The effects of sugars on lipid bilayers during dehydration – SAXS/WAXS measurements and quantitative model.

Thomas Lenné[1†], Christopher J. Garvey[2], Karen L. Koster[3] and Gary Bryant[1,*]

[1]*Applied Physics, RMIT University, Melbourne, Vic, 3001, Australia*

[2]*Australian Nuclear Science and Technology Organization, Menai, Australia*

[3]*Department of Biology, The University of South Dakota, Vermillion, SD, USA*

*Corresponding Author:

Tel: 61-3-99252139

Fax: 61-3-99255290

Email: gary.bryant@rmit.edu.au

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† Current address; Functional Ecology Group, Research School of Biological Sciences, The Australian National University, Canberra, ACT 0200, Australia.

Summary

We present an X-ray scattering (SAXS/WAXS) study of the effects of dehydration on the bilayer and chain-chain repeat spacings of dipalmitoylphosphatidylcholine (DPPC) bilayers in the presence of sugars. The presence of sugars has no effect on the average spacing between the phospholipid chains in either the fluid or gel phase. Using this finding, we establish that for low sugar concentrations only a small amount of sugar exclusion occurs. Under these conditions the effects of sugars on the membrane transition temperatures can be explained quantitatively by the reduction in hydration repulsion between bilayers due to the presence of the sugars. Specific bonding of sugars to lipid headgroups, as proposed by the Water Replacement Hypothesis, is not required to explain this effect.

Introduction

It is well established that sugars and other small solutes are important in improving desiccation and freezing survival for a range of species [1-5]. One property which has been widely studied is the ability of sugars to stabilize membranes in the fluid phase by limiting the dehydration-induced increase in the gel-fluid transition temperature of membranes. This protective effect is observed throughout the dehydration process [6-8], down to the fully dried state [9-11].

For many years it was believed that this ability was due to the ability of disaccharides (trehalose and sucrose) to insert between adjacent lipid head groups during dehydration and hydrogen bond to them, spreading the lipid head groups apart, and thus inhibiting the transition to the more tightly packed gel phase (e.g. see [12-15]). This model, known as the water replacement hypothesis (WRH), is widely cited (e.g. see [15-17] for some recent examples). In recent years, however, an alternative model has been proposed

which explains the observed effect of sugars on the gel-fluid transition temperature in terms of the sugars' effect on the hydration repulsion [Rand, 1989 #29] that develops between opposing membranes during dehydration. In the absence of sugars, the hydration repulsion gives rise to a lateral compressive stress in the bilayer which squeezes adjacent lipids more closely together, resulting in a transition to the gel phase. When solutes such as sugars are present between the membranes, their non-specific osmotic and volumetric effects reduce the hydration repulsion, reduce the compressive stress in the membranes, and hence tend to maintain the average lateral separation between lipids [6, 8, 18-20]. This model, called the hydration forces explanation (HFE), also explains the additional depression of the transition temperature observed if the sugar solution vitrifies while the lipids are in the fluid state [7].

The WRH is a qualitative model, and cannot be used to make quantitative predictions. By contrast the HFE is a mathematical model which has had a great deal of success in semi-quantitatively explaining the observed effects of sugars [21]. Its application is complicated by the fact that partial exclusion of sugars from the interlamellar layers is observed to occur during dehydration [6, 8, 22] . One of the aims of this study is to test the HFE quantitatively under conditions where exclusion is minimal.

A central proposition of the WRH is that during desiccation, sugars partition preferentially into the region near the lipid head groups, displacing water molecules from around, and between, the head groups, and H-bonding with the lipids (e.g. [10, 12]). H-bonding of sugars with lipids is uncontroversial, but the other two aspects of the model are not, and can be tested experimentally. Specifically: do sugars partition preferentially into the region near the lipid head groups; and do the sugars insert between lipid head groups, as has been proposed schematically in a number of cartoons used in explaining the WRH (e.g. [12,])?

An increasing body of evidence shows that sugars are in fact partially excluded from the region near phospholipid headgroups. First, using the Surface Forces Apparatus, Pincet and co-workers [23] showed evidence that sugars (specifically trehalose and sorbitol) were partially excluded from the inter-bilayer space. Second, Yoon and co-workers [24] found, using quantitative solid state NMR, that sugars were excluded from the region near the headgroups. More recently, a hydration forces analysis of membrane dehydration in the presence of sugars found strong indirect evidence for partial exclusion from the inter-bilayer space at low hydrations [6].

