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RESEARCH PAPER

Freeze-Anneal-Thaw Cycling of Unilamellar Liposomes: Effect on Encapsulation Efficiency

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ABSTRACT

Purpose Freeze-thaw cycling is an important processing step in the preparation of liposomes that leads to the encapsulation of drug molecules. There is considerable variability in the number of freeze-thaw cycles reported in the literature. This work is designed to aid in liposomal formulation design by gaining an insight into the drug encapsulation process and an understanding of liposome stabilization during various thawing conditions.

Methods The effects of different thawing temperatures, as well as "annealing" at subzero temperatures on a liposome formulation, are reported here.

Results Two freeze-anneal-thaw ($FA_{NN}T$) cycles (freezing to −196°C, annealing at −1.4°C for ~30 min, thawing at 65°C) resulted in the maximum predicted encapsulation efficiency without causing any significant change in particle size or zeta potential. Annealing at −22°C was shown to be destabilizing due to limited hydration of the liposomes in the frozen state.

Conclusions It was shown that two important processes are occurring during the $FA_{NN}T$ cycling that affect liposome encapsulation efficiency. The first is drug diffusion in the frozen state and the second is fusion/destabilization of the liposomes. This is the first report on the annealing of liposomes and understanding the mechanism of drug encapsulation using the freeze-thaw cycling method.

KEY WORDS annealing · cryo-concentration · freeze-drying · freeze-thaw cycling · Tenofovir

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ABBREVIATIONS

Chol	Cholesterol
Cryo-SEM	Cryogenic scanning electron microscopy
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DSC	Differential scanning calorimetry
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine
DSPG	1,2-dioctadecanoyl-sn-glycero-3-phospho-(1′-rac-glycerol)
EE%	Encapsulation efficiency percentage
$FA_{NN}T$	Freeze-anneal-thaw
FT	Freeze-thaw
PDI	Polydispersity index
PFE	Pre-formed empty

INTRODUCTION

Freeze-thaw (FT) cycling is a technique often used in the preparation of liposomes to increase encapsulation efficiency (1,2). A common procedure is freezing the liposomes with liquid nitrogen (−196°C) and thawing at a temperature above the phase transition temperature of the lipids (3–5). In the preparation of liposomes, freeze-thaw cycling is implemented to reduce the lamellarity of liposomes (6), form a less polydispersed system and/or disrupt the liposomal bilayer (7) to allow drug molecules to diffuse into the liposome, promoting encapsulation (5,8,9). The number of freeze-thaw cycles needed to encapsulate drug molecules varies greatly in the literature, with some papers reporting up to 10 cycles (10,11). The reason for using multiple freeze-thaw cycles is to achieve equilibrium drug concentration conditions (*i.e.* concentration inside the liposomes is equal to the concentration outside of the liposomes). Prior to this study, the mechanism for drug encapsulation was considered to be due to physical disruption of the lipid-bilayer as a result of ice crystal formation (8). Disruption of the lipid-bilayer typically results in vesicle fusion

(12) and increased polydispersity. In this study a second mechanism for drug encapsulation is reported based on cryo-concentration (the phenomenon when water freezes and excludes molecules into concentrated zones). Cryo-concentration of the liposome and drug will increase drug diffusion into the liposome while the dispersion is in a semi-frozen state. Unilamellar, pre-formed empty (PFE) liposomes of a monodispersed population were chosen to eliminate any effects related to polydispersity and/or multiple lipid lamellae. The factors that are studied include thawing and annealing temperatures as well as annealing duration, where annealing refers to holding the samples at constant sub-zero temperatures for a certain duration. The freezing procedure was kept constant.

