Effect of sugar–phosphate mixtures on the stability of DPPC membranes in dehydrated systems

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Abstract

The stabilizing role of sugars on dehydrated membranes is well established. The formation of a glassy matrix and the direct interaction between the sugars and the lipids are some of the mechanisms proposed to be involved in this stabilizing effect. Phospholipidic systems have been studied extensively as models for biological membranes and also due to the practical applications of liposomes as vehicles for drug delivery. In this work, we evaluate the effect of sugar–phosphate mixtures on the transition temperature of dehydrated 1,2-dipalmitoylphosphatidylcholine, and also examine some physical characteristics of these mixtures, such as the glass transition temperature and water sorption properties. The addition of phosphate salts to sugar systems has several interesting features that merit its consideration in formulations to protect dehydrated labile biomaterials. In particular, sucrose–phosphate mixtures provide an interesting alternative to pure saccharide formulations due to their high glass transition temperatures and their increased ability to maintain a low melting transition temperature in the presence of small amounts of water.

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The stabilizing role of sugars on dehydrated labile biomolecules has been recognized for years in the pharmaceutical, food, and biological sciences [2,3,6,16,20,33,35]. The nonreducing disaccharide trehalose has been employed extensively, partly as a result of its effectiveness in protecting membranes and proteins during drying and ensuing storage [1,5,7,8,17,23,35]. Sucrose has attributes similar to those of trehalose, and it is widely used in the pharmaceutical industry as a lyoprotectant for biological materials [13,19,28,34,38]. It has been suggested that the cell membranes are the primary site of desiccation-induced injury [9,36]. Upon desiccation, the gel-to-liquid crystalline phase transition temperature, Tm, of the phospholipids increases. However, when these phospholipids are dehydrated in the presence of saccharides, Tm is actually reduced [11,17,18].

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There are several interpretations for this effect. Crowe proposed a "water replacement hypothesis," in which sugar molecules directly interact with the head groups of the phospholipids, thereby maintaining the lateral spacing between the head groups in the dry state [7]. Another explanation, the vitrification hypothesis, states that desiccated membranes are stabilized through the vitrified state of the sugars [17,18,39]. These ideas, however, are not mutually exclusive; both effects appear to be necessary for conferring stability to the dehydrated membranes [11].

The predominant component of cell membranes, phospholipids, has been studied extensively, primarily to model biological membranes, but also due to the application of liposomes as vehicles for drug delivery. To maintain the stability of dehydrated lipidic vesicles, it is important to keep the \( T_m \) of the membrane below ambient temperature, \( T_a \). For storage at ambient conditions, it is also important that the glass transition temperature, \( T_g \), of the protectant system exceeds \( T_a \). \( T_g \) needs to be as high as possible above \( T_a \) in order to slow relaxation processes in the glassy state. In order to avoid a phase transition, \( T_m \) needs to be below \( T_a \); at \( T_m \) a leakage of vesicle contents is observed, particularly upon rehydration [9].

It is known that ions in solution can form complexes with polyhydroxy compounds [29,31,32]. Oxyanions, such as borate and phosphate, mixed together with sugars have shown increases in viscosity and glass transition temperature with respect to those of sugars alone [27]. For example, the addition of 1 mol of borate ions per mole of trehalose raises the \( T_g \) of the resulting solution by 60 °C [26]. It is believed that the borate accomplishes this by forming a reversibly crosslinked network between trehalose molecules. Borate ions, however, cannot be used for consumer oriented applications as they can be toxic in high concentrations. For this reason, phosphate ions, which are biologically benign and expected to have similar properties as borate ions, were chosen as a replacement. Tests on the protectant effects of sugar–oxyanion (borate and phosphate) mixtures on lactate dehydrogenase and \textit{Lactobacillus acidophilus} upon dehydration have demonstrated improvements in viability and stability compared to those in the absence of oxyanions [4,26,27]. Given the usefulness of these past results, it is of interest to study in more detail the effects of these protectant mixtures on model membranes.

In this work, we evaluate the effect of sugar–phosphate mixtures on the \( T_m \) of dehydrated 1,2-dipalmitoylphosphatidylcholine, as well as some physical characteristics of these mixtures, such as the \( T_g \) and water sorption properties.

### Materials and methods

#### Chemicals

Phospholipid, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) in chloroform was purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Trehalose, raffinose, and sucrose were purchased from Pfistiehl Laboratories (Waukegan, IL). The phosphate salts used were \( \text{KH}_2\text{PO}_4 \) and \( \text{K}_2\text{HPO}_4 \) (Fisher Scientific, NJ). All solutions were prepared in nanopure water.

