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# Phase behavior of freeze-dried phospholipid–cholesterol mixtures stabilized with trehalose

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## Abstract

A study is presented of the role of cholesterol content on the gel-to-liquid crystalline phase transition of freeze-dried liposomes stabilized with trehalose, a well known lyoprotectant. The phospholipids considered in this work, DPPC and DPPE, belong to the two predominant phospholipid species found in numerous biological membranes. Cholesterol is found in abundance in mammalian plasma membranes. DSC measurements reveal that cholesterol-containing liposomes exhibit multiple phase transitions upon dehydration. Addition of trehalose to these systems lowers the phase transition temperature and limits the phase separation of the lipidic components upon freeze-drying. This work provides strong evidence for the effectiveness of trehalose in stabilizing cholesterol-containing membranes upon lyophilization.

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**Keywords:** Cholesterol; DPPC; DPPE; Phase transition; Freeze-dried

## 1. Introduction

Biological systems such as proteins, vaccines, or cells must often be stored for extended periods of time by resorting to freezing in solutions of cryoprotectants. In some cases, it is also possible to freeze-dry such systems in solutions of lyoprotectants, thereby leading to products that are stable at ambient conditions. Achieving long-term stability in biological systems has been a long-standing goal of the food, pharmaceutical, and biomedical industries. Avoiding the need for refrigeration would reduce production and storage costs drastically. From the pharmaceutical point of view, liposomes are used as vehicles for delivery of drugs for medical and cosmetic applications [1–3]. Liposomes, which represent the simplest model of a biological membrane, have also been examined to understand the physical properties of cell membranes upon dehydration [4–6].

Cell membranes are inherently multicomponent systems consisting of various species of lipids and proteins. The physical properties and biological function of such membranes are closely coupled to the particular arrangement and distribution of the components within the phospholipid bilayer. In mammalian plasma membranes, cholesterol (Fig. 1a) is relatively abundant [7]. Since the pioneering work of Ladbroke and Chapman [8], numerous studies have investigated the interactions of cholesterol with various phospholipid species in aqueous suspensions [9–11]. Although cholesterol has various functions in cells, one of its primary roles is that of modulating the physical properties of the plasma membrane [11,12]. Some of the major effects of cholesterol incorporation include: (i) a broadening and eventual elimination of the cooperative gel-to-liquid phase transition ( $T_m$ ) of the phospholipid bilayer, (ii) a marked increase (or decrease) in the orientational order of the phospholipid hydrocarbon chains above (or below) the phase transition, and (iii) a decrease in the phospholipid acyl chain tilt angle in the gel phase [11]. Note that all of the observations listed above correspond to phospholipid–cholesterol systems in the fully hydrated state.

