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Lyophilization of cholesterol-free PEGylated liposomes and its impact on drug loading by passive equilibration

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ABSTRACT

The obstacles in translating liposome formulations into marketable products could be attributed to their physical instabilities upon long-term storage as aqueous dispersions. Lyophilization is the most commonly used technique to improve physical stability of liposomes. The development of stable, lyophilized liposomes is focused primarily on the cholesterol-containing liposomes or pure phosphatidylcholine-based liposomes, with minimal studies on cholesterol-free, pegylated (CF-PEG) liposomes which have emerged as an important class of liposome drug carriers. Hence, it is our interest to investigate the effect of lyophilization on CF-PEG liposomes, and specifically, on drug loading via the passive equilibration method. Three different sugar cryoprotectants were used at two different sugar-to-lipid molar ratios (S/L). Our results demonstrated that CF-PEG liposomes lyophilized with sucrose at S/L = 5:1 yielded the best cryoprotective effect, as characterized by size, polydispersity indices, and microscopic examination upon liposome reconstitution. The lyophilized liposomes had low water content of 2.59 ± 0.18%. Of note, lyophilized CF-PEG liposomes exhibited two-fold increase in drug content when carboplatin was loaded via the passive equilibration method, and the in vitro drug release profile of these liposomes were not different from that of the non-lyophilized counterparts. Taken together, we envisioned that a stable, lyophilized empty CF-PEG liposome system could be coupled to hydrophilic drug loading via the passive equilibration method to produce a liposomal drug kit product.

1. Introduction

Liposomal delivery has been an established technology platform in delivering a wide variety of medicinal agents that has yielded a number of clinically approved products. With its first description by Bangham in 1965, liposome system was quickly recognized as a carrier for drugs with intensive research efforts in the past few decades. One of the landmark advancement in this technology is the inclusion of cholesterol in the liposome bilayer that provides a number of formulation benefits, including good retention of encapsulated drug, reduced plasma protein binding and resilience to macrophage attack. As such, majority of the approved liposomal products including DaunoXome, Doxil, Myocet, AmBisome, and Depocyt all contain cholesterol in the formulation.

While the inclusion of cholesterol has provided multiple formulation benefits, liposomal compositions based on a mixture of phosphatidylcholine (PC) and cholesterol might not be applicable to the encapsulation of all drug compounds, whereby drug retention is, at least in part, dependent on the physicochemical properties of the liposomal membrane composition. This could be illustrated by the Stealth liposome formulation of cisplatin (SPI-077), whereby this cholesterol-containing, pegylated liposome formulation retained the encapsulated cisplatin so well that the amount of cisplatin released from the liposomes was insufficient to elicit good cytotoxic activity (Zamboni et al., 2004), resulting in failure of SPI-077 in Phase I/II clinical trial (Harrington et al., 2001). Therefore, cholesterol free- (CF-) liposomes are important alternatives to the conventional cholesterol-containing liposomes,

Abbreviations: AAS, atomic absorption spectroscopy; Cryo-FESEM, cryogenic field emission scanning electron microscopy; D/L, drug-to-lipid; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DSPC, 1,2- distearoyl-*sn*-glycero-3-phosphatidylcholine; DSPE-PEG$_{1000}$, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)$_{1000}$] (ammonium salt); DSPE-PEG$_{2000}$, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)$_{2000}$] (ammonium salt); DSC, differential scanning calorimetry; FESEM, field emission scanning electron microscope; PBS, phosphate buffered saline; PI, polydispersity index; S.E.M., standard error of mean; S/L, sugar to lipid molar ratio; T_m, phase transition temperature.

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and could provide additional flexibility in developing an appropriate liposome carrier for the drug of interest. More importantly, CF-liposomes would retain a fundamental property of the phospholipid bilayer, the gel-to-liquid crystalline phase transition, which shows increased bilayer permeability when the CF-liposomes are heated above the T_m. This property could be exploited for drug loading by means of passive equilibration of the drug across the liposomal bilayer (Chaudhury et al., 2012; Woo et al., 2008), as well as for the development of thermosensitive liposomes that rely on heat as a means to trigger drug release (Needham et al., 2000; Chiu et al., 2005; Woo et al., 2008). Substituting cholesterol with PEG-lipid conjugates in the binary, PC-based lipid bilayer could yield liposomal compositions that exhibit good drug retention and extended circulation longevity (Chiu et al., 2005; Dos Santos et al., 2005; Woo et al., 2008).

The obstacles in translating more liposome formulations into marketable products could be attributed to their physical instabilities upon long-term storage, such as drug leakage and liposome aggregation, when presented as aqueous dispersions. Many strategies have been described to overcome the physical instabilities, and lyophilization is the most commonly used technique owing to its wide applicability in the pharmaceutical and food industries (Chen et al., 2010a). Lyophilization of liposomes could prevent hydrolysis of the phospholipids and physical degradation of the vesicles during storage (van Winden, 2003), and the addition of cryoprotectants such as sucrose, glucose, trehalose, maltose, or lactose prevents drug leakage or fusion of liposomes (Alexopoulou et al., 2006; Ohtake et al., 2006; Stevens and Lee, 2003; Tang and Pikal, 2004). This knowledge has culminated in the development of a formulation kit that involves empty, lyophilized liposomes cryoprotected by sucrose for the remote loading of doxorubicin via the citrate-based pH gradient, with loading efficiency comparable to that of freshly prepared liposomes (Stevens and Lee, 2003).

In light of the emerging potential of CF-liposomes in the application of drug loading via passive equilibration and in the development of thermosensitive liposomes as described earlier, it is thus important to investigate the effect of lyophilization on such liposome systems, whereby the number of such studies remains limited. As the optimal type and ratio of cryoprotectant used have been shown to be partly dependent on liposomal lipid composition (Stevens and Lee, 2003), the change to using CF-based liposomal composition would call for an optimization of the type and ratio of cryoprotectant for such liposomes. Hence, it is our interest to investigate the effect of lyophilization on cholesterol-free, pegylated (CF-PEG) liposome systems. In particular, we have attempted to identify suitable cryprotectants for producing stable, empty, lyophilized CF-PEG liposomes and to characterize the impact of lyophilization on drug loading into such liposomes via the passive equilibration method.