Finally, for multi-lamellar vesicles in excess water in the presence of sugars, Demé and co-workers [25] used contrast variation Small Angle Neutron Scattering (SANS) to show that sugar concentrations were lower between the bilayers than in the aqueous solution surrounding the membranes. Lenné and co-workers [22] showed that the same technique can be applied at low hydrations, and demonstrated that lipid/glucose/water mixtures undergo microphase separations, with glucose concentrations between the bilayers being considerably lower than in the excluded regions. This mounting evidence strongly suggests that, in the presence of water, down to very low hydration, sugars partition away from phospholipid headgroups, rather than inserting between the headgroups.

In this paper we report results of small and wide angle X-ray scattering experiments that examine how the presence of both mono- and di- saccharides affects the average distance between bilayers and the average distance between lipid chains in the bilayer. Further, the data allow an estimation of the amount of sugar/water exclusion occurring in these systems. These results are compared with the quantitative predictions of the HFE and the qualitative predictions of the WRH to explain the effects of sugars on membrane transition temperatures.

Theory

Effect of sugars on phase transition temperature of phospholipids

At low to intermediate hydrations the force balance between membranes is dominated by the strongly repulsive hydration force [26]:

$$P = P_o \exp\left(-\frac{d_w}{l}\right) \tag{1}$$

where P is the repulsive force per unit area, P_o is the extrapolated value at zero separation, d_w is the separation between opposing bilayers, and λ is the decay length of the force. This repulsive force results in a lateral compressive stress in the membrane [27]:

$$\pi = Pd_w \tag{2}$$

This compressive stress leads to a reduction in the average area per lipid, a, as the hydration is reduced. The area may be written [20]:

$$a = a_o\left(1 + \frac{Pd_w}{k_a}\right) \tag{3}$$

where a_o is the area per lipid at full hydration and k_a is the lateral compressibility of the bilayer. For a system where all the sugar and water are between bilayers [6], d_w is given by:

$$d_w = \frac{2}{a}\left(n_w v_w + n_s v_s\right) \tag{4}$$

where n_w and n_s are the number of water and sugar molecules per lipid, and v_w and v_s are their respective partial molecular volumes.

The compressive stress (Eq. 2) favors the transition to the gel phase, which has a smaller area per molecule. Using the two dimensional version of the Clausius-Clapeyron equation, the corresponding change in transition temperature is given by [28]:

$$\Delta T = \frac{T_o \Delta a}{2L}\pi \tag{5}$$

where T_o is the transition temperature in excess water, Δa is the difference between the area per lipid in the fluid and gel phases, and L is the enthalpy of the transition.

Eqs.1 to 4 can in principle be substituted into Eq.5 to find an expression for the change in the transition temperature ΔT, however there is no analytic solution and the expression can be simplified considerably by making the approximation that the lateral compressibility $k_a >> Pd_w$ in Eq 3, which leads to $a \approx a_o$. This approximation allows for a full solution of Eq 5 in terms of measurable parameters [21]:

$$\Delta T = \frac{T_o}{L}\frac{\Delta a}{a}\left(n_w v_w + n_s v_s\right) P_o \exp\left[-\frac{2}{a}\frac{\left(n_w v_w + n_s v_s\right)}{\lambda}\right] \qquad (6)$$

In the analysis that follows, both the exact solution, (determined iteratively using a program written in Matlab), and the approximate solution will be presented.