#### Lipid–sugar–phosphate sample preparation

Phospholipids were dried under a stream of \( \text{N}_2 \) at 50 °C and then resuspended in a sugar solution to give a 1:1 sugar/lipid weight ratio. The solution also contained \( \text{KH}_2\text{PO}_4 \) and \( \text{K}_2\text{HPO}_4 \) (1:1 mol ratio, unless otherwise stated) to give the specified sugar/phosphate mol ratios (0.5–2). Control samples consisting of lipid, lipid–sugar, and lipid–phosphate mixtures were also prepared. Rehydration was carried out at 50 °C in a water bath, and then the samples were sonicated in a bath sonicator (FS5, Fisher Scientific) for 2 h at 50 °C. The preparation was dried under vacuum at 50 °C for 3 days, then placed in a desiccator with drierite at room temperature, and kept under vacuum of 15 mtorr for 3 additional days. The desiccator containing the samples under vacuum was transferred to a glove box (Vac, Nexus One, Hawthorne, CA), and then the samples were loaded into preweighed DSC pans and sealed for calorimetric analysis, and into preweighed glass vials for water content analysis.

To obtain the desired range of sample hydrations, samples were loaded into DSC pans and
then placed in desiccators over saturated salt solutions that provided constant relative humidities between 11 and 33% [15] at 20 °C for 1 week. Fully hydrated samples were prepared following the same procedure as described above and were loaded into the DSC pans immediately following sonication.

Sugar–phosphate sample preparation

Amorphous systems were obtained by freeze-drying aqueous solutions containing 20% (w/w) sugar. Various ratios of KH₂PO₄ and K₂HPO₄ were added to give the specified pH and sugar/phosphate mol ratios (0.5–2.0). Aliquots of 0.5 ml of the solution were placed in 5 ml glass vials, and then immediately quenched with liquid nitrogen. The samples were then freeze-dried for 48 h in a Viris Genesis 12EL (New York, USA) freeze-dryer at a pressure of 30 mtorr and a condenser temperature of −80 °C. The freeze-dried samples were then placed in vacuum ovens to further remove the residual water, prior to sample preparation in the glove box as described above.

Differential scanning calorimetry

DSC was used to determine the $T_m$ and the $T_g$. $T_m$ represents the peak temperature of the endotherm for the lipid gel to fluid phase transition. $T_g$ was recorded as the onset temperature of the discontinuity in the curve of heat flow versus temperature. The instrument used was a TA Q100 DSC (New Castle, DE). The dehydrated samples were scanned from −20 to 170 °C and the fully hydrated samples from 20 to 50 °C. At least two scans were carried out for each sample. All measurements were made at 10 °C/min, using sealed aluminum pans (crimped pans, TA), and an empty pan was used as a reference. The average value of three replicate samples was reported along with the standard deviation. Data were analyzed using Universal Analysis.

Water content analysis

Residual water measurements for dehydrated samples were made using a Karl Fisher Coulometer Metrohm, Model 737 (Herisau, Switzerland). Three replicates of each sample were analyzed and their average value was reported along with their standard deviation. Variability in errors observed in the residual water contents most likely result due to water absorption by the samples prior to water content measurement.

Results and discussion

Fig. 1 shows the effect of phosphate salt addition on the $T_m$ (analyzed as the transition peak temperature) of DPPC in lipid–sugar–phosphate dehydrated systems. All the samples analyzed in this work showed multiple endotherms in the range of 40–60 °C during the first heating scan; however, only one peak persisted in subsequent scans. Similar results were reported previously by Crowe and Crowe [10]. All $T_m$ values reported henceforth correspond to peak values from the second scan. The $T_m$ of pure dehydrated DPPC was 103 °C, which is in accordance with values of 105–112 °C obtained by Crowe and Crowe [10]. When sugars are added to the lipid formulation, the $T_m$ values are reduced to a different degree depending on the type of sugar used [10,18]. Addition of sugars at sugar/DPPC weight ratio of 1.0

![Graph showing the effect of phosphate salt addition on the $T_m$ (analyzed as the transition peak temperature) of DPPC in lipid–sugar–phosphate dehydrated systems.](image-url)
reduced the $T_m$ of the dry systems to 25, 27, and 25°C in the case of trehalose, sucrose, and raffinose, respectively. Similar $T_m$ values have been reported by various authors [10,18,30]. The addition of phosphate ions to the DPPC–sugar dehydrated mixtures depresses the $T_m$ even further. Equimolar mixtures of potassium phosphate monobasic and dibasic salts were used in order to obtain an initial solution pH of 6.5. All three sugars in the presence of phosphates at a phosphate/sugar molar ratio of 2.0 exhibit $T_m$s in the vicinity of 21°C, i.e., approximately 4°C below those observed in their absence (Fig. 1). The addition of phosphate ions alone to DPPC at the same molar ratios as described above did not affect the dehydrated $T_m$ value (data not shown).

