minutes. Glycine buffer (pH 10, 5 mL) was added to stop enzyme action. The hydrolyzed phenolphthalein absorbance was measured at UV 540 nm.[24,25]

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Lyophilized and micronized liposome powders were mixed with 0.1% Triton X-100. The lipids were extracted in chloroform. The fresh β-glucuronidase (in phosphate buffer), upper phase of lyophilized and jet-milled extracted samples were mixed with NuPAGE sample buffer and NuPAGE reducing reagent. The fresh standard solution of GUS was used as a control. The mixtures underwent the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5%) in SDS-Tris-HCl buffer, 140 V. The gel was stained with Coomassie blue and then destained with ethanol-acetic acid-water destaining solution. The gel image was scanned on a gel scanner (model GS-700 imaging denitomer, Bio-Rad) equipped with Quantity One software.

Emitted Dose Determination

The mass ratios of micronized liposome to carrier (D-mannitol 45-75 um) were 1:0, 1:4, 1:9, and 1:19. The mixtures were blended with mortar and pestle. Powder mixtures of sugar and liposome (40 mg) were weighed into No. 3 gelatin capsule. A dry powder inhaler (Inhalator, Boehringer Ingelheim, Ridgefield, CT) was attached to the mouthpiece of emitted dose apparatus (Dose uniformity apparatus B in *United States Pharmacopeia* [*USP*]). The capsule was pierced and the powder was emitted at 60 L/min for 10 seconds. The tube and filter were rinsed with chloroform. The mannitol was extracted using water to reduce the turbidity in organic phase. Finally the lipid contents were quantified.

Fine Particle Fraction Evaluation

A twin-stage liquid impinger was used to estimate the aerodynamic fine particle fraction of micronized liposome. Six capsules, each containing 40 mg mixed powder (mass ratio 1:4 liposome powder to mannitol carrier), were emptied sequentially from the Inhalator at 60 L/min for 10 seconds. The lipid content and protein activity assays were determined for each liposome powder following rinsing with 20 mM phosphate buffer. The lipid suspensions underwent lipid assay by adding chloroform and the protein activity assays by adding 0.1% Triton X-100.

Phospholipid Quantification Assay

The phospholipid contents of liposomes were determined by a modification of an assay by Stewart, employing an ammoniumferrothiocyanate dye.[26] The absorbance of the phospholipid-dye complexes in chloroform was measured at 470 nm.

Results and Discussion

GUS Encapsulation in Different Liposome Components

Different components of liposomes were studied for the encapsulation of GUS. CH was included in all preparations to confer fluidity to the gel-phase membrane (below its transition temperature) and to strengthen liquid crystal-phase membrane to prevent a membrane phase transition.[27] DOPC-CH (7:3), DOPC-CH-DOTAP (7:2:1, 7:1:2), DOPC-CH-DOPG (7:2:1), DMPC-CH (7:3), and DPPC-CH (7:3) in liposome preparation gave 3.9%, 17%, 13.6%, 19.2%, 19%, and 17.1% encapsulation efficiency, respectively. DMPC-CH (7:3) was selected for further investigation owing to encapsulation efficiency, concern for stability of protein, and ease of use. Storing the mixture of DMPC-CH lipid and aqueous protein solution at 4°C overnight before freezing-thawing cycles increased the encapsulation efficiency to 43%. This may be explained by a time dependent interaction between protein and lipid while the protein encapsulation efficiency depends on this interaction.[28] The size distribution of multilamellar vesicles of DMPC-CH in mannitol-phosphate buffer was 3.07 ± 0.35 μm as measured by dynamic light scattering.

Influences of Lyophilization and Jet-milling on Liposome Encapsulation and Protein Activity

Lyophilization is considered a promising means of extending the shelf-life of liposomes. However, both freezing and drying can induce structural and functional damage to liposomes as well as to proteins. Saccharides are frequently

used as the cryoprotectants in lyophilization for stability of liposomes and proteins. D-Mannitol was used for this purpose in the first trial of lyophilization.

The mass yields from milling lyophilized powder were in the range of 50.1% to 52.1%. Compared with the freshly prepared liposomes, the retained total amount of protein and the activity were 43% and 29% after lyophilization and 36% and 22% after micronization (Figure 1).