2. Materials and method

2.1. Lipids, drugs and reagents

1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)$_{1000}$] (ammonium salt) (DSPE-PEG$_{1000}$), and (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)$_{2000}$] (ammonium salt) (DSPE-PEG$_{2000}$) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Chloroform was obtained from Merck & Co. (Whitehouse station, NJ, USA). Carboplatin (powdered drug), platinum atomic absorption standard solution, Sephadex G-50 size exclusion gel, D-(+)-sucrose, D-(+)-trehalose dihydrate, D-(+)-glucose and all other reagents were obtained from Sigma–Aldrich (St, Louis, MO, USA).

2.2. Liposome preparation

All liposome formulations were prepared by the extrusion method (Mayer et al., 1986). Liposomes were consisted of binary lipid mixtures of PC and PEG-lipid in the molar ratio of 95:5. Lipids were dissolved in chloroform to yield a solution. Chloroform was then evaporated under a stream of nitrogen gas, and the sample was subsequently placed under vacuum for 3 h to remove the residual solvent. The resultant lipid film was hydrated in 0.9% NaCl for 1 h at 55 ± 2 °C (for DPPC-based liposomes) or at 65 ± 2 °C (for DSPC-based liposomes) at a lipid concentration of 80 mg/mL with constant stirring. The liposome samples were then extruded ten times through a thermobarrel extruder (Northern Lipids, Vancouver, BC, Canada) using stacked 100-nm polycarbonate filters. The resultant mean diameter and polydispersity index (PI) of each liposome batch were determined using a Zetasizer model 3000 HS (Malvern Instruments, Malvern, WR, UK) at 632 nm.

2.3. Determination of liposomal lipid concentration

Lipid concentration was determined via the phosphate assay (Fiske and Subbarow, 1925; Meyer, 1930). Briefly, 700 μL of perchloric acid was added to either standards or samples, followed by heating at 190 °C for 1 h. Upon cooling, 700 μL Fiske solution and 7.0 mL of ammonium molybdate solution were added and heated for another 20 min at 100 °C. Absorbance was measured at 830 nm with a UV–vis spectrophotometer (Shimadzu UV1601, Kyoto, Japan) against a standard curve made of known amounts of phosphate.

2.4. Lyophilization of liposomes and subsequent reconstitution

Glucose, sucrose or trehalose was added as cryoprotectant to liposomes at sugar:lipid molar ratios of 3:1 or 5:1. The liposome samples were then frozen at −86 °C for 8 h (ULT Freezer, Thermo Electron Corporation Forma, Waltham, MA, USA), followed by lyophilization at −50 to −55 °C for 48 h at 5 Pa (Christ Alpha 2-4 LD Plus Freeze Dryer, Osterode, Germany). No secondary drying was performed. The lyophilized samples were stored at −20 °C for further characterization and for drug loading, unless otherwise mentioned. Lyophilized samples were taken out from refrigerator and kept at room temperature for 15 min before reconstitution at room temperature to original volume using milli-Q water as and when required. After the addition of water, the reconstituted liposomes were mixed using a vortex mixer at room temperature until the samples were dispersed (without any particulate matter inspected visually). The mean diameter and polydispersity of the reconstituted liposomes were analyzed by ZetaSizer as mentioned in Section 2.2.

2.5. Carboplatin loading into liposomes

Carboplatin loading was done via the passive equilibration method (Woo et al., 2008). Freshly prepared liposomes or freshly reconstituted liposomes were pre-warmed at 55 ± 2 °C (DPPC-based liposomes) or at 65 ± 2 °C (DSPC-based liposomes) before drug loading. Pre-warmed carboplatin powder was added at a drug-to-lipid (D/L) weight ratio of 0.25:1 and incubated at the above mentioned temperature with for 1 h under gentle stirring with a micro magnetic stir bar. Samples were subsequently cooled in a water bath of room temperature for 30 min after drug loading. Unencapsulated carboplatin was separated using 1-mL Sephadex-G50 spin column pre-equilibrated with 0.9% NaCl (pH7.5) at 680 × *g*

for 3 min. Carboplatin concentration was determined by atomic absorption spectroscopy (AAS) using a PerkinElmer analyst 100 model attached to a graphite tube atomiser (PerkinElmer analyst 100 HGA 800 model, Waltham, Massachusetts, USA). The instrument was operated at a wavelength of 265.9 nm following the sequential temperature of 90 °C for 30 s, 120 °C for 10 s, 1100 °C for 15 s, 2700 °C for 5 s (reading time) and 30 °C for 40 s. A standard curve was plotted using the commercially available platinum standard solution. Duplicate measurements were made for all samples.

2.6. Long term stability studies

Drug content, size and PI of carboplatin-loaded DPPC/DSPE-PEG_{1000} liposomes were evaluated for a period of 1 year, using the procedures described in previous sections. For long term stability of lyophilized liposome samples, liposomes were aliquoted in 0.5 mL into small vials, cryoprotected with sucrose (sugar:lipid molar ratio of 5:1) and lyophilized according to the procedure described in previous section. For long-term storage stability, the samples were kept at −20 °C, and at required time points of 1, 15, 30, 60, 120, 180, 240 days and 360 days, an aliquot was reconstituted with milli-Q water as described in Section 2.4. Size and PI were determined as described in previous section. For the stability of the reconstituted liposome samples, these were stored at 2–8 °C and analyzed for size and PI after 1, 15, 30 and 60 days.

2.7. Microscopic examination of liposomes

Surface morphology of lyophilized powdered liposomes with or without different types of cryoprotectants was examined by field emission scanning electron microscope (FESEM). Samples (with or without cryoprotectants) were fixed on the aluminum stubs and coated with platinum for 60 s in an auto fine coater (JFC-1600 (JEOL)). Images were taken at an operating voltage of 5 kV in JOEL field emission scanning electron microscope (JSM-670-1F). Shape and surface morphology of reconstituted liposomes were examined by cryogenic field emission scanning electron microscope (cryo-FESEM). Approximately 2–3 drops of liposomes (∼30 μL) were placed on a copper stub and frozen in nitrogen slush at −196 °C. Samples were subsequently kept in liquid nitrogen and transferred into the cryo preparation chamber (GATAN ALTO 2500, Oxford, UK) attached to the FESEM (JEOL JSM-6700F, Tokyo, Japan). The sample was freeze-fractured and sublimed at −95 °C for 30 s. The fractured sample was sputter-coated with platinum for 120 s in the cryo preparation chamber and then introduced onto the specimen stage at −140 °C and examined at an excitation voltage of 5 kV.