To study the effects of drug diffusion in the frozen state, stable DSPC:Chol:DSPG (6:3:1) liposomes were prepared and the EE% was calculated. Similar DSPC liposomes have previously exhibited minimal particle size changes after freeze-thaw cycling (9). This minimal size change will allow drug diffusion to be studied while avoiding the effects on EE% from changes in particle size/polydispersity. The EE% for this liposome system was then compared to a theoretical maximum EE% based on a mathematical model for the entrapped volume of unilamellar liposomes (4). Briefly, this theoretical EE% is based on parameters such as liposome size, size distribution, bilayer thickness, average molecular area of a lipid at an interface, and the lipid molarity. By supplying these parameters, this mathematical model is able to predict the encapsulation efficiency for any unilamellar, monodispersed liposomal system.

As a model drug, Tenofovir was chosen due to its high polarity (LogP = −1.71) (13) and low membrane permeability. Tenofovir has a pKa of 4.1 ± 0.5 and is negatively charged at the studied pH of 7.4. Since the liposomes were also negatively charged, electrostatic interactions between the drug and liposome surface were avoided. The significance of this research is that it offers physical insight into the behavior of liposomes in the frozen state and provides a method to achieve efficient drug encapsulation into unilamellar, PFE liposomes without the need for multiple freeze −thaw cycling steps and additional downsizing techniques.

MATERIALS AND METHODS

Materials

DSPC, DPPC, cholesterol and DSPG were purchased from Avanti Polar Lipids (Alabaster, AL). Tenofovir (CAS# 147127-20-6) was purchased from Resource Technique Corporation (Laramie, Wyoming). Chloroform and methanol were purchased from Fisher Scientific (Pittsburgh, PA). HEPES sodium salt, and Triton X-100 were purchased from Sigma Aldrich (St. Louis, MO). Amicon Ultra-0.5 (50 kDa) were purchased from Millipore (Billerica, MA).

Preformed Empty Liposomes Preparation

DSPC:Chol:DSPG (6:3:1 molar ratio, 60 mM) PFE liposomes were prepared by the film-hydration method. Briefly, lipid was mixed with chloroform:methanol (2:1 v/v) and evaporated using a Büchi Rotavapor at an elevated temperature (50°C). During evaporation, a low vacuum was applied initially to avoid bursting of the lipid solution. The dried lipid was placed under vacuum overnight to remove residual solvent. The lipid was hydrated at 65°C in 10 mM HEPES buffer, pH 7.4 for approximately 2 h. The liposomes were then sonicated at 80 W for 1 min followed by two freeze-thaw cycles (−196°C for 5 min, 65°C for 10 min). The liposomes were then extruded 8x through a single stack of one 400 nm and two 200 nm polycarbonate membranes using a LIPEX™ Extruder (Northern Lipids Inc.). The extruder was brought to 65°C prior to extrusion.

Drug Encapsulation Process

Tenofovir solution was prepared in 10 mM HEPES buffer, pH 7.4 at approximately 8.7 mg/ml. This solution was mixed with the PFE liposomes, and the mixture was frozen in liquid nitrogen (−196°C) for 3 min. To perform annealing or thawing, the samples were transferred to a temperature controlled water bath maintained at various temperatures (Table I). For direct thawed samples, the thawing time was determined by measuring the temperature of a duplicate sample until the desired thawing temperature was reached. To prevent membrane fouling and facilitate the extrusion process, all samples were equilibrated at 65°C for 10 min just prior to extrusion. Extrusions were performed 6x through a single stack of one 400 nm and two 200 nm polycarbonate membranes at 250 psi. Particle size and zeta potential were monitored before and after the extrusion, and EE% were measured only after extrusion.

Encapsulation Efficiency (EE%)

Encapsulation efficiency was determined by the following equation: $EE\% = 1 - C_{free}/C_{total}$, where C_{free} is the free drug concentration and C_{total} is the total drug concentration. The free drug was separated from encapsulated drug by ultracentrifugation using Amicon 50 kDa filters at 4,000 g for 12 min. Free drug did not interact with the filter membrane and >99% of the free drug passed through the filter. The total drug was determined by lysing the liposomes in a 6% v/v TX-100 solution. The free drug from the filtrate and total drug was then analyzed using a previously developed HPLC analysis (14). All measurements were run in triplicate.