Scanning electron micrographs (SEM) of DMPC-CH-mannitol powder with and without GUS are shown in Figure 2. Figures 2A and 2C show amorphous lyophilized liposome-carbohydrate powders. The porous structure of the cake allows rapid dispersion of the powder for aqueous reconstitution and aids in milling efficiency to micronize powders. The micronized liposome powders are shown in 2B and 2D. The particle sizes from SEM are ~2 to 3 μm, within the respirable particle size range. The particle sizes of 3 μm may be ideal for maximizing pulmonary deposition of dry powders.[13] Micronized powders appeared to be aggregated. The liposome control and sample exhibited similar morphologies. The presence of GUS did not appear to change liposome behavior compared with liposome control. The retentions of protein contents were 43% and 36% in amount and 29% and 22% in activity before and after jet milling. Lyophilization caused protein loss in terms of poor encapsulation efficiency and stability.

β-Glucuronidase is a tetramer. In the presence of reducing reagent and SDS buffer solution, the standard GUS, lyophilized, and jet-milled GUS showed a similar pattern to fresh GUS solution except there were 2 faded bands that had almost disappeared at molecular weights 75 KDa and 55 KDa (Figure 3) with 75 KDa as one monomer in GUS tetramer structure. The lyophilized samples had lower intensity compared with fresh GUS. Flores et al indicated that the quaternary structure disruption is a characteristic of the loss of enzyme activity of GUS.[29] The freeze-drying procedure might interfere with chemical stability of monomer 75 KDa and further interfere with the interactions between the monomers.[30] Therefore, degradation and particularly quaternary structural changes were 2 of the possible reasons for the enzyme activity loss.

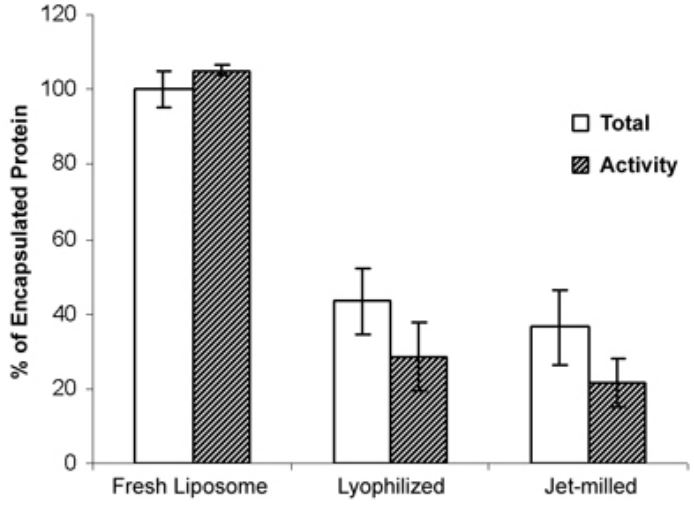

Figure 1. The total amount and retained activity of GUS after liposome preparation, lyophilization, and jet-milling (n = 2, error bars depict range).

Emitted Dose Evaluation at Different Mass Ratio of Liposome to Carrier

To determine the amount of ingredient emitted from the mouthpiece of the dry powder inhaler, a sampling apparatus was used to collect the emitted dose.[31,32] Jet-milled liposome powders were mixed with D-mannitol at the mass ratio of 1:0, 1:4, 1:9, and 1:19. The mannitol was 45- to 75-μm sieved size, which is within the range of sizes shown by Bell et al to be suitable as a carrier.[33,34] Forty milligrams of powders were put into gelatin capsule No. 3, and powders were actuated at 60 L/min for 10 seconds. The inhaler was unable to disperse more than 75% of the dose for any of the powder blends used. There was a trend to a greater proportion liposomes being dispersed as the ratio of mannitol was increased (Figure 4). Percentages of the doses, 53.2%, 58.1%, 66.5%, and 73.6% were delivered by the inhaler at the liposome powder contents of 100%, 20%, 10%, and 5%, respectively.