We have also analyzed the behavior of $T_m$ in fully hydrated systems. As shown in Fig. 2, the $T_m$ value of pure DPPC in the fully hydrated state is 41°C, consistent with previous reports [14]; it can be seen that this value increases slightly (by less than one degree) upon the addition of phosphate or sugars. The addition of phosphate to DPPC increases the $T_m$ more than the addition of trehalose. This suggests that phosphate causes greater changes in the distribution of water around the lipid head groups than trehalose. Even in the fully hydrated state, the added solutes have the ability to affect lipid–lipid interactions. However, the depression of $T_m$ is a phenomenon that occurs upon dehydration.

The sugars are effective in lowering the $T_m$ of dried DPPC by restricting the contraction of the lipids that would otherwise occur upon dehydration. Recent molecular simulations of DPPC–trehalose systems have shown that disaccharide molecules are able to form stable hydrogen bonds with two or three phospholipid head groups, thereby conferring stability to bilayer membranes in the absence of water [37]. As sugar–lipid mixtures are dehydrated, disaccharide molecules compete with bound water to interact with the lipid head groups. The efficacy of the sugars in lowering the $T_m$ depends on how efficient they are in replacing the head group bound water. We propose that one of the effects of phosphate is that of pulling water away from the head groups, thereby enhancing the interaction of disaccharide molecules with lipid head groups and lowering $T_m$ even further. This view is also supported by the fact that, in the presence of phosphates, the amount of sugars needed to depress the $T_m$ of DPPC decreases. This is clearly seen in Figs. 3A and B for trehalose and Figs. 3C and D for sucrose. For the DPPC–trehalose system, a trehalose/DPPC weight ratio of 0.8–1.0 is necessary to obtain the depressed, homogeneous $T_m$ of 25°C. However, upon addition of phosphate at a phosphate/trehalose molar ratio of 1, the required sugar/lipid weight ratio decreases to 0.5. For sucrose, even at a sucrose/DPPC weight ratio of 0.8, the $T_m$ is not depressed; however, in the presence of phosphate at the same sucrose/DPPC weight ratio, the $T_m$ is depressed to 25°C, although a second transition is also seen at 42°C.

When working with dehydrated systems, it is important to take into account the possibility of water sorption from the ambient that can occur during processing or storage. Fig. 4 shows the water sorption behavior of DPPC–trehalose (A) and DPPC–sucrose (B) systems at 20°C in the presence of different amounts of phosphate. Upon addition of phosphate, the water content for any given relative humidity increases for both trehalose and sucrose systems. This observation is consistent.

Fig. 2. Effect of phosphate salt addition on the $T_m$ (analyzed as the transition peak temperature) of DPPC fully hydrated systems. 1:1 weight ratio of lipid/sugar is used for both DPPC–trehalose and DPPC–trehalose–phosphate mixtures. 1:1 molar ratio of phosphate/sugar is used for DPPC–trehalose–phosphate. For DPPC–phosphate mixtures, the same DPPC/phosphate molar ratio is used as for DPPC–trehalose–phosphate. All systems have an initial solution pH of 6.5.
with previous results reported by Mazzobre et al. [25], who showed that the water uptake of mixtures of trehalose and sucrose with various chloride salts is higher than that observed for the pure sugar systems. The effect of water sorption on the \( T_m \) of DPPC in the dehydrated systems (sugar:phosphate molar ratio = 1) exposed to different relative humidities is shown in Fig. 5. It can be seen that for dehydrated DPPC–trehalose systems, a low \( T_m \) can be maintained even at 22% relative humidity; however, this feature is lost upon the addition of phosphate (Fig. 5A). For DPPC–trehalose–phosphate systems at the same relative humidity, for example, we see that the \( T_m \) is 45°C; much higher than the \( T_m \) of 25°C observed in the absence of phosphate. However, for sucrose systems, a different behavior is observed (Fig. 5B). Upon the addition of phosphate, a low \( T_m \) can be maintained at 11% relative humidity, while in the absence of phosphate, \( T_m \) increases to 42°C at the same relative humidity.