Table I Experimental Outline for 60 mM DSPC:Chol:DSPG (6:3:1 Molar Ratio) Liposomes

Sample ID	Annealing temperature (°C)	Thaw/annealing time (min)	Thawing temperature (°C)	Time at 65°C following thawing (min)
FT(65°C)	n/a	10	65	–
FT(1°C)	n/a	90	1	10
FT(4°C)	n/a	45	4	10
FT(23°C)	n/a	20	23	10
$FA_{NN}T(-1.4/65°C)$	−1.4	36, 90, 360, and 960	65	10
$FA_{NN}T(-22/65°C)$	−22	960	65	10

A single freeze-thaw cycle was performed in all cases, with differences in the thawing temperature. The difference in thawing time was needed to accommodate complete thawing. Liposomes were held at 65°C for 10 min before extrusion to prevent membrane fouling

Particle Size and Zeta-Potential

All measurements were performed using a Malvern Zetasizer Nano ZS90. For both particle size and zeta-potential, samples were placed in plastic disposable cuvettes and equilibrated at 25°C. The viscosity of water was assumed since liposome dispersions were below 0.5 mg/ml. Particle size measurements included z-average, PDI, and PDI width. Zeta potential measurements included zeta-potential and zeta deviation. All measurements were run in triplicate.

Cryo-SEM Imaging

In order to determine how annealing can destabilize liposomes in the frozen state, cryo-SEM was performed for samples, (1) frozen to −196°C; and (2) frozen to −196°C then annealed at −20°C. Both samples were immersed in liquid nitrogen before further processing. The −20°C sample was stored in the freezer overnight (~16 h). The samples were then fractured using a Leica EM MED020 with an attached cryo-transfer system (Leica EM VCT100). During fracturing, the sample was held at −140°C and sputter coated with platinum (5 nm thickness). The sample was then transferred at −140°C under vacuum to the scanning electron microscope (FEI Nova™ NanoSEM 450). The accelerating voltage was set to 2.0 kV with a working distance of 5.1 nm and viewed at −140°C.

Differential Scanning Calorimetry

Measurements were carried out using a Q1000 DSC with a Refrigerated Cooling System 90 (TA Instruments). Empty liposome samples with Tenofovir (~20 mg total) were pipetted onto an open aluminum plate, immersed in liquid nitrogen and then transferred to the DSC holder equilibrated at −60°C. The sample was then heated at 50°C/min to either −22°C or −1.4°C. The samples were held at this temperature for 10 min and subsequently cooled to −60°C at 10°C/min. Only the latter thermogram was analyzed. The fast heating rate was used to mimic the thawing conditions of the liposome samples used in the EE% tests.

RESULTS

Particle Size

Particle size analysis was performed to compare the average size and distribution of the particles before and after processing. The PFE liposomes that were used in all the following cases had a particle size diameter of 166.8±39.8 nm (PDI=0.07). As a control, the common procedure of freeze-thaw cycling (in this case, freezing at −196°C and thawing at 65°C for up to ten freeze-thaw cycles) was performed. Since the liposomes were subsequently extruded, the mean particle size diameter for all cycles was 153.3±4.0 nm (PDI=0.06±0.02, PDI width=36.8±7.5). For the test samples, the pre-extrusion particle size and size distribution of FT (thawed at temperatures below 65°C) and freeze-anneal-thaw ($FA_{NN}T$) liposomes frozen at −196°C, annealed at −1.4°C for 16 h and thawed at 65°C [$FA_{NN}T(-196/-1.4_{16h}/65)$] did not change significantly (Fig. 1). The PDI did increase slightly for $FA_{NN}T$ samples held longer than 90 min, but was still ≤0.1 in all cases, which suggests a monodispersed system. The $FA_{NN}T(-196/-22_{16h}/65)$ liposomes had a pre-extrusion particle size of 373.2±263.2 nm and a PDI of 0.50, which indicates high polydispersity. After extrusion, the particle size was similar to all other formulations.