Aerodynamic Fine Particle Fraction Evaluation

The 1:4 ratio of liposome to mannitol powder was used for the aerodynamic fine particle fraction estimation due to its relatively good dispersion potential and greater protein and lipid amount for assay. Six capsules were emptied sequentially from dry powder inhaler to the twin-stage liquid impinger. The lipid assay showed 15.6% of the lipid contents were recovered from the lower stage with aerodynamic particle sizes of less than 6.4 μm. In the upper stage and mouthpiece-throat, 42.7% and 26.9% of the lipids deposited with sizes larger than 6.4 μm (see Figure 5). The total emitted dose was 85% for the 6 capsules, much higher than the single capsule emission of 58.1%. This may be explained by priming the DPI, mouthpiece, and throat. The following doses benefit from this priming. The fine particle fractions from lipid assay were slightly different from that of protein activity assay. Active protein on the upper stage and mouthpiece-throat were 34.0% and 21.3%, respectively, less than the lipid contents percentage, but in the lower stage there was a larger quantity of active protein than the lipids. The total amount of lipids and total amount of active proteins were 85.2% and 78.6%, respectively. The unencapsulated proteins played a role in this particle size fraction difference since they have smaller molecular sizes

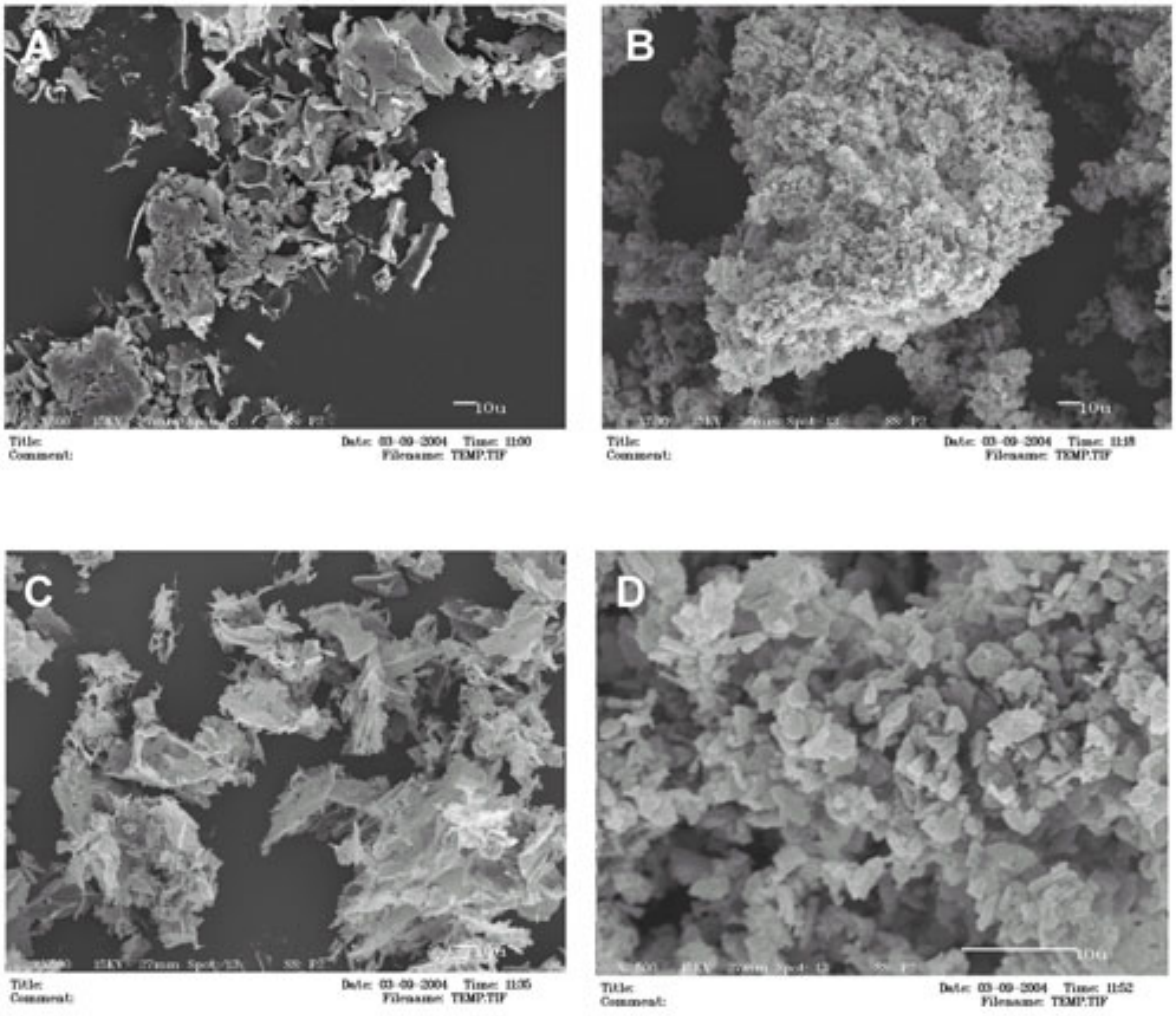

Figure 2. SEM of liposome powders after lyophilization and jet-milling: (A) DMPC-CH-mannitol after lyophilization (original magnification ×500); (B) DMPC-CH-mannitol after jet-milling (original magnification ×500); (C) DMPC-CH-GUS-mannitol after lyophilization (original magnification ×500); and (D) DMPC-CH-GUS-mannitol after jet-milling (original magnification ×2500). The scale bars are 10 μm.