2.8. Moisture content determination by Karl Fischer titration

Moisture content of the lyophilized samples was measured by the Karl Fischer titration instrument. Briefly, the instrument was calibrated for 3 h. Samples were accurately weighed and placed inside the titration chamber. The sample was dissolved at 100 rpm for 200 s. Moisture content was recorded from the instrument.

2.9. In vitro drug release studies

Release studies were done on the freshly prepared carboplatin-loaded liposomes (no lyophilization) and the lyophilized, reconstituted liposomes subsequently loaded with carboplatin. The release of encapsulated carboplatin was determined under sink conditions by dialyzing 0.5 mL samples in mini dialysis cassettes (Pierce, 3.5 K molecular weight cut off) against 500 mL phosphate buffered saline (PBS, pH 7.4) at 37 °C for 72 h with constant stirring. Drug and lipid concentrations were determined as described in previous section.

2.10. Differential scanning calorimetry (DSC)

DSC measurements were performed with a DSC-2920 differential scanning calorimeter (TA Instruments, New Castle, Delaware, USA), and an empty, standard aluminum pan was used as the reference. Liposomes samples (∼5 μL) were placed in the aluminum pan, and measurements were made at a temperature scan range from 25 to 60 °C with a heating rate of 10 °C/min under a stream of air. Lyophilized liposomes were reconstituted with milli-Q water before analysis. The results were analyzed by Thermal Solution Software Version 1.4E (TA Instruments, New Castle, Delaware, USA). The midpoint of the transition was taken as the phase transition temperature (T_m). All scans were done in duplicates, and at least three different batches of liposome formulations were evaluated. The scales of the *Y*-axis for the DSC plots were the same.

2.11. Statistical analysis

All results are presented as mean ± S.E.M. Statistical analysis was performed using unpaired Student's *t*-test or one way analysis of variance (ANOVA) with Newman–Keuls multiple comparison test as post hoc analysis by GraphPad Prism Version 2.00 (San Diego, California). A p-value of <0.05 was considered as statistically significant.

3. Results and discussion

3.1. Stability of CF-PEG liposomes loaded with the model hydrophilic drug carboplatin

Retention of liposome-encapsulated hydrophilic drugs during long-term storage has been a major challenge in the field. Using the hydrophilic drug carboplatin as the model drug, the long-term stability of carboplatin-loaded DPPC/DSPE-PEG_{1000} liposomes was investigated upon storage at 2–8 °C for 300 days. As illustrated in Fig. 1A, the formulation showed significant decrease in drug content within one week of storage (>30%, $p < 0.05$ compared to $t = 0$ day), followed by gradual drug leakage over the rest of the study period to 35% of drug remaining. Substituting DPPC with DSPC, a saturated phospholipid with longer acyl chain length, in the CF-PEG liposomes gave similar results (data not shown). Of note, carboplatin leakage from the CF-PEG liposomes was not due to liposome aggregation, as no significant change in the size and PI of the CF-PEG liposomes was observed over the 300-day study period (Fig. 1B and C).

Based on these observations, we intended to develop stable, lyophilized CF-PEG liposomes for encapsulating carboplatin. Specifically, we envision that a stable, lyophilized empty liposome system could be coupled to hydrophilic drug loading via the passive equilibration method to produce a liposomal drug kit product with good stability. Hence, our investigations were focused on the identification of a suitable cryoprotectant and its ratio to be used as well as the conditions for passive equilibration drug loading.

3.2. Comparison of various cryoprotectants on the lyophilization of CF-PEG liposomes

Polyethylene glycol has been shown to inhibit nucleation of ice and thus ice crystal formation during freezing (Annunziata et al., 2002; Heller et al., 1996). Hence, as the first step, CF-PEG liposomes were lyophilized without the use of cryoprotectants. However, both size and PI of the CF-PEG liposomes were significantly increased in the absence of cryoprotectants (Supplementary data, Fig. 1). Therefore, it was necessary to add cryoprotectants to protect the CF-PEG liposomes from fusion during lyophilization. Preliminary attempts with sucrose added either to the hydration medium for

Table 1
Size and polydispersity index (PI) of different CF-PEG liposomes before or after lyophilization in the presence of different cryoprotectants.

Liposome Composition	Sugar-to-lipid	Before Lyophilization		Sucrose		Trehalose		Glucose	
	Molar ratio	Size	PI	Size	PI	Size	PI	Size	PI
DPPC/DSPE-PEG$_{1000}$	3:1	118 ± 4	0.094 ± 0.008	150 ± 10[a]	0.273 ± 0.038[b]	197 ± 16[a]	0.447 ± 0.034[b]	169 ± 16[a]	0.357 ± 0.067[b]
	5:1	110 ± 0	0.096 ± 0.001	138 ± 2[a]	0.217 ± 0.004[b]	198 ± 8[a]	0.419 ± 0.024[b]	156 ± 8[a]	0.319 ± 0.045[b]
DSPC/DSPE-PEG$_{1000}$	3:1	116 ± 2	0.087 ± 0.007	173 ± 16[a]	0.309 ± 0.033[b]	276 ± 23[a]	0.451 ± 0.018[b]	269 ± 16[a]	0.374 ± 0.029[b]
	5:1	108 ± 5	0.086 ± 0.008	147 ± 5[a]	0.257 ± 0.032[b]	181 ± 8[a]	0.326 ± 0.009[b]	176 ± 8[a]	0.326 ± 0.013[b]
DPPC/DSPE-PEG$_{2000}$	3:1	103 ± 6	0.092 ± 0.01	170 ± 17[a]	0.336 ± 0.041[b]	152 ± 11[a]	0.359 ± 0.031[b]	259 ± 29[a]	0.421 ± 0.051[b]
	5:1	111 ± 6	0.069 ± 0.008	150 ± 5[a]	0.247 ± 0.012[b]	185 ± 22[a]	0.367 ± 0.05[b]	183 ± 3[a]	0.326 ± 0.022[b]

Size and PI were determined by dynamic light scattering (Zetasizer). Results shown are the mean ± S.E.M. from at least three independent batches of formulation upon reconstitution.