Zeta-Potential

In addition to particle size measurements, zeta-potential was compared to pre- and post-extrusion conditions. Pre-extrusion mean zeta potentials were lower than the post-extrusion mean zeta-potentials; however, these values were still within the reported zeta-deviation (Fig. 2). The only sample that did show a major difference in zeta potential was the $FA_{NN}T(-196/-22_{16h}/65)$ liposome sample. This

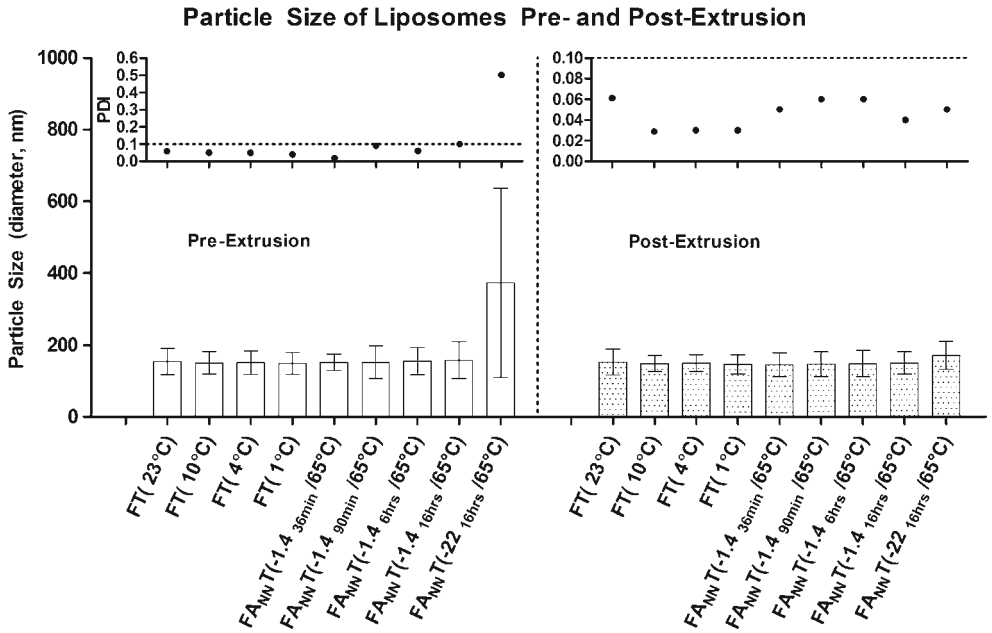

Fig. 1 Particle size of liposomes under various conditions. Samples either underwent a freeze-thaw (*FT*) cycle or a freeze-anneal-thaw ($FA_{NN}T$) cycle. All $FA_{NN}T$ samples were thawed at 65°C. Both pre-extrusion and post-extrusion particle size properties are detailed above. The error bar represents the PDI distribution width. The inset above each group of data represents the PDI of the samples for pre- and post-extrusion.

difference is due to the particle size and particle size distribution increase noted in Fig. 1. In all cases, the zeta-potential did not drop to low levels that would cause instability; however, instability may arise from sample polydispersity (9) (Fig. 2).

Encapsulation Efficiency (Post-Extrusion)

Figure 3 reports on the difference in encapsulation efficiency post-extrusion for all samples. For thawing at 65°C, the first freeze-thaw cycle had an EE% of 3.71±0.23, whereas the EE% was 7.22±0.21 after 10 freeze-thaw cycles. Thawing at various temperatures below 65°C followed a trend in that lowering the thawing temperature increased the encapsulation efficiency. In all cases, annealing increased the EE%. A single $FA_{NN}T(-196/-1.4_{36min}/65)$ cycle had an encapsulation efficiency of 9.83±0.23%, roughly 2.6 times greater than the FT(−196/65) sample. By increasing the annealing duration, slight increases in EE% were observed but reached a plateau around 12%. Lastly, annealing at −22°C increased the EE% into the theoretical maximum EE% range.