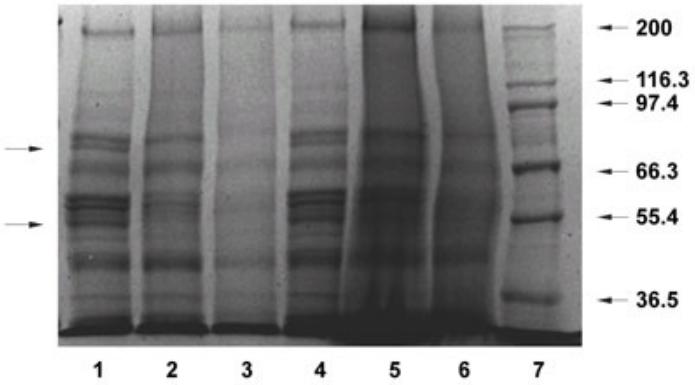

Figure 3. SDS-PAGE image. Lanes 1 and 4, Fresh GUS; lane 2, lyophilized liposome with GUS after lipid extraction; lane 3, micronized liposome with GUS after lipid extraction; lane 5, lyophilized liposome with GUS without lipid extraction; lane 6, micronized liposome with GUS without lipid extraction; lane 7, mark 12 marker. Molecular weights (KDa) are indicated at the right side of the gel. Arrows on the left indicate the bands that have almost disappeared.

than liposomal vesicle diameters, and after being dispersed, they would most likely be deposited on the lower stage of twin-stage liquid impinger.

The carrier mannitol particles have contact points with liposome particles. The greater the quantity of carrier mannitol, the more potential contact points exist between carrier and liposome particles. This potential for distribution on carrier surfaces facilitated liposome delivery. The liposomes that impacted on the upper stage did not completely separate from the mannitol carrier prior to impaction. Therefore, greater energy input would be required for complete separation of the small and large particles.

Lyophilization Optimization

Lyophilization causes loss of GUS activity. Approximately 67% (29%/43%) of the retained encapsulated proteins had activity after lyophilization and 61% (22%/36%) after

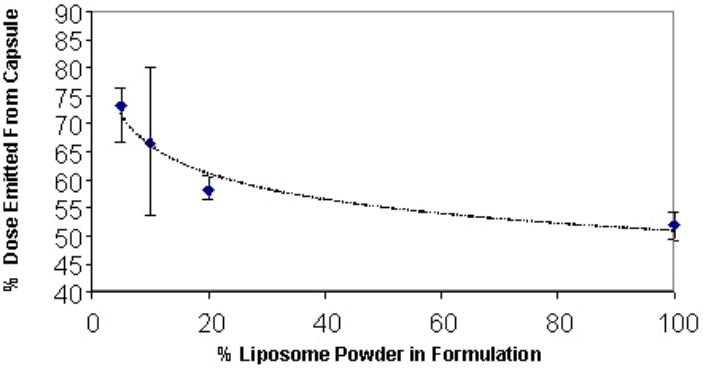

Figure 4. Emitted dose of GUS from a dry powder inhaler sampled at 60 L/min for 10 seconds (n = 3, mean ± SD).

jet-milling. In preparing the liposomes, freeze-thawing did not change GUS activity, which indicated that during lyophilization GUS activity was lost in the drying process. Several studies were conducted attempting to improve GUS stability in order to optimize the lyophilization procedure.