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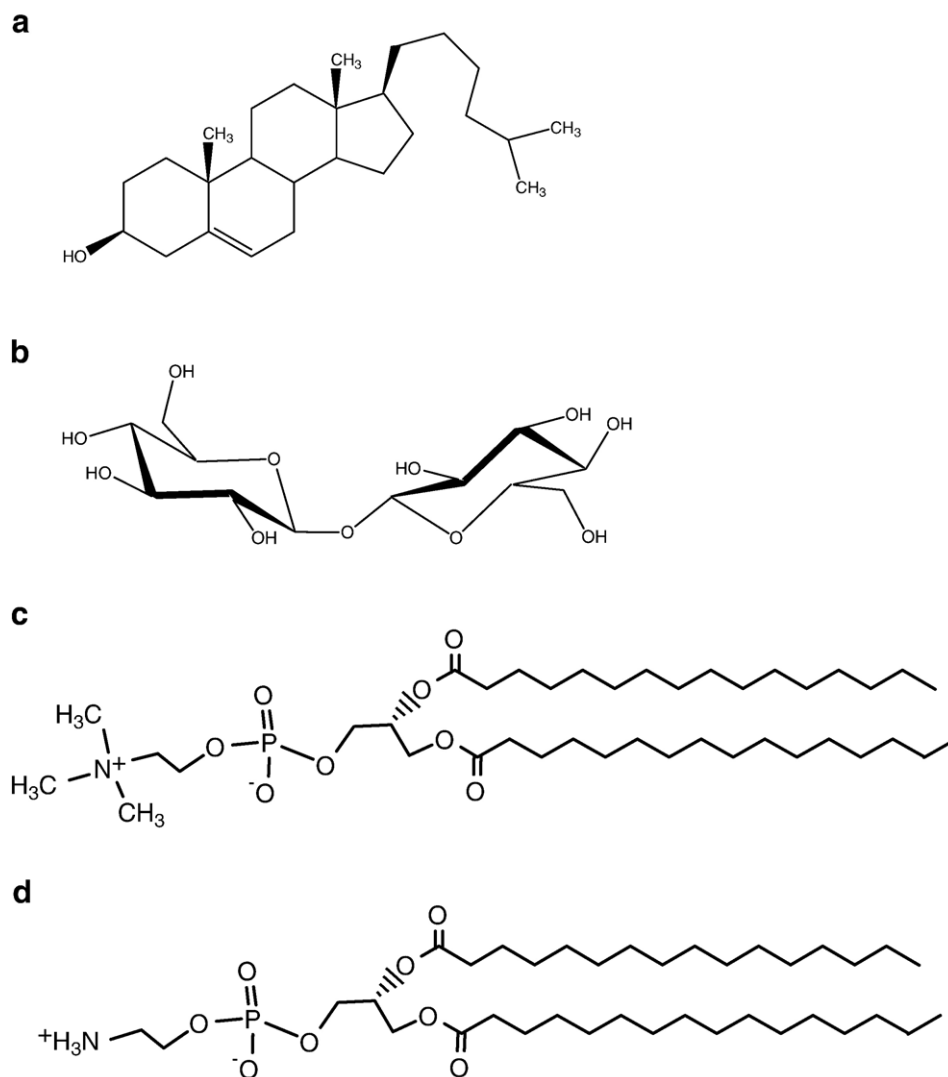


Fig. 1. The chemical structures of (a) cholesterol, (b) trehalose, (c) 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and (d) 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE).

As alluded to earlier, the desiccation of phospholipidic vesicles has been studied in efforts to understand biological membranes under low-water content conditions [4,6,13,14]. Upon dehydration, the phase transition temperature of the phospholipids increases. This occurs due to the decreased headgroup spacing of the lipids, which allows for increased van der Waals interactions between lipid hydrocarbon chains [15]. If the membrane is dried in the presence of trehalose (Fig. 1b), however,  $T_m$  is depressed considerably [13,16]. This effect of trehalose on the biological membranes was explained by Crowe through a “water replacement hypothesis” [17,18], whose main proposition is that sugar molecules interact directly with the polar groups of the phospholipids, thereby maintaining the head group spacing in the dry state and resulting in a depression of  $T_m$ . The stabilization of membranes by trehalose upon dehydration has also been explained through a “vitrification hypothesis”, in which osmotic and volumetric properties of the sugar are responsible for the lowering of  $T_m$  [19,20].

The aim of this work is to understand how cholesterol-containing liposomes behave upon lyophilization. To the best of our knowledge, only a few studies have considered the stability of *dehydrated* mixtures of phospholipids with cholesterol. Van Winden examined the stability of freeze-dried mixtures composed of DPPC, DPPG, and cholesterol in the presence of trehalose, but only at one cholesterol composition [21]. Harrigan et al. studied the influence of cholesterol content on the stability of vacuum-dried liposomes composed of egg phosphatidylcholine and cholesterol [22]. Egg phosphatidylcholine, however, contains several phosphatidylcholines differing in length and saturation; this leads to broad transitions and some ambiguity in the interpretation of experimental data. In this work, we characterize the effects of cholesterol on the phase behavior of lyophilized DPPC (Fig. 1c), DPPE (Fig. 1d), and DPPE–DPPC mixtures in a systematic manner. Particular emphasis is placed on the ability of trehalose to lower the transition temperature and to influence the

demixing of membrane lipids. One of the main findings of this work is that cholesterol, over a well-defined range of concentration, can in fact modulate the phase transition temperature of phospholipidic membranes freeze-dried in the presence of trehalose. This knowledge can be useful in the design of liposomes for entrapment and delivery of pharmaceuticals.