[a] $p < 0.05$ as compared to the size of the corresponding liposome formulation before lyophilization at each sugar-to-lipid molar ratio.

[b] $p < 0.05$ as compared to the PI of the corresponding liposome formulation before lyophilization at each sugar-to-lipid molar ratio.

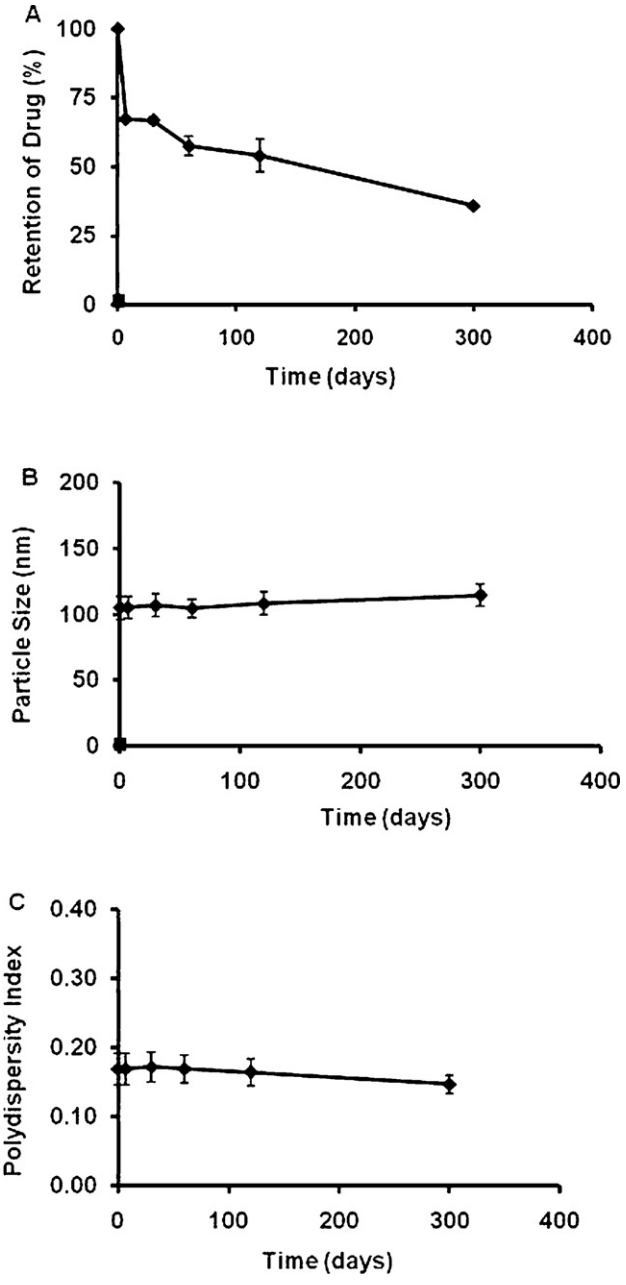

Fig. 1. Stability as reflected by (A) drug content, (B) size, and (C) PI of DPPC/DSPE-PEG$_{1000}$ liposomes over 300 days upon storage at 2–8 °C. Results represent the mean ± S.E.M from at least three batches of liposomes.

hydrating the thin lipid film during liposome formation or after the liposome sample has been extruded to 100-nm size did not yield significant change in size and PI of the reconstituted CF-PEG liposomes. In the selection of cryoprotectants, we have chosen the three most commonly used sugars for lyophilization of cholesterol-based liposomes, and these include sucrose, trehalose or glucose at two different sugar-to-lipid molar ratios (3:1 or 5:1). Table 1 summarizes the effect of cryoprotectants on the size and PI of different CF-PEG liposomes upon lyophilization and reconstitution.

DPPC/DSPE-PEG$_{1000}$ liposomes were lyophilized with the three different sugar cryoprotectants in two different S/L ratios. As reflected by the changes in size and PI (Table 1), the sugar-cryoprotected liposomes exhibited significant increase in size and PI upon reconstitution. Among the three cryoprotectants, sucrose at S/L ratio of 5:1 gave the most promising cryoprotective effect on the DPPC/DSPE-PEG$_{1000}$ liposomes as compared to trehalose and glucose at either 3:1 or 5:1 S/L ratios. The increase in liposome size was in the range of 25–32 nm for DPPC/DSPE-PEG$_{1000}$ liposomes cryoprotected by sucrose, which is the smallest in magnitude produced among the three cryoprotectants used. The PI of the reconstituted liposomes ranged from 0.217 to 0.273, which is within acceptable range. In contrast, the increases in size of DPPC/DSPE-PEG$_{1000}$ liposomes cryoprotected by trehalose or glucose were larger in magnitude (51–79 nm). Substituting DPPC with DSPC or PEG$_{1000}$ with PEG$_{2000}$ did not affect the above trend whereby sucrose at the S/L ratio of 5:1 gave the more promising cryoprotective effect as reflected by the change in size and PI of the reconstituted liposomes. In general, an increase in S/L ratio corresponds to a better cryoprotective effect of the disaccharides. However, higher S/L ratios (>5:1) were not investigated in our study, as previous studies have suggested that CF-PEG liposomes could be more sensitive to transmembrane osmotic gradient as compared to their cholesterol-containing counterparts (Dos Santos et al., 2005), and exceedingly high or low concentrations of sugars could diminish the cryoprotective capability (Miyajima, 1997; Vincourt et al., 2010).