The application of these equations requires literature values for a number of parameters. For the fully hydrated DPPC gel-fluid transition an average of several values in the database Lipidat [42] was used: L = 33.8±3 kJ/mol and T_o = 42.4±0.6°C; The most comprehensive study yet published of lipid areas [30] was used to determine the area parameters. That reference quoted values of: a = 47.9 Å^2 (gel phase @ 20°C) and a = 64 Å^2 (fluid phase @ 50°C), and an area compressibility of 250 mN/m (fluid phase – this is used in the numerical solution, but not in the approximation (equation 6)). These values must be adjusted for the thermal expansivity α = 0.0003 /°C (gel) and α = 0.006 / °C (fluid) [30]. Using these, the adjusted values at the transition temperature are a_g = 48 Å^2, a_f = 61 Å^2 and therefore Δa = 13 Å^2. Finally, the standard values of molecular volumes (from densities) are v_s = 490 Å^3 and v_w = 30 Å^3. The values for the hydration parameters are strongly determined by assumptions used (eg [30, 26]). As we are interested in the effects of sugars, rather than the hydration force *per se*, we use values which give a reasonable fit to the data

without sugar: P_o = 700 MPa, λ = 2 Å. These are consistent with the spread of values measured (eg [30, 26]).

Analysis of X-ray diffraction data

The primary quantity measured in the X-ray scattering experiments is the lamellar repeat spacing, d. It is standard practice to define the boundary between water and the lipid head groups using the volume weighted average interface [29]. By this definition the average inter-bilayer separation d_w is given by:

$$\begin{aligned} d_w &= (1 - f_L)d \\ d &= d_w + d_L \end{aligned} \tag{7}$$

where the lipid volume fraction in the presence of water and sugars is given by:

$$f_L = \frac{m_L \bar{v}_L}{m_L \bar{v}_L + m_w \bar{v}_w + m_s \bar{v}_s} \tag{8}$$

where the m_L, m_w, m_s and $\bar{v}_L$, $\bar{v}_w$ and $\bar{v}_s$ are, respectively, the masses and partial specific volumes of the lipid, water and sugar. The average area per lipid head group, in the gravimetric approximation, is then given by:

$$a = a_{grav} = \frac{2M_L \bar{v}_L}{d_L N_A} \tag{9}$$

where M_L = 734 kg/kmol for DPPC is the lipid molecular mass and N_A is Avogadro's constant. While Eq. 9 relies on the gravimetric approximation, which has a number of limitations (as discussed in detail in [30]), the approximation is adequate for the purposes of the discussion here.

In addition, the experiments yield the wide angle reflection, which corresponds to the average lateral chain-chain separation, d_c. In the gel phase, where the chain packing is approximately hexagonal, this can be used to determine the average area per lipid chain, a_c:

$$a_c = \left(\frac{2}{\sqrt{3}}\right) d_c^2 \tag{10}$$

The gel phase of DPPC is known to have the lipid chains tilted at an angle θ_t relative to the bilayer normal (designated the $L_{\beta'}$ phase). The tilt of the lipid chains with respect to the bilayer normal is then given by [31] :

$$q_t = \cos^{-1}\left(\frac{2a_c}{a}\right) \tag{11}$$

The above equations can be applied only if there is a single phase – i.e. all of the water and sugars are between bilayers, and there is no excluded phase.

Methods

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

Dry DPPC was suspended in an appropriate amount of sugar solution to achieve the desired sugar: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) (KNO_3, 91%; NaCl, 75%; NaBr, 57.5%, $MgCl_2$, 32.5%, LiCl, 13%, $ZnCl_2$, 5.5%, P_2O_5, ~0.1%) [32, 33] for a period of 1-3 weeks. The RHs were monitored with a Hastings humidity data logger (Hastings, Port Macquarie, Australia). Sample masses were monitored during the dehydration process. Samples were considered to be near equilibrium when the mass remained constant over several days. Mixing continued until samples were visually homogeneous. Previous work [8] has established that these preparation methods produce well mixed, homogeneous samples. For the present experiments the sugar concentrations are much lower than in most previous studies, and mixing is relatively easy. Nonetheless, good mixing was confirmed

by the reproducibility of the method (at least 2 replicates), and the repeatability of the DSC transitions. Once equilibrated, samples for X-ray analysis were transferred into 1.5 mm quartz X-ray capillaries (Wolfgang Muller Glas Technik, Berlin) and sealed using silicone (Pro Sea instant gasket, Racer Technology, USA). Samples for DSC were loaded into volatile sample pans and Differential Scanning Calorimetry (DSC) was carried out to determine transition temperatures, as described in [21, 34]. Sample weights were recorded at each stage of the sample preparation, enabling the calculation of the masses of each component.