## 2. Materials and methods

### 2.1. Chemicals

Phospholipids, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 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 41 mol% Ch. Chloroform was evaporated under a stream of N<sub>2</sub>, and further drying was done in a vacuum oven. The dried lipids were resuspended (50 mg/mL) at a temperature above the phase transition temperature of each phospholipid sample (50 °C for DPPC and 70 °C for samples containing DPPE) in a solution containing trehalose to give a 1:1 sugar/lipid weight ratio. Control samples consisting of phospholipids without trehalose and pure cholesterol were also prepared. The samples were extruded through membrane filters (100 nm pore diameter) 19 times using an extruder (Avanti polar lipids, Alabaster, AL) at the same temperature employed for rehydration. Aliquots of 0.5 mL of the extruded solutions were placed in 4 mL glass vials and immediately submerged in liquid nitrogen. The samples were then 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.

### 2.3. Differential scanning calorimetry

DSC was used to determine the phase transition temperature ( $T_m$ ) of the phospholipids.  $T_m$  represents the peak temperature of the endotherm for the lipid gel-to-liquid crystalline phase transition recorded during the heating scan. The instrument used was a TA Q100 DSC (New Castle, DE). 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. No changes in transition temperatures were observed following the second heating cycle, hence all reported data correspond to those obtained during the second heating scan. All measurements were made using sealed aluminum pans, and an empty pan was used as a reference. Data were analyzed using Universal Analysis.

### 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.

## 3. Results and discussion

### 3.1. DPPC–Ch mixtures

The thermotropic phase behavior of mixtures composed of DPPC and cholesterol (Ch) was studied using differential scanning calorimetry (DSC). Thermograms (obtained during the second heating cycle) for freeze-dried DPPC–Ch mixtures are shown in Fig. 2a. The phase transition temperature ( $T_m$ ) for dehydrated DPPC is 105 °C [13,16], which is much higher than that observed in the fully hydrated state (42 °C) [11,13,16,23,24]. In the absence of water, the spacing between the phospholipid head groups decreases, thereby giving rise to increased van der Waals interactions between the lipid hydrocarbon chains, and a higher  $T_m$  [25]. Upon the addition of cholesterol, the peak for the transition observed at 105 °C decreases both in magnitude and in temperature. At 33 mol% Ch, the DPPC–Ch mixture shows a decrease in temperature of 20 °C and the transition peak area diminishes by a factor of 7 with respect to pure DPPC liposomes (Table 1). A second, broad endothermic transition at 70 °C becomes observable at 23 mol% Ch and its peak intensity increases as the cholesterol proportion is increased. The enthalpy of this low temperature transition was not analyzed, as the peak is very broad (and in fact consists of overlapping features) (Fig. 2a). In the fully hydrated state, the addition of cholesterol to DPPC does not change the  $T_m$  significantly, while the enthalpy of the transition decreases considerably [11,23]. The intercalation of cholesterol between the lipids destabilizes the phospholipid packing in the gel phase, leading to a depression in the  $T_m$  of the dehydrated samples. These results suggest that the dehydrated membrane is in a more fluid state in the presence of cholesterol than without it. The presence of the two endothermic peaks can be explained by the existence of Ch-rich and Ch-poor domains, as described for these same systems in the fully hydrated state [11,23]. The Ch-poor region is characterized by a high  $T_m$ , exhibiting a sharp transition in the thermogram; the Ch-rich region is characterized by a broad transition in the