The addition of disaccharides such as sucrose or trehalose could reduce the gel-to-liquid crystalline phase transition temperature, T_m, of the lipid bilayer due to the direct interaction between the hydroxyl groups of the disaccharides and the polar head group of the phospholipids via hydrogen bonding (van Winden et al., 1997). Hence, the T_m values of the liposomes lyophilized with different cryoprotectants were determined by DSC, and the DSC profiles are shown in Supplementary data, Fig. 2. As the pre-transition of each DSC curve was broadened and not very well defined, the more identifiable midpoint of each curve was determined and used for comparison. Previous studies have indicated that this broadening effect could be due to the thermotropic behavior of liposomes, which is usually dependent on the packing of the lipids within the bilayer (Biltonen and Lichtenberg, 1993; Stark et al., 2010). As shown in Table 2, addition of the three cryoprotectants lowered the

Table 2
Effect of different cryoprotectants on the phase transition temperature (T_m) of liposomes.

Samples	T_m
Freshly prepared, non-lyophilized DPPC/DSPE-PEG$_{1000}$ liposomes	43.06 ± 0.05
Lyophilized DPPC/DSPE-PEG$_{1000}$ liposomes without cryoprotectant	42.94 ± 0.03
Lyophilized DPPC/DSPE-PEG$_{1000}$ liposomes with sucrose (S/L = 5:1)	42.58 ± 0.01[a]
Lyophilized DPPC/DSPE-PEG$_{1000}$ liposomes with glucose (S/L = 5:1)	42.88 ± 0.02
Lyophilized DPPC/DSPE-PEG$_{1000}$ liposomes with trehalose (S/L = 5:1)	42.96 ± 0.08

T_m values were determined from the DSC curves of the liposomes presented in Supplementary Fig. 2.
All scans were done in duplicates and at least three different batches of liposome formulations were evaluated.
[a] $p < 0.05$ for liposomes lyophilized with Sucrose 5:1 vs. all other groups.

T_m of the liposomes when compared to non-lyophilized liposomes and lyophilized liposomes without any cryoprotectant. However, sucrose at S/L ratio of 5:1 was the only sample that produced statistical significant reduction in T_m.

3.3. Microscopic examination of lyophilized and reconstituted CF-PEG liposomes

Microscopic examination of freeze-dried product is a direct way to observe the microstructure of the freeze-dried matrix, and the changes in the morphology that might have occurred. FESEM studies were performed on the lyophilized powdered form of the CF-PEG liposomes to elucidate the effect of different cryoprotectants on the surface morphology of the lyophilized product (Fig. 2). From the FESEM images, the lyophilized CF-PEG liposomes showed intact liposome structures when protected with different sugars; however, in the absence of any cryoprotectant, lyophilized liposomes appeared to be much larger and without any discrete liposome structures (Fig. 2A). In contrast, lyophilized liposomes cryoprotected with sucrose at S/L ratio of 5:1 showed intact liposome structures (Fig. 2B). Lyophilized liposomes cryoprotected with glucose or trehalose at S/L ratio of 5:1showed liposomal structures that were less discrete and found to be fused with one another (Fig. 2C and D). Similar to FESEM analysis, the images from cryo-FESEM showed that non-cryoprotected, lyophilized liposomes appeared to be larger than the sugar-protected liposomes after reconstitution (Fig. 3B). Liposomes cryoprotected by sucrose or trehalose at S/L ratio of 5:1 seemed to show spherical shape and smooth surface morphology, similar to the non-lyophilized liposomes (Fig. 3C and E), whereas glucose-protected liposomes seemed to show less spherical and less smooth surface morphology (Fig. 3D).

3.4. Determination of moisture content by Karl Fischer titration

Low moisture content is an essential criterion of any lyophilized product, as this confers improved stability of the product by preventing any chemical reactions that can occur due to hydrolysis. The maximum water content in freeze-dried products acceptable to regulatory authorities is within 3% (w/w) (Mohammed et al., 2007); therefore, moisture content in the sucrose-protected, DPPC/DSPE-PEG$_{1000}$ lyophilized liposomes was determined by Karl Fischer

