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Effects of trehalose on the phase behavior of DPPC–cholesterol unilamellar vesicles

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Abstract

A systematic study is presented of the effects of trehalose on the physical properties of extruded DPPC–cholesterol unilamellar vesicles. Particular emphasis is placed on examining how the interactions present in the hydrated state translate into those in the dehydrated state. Observations from HSDSC and DSC are used to examine the phase behavior of hydrated and dehydrated vesicles, respectively. The concentration of trehalose inside and outside the vesicles is manipulated, and is shown to affect the relative stability of the membranes. Our results show for the first time that a combination of high inner and low outer trehalose concentration is able to decrease the gel-to-liquid crystalline phase temperature (T_m), while any other combination will not. Upon dehydration, the T_m of all lipid mixtures increases. The extent of the increase depends on the trehalose distribution across the bilayer. The T_m changes in the same direction with trehalose concentration for both freeze-dried and fully hydrated samples, suggesting that the trehalose distribution across the vesicle membrane, as well as the trehalose–phospholipid interaction, is maintained upon lyophilization. The results presented in this work may aid in the formulation of systems to be used in the lyophilization of liposomes for drug delivery applications.

Keywords: Trehalose; DPPC; Cholesterol; Liposome

1. Introduction

Sugars are found in a wide variety of organisms that survive osmotic stress, severe dehydration, and low temperature stress. The effects of sugars on biological systems, such as proteins [1] and membranes [2–4], have been examined extensively in order to elucidate the mechanisms by which they confer stability. Particular emphasis has been placed on trehalose, a non-reducing disaccharide of glucose. Several studies have examined the effects of sugars on the physical properties of phospholipid membranes in aqueous solutions. Studies performed on monomolecular films of different phospholipids suggest that, in the presence of bulk water, trehalose causes an expansion of the monolayer [5–7]. FTIR experiments indicate that a direct interaction arises between trehalose and DMPC bilayers in solution [8]. The above two studies are consistent with recent simulation results obtained from our group, which reveal the formation of hydrogen bonds between trehalose and DPPC molecules, thereby resulting in a slight increase of the head group spacing [9,10]. Simulation studies by other groups also report direct interaction between trehalose and DPPC bilayers [11,12]. These conclusions are in apparent contradiction with a study by NMR, which suggests that trehalose increases the packing density of DPPC polar head groups in unilamellar vesicles [13]. These NMR measurements are consistent with calorimetric studies of bilayers that show a slight increase of the phospholipid gel-to-liquid crystalline phase transition temperature (T_m) upon addition of trehalose [14,15]. Several interpretations have been drawn to explain the latter observation, including osmotic dehydration [16], a kosmotropic effect of trehalose [15,17], and a preferential exclusion of trehalose from the vicinity of the membrane [14]; the latter interpretation, however, appears to be an extrapolation of observations and conclusions pertaining to proteins in

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solution [1] to the case of hydrated bilayers, and have been called into question by results from molecular simulations [9].

In the dehydrated state, sugars inhibit the fusion of biological membranes (phospholipid liposomes) [18] and help maintain the lipid distribution that was present in the hydrated state [19,20]. In addition, the gel-to-liquid crystalline phase transition temperature (T_m) of the phospholipid is decreased [4,20–23], allowing the membrane lipids to remain in the same phase throughout the dehydration and subsequent rehydration processes. By avoiding a phase transition, it is possible to increase the retention of entrapped solutes in lyophilized liposomes [18]. The stabilizing effect of sugars on the dry membrane has been explained by the so-called "water replacement hypothesis", in which the sugar molecules interact directly with the membrane phospholipids (through hydrogen bonding), and essentially "replace" the water molecules in their vicinity upon dehydration [2,3]. In doing so, the sugars are able to maintain a semblance of the hydrated environment, but in the dried state. It has also been proposed that the membrane is stabilized through the formation of a glass, good glass formers, such as trehalose, can encase the membrane in an amorphous matrix and restrict its mobility [4,24]. The water replacement and vitrification hypotheses are not mutually exclusive; both effects appear to be necessary for conferring stability to the dehydrated membranes [3].

Previous work in the field of biomembrane stabilization has focused mainly on simple phospholipid systems, often consisting of only one component or a binary mixture of phospholipids. Cell membranes, however, are inherently multicomponent systems consisting of various species of lipids and proteins. The physical properties and biological functions of such membranes are closely coupled to the particular arrangement and distribution of the components within the phospholipid bilayer. Cholesterol is relatively abundant in mammalian plasma membranes [25], and numerous studies have investigated its interactions with various phospholipid species in fully hydrated model membranes [20,26–29]. Although cholesterol has various functions in cells, one of its primary roles is that of modulating the physical properties of the plasma membrane [29,30]. Studies of dry membranes with cholesterol have been scarce [20,31,32].

This work presents results for the melting transitions of DPPC liposomes with and without cholesterol. In a departure from previous work, unilamellar vesicles are prepared with various even and uneven trehalose distributions across the membrane bilayer. In addition, we address how the interactions present between trehalose and the cholesterol–DPPC liposomes in the hydrated state translate into those upon the removal of water. It is shown that vesicles prepared with high inner/low outer trehalose concentration exhibit a near optimal behavior for lyophilization and drug delivery applications.