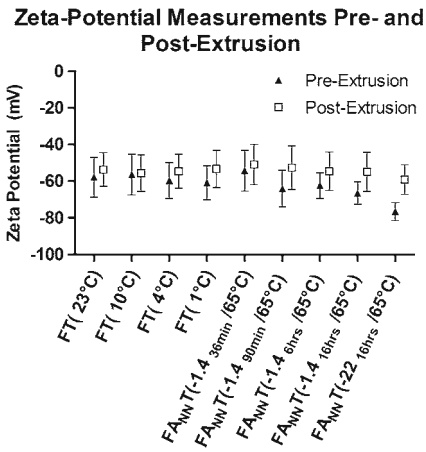

Fig. 2 Zeta-potential of the liposome formulation. The zeta-potential is provided for both pre- and post-extrusion for the samples listed in Fig. 1.

Two Freeze-Anneal-Thaw Cycles (Pre-Extrusion)

Another sample was prepared to determine whether the maximum EE% could be reached without the need for extrusion while maintaining the particle size characteristics of the extruded liposomes. In order to achieve this, two cycles of $FA_{NN}T(-196/-1.4_{36min}/65)$ were performed (Table II). After two cycles, the particle size characteristics were not significantly different than the post-extruded samples. The EE% increased to values within the maximum EE% range for this formulation. A direct comparison of these results to the EE% results for two freeze-thaw cycles (thawed at 65°C without annealing, Fig. 3) suggests that sub-zero temperatures near the freezing point of the solution allows for increased drug encapsulation.

Cryo-SEM Imaging

It can be seen from Fig. 4 that ice formation (darker, solid regions) causes the liposomes to be excluded from regions occupied by ice crystals thus forming concentrated clusters (lighter regions). The samples that were only frozen (not annealed) appear to have well-defined liposomes with larger clusters of liposomes (Fig. 4a, b). For the −20°C annealed sample, the liposomes were more difficult to find and either had an overall less-defined/fused structure and/or were within narrow bands between the ice-phase (Fig. 4c, d).

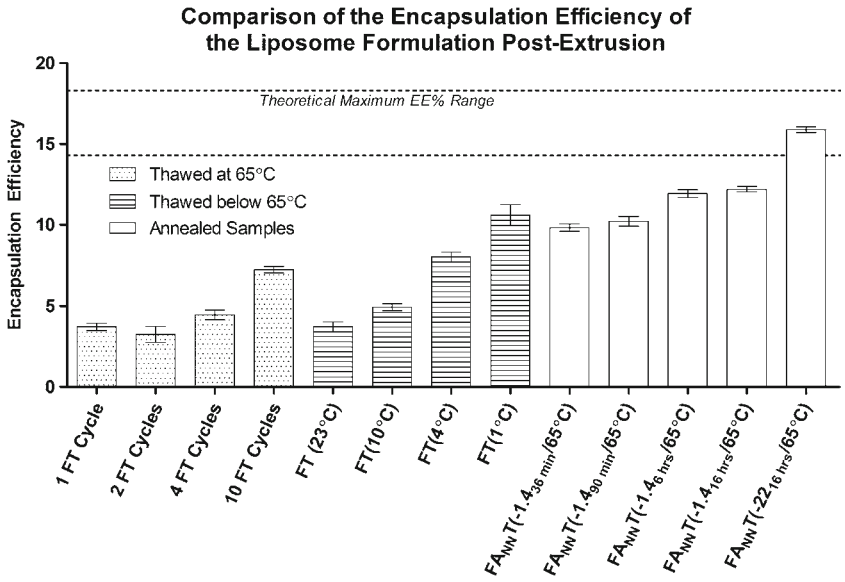

Fig. 3 Encapsulation efficiency for samples thawed under various conditions. All samples were frozen in liquid nitrogen at −196°C. The samples thawed directly at 65°C represent normal freeze-thaw cycling. All other samples underwent a single freeze-thaw or freeze-anneal-thaw cycle. The effects on EE% of thawing at temperatures above 0°C and annealing at subzero temperatures is demonstrated ($n = 3$).