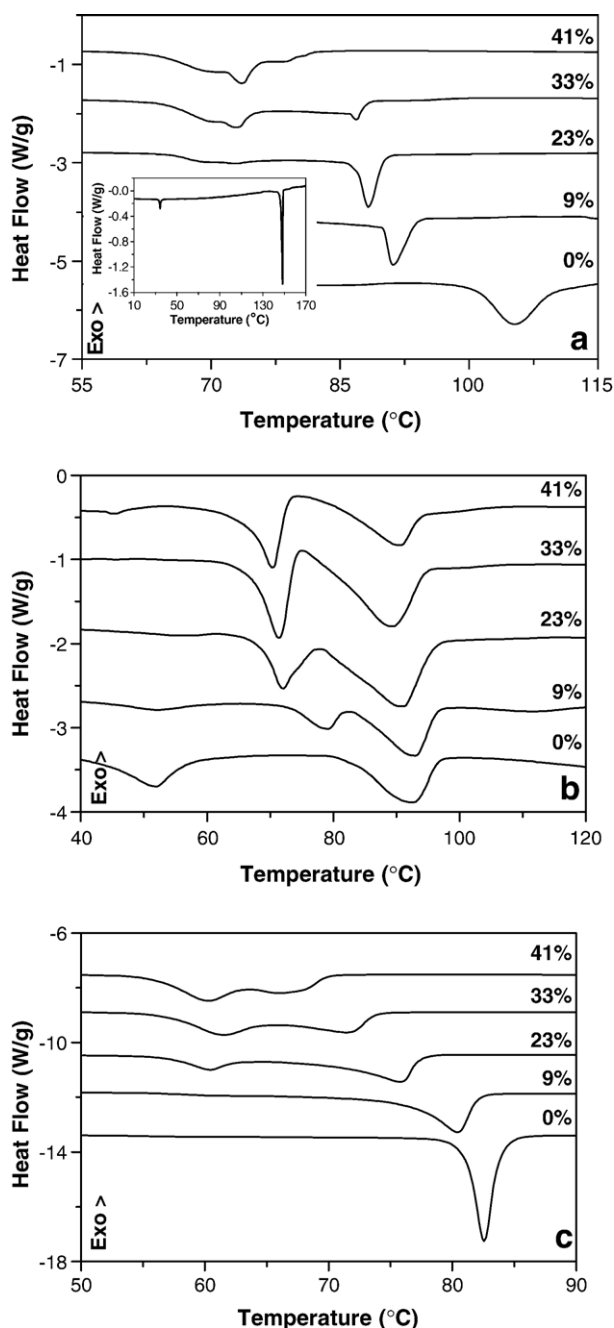


Fig. 2. DSC thermograms for cholesterol-containing liposomes composed of (a) DPPC, (b) DPPE, and (c) DPPE–DPPC (1:1 molar ratio) upon freeze-drying. The cholesterol molar ratios examined are indicated in each thermogram. Samples contain less than 1.5% (wt) residual water content and were scanned at 10 °C/min. The DSC thermograms were corrected for total mass of phospholipid. Inset for (a): Phase transition temperatures of freeze-dried cholesterol.

thermogram at lower temperatures. As the proportion of cholesterol is increased in DPPC–Ch mixtures, the enthalpy of melting of the Ch-poor region decreases (Table 1), while that of the Ch-rich region increases.

There is an upper limit to the concentration of cholesterol that can be accommodated in a hydrated phospholipid bilayer; any excess cholesterol will tend to precipitate,

forming crystals. Although the cholesterol contents considered in this work are below the reported solubility limit (ranging from 50 to 66 mol%) [26], it is important to determine whether cholesterol crystallization can occur upon dehydration, and thus contribute to the peaks observed in the thermograms. Cholesterol crystals can be detected using the DSC. At 37 °C, anhydrous cholesterol crystals undergo a polymorphic phase transition from one triclinic form to another and, at approximately 150 °C, the cholesterol crystals melt [27]. We observe two endothermic transitions at 37 °C and 150 °C for freeze-dried pure cholesterol samples (Fig. 2a inset). Neither of these transitions is present in the DPPC–Ch mixtures that we have analyzed, suggesting that cholesterol did not crystallize.

### 3.2. DPPE–Ch mixtures

Dehydrated DPPE exists either entirely in the gel phase or as a mixture of two phases (crystalline and gel phases) [28]. The gel-to-liquid crystalline phase transition of DPPE was reported at 52 °C and the crystal-to-liquid crystalline phase transition was observed at 95 °C [28]. Fig. 2b shows the thermograms for freeze-dried DPPE–Ch mixtures. Two transitions at 52° and 94 °C are observed for dehydrated DPPE, which are in accordance with previously reported data [28]. At ambient conditions, our freeze-dried DPPE samples consist of gel and crystalline phases. Handa reported that the crystalline phase can only be attained through the annealing of the gel phase at elevated temperatures (65–70 °C) [28]. In our case, however, we observe both transitions without annealing. This difference may be attributable to the different sample preparation techniques.