2. Materials and methods

2.1. Chemicals

1, 2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine (DPPC) and cholesterol (Ch) in chloroform were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Trehalose was purchased from Pfanstiehl Laboratories (Waukegan, IL). All solutions were prepared in nanopure water.

2.2. Sample preparation

Phospholipids and cholesterol were mixed in chloroform up to 33 mol% Ch. Chloroform was evaporated under a stream of N_2, and further drying was conducted in a vacuum oven. The dried lipids were resuspended at 50 °C in a solution containing trehalose to achieve several different sugar/lipid ratios. The samples were then extruded through membrane filters (50 and 100 nm pore diameters) 19 times using an extruder (Avanti polar lipids, Alabaster, AL) at 50 °C. The sample was then diluted to reach a final lipid concentration of 5 mM. The dilution was made with nanopure water or with trehalose solutions to obtain the desired sugar concentration on the two sides of the vesicle membrane (Table 1). These samples were analyzed using HS-DSC. Aliquots of 0.5 mL of the extruded solutions were then placed in 4 mL glass vials and immediately submerged in liquid nitrogen. The samples were freeze-dried for 48 h in a Virtis Genesis 12EL (New York, USA) freeze-dryer at a pressure of 30 mTorr and a condenser temperature of −80 °C. The samples under nitrogen atmosphere were loaded into preweighed DSC pans and sealed for calorimetric analysis, and into preweighed glass vials for water content analysis. Throughout the text, a shorthand notation will be used to designate the trehalose concentration on the inside and outside of the vesicle (e.g., 146/11 mM denotes 146 mM trehalose inside and 11 mM trehalose outside).

2.3. Differential scanning calorimetry

High Sensitivity DSC (HS-DSC) was used to determine the phase transition temperatures of fully hydrated phospholipid samples. The main transition (at T_m) represents the rippled gel-to-liquid crystalline phase transition. The transition temperatures correspond to the peaks of the endotherms during the heating scans. Microcal MCS calorimeter (Northampton, MA) at a scan rate of 10 °C/h was used. The samples consisted of 1.2 mL of 5 mM phospholipid solution (for the analysis, cholesterol-containing samples were normalized to phospholipid mass); nanopure water was used as a reference. Sample runs were repeated at least 5 times on three or more different batches to ensure reproducibility. The standard deviation was lower than 0.06 °C. The analysis of the HS-DSC endotherms was conducted using Microcal's Origin, version 2. The size of the cooperative unit (CU) was calculated following the Zimm and Bragg theory of cooperative phase transitions [33].

For freeze-dried samples, a TA Q100 DSC (New Castle, DE) was used to measure the phase transition temperatures. The dehydrated samples were scanned from −20 °C to 150 °C at 10 °C/min. More than three replicate samples were measured, and at least three scans were carried out for each sample. All measurements were made using sealed aluminum pans, and an empty pan was used as a reference. Data were analyzed using Universal Analysis.

Thermograms for hydrated samples, obtained from HSDSC, are reported with the exothermic direction down. Thermograms for dehydrated samples are reported with the exothermic direction up.

Table 1
The various trehalose concentrations considered in this work

Trehalose distribution	Trehalose inside (mM)	Trehalose outside (mM)
Symmetric	3	3
	6	6
	11	11
	25	25
	43	43
	88	88
	146	146
	292	292
Asymmetric	11	146
	146	11

2.4. Water content analysis

Residual water measurements for dehydrated samples were made using a Karl Fisher Coulometer Metrohm, Model 737 (Herisau, Switzerland). All freeze-dried samples contained less than 1.5 wt.% water.

2.5. Vesicle size determination

Dynamic light scattering was used to determine the distribution of hydrodynamic radii of the vesicles. Quasi-elastic light scattering measurements were performed using a Brookhaven light scattering apparatus (Brookhaven Instruments, Holtsville, NY), consisting of a BI-9000AT digital autocorrelator, a BI-200SM goniometer, and a 100 mW laser (532 nm, Coherent Compass 315 M-100). The detector angle was set to 90°. Aqueous solutions were prepared by diluting 40 μL of the freshly extruded lipid sample in 5 mL of nanopure water, which was then allowed to equilibrate at 20 °C. At least three separate batches were measured for each sample analyzed. The autocorrelation curves were analyzed by using Contin [34].

3. Results and discussion

3.1. Effects of Trehalose on the Phase Transitions of Hydrated DPPC Vesicles

Fig. 1 shows the thermotropic phase behavior of 100 nm DPPC unilamellar vesicles in the presence of trehalose, ranging from 3 to 292 mM (on both sides of the vesicle). The addition of small amounts of trehalose does not modify the gel-to-liquid crystalline phase transition temperature (T_m) of DPPC significantly, although the peak is broadened (Fig. 1). At 11 mM trehalose and above, a shoulder becomes observable on the high temperature side of the peak, which develops into a distinct peak at 43 mM trehalose. The peak continues to grow in magnitude up to 292 mM trehalose (the highest concentration examined in this work), at which point the T_m (41.5 °C) is higher than that of pure DPPC (41.1 °C). A similar T_m increase was reported for multilamellar [15] and for extruded [14] DPPC vesicles in the presence of 1 M trehalose. In addition to the development of the new peak, the main transition peak broadens and decreases in temperature above 11 mM trehalose (Fig. 1). The decrease in the T_m of hydrated DPPC unilamellar vesicles has been observed previously, but only at a much higher trehalose concentration (1 M) [14].