Moreover, for the −20°C annealed samples, there were some locations in the sample that did have clusters of moderately-defined liposomes. The existence of both clusters of moderately-defined liposomes and large regions of fused liposomes would contribute to the increase in polydispersity as shown in Fig. 1 for the −22°C annealed sample.

Differential Scanning Calorimetry Thermogram

DSC was performed to determine whether water melted during the annealing process. Heating from −196°C to either −1.4°C or −22°C and annealing for 10 min before re-cooling to −60°C, a peak only appears for the −1.4°C sample (Fig. 5). This indicates a portion of the water in the −1.4°C sample melted and then refroze. HEPES buffer without liposomes was measured as a control. The increase in enthalpy for liposomes −1.4°C *vs.* HEPES −1.4°C suggests additional water melted and froze upon re-cooling for the liposome samples.

DISCUSSION

For the liposome formulation investigated here, the theoretical maximum EE% is 16.3 ± 2.0% (4). Accordingly, it would appear that more than half of the predicted drug amount was not encapsulated even after 10 freeze-thaw cycles. In order to understand how freeze-thaw cycling influenced liposome EE%, all of the liposome samples [except for $FA_{NN}T(-196/-1.4_{36min}/65)$] underwent a single freeze-thaw cycle. Liposomes were immersed in liquid nitrogen and thawed at various temperatures to determine whether the thawing temperature influenced liposome physical characteristics. As seen above, thawing at temperatures above 0°C did not significantly alter liposome particle size both pre- and post-extrusion (Fig. 1), which indicates liposome stability. Additionally, the physical characteristics of the samples that underwent $FA_{NN}T$ cycles did not change significantly except for the $FA_{NN}T(-196/-22_{16h}/65)$ sample. The reason for the liposome stability at −1.4°C and at temperatures above 0°C may be due to the hydration of the liposomes as discussed later.

The EE% was analyzed to determine whether drug molecules entered the liposomes during the frozen state. As compared to the control freeze-thaw cycles, adjusting the thawing temperatures to less than 65°C showed an increase in EE% as the thawing temperature was lowered. An explanation is that at lower temperatures, it takes more time for ice to melt, thus ice growth and subsequent cryo-concentration of drug and liposomes increases the amount of drug entrapped once the liposomes become rehydrated. Furthermore, complete thawing at 1°C took about 55 min, and the EE% is comparable to annealing at −1.4°C for both 36 and 90 min (Fig. 3). As for the annealed samples, increased drug diffusion was evident (EE% >9% after 1 cycle). However, the maximum encapsulation (~16.3%) was still not reached, which indicates that there was either limited drug diffusion into the liposomes or that only a percentage of the liposomes had drug encapsulated. A possible explanation is that under these freezing conditions, densely populated liposome phases (Fig. 4a, b)

Table II Pre-Extrusion Results for Two $FA_{NN}T(-196/-1.4_{36min}/65)$ Cycles ($n = 3$)

Particle Size ± PDI Width (d. nm)	151.9 ± 35.0
PDI	0.05
Zeta-Potential ± Deviation (mV)	−56.77 ± 10.17
Encapsulation Efficiency	15.42 ± 0.08%

