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How much solute is needed to inhibit the gel to fluid membrane phase transition at low hydration?

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Summary

We present a quantitative study of the effect of sugars on the membrane gel-fluid phase transition as a function of sugar:lipid ratio. We show that the maximum effect occurs at around 1.5 sugar rings per molecule for both mono- and di-saccharides. We present a theoretical model to try to explain these results, and discuss the assumptions inherent in the model.
It is considered that sugars and other small solutes are important in improving desiccation and freezing survival for a range of species [1-3]. One of the well known effects of sugars is that they can prevent the dehydration induced increase in the temperature at which membranes undergo the gel-fluid phase transition. This effect is observed throughout dehydration [4-6], down to the fully dried state [7-9]. The mechanisms for this can be understood in terms of the effects of solutes on hydration forces between membranes [6, 10, 11], with specific interactions (e.g. water replacement) playing a role in the fully dry state [12]. The effects of dehydration on phosphatidylcholines as a function of sugar type [5, 6] and different tail groups [4-6] are now well understood.

There remain, however, some unanswered questions, one of which is the question of how much sugar is needed to protect membranes, and how the effects change with changing sugar concentration. Naively, one might expect that this ability would be a direct function of sugar concentration, and the effects should increase as the amount of sugar increases. However, the real situation is more complex. Previous work [6] has shown that there are two distinct mechanisms for reduction in the transition temperature: First, if the sugar concentration is too low to form a glass, then the transition temperature can be reduced to (at best) the full hydration value; and second, if a glass forms, the transition temperature can be depressed to a fixed value, largely independent of sugar concentration.

In this letter we report a systematic study of the effects of increasing amounts of sugar on membrane phase transitions as a function of dehydration. We show that in the absence of a glass transition, the maximum reduction in the membrane phase
transition temperature is reached at a limiting sugar:l lipid ratio of approximately 1.5 sugar rings per phosphatidylcholine. Beyond that value, the addition of further sugar no longer alters the membrane phase transition temperature. A simple theoretical model is developed to explain the effects. The model qualitatively reproduces the experimental results, but differences between the model and the experiments point to partial exclusion of solutes from the interlamellar spaces during dehydration, as suggested by recent preliminary experiments [13].

The phosphatidylcholines DPPC (1,2-dipalmitoylphosphatidylcholine) (powder) and DMPC (1,2-dimyristoylphosphatidylcholine) (in chloroform) were obtained from Avanti Polar Lipids (Birmingham, AL, USA) and were used without further purification. The sugars sucrose (SigmaUltra >99.5% purity) and glucose (>99% purity) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Two separate experiments were performed to test the model using different sugars and lipids. DPPC was tested using several sugar:l lipid molar ratios at a range of hydrations, while DMPC was tested using more numerous sugar:l lipid ratios at a single hydration level.

Dry DPPC was suspended in an appropriate amount of sucrose solution to achieve the desired sucrose:DPPC molar ratio in the range from 0:1 to 1:1. Further milli-Q water was added as necessary to ensure the sample was in excess water. Samples were mixed by repeated freeze-thawing, vortex mixing and centrifugation, then equilibrated at 23 °C over saturated salts that generate known Relative Humidities (RH) [14] for a period of 1-3 weeks. The RHs were monitored with a Hastings humidity data logger.
(Hastings, Port Macquarie, Australia) to ensure equilibrium had been reached. Once equilibrated, the samples were placed into pre-weighed DSC volatile sample pans and hermetically sealed. Differential Scanning Calorimetry was performed using a Perkin Elmer Pyrus 7 DSC (Norwalk, CT, USA). Samples were loaded at 20 °C. Prior to scanning, samples were heated at a nominal rate of 200 C/min to 80 °C, then cooled to 0 °C and allowed to equilibrate. This procedure was used to ensure that equilibrium behaviour was being measured, rather than the metastable behaviour previously observed on the first scan when lipids in the gel phase are dried in the presence of sugars [6, 15]. Samples were then scanned at 10 °C/min to 80 °C, then cooled at 10 °C/min to 0 °C, and then the cycle was repeated. No significant differences were noted between the two scans.