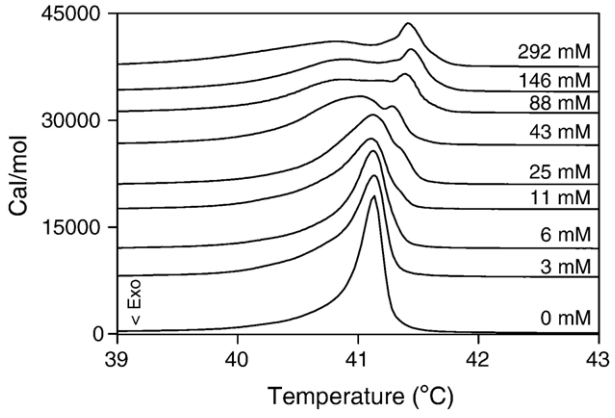

Fig. 1. Thermograms for DPPC liposomes (diameter = 100 nm) in trehalose solutions. Trehalose concentration (mM) is indicated for each curve.

Trehalose addition does not modify the enthalpy of the phase transition significantly (Table 2), which is in accordance with previous reports [15]. Table 2 also shows the average diameter and the cooperative units of melting [33] for liposomes containing various trehalose concentrations. The size distribution of the vesicles does not change significantly in the presence of trehalose, suggesting that the vesicles do not undergo fusion or aggregation (data not shown). Cooperativity decreases significantly with increasing trehalose concentration, as reported previously [14].

The presence of a peak with a T_m lower than that for pure DPPC (Fig. 1) suggests that the neighboring phospholipids are packed less tightly; this could be achieved by their interaction with trehalose molecules through hydrogen bonds. On the other hand, the presence of a high T_m peak suggests that trehalose can exert its influence through a kosmotropic effect. We attribute the appearance of two transitions to a difference in the trehalose–phospholipid interactions on the two leaflets of the bilayer. For this reason, we have examined systems in which an asymmetric concentration of trehalose is present across the bilayer (Fig. 2).

The bilayer is composed of two symmetrical monolayers of phospholipids, one forming the inside of the vesicle and the other forming the external surface. A literature report indicates that in small lipid vesicles (15–30 nm) the molecular packing of the inner and outer monolayers is different [35]. The phospholipid packing geometry of the outer membrane monolayer closely parallels that of a planar bilayer, in contrast to the inner monolayer, which is highly curved and subject to packing constraints [36]. In the case of large vesicles (>100 nm), there is little evidence for differences in molecular packing of the two monolayers [36]. To determine whether the vesicle size is of importance in affecting the ability of trehalose to modify the T_m, we examined both 100 and 50 nm vesicles at various trehalose distributions. The osmotic stress imposed on the vesicle does not cause vesicle rupture/fusion, as confirmed by light scattering analysis; the sizes of vesicles containing trehalose (105.1 nm and 101.9 nm for 11/146 mM and 146/11 mM, respectively) are similar to those of pure DPPC vesicles (105.5 nm). As described previously, the addition of low amounts of trehalose to both sides of the 100 nm vesicle (11/11 mM) has no effect on the T_m, which remains at 41.1 °C (Fig. 2a). Upon further increasing the trehalose concentration inside the vesicle to 146 mM (146/11 mM), the T_m decreases to 39.9 °C with a shoulder appearing at 41.1 °C. On the other hand, increasing the trehalose concentration outside the vesicle to 146 mM (11/146 mM) causes the T_m to decrease to 40.8 °C, with another peak appearing at 41.4 °C. In addition, the peak at 40.8 °C grows in magnitude by further increasing the intravesicular trehalose concentration to 146 mM (146/146 mM). The decrease in vesicle size (from 100 to 50 nm) does not modify the effect of trehalose on the T_m; for both vesicle sizes, the T_m decreases from 41.0 °C to 39.9 °C upon the addition of 146/11 mM trehalose, while at 11/146 mM trehalose, two transitions are observed (at 40.8 °C and 41.4 °C) (Fig. 2b). The symmetry of the peak for the 50 nm DPPC liposome, however, is slightly

Table 2
The enthalpy of the phase transition (ΔH), the vesicle diameter (d), and the size of the cooperative unit of the transition (CU) for 100 nm DPPC liposomes containing the indicated trehalose concentrations

	Trehalose concentration (mM)		
	0	11	146
ΔH (Kcal/mol)	7.2±0.4	7.1±0.2	7.3±0.8
d (nm)	105.5±3.0	95.4±1.3	102.2±3.4
CU	103.6±1.1	25.9±0.2	15.9±2.1

Standard deviation data are indicated for each ΔH, d and CU value.

skewed on the low temperature side. The size distribution of the 50 nm vesicles is not affected by the addition of trehalose (data not shown). In addition, trehalose does not cause any changes to the transition enthalpy (data not shown). The size of the unilamellar vesicle (50 and 100 nm diameter) does not influence significantly the effect of trehalose on the phase transitions of DPPC membranes.

