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The effects of freeze-drying on the stability of liposomes to jet nebulization

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Abstract

Egg phosphatidylcholine liposomes were freeze-dried in the presence and absence of trehalose. The lyophilized liposomes were rehydrated and aerosolized using a Pari LC jet nebulizer. The size of the aerosols generated was determined by laser diffraction, which was also used to determine the size distribution of the liposomes before lyophilization, post-rehydration, in the nebulizer post-aerosolization and those deposited in the two stages of a twin impinger. In the absence of trehalose, large liposomes and vesicle aggregates were produced on rehydration, which were rapidly reduced in size on nebulization. Liposomes with a mean size of 1 or 2.5 μm, freeze-dried with trehalose, had a mean size less than 3 μm following rehydration and exhibited enhanced stability to nebulization. Liposomes of 1 μm before freeze-drying were evenly distributed within aerosols generated by the nebulizer, whilst aerosols generated from 2.5 μm liposomes were fractionated in the twin impinger with the largest liposomes collected in the upper stage.

Introduction

Nebulizers have been extensively studied for the delivery of liposomes to the airways (Bridges & Taylor 1998). However, liposomes exhibit physical and chemical instability. Freeze-drying affords protection against many of the processes causing liposome instability in aqueous dispersions, yielding a product with a greatly extended shelf-life. However, the freezing and dehydration processes exert stresses on the liposomes. Freezing may cause phase transition changes, osmotic stress and the expansion of bilayers, due to ice formation (Talsma & Crommelin 1992; Hauser 1993). Liposome bilayers depend on hydrogen bonding between water molecules and the polar head groups of the constituent phospholipids for stability. Loss of water on drying produces changes in bilayer behaviour, and loss of integrity, leading to bilayer fractures, fusion, and vesicle aggregation, resulting in loss of entrapped material (Crowe et al 1985), and changes in liposome size distribution (Crowe et al 1986). Such deleterious changes may be minimized by the inclusion of cryoprotectants, usually disaccharide sugars, within the formulation. Trehalose and similar sugars are thought to protect liposomes through their action on two destabilizing mechanisms, which occur during freeze-drying. The first involves the inhibition of vesicle fusion, and aggregation, during dehydration (Rudolph 1988). The second is modification of the phase changes occurring in bilayers during dehydration (the "water replacement hypothesis" (Rudolph 1988; Crowe & Crowe 1992)).

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The ability to produce a lyophilized liposomal formulation, suitable for rehydration before nebulization, would offer obvious advantages from the viewpoint of stability. Therefore, we have investigated the effects of freeze-drying, in the presence and absence of a cryoprotectant, on the physical stability of rehydrated liposomal formulations during jet nebulization.

Materials and Methods

Materials

Unless otherwise stated, materials were AnalaR grade and obtained from BDH (UK). Egg phosphatidylcholine (eggPC, Lipoid Ltd, Italy) was refined by chromatographic purification (Bangham et al 1974). Water was deionized (Whatman WR50 RO/Deioniser, Whatman, UK).

Preparation of liposomes

EggPC liposomes, with or without equimolar cholesterol, were prepared using the thin-film method (Leung et al 1996), by hydrating lipid films with water or aqueous solutions of trehalose (1 g trehalose to 1g lipid) to give liposomes having a total lipid concentration of 10 mg mL^{-1}. A ratio of 1:1 cryoprotectant:lipid had been shown previously to inhibit the aggregation and fusion of liposomes during freeze-drying (Crowe et al 1986). The size of the liposomes was reduced by repeated extrusion through polycarbonate membrane filters (Nucleopore, US), pore size 1, 2.5 or 5 μm, held in 25-mm holders, until the volume median size, as measured by laser Fraunhofer diffraction (Malvern 2600c, Malvern Instruments, UK) with a 63-mm lens, approximated the pore size of the filter (Bridges & Taylor 2000).

Freeze-drying liposomes

Liposomes were freeze-dried using the Edwards Micro Modulyo freeze-drier (Edwards Ltd, UK). Liposome dispersions were added to glass drying vessels, and frozen in a freezer at -20°C. The vessels were then attached to the freeze-drier and dried for 12 h. The freeze-dried samples were stored in vacuum-sealed vessels, at 1–5°C in darkness, until required. Before use, the freeze-dried liposomes were rehydrated with deionized water at ambient temperature, followed by 2-min vortex mixing to redisperse the liposomes. The size distribution of the liposome samples, before and after freeze-drying and rehydration, was determined using the Malvern 2600c laser diffraction sizer. The instrument's software expressed particle size as the volume median diameter and the size distribution was expressed as a span value ((90 % undersize $-$ 10 % undersize)/50 % undersize).