For samples using DMPC, aliquots of the lipid in chloroform were first dried under a stream of N₂, then were resuspended in solutions of glucose in water:methanol (1:1, v/v) to achieve the desired glucose:lipid molar ratios ranging from 0-3. After thorough mixing to disperse the lipid and solutes, the samples were dried in vacuo at 60 °C to remove the methanol [5, 6]. The dry DMPC-glucose mixtures were resuspended in purified water (Nanopure, Barnstead, Inc., Dubuque, IA, USA) and mixed thoroughly by repeated freeze-thawing and vortex mixing. The samples were then loaded into pre-weighed volatile sample pans and incubated above a saturated solution of LiCl at 28 °C (RH = 13%) [14] for a period of 3-12 months. Differential Scanning Calorimetry was performed using a Perkin Elmer DSC-7 (Norwalk, CT, USA). Samples were loaded at 20 °C, cooled at a nominal rate of 200 C/min to -30 °C, and allowed to equilibrate prior to scanning. Samples were scanned at 20 C/min to 80 °C, cooled to -30 °C, and reheated to 80 °C.
For both experiments, in most cases only single peaks were observed. Overlapping transitions, believed to arise from the melting of the glassy solution and the lipid, were sometimes observed in heating scans [5]. To avoid ambiguity, $T_m$ was determined from the midpoint peak values of the cooling scans. In some cases the samples showed two closely spaced maxima, completely reproducible on repeated scans. This is possibly related to the pre-transition ripple phase observed in long chain phosphatidylcholines [16]. In these cases the larger peak was chosen to represent $T_m$, and the resulting uncertainty in $T_m$ is smaller than the error bars shown.

Figure 1 shows the measured transition temperatures for the gel-fluid transition of DPPC, as a function of sucrose:lipid ratio, for several different RH. For the pure lipid (x axis = zero) the effect of dehydration is clearly seen as an increase in the transition temperature, $T_m$, from around 47 °C at 91% RH to 75 °C at 1.1% RH, consistent with previous results [15]. As a small amount of sugar was added (0.1 mole sugar per lipid), the effect of dehydration was reduced substantially, with the maximum transition temperature reduced from 75 °C to around 63 °C. As progressively more sugar was added, the range of transition temperatures narrowed, until at around 0.8 sugars per molecule, there was no significant difference among the transition temperatures at different humidities. Increasing the sugar:lipid ratio to 1:1 caused no further change in $T_m$.

To investigate if the type of lipid or sugar makes a difference, a similar experiment was carried out using the monosaccharide glucose and a different phosphatidylcholine, DMPC. In this experiment one RH was tested, but a larger
number of sugar:lipid ratios was investigated. Figure 2 shows the measured midpoint transition temperatures for the gel-fluid transition. Clearly the same trend is observed, with the maximum effect of sugar on $T_m$ found at about 1.5 glucose molecules per lipid. This corresponds very well with the results shown in figure 1, when one takes into account the fact that sucrose is a disaccharide and glucose is a monosaccharide. Figures 1 and 2 both indicate that the effect of the sugars is maximized when there are about 1.5 sugar rings per lipid molecule.

The results reported here are similar to those reported previously on the stoichiometry of trehalose-DPPC interactions in the dry state [15, 17]. In those studies, increasing molar ratios of trehalose were found to progressively lower the $T_m$ of dry DPPC. Crowe and Crowe [15] found that the maximum effect was observed at a trehalose:DPPC ratio between 0.65 and 1.5 (intermediate ratios were not studied). Nakagaki et al. [17] tested numerous ratios and found that a mole ratio of approximately 0.7 trehalose per DPPC was needed to lower $T_m$ maximally.

In order to understand this effect, we present a simple model which semi-quantitatively explains the effect of sugar on the membrane transition temperature. While the phase equilibrium of lipid/water mixtures is complex, at low to intermediate hydrations the force balance is dominated by the strongly repulsive hydration force [18], which may be written as:

$$P = P_o \exp \left( -\frac{d_m}{\lambda} \right)$$

(1)

where $P$ is the force per unit area, $P_o$ is the extrapolated value at zero separation, $d_m$ is the separation between opposing bilayers, and $\lambda$ is the decay length of the force. This
The repulsive force is balanced in the water phase by a suction (negative pressure) $P' = -P$. This suction results in a lateral compressive stress in the membrane, given by [19]:

$$\pi = -P'd_w = Pd_w$$  \hspace{1cm} (2)