Results shown in Fig. 2 demonstrate that there is a marked difference in the way trehalose interacts with DPPC, depending on which side of the vesicle it is present in. The direction of the T_m shift varies according to the proportion of trehalose inside and outside the vesicle; high inner and low outer trehalose concentrations decrease the T_m by approximately 1 °C, but high external trehalose concentration increases the T_m by up to 0.5 °C, irrespective of the inside trehalose concentration (Fig. 2). It has been argued that the local availability of water differs on the two leaflets of the bilayer of small unilamellar vesicles [36], suggesting that the inner monolayer is partially dehydrated compared to the outer monolayer. The reduced hydration (of the inner monolayer) may favor the hydrogen bonding of trehalose with the phospholipids, thereby leading to a water replacement mechanism even in the presence of bulk water. The interaction is most likely achieved during the liposome preparation in the liquid crystalline phase, as the tighter packing of phospholipids in the gel phase would prevent such interactions.

It is interesting to note that the trehalose molecules are not displaced upon cooling the sample to the gel phase. The trehalose–phospholipid interaction, however, is not observed for samples containing high concentrations of trehalose outside the vesicle. The outer monolayer, on the other hand, is less subject to packing constraints. Hydration, rather than the binding of trehalose, is favored. Although it is possible for trehalose to bind to the outer leaflet phospholipids, our results suggest that this is overshadowed by the kosmotropic effect of trehalose, as we do not observe a decrease in T_m. Results shown in Fig. 2 indicate that the size change from 100 to 50 nm does not modify the phospholipid packing significantly. It should be noted that the initial trehalose distribution across the bilayer is maintained throughout the duration of the experiment, as the thermogram obtained from the first heating scan is identical to those obtained during subsequent heating scans. This is in contrast to a literature report, in which the transition peak shifted with repeated heating, suggesting the equilibration of trehalose across the bilayer [14].

3.2. Effects of trehalose on the phase transitions of hydrated DPPC–cholesterol vesicles

In the hydrated state, the T_m remains at approximately 41 °C for vesicles containing cholesterol up to 17 mol% (data not shown). McMullen and others have reported the presence of two transitions upon the deconvolution of a single transition peak, which appears asymmetric in the thermogram [29]. We also observe asymmetry in the transition peak, but we do not consider peak deconvolution, as our primary interest lies in examining the change in the peak T_m and in the total enthalpy of the transition. There is no significant change in T_m upon the addition of 11 mM trehalose to DPPC–cholesterol liposomes containing 9 (Fig. 3a) and 17 (Fig. 3b) mol% cholesterol. Upon further increasing the trehalose concentration outside the vesicle to 146 mM (11/146 mM), the T_m increases by approximately 0.3 °C for both cholesterol-containing vesicles. In addition, the peak observed at 40.8 °C for DPPC at the same trehalose concentration (Fig. 2) is barely visible in the presence of cholesterol (Fig. 3). The magnitude of the decrease in T_m at 11/146 mM trehalose, however, is diminished in the presence of cholesterol; T_m decreases by 1.3 °C for pure DPPC and by 0.8 °C for both 9 and 17 mol% cholesterol. In addition, a shoulder at approximately 40.8 °C is visible for both 9 and 17 mol% cholesterol, while another shoulder (at approximately 39.5 °C)

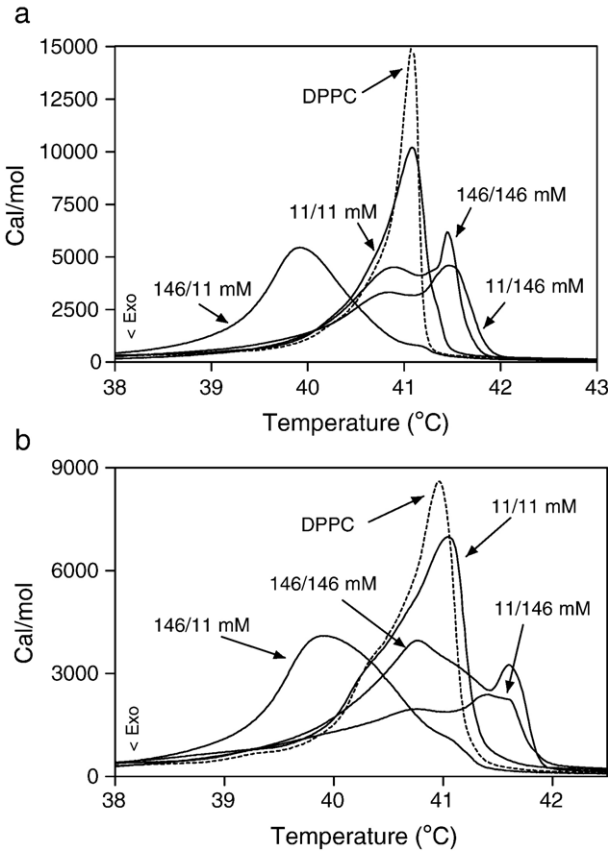

Fig. 2. Thermograms for (a) 100 nm and (b) 50 nm diameter DPPC liposomes containing asymmetric trehalose concentrations (values indicated in the figure denote inner/outer mM trehalose concentrations) across the vesicle bilayer.