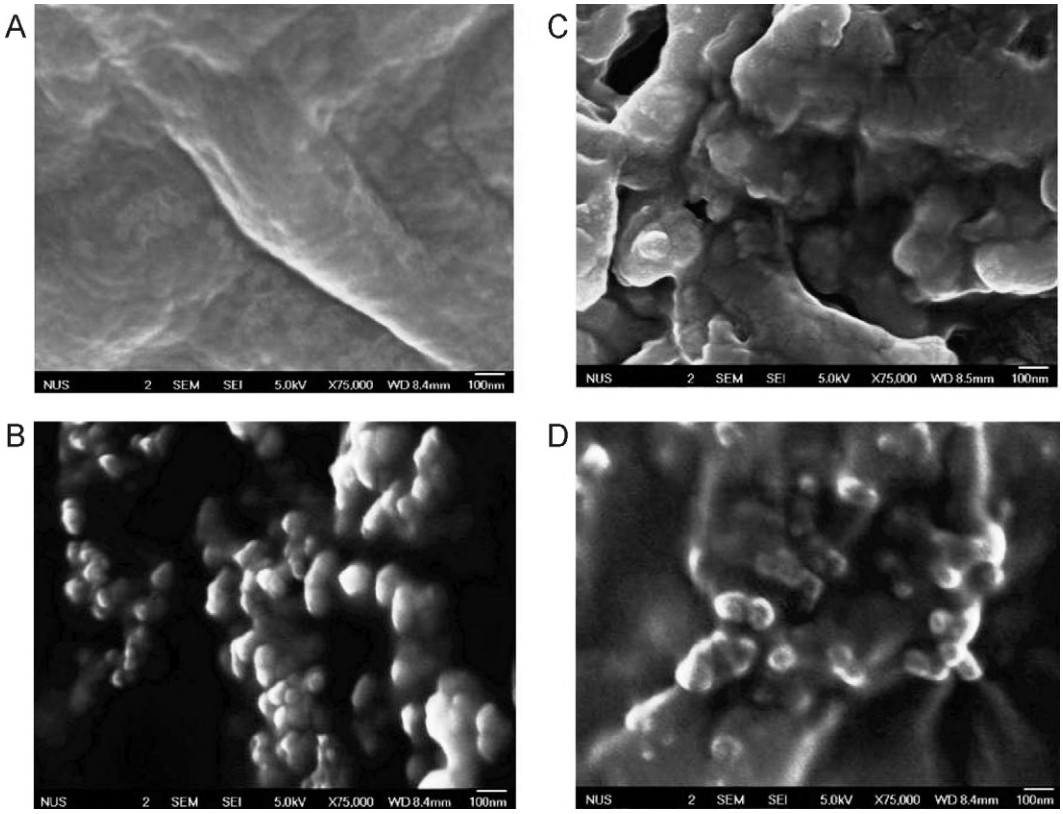

Fig. 2. FESEM images of lyophilized DPPC/DSPE-PEG$_{1000}$ liposomes in the absence of cryoprotectant (A), or in the presence of sucrose (B), glucose (C), and trehalose (D). All cryoprotectants were used at an S/L ratio of 5:1. For each experimental condition, 5–10 images were collected, and each condition was repeated with three independent batches of liposomes.

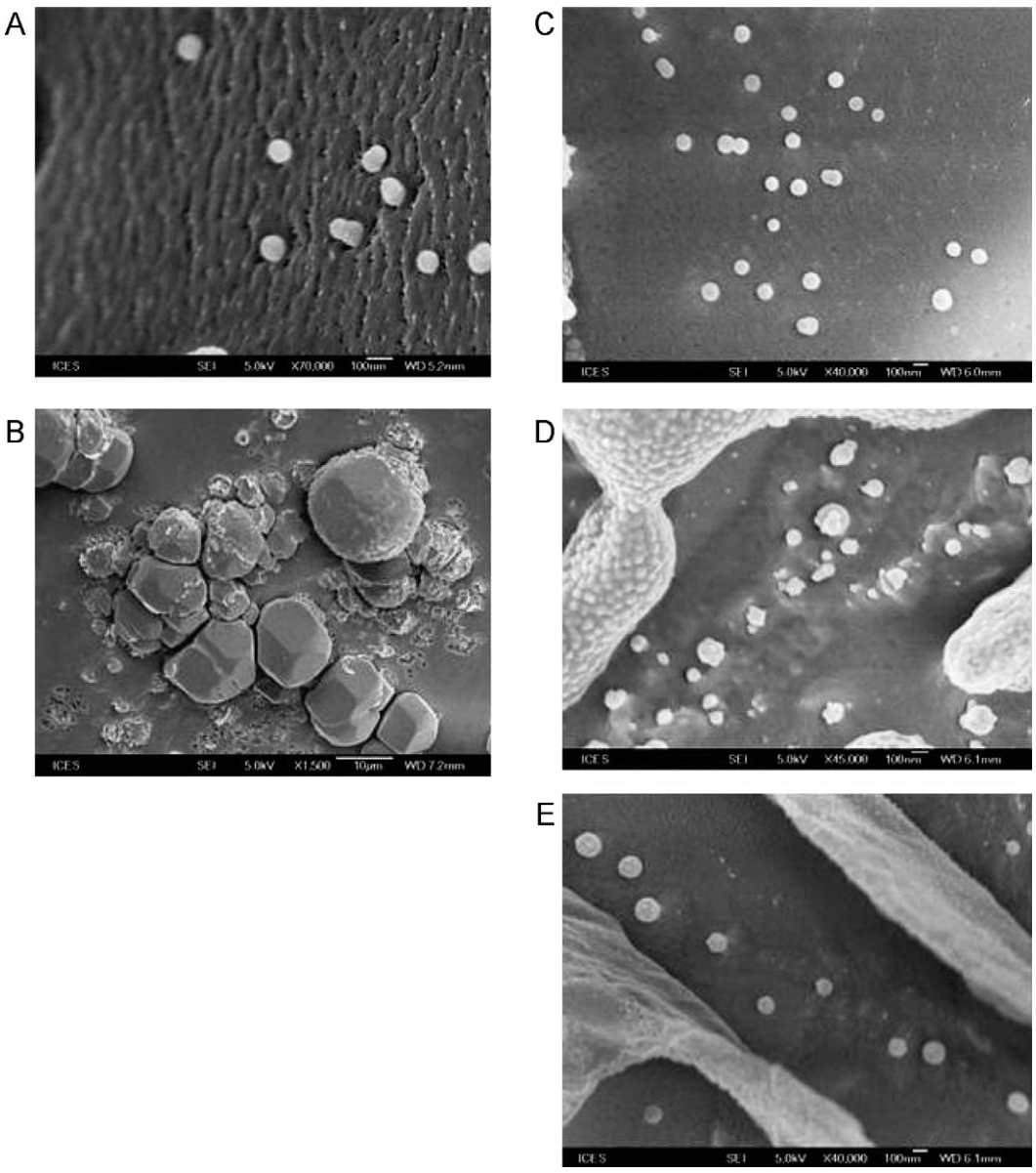

Fig. 3. Cryo-FESEM images of non-lyophilized liposomes (A), lyophilized liposomes in the absence of cryoprotectant (B) or in the presence of sucrose (C), glucose (D), and trehalose (E). All lyophilized liposomes were reconstituted with milli-Q water at room temperature before imaging. Liposomes were composed of DPPC/DSPE-PEG_{1000} and the S/L ratio was 5:1. For each experimental condition, 5–10 images were collected, and each condition was repeated with three independent batches of liposomes.

titration method. The samples used for the determination of moisture content were stored at −20 °C for 2 months before analysis, and the post-lyophilization product was found to have a low moisture content of 2.59 ± 0.18%, which was within the allowable limit of 3% (w/w).

3.5. Long-term stability of lyophilized CF-PEG liposomes

Based on the results in previous sections, sucrose at S/L ratio of 5:1 was selected as the cryoprotectant of choice as it appears to be the most promising. Thus, the long-term storage stability of CF-PEG liposomes cryoprotected with sucrose (S/L = 5:1) was examined for 1 year, with the lyophilized samples stored at −20 °C. As shown in Fig. 4A and B, no significant change in size and PI was observed upon the reconstitution of the sucrose-cryoprotected CF-PEG liposomes over the course of 1 year. In addition, no significant change in size and PI was observed when the reconstituted CF-PEG liposomes were stored for 60 days at 2–8 °C as reconstituted aqueous liposomal dispersions (Fig. 4C and D). Taken together, the sucrose-cryoprotected CF-PEG liposomes demonstrated good stability as lyophilized powder and also upon reconstitution.