All of the DPPE–Ch mixtures exhibit multiple transitions. The phase transition at approximately 92 °C is always present, irrespective of the proportion of cholesterol. The other transition (at 52 °C), however, decreases both in magnitude and in temperature with increasing cholesterol content (Table 1). A new transition at approximately 72 °C appears upon the addition of cholesterol, and is present at all of the cholesterol concentrations examined in this work. The consistency of the peak observed at 92 °C suggests that the crystalline phase of DPPE does not incorporate cholesterol. The gel phase, however, is more prone to interact with cholesterol, as the enthalpy of the peak at 52 °C diminishes considerably with increasing cholesterol proportion (Fig. 2b, Table 1). We speculate that the transition at 72 °C represents an intermediate DPPE phase (between gel and crystal) enriched in cholesterol. The peak enthalpy values of the transitions for DPPE–Ch mixtures are not always consistent (although the direction of the peak enthalpy change is consistent), indicating that the system is metastable (Table 1).

We have also considered the possibility of cholesterol crystallization in the freeze-dried DPPE–Ch mixtures. The solubility limit of cholesterol in the hydrated DPPE bilayers is 51 mol% [26], which is higher than the cholesterol contents considered in this work. As we do not observe the

Table 1  
Transition temperature and enthalpy of freeze-dried liposomes containing cholesterol and DPPC, DPPE, or DPPE–DPPC (1:1 molar ratio)

Phospholipid	Ch (mol%)	Phase transition (°C)			$\Delta H$ (KJ/mol)	
DPPC <sup>a,b</sup>	0	104.6 ± 1.3			22.3 ± 3.8	
	9	91.4 ± 1.6			18.6 ± 5.5	
	23	87.5 ± 1.8			8.6 ± 2.4	
	33	84.8 ± 1.9			2.6 ± 2.2	
DPPE <sup>b</sup>	0	52.3 ± 0.5	93.8 ± 1.3	9.8 ± 12.9	10.3 ± 8.4	
	9	51.9 ± 1.0	80.0 ± 3.5	92.5 ± 0.4	10.8 ± 10.8	20.2 ± 10.4
	23		73.0 ± 1.8	90.6 ± 1.9		20.1 ± 10.4
	33		71.4 ± 0.5	89.4 ± 1.2		29.6 ± 22.2
	41		70.5 ± 0.2	90.2 ± 0.3		12.7 ± 7.6
DPPE–DPPC <sup>b</sup>	0		82.4 ± 0.3			37.4 ± 4.0
	9	59.7 ± 0.9	80.4 ± 0.2		0.4 ± 0.3	25.9 ± 2.0
	23	60.3 ± 1.0	75.8 ± 0.1		2.4 ± 2.0	16.3 ± 4.3
	33	59.7 ± 1.5	71.8 ± 0.1		8.0 ± 5.7	6.6 ± 4.1
	41	60.0 ± 0.1	66.5 ± 0.2		3.4 ± 3.3	2.0 ± 0.8

Samples contain less than 1.5% (wt) residual water.

<sup>a</sup> Only the high temperature transition shown.

<sup>b</sup> In cases where more than one transition is observed, the various transition temperatures are reported along with the corresponding enthalpy changes.

transitions at 37 °C and 150 °C (Fig. 2a inset), our results indicate that DPPE–Ch mixtures do not exhibit cholesterol crystallization upon dehydration at any of the cholesterol proportions examined here.