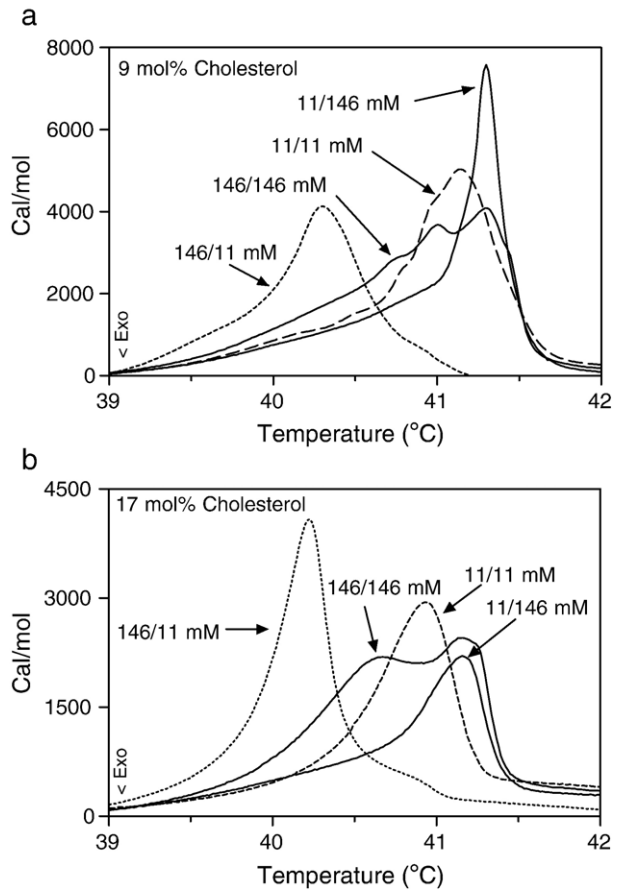

Fig. 3. Thermograms for 100 nm diameter DPPC–cholesterol liposomes containing (a) 9 mol% and (b) 17 mol% cholesterol. The values indicate the concentration (mM) of trehalose inside/outside the vesicle. The thermograms for DPPC–cholesterol liposomes without trehalose are identical to those obtained at 11/11 mM trehalose (data not shown).

is present only at 9 mol% cholesterol (Fig. 3). The shoulders observed in these samples may correspond to domains of lipids containing various cholesterol proportions. For samples containing 146 mM trehalose on both sides of the vesicle, two transitions are observed, both of which decrease in temperature by increasing the cholesterol proportion from 0 (41.4 °C and 40.9 °C) to 17 mol% (41.2 °C and 40.5 °C) (Fig. 3). At cholesterol proportions greater than 17 mol% (but up to 41 mol %), trehalose ceases to impart any significant effect on the T_m of DPPC–cholesterol vesicles (data not shown). Overall, the effect of trehalose on the T_m of cholesterol-containing vesicles is dependent on the distribution of trehalose across the bilayer and on the amount of cholesterol in the membrane. The most remarkable difference between the samples containing cholesterol and the pure DPPC vesicles is the behavior at high external trehalose concentration (11/146 mM trehalose). Cholesterol-containing liposomes only show one peak (Fig. 3), while DPPC liposomes show two peaks (Fig. 2a).

The enthalpy of the main phase transition of DPPC unilamellar vesicles decreases upon the addition of cholesterol (data not sown). The enthalpy values obtained for the DPPC–cholesterol mixtures (in the absence of trehalose) are comparable to those reported by McMullen et al. [29]. The decrease in the transition enthalpy with increasing cholesterol proportion is caused by the dilution of DPPC concentration in the vesicles, thereby reducing the number of phospholipid–phospholipid interactions that are responsible for the occurrence of the phase transition itself [20]. Trehalose addition modifies the temperature at which the DPPC–cholesterol mixtures undergo a phase transition (Fig. 3), but it does not modify the enthalpy of the transition. The transition enthalpy of cholesterol-containing vesicles remains unchanged at all the trehalose distributions examined in this work (data not shown).

Table 3
Average diameters (nm) of DPPC–cholesterol vesicles containing various trehalose concentrations

Cholesterol	Trehalose concentration (mM) (inner/outer)				
	0/0	11/11	11/146	146/11	146/146
9 mol%	103.5±7.6	106.8±4.1	96.3±4.2	106.6±6.0	105.3±7.9
17 mol%	108.6±1.8	104.3±4.2	103.1±4.3	105.4±5.1	106.2±5.7
23 mol%	104.4±1.7	102.4±1.3	107.1±1.9	104.2±7.0	107.9±1.3
33 mol%	98.5±3.2	107.2±7.4	103.7±2.8	106.4±7.2	104.8±3.6

Standard deviation data are indicated for each size value.