Synchrotron Small and Wide angle X-ray scattering (SAXS and WAXS) experiments were carried out on the ChemMatCARS 15ID-D beamline at the Advanced Photon Source (APS), Argonne National Laboratory. Diffraction patterns were recorded on a Bruker 6000 CCD detector over the Q range 0.046 to 1.7 $Å^{-1}$, covering the length scales of interest for the primary repeat distance (in the SAXS regime) and the wide angle reflection (in the WAXS regime). For further details see [34, 35]. Kinetic (temperature scanning) measurements were made during both cooling and warming between 70 °C and 20°C. Equilibrium measurements were made at fixed temperatures, after incubating at that temperature for 5 minutes.

Results

Figure 1 shows a typical example of a set of intensity versus scattering vector plots as temperature was scanned down from 70 °C to 20 °C at a rate of ~15 °C/min (sucrose:DPPC 0.2:1, RH=13%). At 70 °C the sample was in the fluid phase (back of figure), and as it was cooled it underwent a transition to the gel phase (front of figure). The transition can be clearly seen by the increase in the number of small angle inter-lamellar reflections, characteristic of an ordered lamellar phase. The change in the character of the

wide angle reflection from a broad fluid peak at q~1.4 $Å^{-1}$, to a sharper peak at q~1.5 $Å^{-1}$ is characteristic of the fluid-gel phase transition. This behaviour is completely consistent with the known behaviour of phospholipids [36, 37], and the presence of sugars did not qualitatively change this behaviour; it only lowered the temperature at which the transition occurred.

In order to investigate what effect the presence of sugars has on the structure of bilayers, equilibrium measurements were made at several fixed temperatures. The repeat spacings ($d=2\pi/q$) for both the primary reflection (d) and the wide angle reflection (d_c) are plotted as functions of sugar content for several different relative humidities (Fig. 2).

Figs 2a and 2b, show respectively the effects of glucose and sucrose on DPPC in the gel phase at 20 °C (note the data are plotted with the number of sugar rings per lipid on the x axis). As can be seen the primary repeat spacing, d, increases almost linearly with increasing sugar volume, while the chain-chain distance d_c is essentially independent of sugar ratio and relative humidity (note the highly expanded scale on the right hand axis). The results for sucrose and glucose are essentially identical. The increase in d spacing is consistent with the presence of sugar between bilayers. The fact that glucose and sucrose give identical results (when plotted as d vs. number of sugar rings/lipid) supports the idea that it is the volume of sugar, rather than the specific nature of the sugar, that determines its effect on the membrane [20].

Figure 2c shows the effects of sucrose on DPPC in the fluid phase at 70 °C. The effects are similar to the gel phase, with some small differences: first d increases much less with increasing sugar ratio than is the case in the gel phase; second there is more scatter in the values of the average chain-chain spacing, due to the broad nature of the reflection in the fluid phase (see figure 1). However, again there is no effect of the sugar on the chain-chain spacing.

Looking more closely at the wide angle chain-chain spacing, for the samples in the gel phase (at 20 °C), the wide angle reflection was 4.15±0.2 Å, regardless of the type or concentration of sugar present. For DPPC in the fluid phase (at 70 °C) the average chain separation was 4.51±0.02 Å, again, regardless of the concentration of sucrose present. The results shown here are consistent with previous experiments on DPPC without sugar, where values of ~4.2 Å and ~4.6 Å are found in the gel and fluid phases respectively (e.g. [36, 38]). Experiments at temperatures between 20 °C and 70 °C show similar trends (data not shown). The data shown are for sucrose:lipid ratios up to 0.5:1, and glucose ratios up to 1:1. In both cases the maximum sugar ratio used was 1 sugar ring per lipid. As has been shown previously [21] concentrations beyond this level do not provide any additional effect on the membrane phase transition temperatures.

In order to apply the HFE model, we need to make an estimate of how much sugar/water is excluded from between the bilayers. To do this we can make two independent estimates of the area per lipid. For the gel phase lipid, Eq. 10 can be used to calculate the average area per lipid chain, giving a value of $a_c = 20\pm0.2$ Å^2. (For the fluid phase, where the chain packing is not hexagonal, Eq. 10 does not strictly apply, but gives an indicative value of $a_c = 23.4\pm0.6$ Å^2).