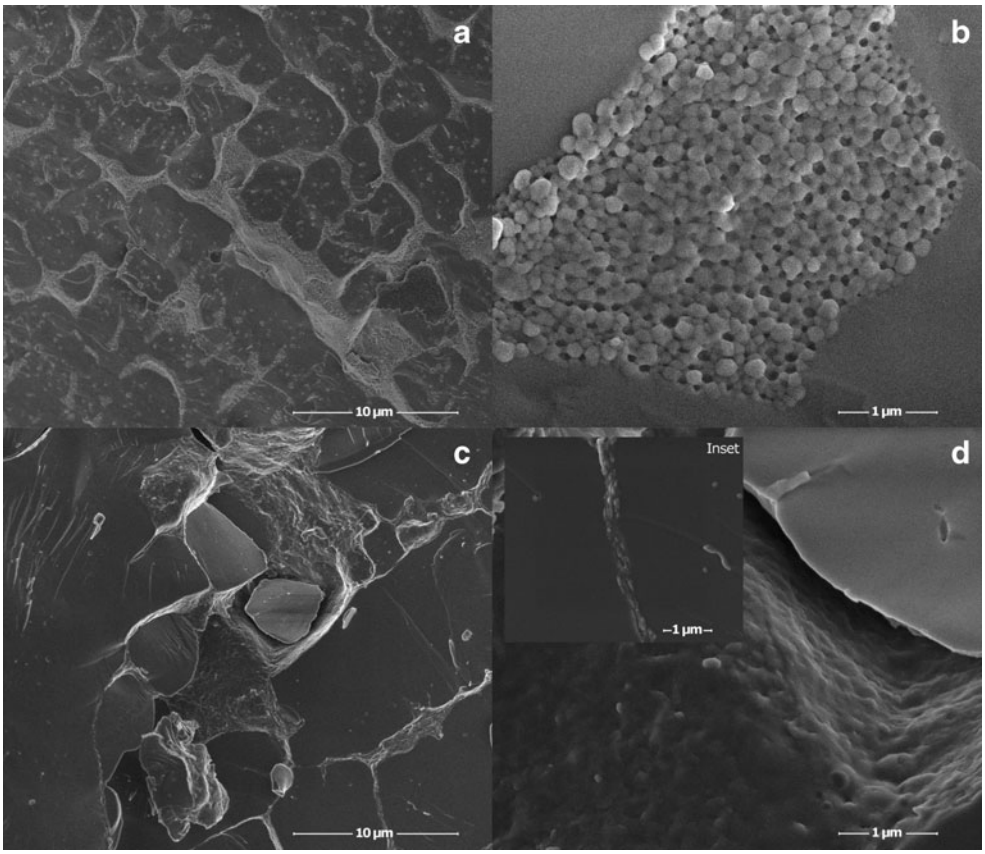

Fig. 4 Cryo-SEM images of PFE-liposomes with tenofovir. In all the images, the smooth/darker areas represent the ice phase while the non-smooth/lighter regions are liposomes. (**a**) Sample frozen to −196°C, magnification (mag.) 5,000×. (**b**) 30,000× mag. of sample from (**a**) with liposomes clearly visible. (**c**) Sample frozen to −196°C, annealed at −20°C overnight, and then refrozen to −196°C prior to imaging, mag. 5,000×. (**d**) 30,000× mag. of sample from (**c**). In (**d**), the *top right hand corner* is ice while the rest of the image is of liposomes in an apparent fused state. (**d**) Inset 30,000× mag. of a thin channel of liposomes between the ice-phases.

are present. These dense phases may inhibit the diffusion of the water-soluble drug within the liposome phase. Liposomes located more towards the center of this phase and/or liposomes entrapped in the bulk ice would have limited contact with the drug. It is suggested that two $FA_{NN}T$ cycles are necessary since a second cycle redistributes the liposomes, facilitating contact of the drug with all liposomes. After the two $FA_{NN}T$ cycles, the EE% was similar to the theoretical maximum and the physical characteristics did not change.

In addition to increased drug diffusion, liposomal disruption/fusion is another mechanism of drug encapsulation.