Table 3 shows the average vesicle sizes for liposomes containing cholesterol (0–33 mol%) at the various trehalose concentrations. Neither the presence of cholesterol nor the osmotic gradient of trehalose across the bilayer have any significant effect on the vesicle diameter, as observed by the consistency of the vesicle sizes at approximately 100 nm (deviation within 10% of the average size). The cooperativity of the phase transition decreases with increasing trehalose concentration and cholesterol proportion (Table 4). The addition of solutes (whether it be trehalose, cholesterol, or both) decreases the cooperative melting of the phospholipids by interfering with the phospholipid–phospholipid interaction, thus broadening the temperature range over which the phospholipids undergo a phase change. The effect of solutes on the cooperativity of melting is additive; the greater the total amount of solutes, the less cooperative the transition. An exception is observed at 146/11 mM trehalose; the cooperative unit of transition for DPPC is lower than for those containing cholesterol, which suggests that trehalose is better able to disrupt the interactions present between the phospholipids in the absence of cholesterol. This idea is supported by our results shown in Fig. 3, in which the presence of cholesterol diminishes the amount of T_m decrease observed (caused by the compression of the bilayer upon the incorporation of cholesterol). The above-mentioned effect is not observed at 11/11 mM trehalose, as there is not enough trehalose to observe a direct interaction,

Table 4
Size of the cooperative unit of the phase transition (CU) for DPPC and DPPC–cholesterol vesicles at 9 and 17 mol% cholesterol

Inner/outer trehalose concentration (mM)	DPPC CU	9 mol% Cholesterol CU	17 mol% Cholesterol CU
0/0	89.8±7.1	68.6±7.9	63.9±8.9
11/11	66.4±1.6	59.2±1.2	51.1±1.4
146/11	27.0±1.0	45.4±1.6	32.8±1.5

Standard deviation data are indicated for each CU value.

or the disruption of phospholipid–phospholipid interactions, to such a great extent.

3.3. Effects of trehalose on the phase transitions of freeze-dried DPPC–cholesterol vesicles

While a number of studies have been conducted to examine the interactions of trehalose with the phospholipid membrane at various hydration levels, there is a lack of data pertaining to how the interactions present in the hydrated state translate into those in the dehydrated state. In previous sections, we have examined the effects of trehalose on the transition behavior of DPPC and DPPC–cholesterol mixtures in excess water. In this section, we examine how these interactions are manifest upon freeze-drying.

Upon dehydration, the phase transition temperature of DPPC increases from 41 °C to 105 °C [21,23]. Removal of water leads to a decrease in the head group spacing of the lipids, thus allowing for increased van der Waals interactions between the lipid hydrocarbon chains [37]. If the membrane (in the liquid crystalline phase) is dried in the presence of trehalose, the contraction of the phospholipids is avoided and the T_m decreases considerably [3,21]. Fig. 4 shows the thermograms for the freeze-dried DPPC vesicles (100 nm) containing various inner/outer trehalose concentrations. The lipid concentration for all of our samples is 11 mM, thus the lowest amount of trehalose present in any of these systems (11 mM) corresponds to 1:1 molar ratio (trehalose/DPPC). During the first heating scan, the transitions are observed in between 60 and 80 °C, depending on the trehalose distribution (Fig. 4a). During the freezing step of lyophilization, the membrane (prepared in the liquid crystalline phase) undergoes a phase transition to the gel phase. The tighter packing of the gel phase lipids results in a smaller number of trehalose molecules to interact (via water replacement) with the phospholipids during the drying process. Therefore, only a partial decrease in T_m (from 105 °C to 60–80 °C) is observed. Crowe observed similar results for small unilamellar DPPC vesicles containing different amounts of trehalose [21]. For the sample containing 11 mM trehalose on both sides of the vesicle, the T_m is observed at approximately 79 °C (Fig. 4a). The transition is decreased to 67 °C upon increasing the trehalose concentration inside the vesicle to 146 mM (146/11 mM), while it decreases to approximately 83 °C for the samples containing 146 mM trehalose outside the vesicle (for both 11/146 mM and 146/146 mM). As described in previous sections, in the fully hydrated state the decrease in T_m is mainly observed for the sample containing high trehalose concentration inside the vesicle (146/11 mM trehalose). Note that the shift in the T_m, observed at the various trehalose distributions, is amplified significantly upon dehydration. The largest T_m change, observed upon the addition of 146/11 mM trehalose to DPPC, increases from 1 °C in the fully hydrated state to 38 °C in the dried state. The transition width also increases from approximately 3 °C to greater than 15 °C upon dehydration. Additionally, a second peak, or a shoulder, is observed for the dry sample containing 146/146 mM