In the absence of cholesterol, encapsulation efficiency of DMPC liposomes was 38.7%, but after lyophilization, only 9.6% GUS retained activity compared with freshly prepared liposomes. The presence of cholesterol may increase the resistance of liposomal bilayers to freeze-drying stress making it a stabilizing compound. This result is consistent with the data of van Winden et al.[35]

For freeze-drying optimization, sucrose was used instead of mannitol. The molar ratio of sucrose to lipid was 10:1. Sucrose was selected as an alternative excipient for its capacity to act as a cryoprotectant and dehydroprotectant. As a disaccharide, sucrose has glass formation with high T_g (77°C) in amorphous state after lyophilization.[36] Sucrose can interact with membrane phospholipid head groups via hydrogen-bonding to replace the water-lipid interaction to stabilize the dry membrane. Mannitol is a monosaccharide

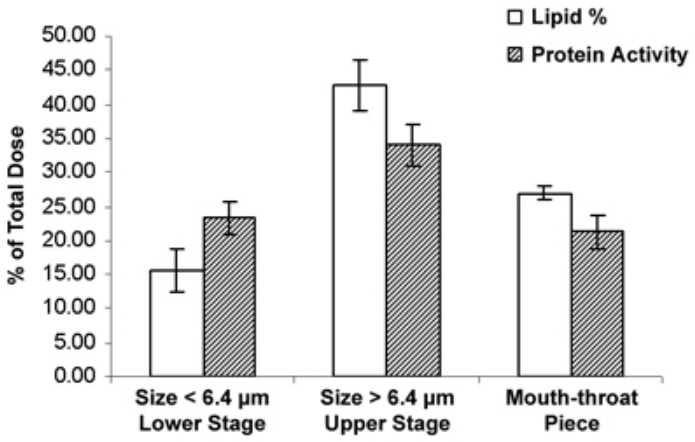

Figure 5. Aerodynamic fine particle fractions in twin-stage liquid impinger (n = 2, error bars depict range).

Table 1. GUS Retained Activity Immediately After Lyophilization and Upon Storage for 50 Days*

	Retained Activity (%)	
	0 days	50 days
DMPC in mannitol	9.6 (0.6)	5.7 (0.3)
DMPC:CH (7:3) in mannitol	41.4 (5.84)†	16.9 (1.2)
DMPC:CH (7:3) in PVP (2.5% wt/vol)-mannitol	67.3 (5.6)†	39.4 (3.5)
DMPC:CH (7:3) in sucrose	55.8 (0.3)†	27.4 (0.9)

*DMPC indicates dimyristoyl phosphatidylcholine; CH, cholesterol; and PVP, polyvinylpyrrolidone. n = 2, mean (error depicts range).
†n = 3, mean (SD).

alcohol that easily crystallizes and has the tendency of phase separation in the lyophilized cake, which is deleterious to the stability of dry liposomes. Crystallization of saccharides may be inhibited in the presence of povidone due to its ability to increase the T_g of the saccharides, and the larger molecular weight PVP, the stronger prevention of crystallization.[37,38] Therefore, povidone (Mw 40 K) was added to mannitol to optimize the lyophilization process. The povidone assay, used 2.5% (wt/vol) povidone in mannitol solution followed by liposome preparation and lyophilization. All other parameters were kept the same as those in the preparation of mannitol with lipid.

After freeze-drying, 41.4%, 67.3%, and 55.8% of encapsulated GUS retained activity in the presence of mannitol, PVP-mannitol, and sucrose, respectively. After 50 days of storage in desiccator, the retained activities were 16.9, 39.4, and 27.4, respectively (Table 1). These results indicate that the presence of PVP in mannitol gave the best retained activity for lyophilization and storage stability after 50 days. Sucrose improved the GUS stability compared with mannitol. Therefore, crystallization is most likely one of the important reasons for GUS activity loss in lyophilization.

Since sodium phosphate buffer may induce a large pH shift in lyophilization, the same pH 7.4 HEPES buffer system was evaluated. 58.5% activity was retained after freeze-drying and there was no significant difference in the GUS stability between HEPES and sodium phosphate buffer. The existence of saccharides alone may effectively reduce the pH shift of sodium phosphate buffer at a quite low concentration of 20 mM.

CONCLUSION

The feasibility of lyophilization and milling for preparation of protein, liposome dry powders for pulmonary delivery was evaluated. β-glucuronidase was encapsulated in liposomes, lyophilized, micronized, and characterized in terms of emitted dose and aerodynamic fine particle fraction.

Further optimization of the manufacturing processes is required in the context of specific therapeutic proteins to maximize stability and aerosol delivery efficiency.

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