In principle, Eqs. 7-9 can then be used to calculate the area per lipid head group a, and Eq. 4 can be used to calculate the inter-bilayer separation d_w. The application of these equations assumes that all of the sugar and water lie between the bilayers. However, recent neutron scattering experiments have shown that at high concentrations sugars are partially excluded during dehydration [22], confirming previous circumstantial evidence [6]. In order to quantify the effects observed here, it is necessary to ascertain to what extent partial exclusion is taking place in these systems.

As DPPC has been so well characterized in the absence of sugar, and as we have shown that the presence of sugar has no effect on the chain packing, we can use this information to make estimates of both a and d_w. In the gel phase, Eq. 11 relates the angle of tilt of the lipid chains relative to the bilayer normal, θ_t, to the chain area a_c and the area per headgroup a. The best estimated value for θ_t for fully hydrated DPPC in the gel phase is that of Sun et al. [39], who determined a value of $\theta_t = 31.6°$. It is known that the value of θ_t decreases as the water content is reduced [40], so for the purposes of the discussion here, this value can be regarded as an upper limit. Using the calculated value of a_c and Eq. 11 therefore leads to an upper limit to the average area per lipid of $a_{max} = 47$ Å^2 (averaged over all samples). This value is consistent with the fully hydrated value of a=47.9 Å^2 [30].

Alternatively, by assuming there is no phase separation, and that all the sugar and water are between the lamellae, we can use the measured mass fractions of the components, the known value of $\bar{v}_L$ (0.939 ml/g in the gel phase [30]) along with Eqs. 7-9, to calculate the area a_{grav}. Figure 3 shows both a_{grav} and a_{max} for the samples in the gel phase. Although there is some scatter in the data, a_{grav} is lower than a_{max} by a small amount (<10%) in all cases, and the difference decreases as the sugar ratio increases. These results suggest that there may be some phase separation, but it is relatively small at these sugar ratios. The error introduced by the assumption that all the sugar and water is between the bilayers, in the calculation of d_w in Eq. 4 is less than 10%.

Having demonstrated that exclusion is a relatively small effect for these systems, the experimental data on the effects of sugars on the membrane transition temperature can now be compared with the theory. Figure 4 shows experimental transition temperatures measured previously [21] as a function of water:lipid ratio, along with the results of the model (both the iterative solution (solid lines) and the approximation (Eq. 6 - dashed lines).

The model gives give good agreement with experimental results, with the iterative solution slightly higher than the approximation. With the exception of the hydration force parameters, small changes in the values for the parameters do not significantly affect the results. Changing these parameters affects the absolute position and shape of the curve without sugar, but does not qualitatively change the effect of the sugars. Further refinement of the hydration force parameters would help to determine the accuracy of the model.

Discussion

Figure 4 shows that the HFE quantitatively explains the effects of low concentrations of sugars on membrane transition temperatures over a wide hydration range. As discussed in a previous paper [21], the model is quantitatively valid only if all the sugar is located in the inter-membrane layers. For sugar/lipid ratios higher than 0.2:1, partial exclusion can occur [21, 22]. However, the results presented here have shown that this exclusion is relatively small for the samples measured here, allowing the model to be applied. More precise data regarding the extent of exclusion would be needed to enable the model to be used at higher sugar:lipid ratios.

Previously we have shown that the maximum effect of sugars on reducing the transition temperature is achieved at a ratio of ~1 sugar rings per lipid [21], so the results presented here cover the most relevant range of sugar:lipid ratios. The evidence presented here strongly suggests that the presence of sugars does not significantly affect the membrane structure during dehydration, in either the fluid or gel phase, except for changing the transition temperature via the Clausius-Clapeyron effect. This is indirect evidence that insertion of sugars between lipid head groups in the plane of the bilayer (as proposed in some versions of the WRH) is not responsible for their effects on membrane transition temperatures. The fact the effects of sucrose and glucose are almost identical

further supports this view, confirming previous studies showing the non-specific nature of the effect of a wide range of sugars [7, 41] and maltodextrins [8] on membrane phase transition temperatures. The WRH is not quantitative and so has no quantitative predictions to be compared with experiments. In contrast, the Hydration Forces Explanation is quantitative, and provides excellent agreement with experiment, as shown in figure 4.