### 3.3. DPPE–DPPC–Ch mixtures

A freeze-dried DPPE–DPPC mixture (1:1 molar ratio) exhibits a single transition at 83 °C (Fig. 2c). In the hydrated state, the DPPE–DPPC mixtures are miscible, exhibiting a single transition peak (54 °C) [29,30]. This suggests that the two phospholipids remain miscible upon dehydration. As the cholesterol proportion is increased in the DPPE–DPPC–Ch mixtures, the  $T_m$  at 83 °C decreases both in temperature and in magnitude (Fig. 2c, Table 1). A new transition at 60 °C appears above 9 mol% Ch and grows in magnitude with increasing cholesterol content. As was previously described for DPPC–Ch systems (Fig. 2a), the two peaks shown in Fig. 2c can be ascribed to Ch-rich and Ch-poor regions. In the presence of water, the DPPE–DPPC–Ch mixtures exhibit a single peak, although Blume suggests that it is possible to deconvolute the peak into two transitions [29]. Our results show that the heterogeneity of the sample, indicated by the presence of two distinct peaks, becomes clearer upon the removal of water (Fig. 2c). Cholesterol has been reported to not interact preferentially with either PE or PC in the DPPE–DPPC–Ch mixtures in excess water [29]. As we did not observe phase separation of DPPC from DPPE upon dehydration, we expect the two peaks in the DPPE–DPPC–Ch mixtures to contain a constant DPPE/DPPC ratio (although containing different amounts of cholesterol).

### 3.4. Effects of trehalose

Trehalose is effective in protecting biological membranes upon freeze-drying, and has been widely used to preserve the integrity of phospholipid liposomes. However, the

effects of trehalose on dehydrated Ch-containing liposomes have not been examined in detail. Fig. 3 shows the DSC thermograms (obtained during the second heating cycle) for various phospholipid–Ch mixtures freeze-dried from trehalose solutions. As reported in previous studies, including our own, the  $T_m$  of freeze-dried pure DPPC decreases from 105 °C (Table 1) to 25 °C upon the addition of trehalose (Table 2) [13,16,19]. Trehalose also reduces the  $T_m$  of pure DPPE from 52 °C to 39 °C, and that of the DPPE–DPPC (1:1 molar ratio) mixture from 83 °C to 21 °C. The  $T_m$  depression is explained by the ability of trehalose to restrict the contraction of the lipids that would otherwise occur upon dehydration [13]. More specifically, this is achieved by the direct interaction of trehalose with the phospholipids, an observation supported by experiments and recent computer simulations [31–36].

The amount of  $T_m$  depression caused by the addition of trehalose depends on the type of phospholipid considered. The  $\Delta T_m$  ( $T_m$  of dry phospholipid– $T_m$  of phospholipid dried with trehalose) is approximately 80 °C, 13 °C and 61 °C for DPPC, DPPE, and the DPPE–DPPC mixture, respectively; the effect of trehalose in depressing the  $T_m$  is the largest for DPPC and the smallest for DPPE. This behavior can be accounted for by the difference in the ability of the two phospholipids to form hydrogen bonds with their neighbors. The formation of hydrogen bonds between the  $\text{NH}_3^+$  protons of the PE head group and the neighboring phosphate oxygens (Fig. 1d) causes higher transition temperatures in water for PE's compared to PC's (63 °C for DPPE and 42 °C for DPPC) [29]. We speculate that this strong PE–PE interaction reduces the ability of trehalose to hydrogen bond with DPPE molecules, resulting in a lower  $\Delta T_m$  for DPPE. The  $\Delta T_m$  of the DPPE–DPPC mixture, which shows an intermediate value between those observed for the pure phospholipids, suggests that the inter-head group hydrogen bonding strength of the dried mixture is in between those of DPPE and DPPC.