3.6. Drug loading and drug release of lyophilized CF-PEG liposomes

After exploring the feasibility of lyophilizing CF-PEG liposomes, the main interest of our study is to examine if such lyophilized liposomes could remain functional for loading drug compounds via the passive equilibration method. It is of note that the passive equilibration drug loading method could only be applied to CF-liposomes, as the presence of cholesterol (at a level similar to that used in Stealth liposomes) abolished carboplatin loading into the DPPC/Cholesterol/DSPE-PEG1000 50:45:5 (molar ratio) via passive equilibration (Supplementary data, Fig. 3). It is thus anticipated that empty, lyophilized CF-PEG liposomes coupled with drug loading by

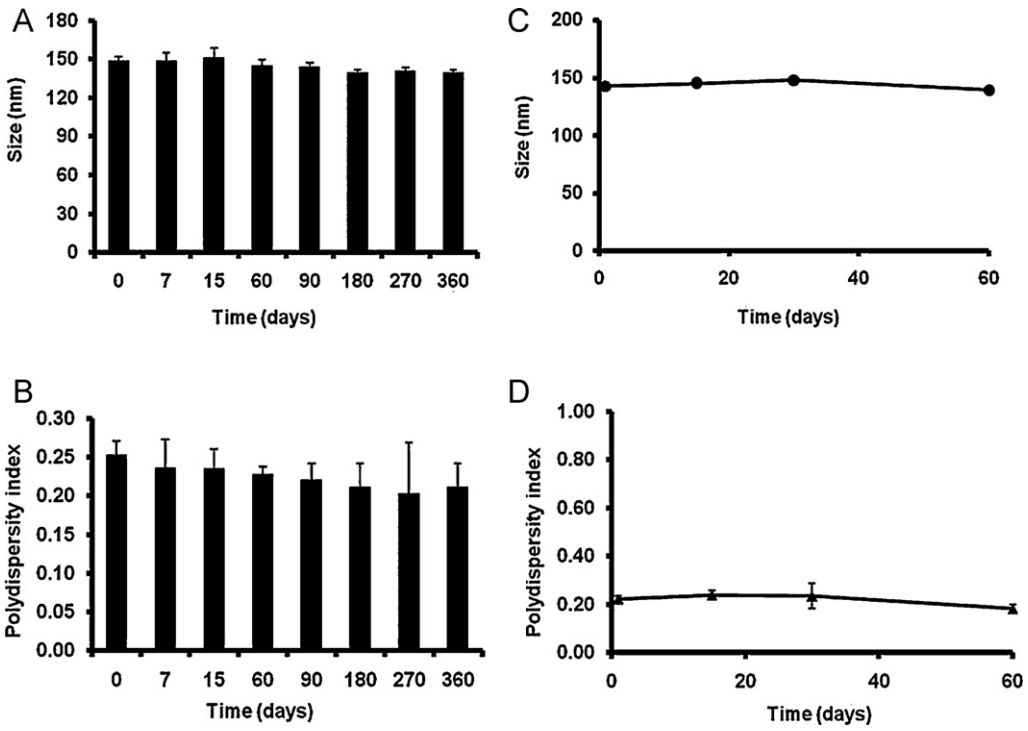

Fig. 4. Change in size (A and C) and polydispersity (B and D) of lyophilized DPPC/DSPE-PEG$_{1000}$ liposomes upon storage at −20 °C for a period of 360 days, or after reconstitution and storage at 2–8 °C for a period of 60 days. Results represent the mean ± S.E.M from at least three batches of liposomes. Some error bars are too small to be seen.

passive equilibration method could present a unique opportunity to develop liposomal drug product kit.

Similar to other studies on the lyophilization of liposomes containing hydrophilic drugs (Chen et al., 2010b; Glavas-Dodov et al., 2005), lyophilization and reconstitution of carboplatin-loaded DPPC/DSPE-PEG$_{1000}$ liposomes significantly decreased the carboplatin content in the formulation by ~80% (Fig. 5A, group 2), as compared to the freshly drug-loaded, non-lyophilized liposomes (Fig. 5A, group 1). In contrast, loading of carboplatin into these reconstituted, sucrose-cryoprotected CF-PEG liposomes exhibited significantly higher carboplatin content than the non-lyophilized liposomes (Fig. 5A, group 3), with ~80% more drug loaded in the reconstituted liposomes as compared to the non-lyophilized sample. Exogenously added sucrose (in S/L ratio of 5:1) to fresh, non-lyophilized CF-PEG liposomes did reduce carboplatin loading into the liposomes by passive equilibration (Fig. 5A, group 4), whereas sucrose added as cryoprotectant and gone through the lyophilization-reconstitution process increased carboplatin loading. It is speculated that when sucrose was added as a cryoprotectant for the lyophilization and reconstitution of the CF-PEG liposomes (group 3, Fig. 5A), it could be present within the liposome core as it went through the lyophilization-reconstitution process. The hydroxyl group of sucrose within the liposome core may have interacted with the carboxyl group of carboplatin via hydrogen bonding, and this interaction may have facilitated drug retention and prevented drug leakage during and after loading into the reconstituted liposomes. Previous studies have indicated the role of hydrogen bonding in improving drug retention after lyophilization of liposomes (Boggs, 1987; Smirnova et al., 1986). Further investigations involving FT-IR studies would help dissect the effect of sucrose on the liposomes prepared either freshly or with lyophilization and reconstitution.