The physical state of the matrix is an important factor to consider regarding the stability of biologicals in the dehydrated state. Fig. 6 shows the effect of phosphate addition on the \( T_g \) of different sugar–DPPC systems having approximately 0.5 wt% residual water content. In the absence of phosphate, the \( T_g \) values are 118, 75, and 109°C for trehalose–, sucrose–, and raffinose–DPPC systems, respectively. The observed value of \( T_g \) for trehalose–DPPC is higher than that reported previously [18]; however, this may be explained by the fact that our samples are more anhydrous than those studied in the literature, which had approximately 1 wt% residual water content. The \( T_g \) for DPPC–sucrose systems increases significantly with increasing phosphate concentration up to a phosphate/sugar molar ratio of 1, where the \( T_g \) value is
This behavior was not observed for trehalose and raffinose systems where, at a pH of 6.5, the \( T_g \) values remained relatively constant with increasing phosphate salt concentration (Fig. 6).

Miller et al. [26] showed a marked effect of pH on \( T_g \) for aqueous solutions of trehalose–borate. They showed that a high pH facilitates crosslinking between borate and trehalose by shifting the equilibrium between borate ions and boric acid toward borate [26]. The effect of pH on the \( T_g \) of dehydrated sugar–phosphate systems and lipid–sugar–phosphate systems is shown in Figs. 7 and 8.

Fig. 7 shows the glass transition temperature as a function of pH for freeze-dried trehalose–phosphate and sucrose–phosphate samples containing phosphate/sugar molar ratio of 0.5. It can be seen that at high pH values, the \( T_g \) is increased both for trehalose and sucrose systems. At higher pH values, more phosphate is available to interact with the sugars, thereby resulting in a system which has higher \( T_g \), as was seen for sugar–borate mixtures [27]. It is desirable to reach as high a \( T_g \) as possible for a protectant formulation, but the corresponding high pH value can be detrimental to the biological system to be preserved, as was shown previously for *Lactobacillus acidophilus* [4,12].

Fig. 8 shows the effect of phosphate on \( T_m \) and \( T_g \)
at different pH values for DPPC–trehalose–phosphate (A) and DPPC–sucrose–phosphate (B) dehydrated systems. It can be seen that for DPPC–trehalose–phosphate samples, the $T_m$ values were not significantly affected by changes in the pH (Fig. 8A). The results for DPPC–sucrose–phosphate samples were different from those observed for the DPPC–trehalose–phosphate systems (Fig. 8B). The thermograms showed inhomogeneities at the two pH extremes (pH ~ 4 and pH ~ 9). There are clearly different interactions between trehalose–phosphate and sucrose–phosphate with DPPC. The $T_g$ values obtained for both DPPC–trehalose–phosphate and DPPC–sucrose–phosphate increased at higher pH values (Fig. 8), which is in agreement with the data obtained for sugar–phosphate samples (Fig. 7).

It is of interest to note that several authors have analyzed the effect of cations on the glass transition temperatures of polyhydroxy compounds. Vacuum-dried mixtures of glycerol with MgCl$_2$ show a pronounced increase in $T_g$ with increase in the salt/glycerol molar ratio [22]. However, freeze-dried mixtures of trehalose and sucrose with MgCl$_2$ at a sugar/salt molar ratio of 5:1 do not show changes in $T_g$ compared to the pure sugars [21, 24]. We have also analyzed the effect of the amount of potassium on $T_g$. Table 1 shows the $T_g$ and pH values obtained for samples containing
different amounts of phosphate and potassium ions. Trehalose–phosphate systems with the same potassium concentration (i.e., 1.0 potassium/sugar molar ratio) but at a different pH have a difference in \( T_g \) of 27°C. Sucrose–phosphate systems under the same conditions show a difference in \( T_g \) of 44°C. However, systems that have the same pH (~6.5) but a different concentration of potassium ions have similar \( T_g \): 115°C for trehalose–phosphate and 76°C for sucrose–phosphate. The effect of pH on the \( T_g \) seems more pronounced and is the likely cause for the observed difference in the \( T_g \) values. Systems that have similar pH values have similar \( T_g \), while those having higher pH values have higher \( T_g \), irrespective of the amount of counterions present.

The addition of phosphate salts to sugar systems has several interesting features to merit their consideration as potential formulation to protect dehydrated labile biomaterials. In particular, sucrose–phosphate mixtures provide a good alternative to pure saccharide formulations; these mixtures exhibit a high \( T_g \) and an increased ability to maintain a low \( T_m \) when small amounts of water are present. It would therefore be of interest to examine the effectiveness of such mixtures for lyophilization of cellular systems.

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Table 1

<table>
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<th>Sample</th>
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<th>( \text{K}^+/\text{sugar molar ratio} )</th>
<th>( T_g ) (°C)</th>
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<td>1.00</td>
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<td></td>
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<td>1.50</td>
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References