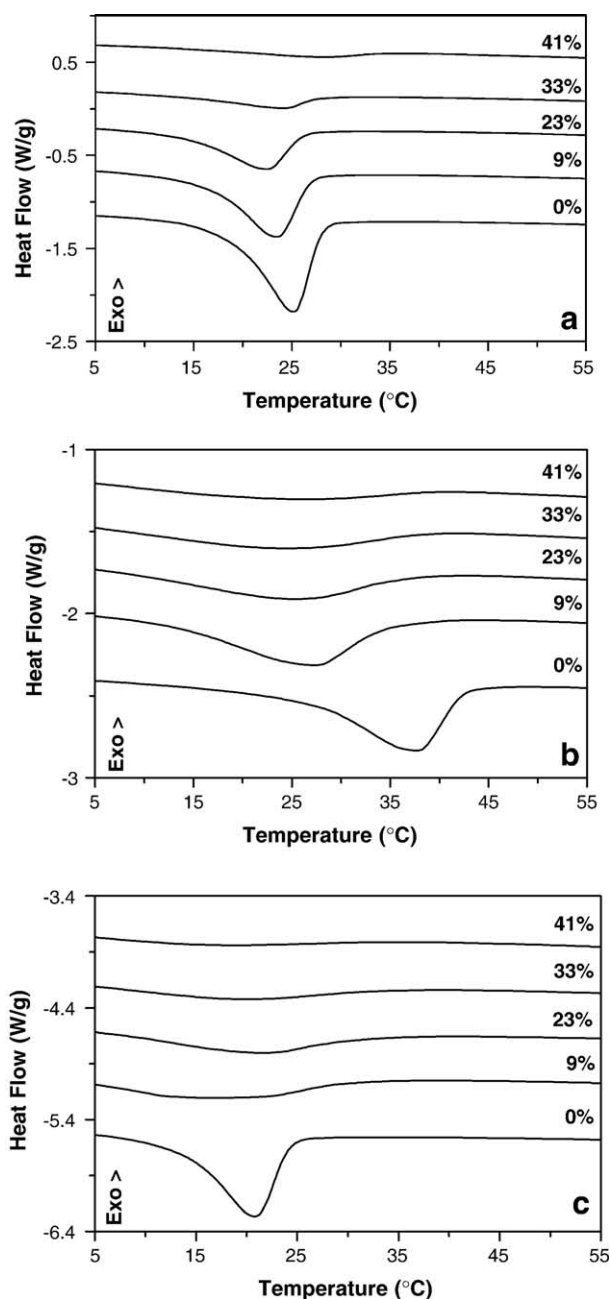


Fig. 3. DSC thermograms for cholesterol-containing liposomes composed of (a) DPPC, (b) DPPE, and (c) DPPE–DPPC (1:1 molar ratio) freeze-dried in the presence of trehalose (1:1 lipid/trehalose wt ratio). The cholesterol molar ratios examined are indicated in each thermogram. Samples contain less than 1.5% (wt) residual water content and were scanned at 10 °C/min. The DSC thermograms were corrected for total mass of phospholipid.

DPPE freeze-dried with trehalose exhibits a single transition (39 °C) (Fig. 3b), which is lower than either of the two transitions present in the absence of trehalose (52 and 94 °C) (Fig. 2b). Lyophilization with trehalose allows the membrane to remain in a more fluid state, hindering the formation of the crystalline phase. This explains the absence of a high temperature transition. Although we do not observe multiple peaks for any of the phospholipid–Ch mixtures (as observed for samples dehydrated without

trehalose), the samples exhibit broad transitions. This indicates that the distribution of the lipidic components in the mixtures is heterogeneous.

The phase transition temperatures of all Ch-containing liposomes are reduced when lyophilized with trehalose (Table 2). The addition of cholesterol further influences the  $T_m$  depending on the phospholipids present in each mixture. Cholesterol does not affect significantly the  $T_m$  of DPPC–Ch (Table 2) mixtures. For DPPE–Ch mixtures, however, the  $T_m$  decreases from 39 °C (pure DPPE) to approximately 21 °C as increasing amounts of cholesterol are incorporated into the liposomes (Table 2).  $T_m$  corresponds to the melting of neighboring phospholipid molecules. As previously described, cholesterol addition can affect its surrounding environment, which in turn can affect the  $T_m$ . Our results suggest that for DPPC–Ch mixtures, cholesterol incorporation does not affect their  $T_m$  because the PC–PC head group interactions are not significantly modified. Trehalose interaction with DPPC molecules, hence, is not affected by the presence of cholesterol. For DPPE–Ch mixtures, however, the  $T_m$  decreases considerably (12 °C) upon the addition of low amounts of cholesterol (9 mol% Ch), and  $T_m$  continues to decrease with further addition of cholesterol. This suggests that trehalose is better able to interact with the PE molecules upon the intercalation of cholesterol between the phospholipid molecules. This results from the perturbation of the PE–PE hydrogen bonding network by cholesterol, as described earlier. For DPPE–DPPC–Ch mixtures, the depression of  $T_m$  by the addition of cholesterol is more pronounced than that observed for DPPC, but less than that observed for DPPE. This suggests that DPPC is able to disrupt the strong PE–PE interaction, thereby facilitating the interaction with trehalose.