Carboplatin loading into reconstituted CF-PEG liposomes was further studied with DSPC/DSPE-PEG$_{1000}$. As shown in Fig. 5B, the lyophilization/reconstitution process did not compromise carboplatin loading into the DSPC/DSPE-PEG$_{1000}$ liposomes, wherby carboplatin content in the reconstituted sample was slightly higher as compared to the non-lyophilized sample although the difference was not statistically significant. The lower loading of DSPC-based liposomes might be attributable to the increased chain length of the phospholipid (from DPPC to DSPC) leading to an increase in hydrophobicity of the liposomal membrane which may not favor the entry of the hydrophilic drug carboplatin as previously reported (Chaudhury et al., 2012). Alternatively, it could be due to the presence of non-bilayer structures (e.g. disc-shaped structures or "deformed/collapsed" liposomes) which do not exhibit an aqueous liposomal core thus leading to a lower amount of carboplatin loaded in the DSPC-based liposomes (Kuntsche et al., 2010).

Collectively, our results on carboplatin loading into the reconstituted CF-PEG liposomes provide further supporting evidence to recent studies that have demonstrated the potential of the lyophilization/rehydration method to increase the encapsulation of the highly charged, hydrophilic siRNA molecules into cholesterol-containing liposomes (Gao et al., 2010; Peer et al., 2008). The lyophilization/rehydration method in liposome encapsulation of siRNA could minimize siRNA degradation and loss of encapsulated siRNA during the encapsulation process, thus yielding improved gene silencing ability of the formulation.

Next, the in vitro drug release profiles of carboplatin-loaded liposomes were compared between those that were lyophilized (sucrose-cryoprotected) and reconstituted with those that were freshly prepared without lyophilization. Fig. 6 presents these drug release profiles obtained at 37 °C by dialysis method. Notably, the differences in the release profiles of lyophilized and non-lyophilized liposomes were not statistically significant, and both types of liposome samples displayed sustained release of carboplatin. Taken together, carboplatin loading could be satisfactorily achieved without compromising the amount of drug that could be loaded into the lyophilized liposome samples, and the process of lyophilization and reconstitution did not alter the drug release kinetics from the CF-PEG liposomes.

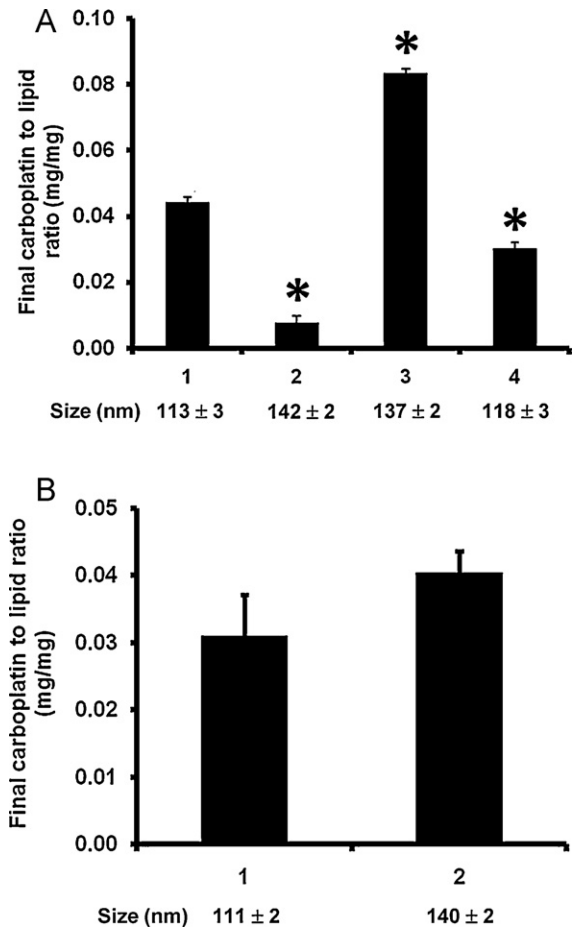

Fig. 5. (A) Carboplatin loading into DPPC/DSPC-PEG$_{1000}$ liposomes under various conditions: (1) loading into freshly prepared liposomes without lyophilization; (2) drug-loaded liposomes that have gone through lyophilization and reconstitution; (3) loading into reconstituted lyophilized liposomes; (4) loading into freshly prepared liposomes without lyophilization and with sucrose added exogenously at S/L ratio of 5:1. *Represents $p < 0.05$ as compared to condition (1). (B) Carboplatin loading into DSPC/DPSE-PEG$_{1000}$ liposomes under various conditions: (1) loading into freshly prepared liposomes without lyophilization; (2) loading into reconstituted lyophilized liposomes. The size of the liposomes for each condition in panel A and B was given at the bottom. Results represent the mean ± S.E.M from at least three batches of liposomes.

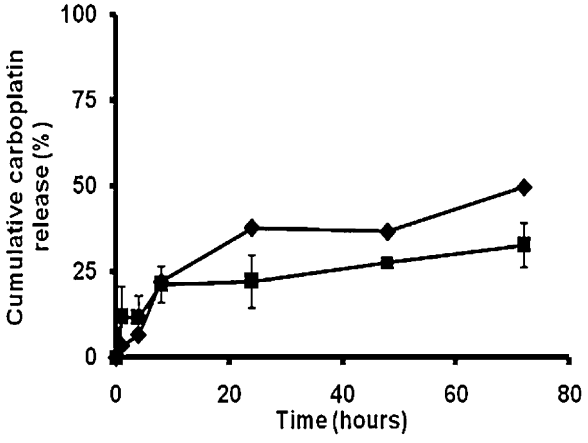

Fig. 6. Cumulative carboplatin release from lyophilized (■) or non-lyophilized (♦) DPPC/DSPE-PEG$_{1000}$ liposomes. Results shown are the mean ± S.E.M from at least three batches of liposomes. Some error bars are too small to be seen.

4. Conclusion

Taken together, our results indicated that lyophilization with sucrose at sugar-to-lipid molar ratio of 5:1 was suitable for long-term storage of CF-PEG liposomes. Furthermore, lyophilization of the CF-PEG liposomes conferred improved drug loading properties using carboplatin as the hydrophilic model drug, without altering the drug release profile from these liposomes. Our results could provide valuable insights for future studies and development of CF-PEG liposome drug carriers which represent an important alternative to the conventional cholesterol-containing liposomes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2012.04.036.

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