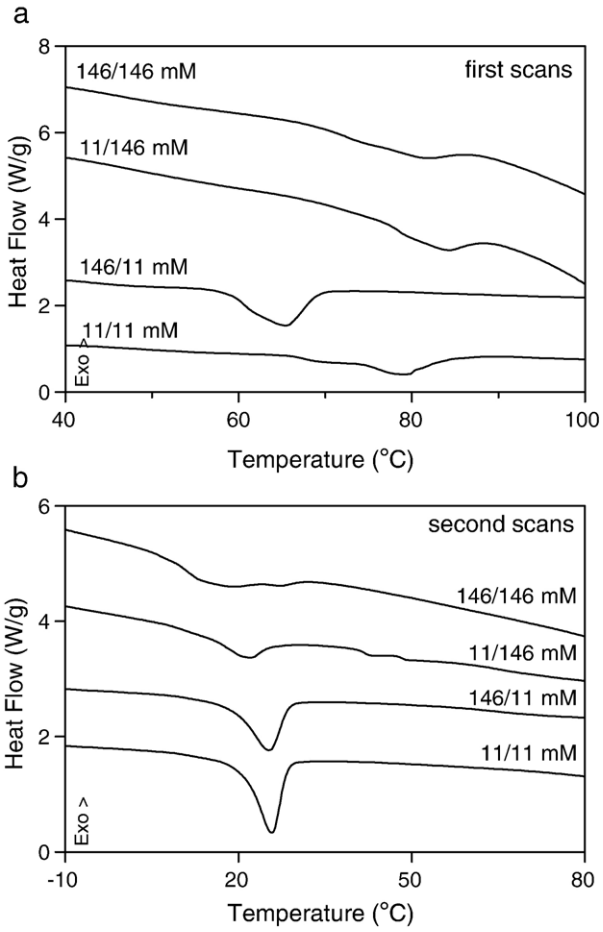

Fig. 4. Thermograms obtained from the first (a) and second (b) heating scans for 100 nm diameter DPPC vesicles freeze-dried in the presence of various trehalose concentrations (values indicated in the figure). The samples contain less than 1% (wt) water.

trehalose, mirroring the two transitions observed in the hydrated state (Fig. 2). Collectively, our results demonstrate that the trehalose distribution in the fully hydrated state is maintained upon lyophilization. More importantly, the direct interaction between trehalose and phospholipid (via hydrogen bonding) in the hydrated state (at 146/11 mM trehalose) is not only maintained, but amplified upon dehydration.

Although the first heating scan gives a more accurate description of the freeze-dried membrane–sugar system, the second heating scan (and subsequent scans) can be viewed to examine the *equilibrium* state of the membrane–sugar system. Upon heating and cooling the samples once through the transition, the T_m is decreased to approximately 25 °C (Fig. 4b). It has been reported that the maximal effect of trehalose on the depression of T_m is only observed if the membrane is in the liquid-crystalline state, in which the head group spacing is large enough to accommodate the trehalose molecule [21]. This is accomplished in our experiments by heating the freeze-dried sample beyond its phase transition (during the first heating scan). This allows the sugar molecules to rearrange themselves to orientations that maximize their interactions with the phospholipids, which accounts for the

significant decrease in the T_m observed between the first and second heating scans. The sample containing 11/11 mM trehalose shows a sharp transition at 26 °C (Fig. 4b), in contrast to the broad transition (at approximately 79 °C) observed during the first heating scan (Fig. 4a). The transition remains at the same temperature (26 °C) for the sample containing 146/11 mM trehalose. Upon increasing the exterior trehalose concentration to 146 mM (11/146 mM), the T_m decreases to 18 °C (Fig. 4b), which is decreased further and its peak broadened (also the peak becomes heterogeneous by the appearance of a shoulder at approximately 29 °C) by increasing the interior trehalose concentration to 146 mM (146/146 mM). Additional heating and cooling scans did not change the enthalpy or the temperature of transitions observed in any of the thermograms (data not shown). For 50 nm DPPC vesicles, the thermograms are identical to those obtained for 100 nm vesicles (data not shown).

Dehydration of DPPC–cholesterol mixtures leads to an increase of T_m and phase separation; cholesterol-containing vesicles show multiple peaks, revealing the heterogeneity of the membrane lipid distribution (i.e., domains enriched/depleted in cholesterol) [20]. Fig. 5 shows the first and second heating scans for several DPPC–cholesterol mixtures

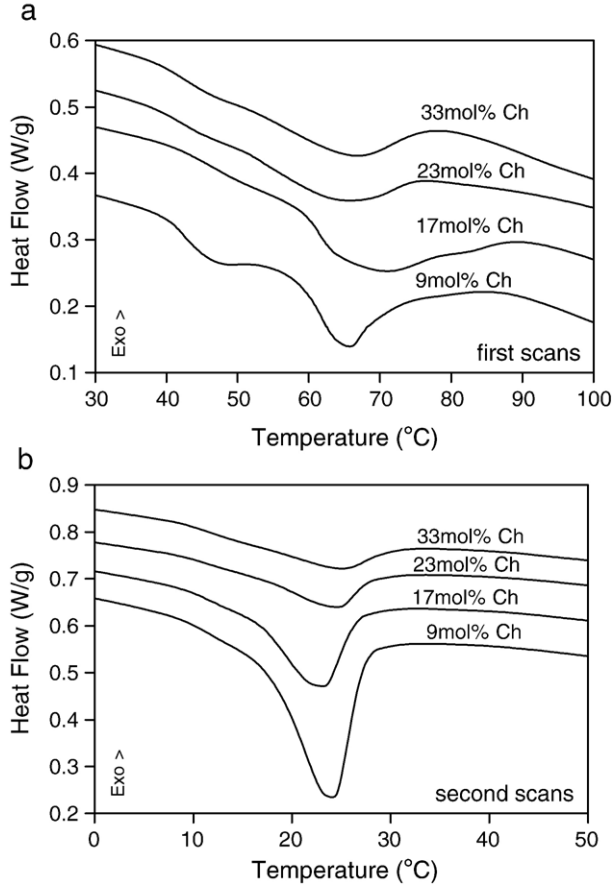

Fig. 5. Thermograms obtained from the first (a) and second (b) heating scans for various 100 nm diameter DPPC–cholesterol (Ch) vesicles (values indicated in the figure) freeze-dried in the presence of 146/11 mM trehalose. The samples contain less than 1% (wt) water.