Nebulization of rehydrated freeze-dried liposomes

Pari-LC air jet nebulizers (Pari-Werk, Germany) were used to aerosolize reconstituted liposomes at a gas flow rate of 7 L min^{-1}, from a nitrogen gas cylinder. Five millilitres of the liposomes under study was added to each of three Pari-LC nebulizers. The nebulizers were clamped 25 mm from the centre of the laser beam, and adjusted so that the aerosol traversed the beam at a distance of 5 mm from the 63 mm lens. Aerosol was drawn through the laser of the Malvern 2600c instrument, and the size distribution of the aerosol determined.

In further experiments, 5 mL liposome dispersion was added to each of three Pari-LC nebulizers. The nebulizers were operated consecutively, at a driving gas flow rate of 7 L min^{-1}, until 50 % of the sputtering time i.e. the time when aerosol output became intermittent, determined in preliminary experiments (approximately 330 s for this nebulizer). Aerosols produced by nebulizers were collected in a two-stage (twin) impinger, a device used routinely for characterizing aerosols (Hallworth & Westmoreland 1987), by drawing the aerosol through at 60 L min^{-1} using a vacuum pump. The liposomes remaining within the nebulizer reservoir, and those deposited in the upper and lower stages of the twin impinger, were sized by laser diffraction. The twin impinger was cleaned and reassembled. Each nebulizer was then operated once more from 50 % to 100 % of the nebulization time and generated aerosols collected in the twin impinger. The size distribution of liposomes in the residual fluid remaining in the nebulizer and the liposomes deposited within each stage of the twin impinger was determined.

Results and Discussion

Freeze-drying of liposomes

Freeze-drying caused the mean vesicle size of all liposomes to increase significantly ($P < 0.05$), upon subsequent redispersion, with final mean sizes between 1.6

Table 1 Size characteristics of eggPC and eggPC/cholesterol (1:1) liposomes, freeze-dried in the absence or presence of trehalose.

Liposome (initial size)	Before freeze-drying		After freeze-drying and rehydration	
	Size (μm)	Span	Size (μm)	Span
eggPC (5 μm)	5.04 (0.04)	1.47 (0.04)	7.67 (0.54)	2.98 (0.20)
eggPC (2.5 μm)	2.53 (0.05)	1.30 (0.02)	6.92 (0.53)	2.29 (0.33)
eggPC (1 μm)	1.02 (0.06)	0.98 (0.09)	7.00 (0.64)	2.13 (0.09)
eggPC/cholesterol (5 μm)	5.00 (0.02)	1.64 (0.09)	7.21 (0.68)	2.35 (0.32)
eggPC/cholesterol (2.5 μm)	2.51 (0.07)	1.03 (0.07)	7.23 (0.61)	2.30 (0.16)
eggPC/cholesterol (1 μm)	1.06 (0.11)	0.94 (0.03)	6.96 (0.66)	2.12 (0.23)
eggPC/trehalose (5 μm)	5.04 (0.09)	1.94 (0.09)	6.06 (0.20)	1.36 (0.07)
eggPC/trehalose (2.5 μm)	2.50 (0.04)	1.21 (0.06)	2.79 (0.26)	1.29 (0.04)
eggPC/trehalose (1 μm)	1.01 (0.05)	1.01 (0.04)	1.71 (0.07)	1.19 (0.05)
eggPC/cholesterol/trehalose (5 μm)	5.05 (0.02)	1.09 (0.09)	6.09 (0.11)	1.31 (0.10)
eggPC/cholesterol/trehalose(2.5 μm)	2.53 (0.07)	1.04 (0.11)	2.94 (0.23)	1.09 (0.05)
eggPC/cholesterol/trehalose (1 μm)	1.00 (0.05)	0.82 (0.08)	1.61 (0.09)	1.24 (0.03)

Values are mean ($\pm$s.d.); n = 6.

and 7.7 μm, representing an increase of between approximately 12 and 586% (Table 1). These increases resulted from the aggregation and fusion of liposomes during freeze-drying without cryoprotectant (Engel et al 1994; Mobley & Schreier 1994). Increases were greatest for liposomes freeze-dried in the absence of trehalose. The inclusion of cholesterol had no significant effect on the maintenance of the original size characteristics of liposomes during freeze-drying ($P < 0.05$). This was consistent with previous studies of freezing and dehydrating cholesterol-containing liposomes (Crommelin & Van Bommel 1984; Strauss 1992) and indicated that although cholesterol modified the phase behaviour of bilayers, this did not improve the stability of liposome formulations to freeze-drying. Each of the liposome formulations freeze-dried in the absence of trehalose yielded a reconstituted size of approximately 7 μm. Thus, without cryoprotectant the size of reconstituted liposomes was independent of the initial size, suggesting that the initial liposome structure was lost and new vesicles were formed.