Conclusions

In this paper we have demonstrated that the presence of sugars has no significant effect on lipid chain packing in either the gel or fluid phases, at any hydration. We have also shown that the lamellar repeat spacing increases monotonically with sugar concentration up to ratios of ~1 sugar rings per lipid, for both sucrose and glucose. The results show that exclusion of sugars from the inter-bilayer space is modest for the systems studied, allowing a comparison between experimental results and the Hydration Forces Explanation This comparison shows that, unlike the Water Replacement Hypothesis, the Hydration Forces Explanation quantitatively explains the effects of sugars on membrane transition temperatures, using only their non-specific volumetric properties.

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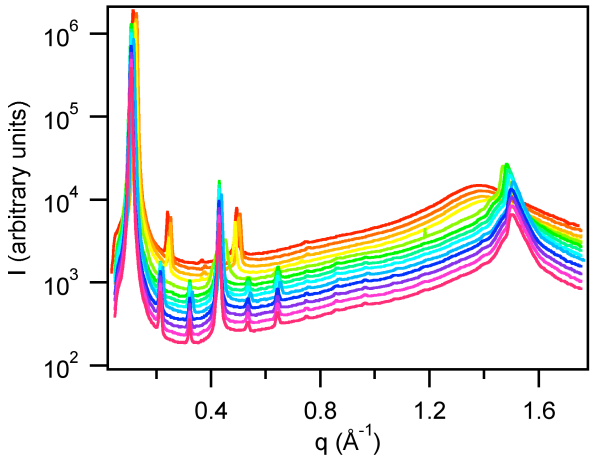

Figure 1: A typical example of a set of intensity versus scattering vector plots during cooling from 70 °C to 20 °C at a rate of ~15 °C/min (sucrose:DPPC 0.2:1,RH=13%). The transition from the fluid phase (70 °C, back of figure) to the gel phase (20 °C, front of figure) is indicated by (i) an increase in the number of inter-lamellar reflections in the gel phase, indicating increasing order; and (ii) a change in character of the wide angle intra-lipid reflection from a broad fluid peak at q~1.4 $Å^{-1}$, to a sharper gel peak at q~1.5 $Å^{-1}$.