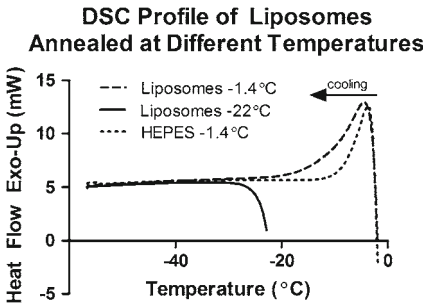

Fig. 5 DSC profile of annealed liposomes. Samples were first frozen in liquid nitrogen (−196°C) and heated to the annealing temperature (−22°C or −1.4°C) and held at that temperature for 10 min (not shown). The sample was then re-cooled to −60°C (shown above). HEPES buffer without liposomes was shown as a control. All samples were run in triplicate and the average standard deviation was <0.4 in all cases.

This is evident from the $FA_{NN}T(-196/-22_{16h}/65)$ sample, which had high polydispersity pre-extrusion. The post-extrusion polydispersity returned to a monodispersed system and the EE% was near the theoretical maximum value. This is due to the complete disruption and fusion of the liposomes during the thaw process –after extrusion, the drug is equally dispersed between the intraliposomal and extraliposomal space. As suggested by Talsma *et al.* and supported by cryo-SEM imaging (Fig. 4c, d), ice crystal growth may be destabilizing to the liposomes (15) by forcing the vesicles into close contact. At such close distances between liposomes, electrostatic repulsive forces and hydration forces (16) are no longer sufficient to prevent particle fusion. Thus, particle size, size distribution and polydispersity increased for the $FA_{NN}T$ $(-196/-22_{16h}/65)$ samples. For the $FA_{NN}T(-196/-1.4/65)$ and FT samples, it may be that the high concentrations of drug, liposomes and buffer between the ice-phase is allowing for intra-liposomal and/or extra-liposomal ice within the vicinity of the liposomes to melt, thus keeping the liposomes hydrated and preventing them from fusing. Thus, at temperatures close to 0°C, such high concentrations would result in a freezing point depression causing the ice to melt.

To further investigate whether ice is melting and possibly hydrating/stabilizing the liposomes at subzero temperatures, DSC was used to detect exothermic peaks that indicate water refreezing after annealing. Reported DSC measurements for

DPPC liposomes demonstrates that heating a sample from −50°C to −15°C and re-cooling to −50°C does not show the intra-liposomal water freezing peak (at −43°C) in the DSC profile (17), thus intra-liposomal and extra-liposomal ice is not melting upon heating to moderate temperatures above the intra-liposomal freezing temperature. In the current case, the DSC thermogram from Fig. 5 shows that ice melted and refroze for the −1.4°C sample. This suggests that melted water is hydrating the liposomes and preventing liposome fusion. In the case of the −22°C sample, no exothermic peak was visible, which indicated water did not melt during the annealing stage and did not stabilize the liposomes.

Lastly, the results reported here were only for encapsulating a single hydrophilic molecule. As previously mentioned, lipid-drug interactions were avoided by choosing a model drug that was hydrophilic and exhibited low permeability, and by choosing lipid of the same charge as the drug. Therefore, these results can be extended to other small molecules and/or proteins (depending on protein stability during freezing/thawing). Moreover, further testing needs to be done on lipid composition (*e.g.* mixing lipids that have various hydrocarbon chain lengths, that are saturated *vs.* unsaturated and/or that have different lipid head groups). All of these adjustments in the lipid composition may form voids in the lipid bilayer, causing the liposomes to fuse during the thawing cycle and resulting in a polydispersed system rather than a monodispersed system after freeze-thaw cycling.

CONCLUSION

The significance of this work is that the results provide further insight into conditions that destabilize liposomes and induce fusion (due to dehydration and ice growth). The results also suggest a second mechanism for drug encapsulation (*i.e.*, cryo-concentration of drug and liposomes in the frozen state) that increases the EE% of the drug. Moreover, for this DSPC:Chol:DSPG liposome formulation, it is possible to encapsulate drug into pre-formed empty liposomes without the need for extrusion or other downsizing techniques. As for other applications, such as freeze-drying of liposomes, this mechanistic insight provides an understanding of why liposomes become destabilized under various conditions.

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