The polydispersity of liposome populations was also increased by freeze-drying and subsequent rehydration, without trehalose (Table 1). Span values for rehydrated liposomes increased from an initial 1 to 1.5, to approximately 2 to 3, representing an increase of between 70 and 125%.

Liposomes freeze-dried in the presence of trehalose had a significantly smaller ($P < 0.05$) mean size, following rehydration, compared with those without the disaccharide. Formulations containing trehalose gave a reconstituted size that was dependent on the mean size before freeze-drying. Thus, inclusion of trehalose allowed the preparation of a dried liposome product which, upon rehydration, possessed vesicle size characteristics that approximated to those of the initial liposome formulation, though in each case, particularly for initially 1 μm liposomes, the size following reconstitution was larger than the initial size.

Nebulization of rehydrated freeze-dried liposomes

Nebulization of 1- and 2.5-μm mean size eggPC/cholesterol (1:1) liposomes, which had not been freeze-dried, yielded aerosols which did not differ significantly in size ($P < 0.05$) (Table 2). This indicated that droplet size produced by the nebulizer was independent of the mean liposome size in this size range. However, the droplets produced when nebulizing liposomes previously freeze-dried without trehalose were slightly larger than those produced from the other formulations, though this was only statistically significant ($P < 0.05$) for the initially 1 μm mean size formulation.

Previous freeze-drying of the formulation and the inclusion of trehalose determined the size of the liposomes remaining in the residual fluid post-nebulization (Figure 1). The size of residual, non-cryoprotected liposomes decreased rapidly during the first half of nebulization. Indeed, the rate of liposome processing seen in the earlier stages of nebulization was greater than that observed for 5 μm mean size, non-freeze-dried, eggPC/cholesterol (1:1) liposomes (Table 3). This reflected the large mean size (approximately 7 μm) of liposomes

Table 2 Size characteristics of aerosols produced on nebulization of freeze-dried and rehydrated liposomes, and non-freeze-dried liposomes. Liposomes were prepared in the absence or presence of trehalose.

	eggPC/cholesterol (1:1)	eggPC/cholesterol (1:1) (freeze-dried)	eggPC/cholesterol (1:1) (freeze-dried + trehalose)
Liposomes with mean size 1 μm before freeze-drying			
Volume median diameter (μm)	2.19 (0.05)	2.31 (0.03)	2.18 (0.07)
Span	2.57 (0.05)	2.67 (0.05)	2.61 (0.04)
Liposomes with mean size 2.5 μm before freeze-drying			
Volume median diameter (μm)	2.31 (0.08)	2.37 (0.03)	2.21 (0.03)
Span	2.63 (0.03)	2.74 (0.04)	2.62 (0.05)

Values are mean ($\pm$s.d.); n = 3.

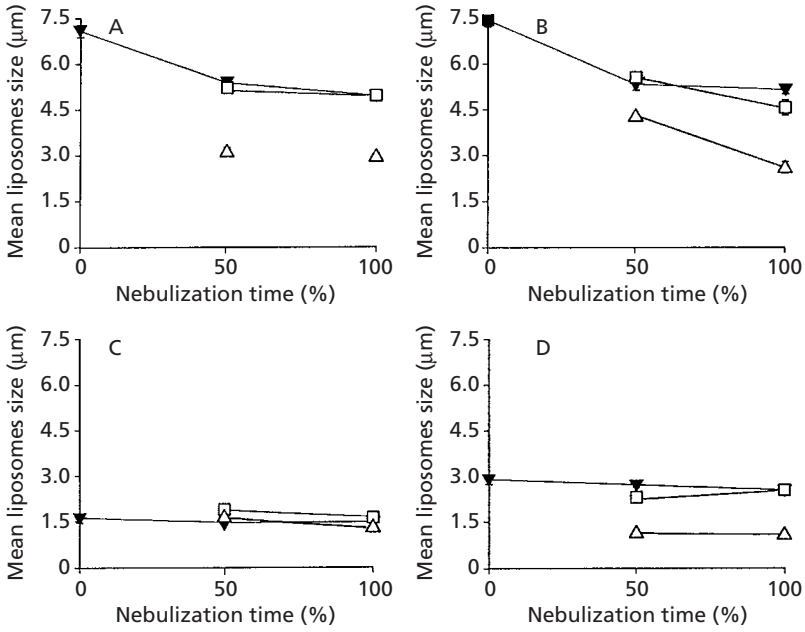

Figure 1 Mean size of freeze-dried and reconstituted eggPC/cholesterol (1:1) liposomes during nebulization into a twin impinger: A. 1-μm initial mean liposome size; B. 2.5 μm initial mean liposome size; C. 1 μm initial mean liposome size, containing trehalose; D. 2.5 μm initial mean liposome size, containing trehalose. The liposomes were sampled from (▼) the nebulizer chamber, (□) the upper stage and (△) the lower stage of the impinger. Values are the mean $\pm$ s.d., n = 3.

within the freeze-dried formulation. Large liposomes were less able to be included within secondary aerosol droplets, becoming retained for extended periods within the nebulizer chamber, and, due to shearing within the nebulizer, size reduction of vesicles occurred. Taylor et al (1990) demonstrated that the shearing of liposomes during nebulization resulted in the loss of entrapped hydrophilic materials.