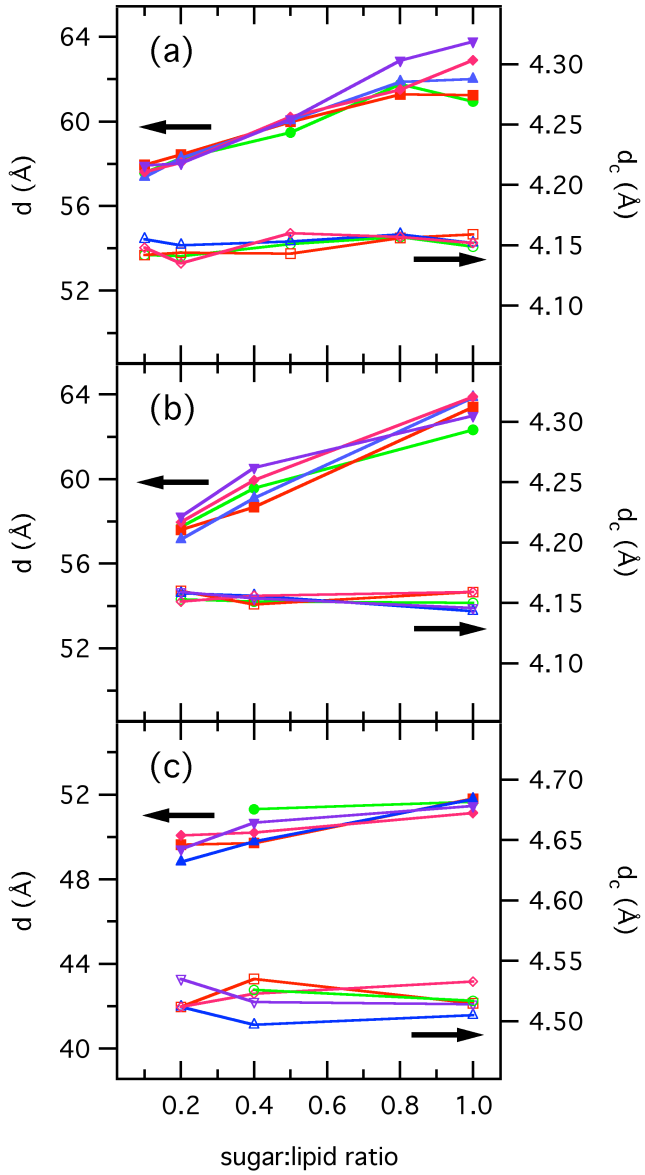

Figure 2: Shows the main repeat spacing (d) and the chain-chain spacing (d_c) for DPPC as a function of sugar:lipid ratio, for several values of relative humidity (RH): (a) 20 °C (gel phase) in the presence of sucrose; (b) 20 °C (gel phase) in the presence of glucose; and (c) 70 °C (fluid phase) in the presence of sucrose. The sugar:lipid ratio is the ratio of sugar rings to lipid molecules. The symbols are for RH values of: ~0.1% (circles); 5.5% (squares); 13% (up triangles); 32.5% (diamonds); 57.5% (down triangles). Filled symbols are the repeat spacing d (left axis) and open symbols are the chain-chain spacing d_c (right axis). Lines are a guide to the eye.

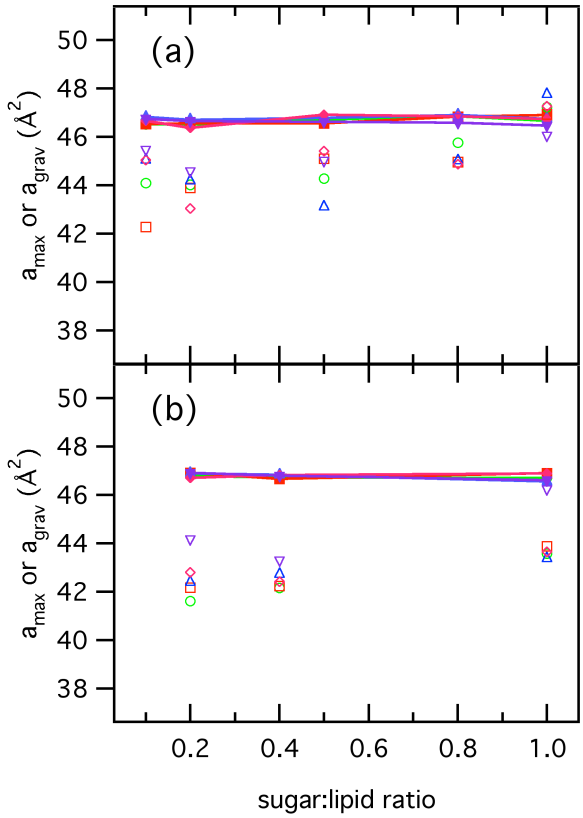

Figure 3: Shows two estimates of the area per lipid, a_{max} (closed symbols) and a_{grav} (open symbols), as functions of sugar:lipid ratio: (a) 20 °C (gel phase) in the presence of sucrose; (b) 20 °C (gel phase) in the presence of glucose. Symbols are as in figure 2. Lines are a guide to the eye.

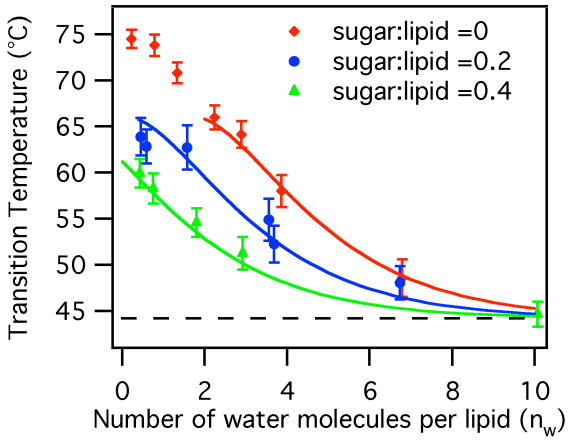

Figure 4: Shows the transition temperatures measured using DSC [21] for three sucrose/lipid ratios (shown in the legend). The theoretical predictions, using literature values are given by the bold lines. The horizontal dashed line represents the fully hydrated transition temperature.