$T_m$  change with cholesterol addition observed in the dry state (with trehalose) is similar to that reported in the

Table 2

Transition temperature and enthalpy of cholesterol-containing liposomes composed of DPPC, DPPE, and DPPE–DPPC (1:1 molar ratio) freeze-dried with trehalose (1:1 lipid/trehalose wt ratio)

Phospholipid	Ch (mol%)	Phase transition (°C)	$\Delta H$ (KJ/mol)
DPPC	0	25.0 ± 0.2	31.8 ± 0.7
	9	23.6 ± 0.2	24.6 ± 2.6
	23	22.7 ± 0.3	16.6 ± 1.9
	33	26.7 ± 1.0	4.6 ± 3.0
	41	28.5 ± 0.6	3.0 ± 0.8
DPPE	0	39.0 ± 1.4	23.3 ± 4.0
	9	26.8 ± 0.4	17.9 ± 3.5
	23	25.1 ± 0.6	18.4 ± 4.1
	33	22.7 ± 0.5	12.8 ± 2.3
	41	21.2 ± 1.6	11.8 ± 4.5
DPPE–DPPC	0	20.7 ± 0.2	24.2 ± 2.8
	9	14.6 ± 0.2	21.5 ± 1.0
	23	21.2 ± 0.7	19.0 ± 1.1
	33	18.1 ± 0.2	18.7 ± 2.5
	41	13.9 ± 0.1	16.6 ± 2.3

Samples contain less than 1.5% (wt) residual water.

presence of water. For DPPC–Ch and DPPE–DPPC–Ch mixtures, minimal change in  $T_m$  with cholesterol addition was observed in the presence of water [11,29]. Hydrated DPPE–Ch mixtures exhibit a  $T_m$  decrease by approximately 10 °C upon the addition of 30 mol% Ch [37]. This observation reinforces the ability of trehalose to maintain the lipidic mixture in the fluid state upon dehydration.

For all of the phospholipid–Ch mixtures analyzed, the enthalpy of the thermotropic transition decreases with increasing cholesterol proportion (Table 2). Reduction in the transition enthalpy was also observed in the aqueous phase for the same lipid mixtures (data not shown). Increasing the cholesterol proportion leads to “dilution” of the phospholipids, thus, there are less phospholipid–phospholipid interactions. This causes the decrease and eventual abolition in the heat of transition.

We have analyzed the effects of trehalose on several phospholipid–Ch mixtures and observed: (i) a lowering of the  $T_m$  and (ii) prevention of phase separation (i.e., only one transition is present) of the lipidic components upon freeze-drying. The lack of multiple transitions suggests that trehalose limits the phase separation of phospholipids from cholesterol and also limits the transition of phospholipids to other phases (for DPPE).

#### 4. Conclusion

Cholesterol is a major component in cell membranes and plays an important role in the organization of membranes. In this work, we have analyzed the phase behavior of various freeze-dried mixtures of DPPE, DPPC, and cholesterol and have examined the effects of trehalose addition to these liposomes. The main focus of the use of trehalose has been to reduce the  $T_m$  of phospholipids upon dehydration. This is an important aspect since liposomes undergoing phase transition during rehydration can lead to leakage of encapsulated components [31]. In this study, we show that for dehydrated systems containing cholesterol, however, trehalose is also necessary to limit phase separation. Despite the abundance of reports examining the Ch-containing liposomes in the fully hydrated state, there is a shortage of studies on these systems in the dried state. This work provides strong evidence for the effectiveness of trehalose in stabilizing cholesterol-containing membranes upon lyophilization and could be useful in the design of liposomes for entrapment and delivery of pharmaceuticals.

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