Table 5
T_m of DPPC–cholesterol vesicles obtained during the first and second heating scans

	Cholesterol (mol%)	Trehalose concentration (mM) (inner/outer)			
		11/11 T_m (°C)	11/146 T_m (°C)	146/11 T_m (°C)	146/146 T_m (°C)
First scan	9	73.6±1.5	78.8±4.4	65.3±0.4	72.0±2.8
	17	72.5±0.6	78.7±7.4	66.8±0.3	70.3±3.2
	23	70.0±0.1	77.8±±9.7	63.4±0.5	63.4±1.5
	33	62.7±3.0	72.8±4.7	64.0±2.2	51.0±2.5
Second scan	9	23.8±0.1	16.3±0.3	23.9±0.0	16.4±0.3
	17	23.7±0.1	15.1±0.9	23.0±0.1	17.0±0.0
	23	23.8±0.1	17.1±0.0	24.1±0.2	15.5±0.2
	33	25.1±0.2	18.6±0.0	24.3±0.2	19.5±0.8

Standard deviation data are indicated for each T_m value.

in the presence of 146/11 mM trehalose. The first heating scans show broad transitions (Fig. 5a) with temperatures even lower than those for pure DPPC liposomes (67 °C). The second heating scans (Fig. 5b) reveal a slight decrease in T_m upon the incorporation of cholesterol, and a decrease in the magnitude of the transition peak. Table 5 summarizes the T_m values for several DPPC–cholesterol mixtures at all the trehalose distributions analyzed in this work. The T_m values differ depending on the trehalose concentration and on the cholesterol proportion present in the vesicle. During the first heating scan, all samples exhibit transitions in between 50 °C and 79 °C. For the samples containing low amounts of cholesterol (up to 17 mol%), the same relation observed in the hydrated state (between T_m and trehalose distribution) (Fig. 5) is observed in the freeze-dried state (Table 5); the lowest T_m is observed at 146/11 mM trehalose. At higher cholesterol compositions, the lowest T_m is observed at 146/146 mM. As alluded to earlier, cholesterol incorporation into the liquid crystalline phase phospholipid condenses the membrane, thus reducing the phospholipid–trehalose interaction. During the freezing step of lyophilization, however, the membrane undergoes a phase transition to the gel phase. The presence of cholesterol in the gel phase phospholipid *fluidizes* the membrane structure, thus allowing for more efficient interaction with trehalose via water replacement during the drying process. This may explain the large T_m decrease observed at 146/146 mM trehalose (in the presence of high cholesterol proportion).

Upon heating the samples past their phase transition temperatures once, all the samples show a decrease in their T_m's (Table 5). For the sample containing 11 mM trehalose on both sides of the vesicle, the T_m changes with cholesterol proportion in a parabolic manner; the T_m decreases from 25.9 °C (for pure DPPC) to 23.7 °C upon increasing the cholesterol proportion to 17 mol%, however, upon further increasing the cholesterol proportion to 33 mol%, the T_m increases to 25.1 °C. Similar change in T_m with cholesterol proportion is observed for the samples containing 11/146 mM and 146/11 mM trehalose. There is no clear trend, however, for the sample containing 146 mM trehalose on both sides of the vesicle. It should be noted that the samples containing high extravesicular trehalose concentration have lower T_m's

compared to those with high intravesicular trehalose concentration, as is observed for DPPC liposomes (Fig. 4b). In addition, the difference in T_m with cholesterol proportion (at the same trehalose concentration) is much larger for the freshly lyophilized sample (first heating scan) compared to the *equilibrated* sample (second heating scan); by increasing the cholesterol proportion from 9 to 33 mol% at 146/146 mM trehalose, a 20 °C difference in the T_m is observed during the first heating scan, compared to the 3 °C difference observed during the second heating scan. Also, note that the T_m difference observed in the dehydrated state is much larger than that observed in the hydrated state (maximum T_m difference is 1 °C) (Fig. 3).

4. Conclusions

A systematic study has been presented of the differential effect of trehalose on the two sides of the vesicle membrane, both in the hydrated and dry states. A combination of high inner/low outer trehalose concentrations is shown to decrease the T_m of both DPPC and DPPC–cholesterol liposomes in aqueous media. Upon dehydration, the amount of T_m decrease is amplified significantly; it is found that loading high concentrations of trehalose inside the liposomes offers considerable benefits for their subsequent effective stabilization. The outside concentration of trehalose, however, must be minimal (enough to prevent fusion/aggregation of liposomes) as the amount of T_m decrease is diminished with increasing exterior concentration. These results are counterintuitive, as trehalose (as part of a lyoprotectant formulation) is generally added in large quantities on the outside of liposomes prior to lyophilization. Taken together, the findings reported in this work should prompt a reevaluation of current formulations for drug delivery.

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