The effect of the compressive stress is to favour the transition to the gel phase, which has a smaller area per molecule. This effect can be modelled by a two dimensional version of the Clausius-Clapyron equation [20]:

$$\Delta T = \frac{T \Delta a}{2L} \pi$$  \hspace{1cm} (3)

where $T_o$ is the transition temperature in excess water, $\Delta a$ is the difference between the area per lipid in the fluid and gel phases, and $L$ is the enthalpy of the transition. In order to relate the change in transition temperature to a water content, we need to relate the water separation, $d_w$, to the number of water molecules per lipid, $n_w$. This is given by:

$$\frac{d_w}{2} = \frac{n_wv_w}{a}$$  \hspace{1cm} (4)

Substituting equations 1, 2 and 4 into 3, gives an expression for the change in transition temperature as a function of water content:

$$\Delta T = \frac{T}{L} \frac{\Delta a}{a} (n_wv_w)P_w \exp\left[-\frac{2(n_wv_w)}{a \lambda}\right]$$  \hspace{1cm} (5)

Equation 5 assumes that there is only water present between the membranes. If there is also solute present, then term $(n_wv_w)$ must be replaced by the total intermembrane volume (water plus solutes = $(n_wv_w + n_sv_s)$), giving:

$$\Delta T = \frac{T}{L} \frac{\Delta a}{a} (n_wv_w + n_sv_s)P_w \exp\left[-\frac{2(n_wv_w + n_sv_s)}{a \lambda}\right]$$  \hspace{1cm} (6)
Using literature values ([18, 21] and references therein) for the parameters (DPPC: $T_o = 44.2^\circ$C, $L = 35$ kJ/mol, $\Delta a = 0.19$ nm$^2$, $a = 0.5$ nm$^2$, $\lambda = 0.2$ nm; Sucrose: $v_s = 0.5$ nm$^3$; Water: $v_w = 0.03$ nm$^3$), the transition temperatures for DPPC as a function of water content are shown in figure 3.

Clearly the results qualitatively agree with the experiments - without sugar, the transition temperature $T_m$ rises towards 70 °C in the dehydrated samples. As sugar is added, this maximum value of the $T_m$ is reduced. At around 0.4 sugar molecules per lipid, $T_m$ rises only one degree, and for 0.5 sugars per lipid and higher, $T_m$ remains effectively constant.

Interestingly, a related study was recently carried out by Cacela and Hincha [22] who reported for fully dehydrated sucrose:Egg Phosphatidylcholine (EPC) mixtures that the maximum effect on $T_m$ was observed at a sucrose:EPC ratio of 0.4 sugars per lipid. However, those results cannot be compared directly with the model described here, as the value of the lipid $T_m$ they reported for dried samples was depressed below the $T_m$ of the fully hydrated lipid by the presence of vitrified sugars and, therefore, did not result solely from the effect of sugars on hydration forces. The effect of vitrified sugars on membrane gel-fluid phase transitions has been quantitatively explained previously [6].

While the experimental and theoretical results presented here are in qualitative agreement, the model suggests that only around 0.5 sucrose molecules per lipid are sufficient to inhibit the rise in the transition temperature, compared with around 0.8 molecules per lipid from the experiments. One possible explanation for this is that
some of the sugars are excluded from between the membranes [4, 5], something that is not taken into account by the model. This possibility is supported by recent preliminary small angle neutron scattering experiments [13] which suggest that solutes are indeed partially excluded during dehydration. The extent of exclusion may depend upon sample preparation methods. Further experiments are currently underway in order to quantify the exclusions. These results should allow the development of a full quantitative model to describe the effects of sugars on the gel-fluid membrane transition during dehydration.

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References


Figure 1. Membrane gel-fluid transition temperature for DPPC as a function of sucrose:DPPC molar ratio, equilibrated at for several different values of relative humidity. Values are the mean peak transition temperature during cooling. Error bars are deliberately overestimated using both peak and onset temperatures from both heating and cooling scans. The dashed line represents the DPPC fluid-gel transition temperature at full hydration in the absence of sugar at the same cooling rate. Without sugar, the transition temperature of fully dehydrated DPPC is approximately 105°C [15].
Figure 2. Membrane gel-fluid transition peak temperature for DMPC, equilibrated to a relative humidity of 13%, as a function of glucose:DPPC molar ratio. Values are the mean peak temperatures from the cooling scans, and error bars are the standard deviations of 2-3 samples. The dashed line represents the DMPC fluid-gel transition temperature at full hydration in the absence of sugar at the same cooling rate. Without sugar, the transition temperature of fully dehydrated DMPC is approximately 70°C.
Figure 3. DPPC membrane phase transition temperature as a function of water content, for different sucrose:DPPC ratios, calculated using equation 6.