Once 50% of the nebulization time had been reached, large aggregates and liposomes had been disrupted to produce smaller vesicles. As a result, the trehalose-lacking, freeze-dried formulations experienced a decrease in the residual liposome size in the latter stages of nebulization, which was of a magnitude similar to that for non-freeze-dried eggPC/cholesterol (1:1) liposome formulations (Table 3). Size reduction of conventional

Table 3 Rate of liposome size reduction within the nebulizer during nebulization of liposomes freeze-dried in the absence or presence of trehalose, and of non-freeze-dried eggPC/cholesterol (1:1) liposome formulations.

Liposomes	Measured rate of liposome size reduction (nm min^{-1})	
	0 to 50% of nebulization	50 to 100% of nebulization
Initial size 2.5 μm (non-freeze-dried)	184 (27)	127 (25)
Initial size 5 μm (non-freeze-dried)	233 (30)	46 (50)
Initial size 1 μm (freeze-dried)	622 (36)	156 (78)
Initial size 2.5 μm (freeze-dried)	767 (11)	64 (70)
Initial size 1 μm (freeze-dried + trehalose)	58 (25)	-1.3 (12)
Initial size 2.5 μm (freeze-dried + trehalose)	65.5 (29)	62 (25)

Values are mean ($\pm$s.d.); n = 3.

liposomes during nebulization is greatest in the initial stages, when the liposomes have a large initial mean size (Bridges & Taylor 2000). As the liposomes became reduced in size, so the processing of the liposomes became size-limiting.

Inclusion of trehalose reduced the size of vesicles at the commencement of nebulization. Consequently, the rate of size reduction during nebulization was greatly reduced compared with preparations freeze-dried without trehalose (Table 3). This indicated that the presence of the cryoprotectant during freeze-drying permitted nebulization of reconstituted vesicles, with minimal disruption of bilayers. Freeze-drying had no significant effects on the changes in the polydispersity of residual liposomes that occurred during nebulization, regardless of whether trehalose was present.

The pattern of liposome deposition of freeze-dried liposomes within each stage of the twin impinger is shown in Figure 1. Cryoprotected liposomes gave small liposomes upon rehydration. These were more stable to nebulization, and produced deposited liposomes with a consistent size at both times of sampling during nebulization, compared with those freeze-dried without cryoprotectant. The 1 μm initial mean liposome size formulation, freeze-dried with trehalose, yielded liposomes which deposited within the twin impinger with a mean size that did not significantly differ between the upper and lower stages. Aerosols were produced from liposomes having a relatively small mean size (1.61 μm) and thus aerosolized liposomes had an approximately equal distribution between the large and small aerosol droplets deposited in the upper and lower stages, respectively. Similar findings have been reported for nebulized polymer sphere suspensions (Lashmar et al 1994). 'Polybeads' with a diameter of 1 μm were evenly distributed between the stages of a multistage liquid impinger, whilst with 3 μm beads a greater percentage deposited in the upper stages of the impinger. A comparable effect was observed in this study. The larger liposomes, i.e. the 2.5 μm initial size liposomes, freeze-dried with trehalose and those freeze-dried in the absence of trehalose were effectively fractionated within the twin impinger, which had an effective cut-off diameter between stages of 6.4 μm (Hallworth & Westmoreland 1987). The mean size of liposomes deposited in the lower stage of the twin impinger from freeze-dried, but not cryoprotected liposomes was much smaller than that of the liposomes remaining in the nebulizer reservoir, indicating the reduction of the vesicles during nebulization and retention of larger vesicles within the nebulizer.

In conclusion, freeze-drying liposomes without a cryoprotectant resulted in large liposomes that were unstable to nebulization. Inclusion of a cryoprotectant largely overcame this problem. Liposomes having small initial mean size may be freeze-dried and redispersed in the presence of trehalose, with relatively unchanged size characteristics. The resultant liposome dispersions were comparatively stable to nebulization, suggesting that freeze-drying was an appropriate approach to producing liposome preparations, having long-term chemical and physical stability, which may be rehydrated before delivery from a jet nebulizer.

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