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ARTICLE

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Exclusion of maltodextrins from phosphatidylcholine multilayers during dehydration: effects on membrane phase behaviour

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Abstract The effect of increasing solute size on phosphatidylcholine phase behaviour at a range of hydrations was investigated using differential scanning calorimetry. Dehydration of phospholipid membranes gives rise to a compressive stress within the bilayers that promotes fluid-to-gel phase transitions. According to the Hydration Forces Explanation, sugars in the intermembrane space minimize the compressive stress and limit increases in the fluid-gel transition temperature, T_m, by acting as osmotic and volumetric spacers that hinder the close approach of membranes. However, the sugars must remain between the bilayers in order to limit the rise in T_m. Large polymers are excluded from the interlamellar space during dehydration and do not limit the dehydration-induced rise in T_m. In this study, we used maltodextrins with a range of molecular weights to investigate the size-exclusion limit for polymers between phosphatidylcholine bilayers. Solutes with sizes ranging from glucose to dextran 1000 limited the rise in lipid T_m during dehydration, suggesting that they remain between dehydrated bilayers. At the lowest hydrations the solutions vitrified, and T_m was further depressed to about 20 °C below the transition temperature for the lipid in excess water, T_o. The depression of T_m below T_o occurs when the interlamellar solution vitrifies between fluid phase bilayers. The larger maltodextrins, dextran 5000 and 12,000, had little effect on the T_m of the PCs at any hydration, nor did vitrification of these larger polymers affect the lipid phase behaviour. This suggests that the larger maltodextrins are excluded from the interlamellar region during dehydration.

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Abbreviations *DMPC* 1,2-dimyristoylphosphatidyl-choline · *dp* degree of polymerization · *DSC* differential scanning calorimetry · M_n number average molecular weight · M_w average molecular mass · *POPC* 1-palmitoyl-2-oleoylphosphatidylcholine · T_g glass transition temperature · T_m lipid gel-to-fluid phase transition temperature · T_o gel-to-fluid phase transition temperature of fully hydrated lipid

Introduction

Dehydration of membrane-rich systems such as cells and liposomes can cause physical stresses that result in demixing of membrane components, fluid-gel phase transitions, and formation of non-bilayer phases (Wolfe 1987; Wolfe and Bryant 1999). These changes in the membrane can alter membrane permeability, which damages the cells or liposomes by allowing their contents to leak to the external environment (e.g. Steponkus and Webb 1992; Sun et al. 1996). Physical stresses in the dried membranes arise from the increasing importance of strong hydration forces near membranes and other hydrophilic surfaces that are brought into close apposition (LeNeveu et al. 1976; Rand and Parsegian 1989). As membrane-rich systems are dehydrated, water is removed from the interlamellar space, and membranes come into close approach. The suction needed to overcome the hydration force between opposing membranes leads to a compressive stress in the plane of the bilayer, which favours the fluid-gel phase transition (Wolfe 1987; Wolfe and Bryant 1999). Dehydration thus increases the fluid-gel transition temperature, T_m.

Numerous studies have documented the ability of polyhydroxy compounds, such as sugars, to hinder the rise in T_m during dehydration of liposomes and multilamellar vesicles (e.g. Crowe and Crowe 1988; Crowe et al. 1992; Koster et al. 1994, 2000). The Hydration Forces

Explanation (HFE) states that nonspecific osmotic and volumetric effects are responsible for the observed membrane phase behaviours in the presence of sugars (Bryant and Wolfe 1992; Bryant et al. 2001; Koster et al. 1994, 2000; Wolfe and Bryant 1999). According to the HFE, the presence of small solutes between the membranes during dehydration hinders the close approach of membrane bilayers, reducing the hydration force between the surfaces, and thereby reducing the mechanical stresses that arise when the bilayers are in closer proximity (Bryant and Wolfe 1992; Bryant et al. 2001; Wolfe and Bryant 1999). The increased osmotic pressure generated by the solutes between the bilayers limits the removal of water from between the membranes, keeping the bilayers further apart. The volumetric effects derive from the fact that the solutes themselves have a molecular volume that keeps the membranes separated. Thus, stresses and strains in membranes at low hydrations may be diminished by interlamellar solutes, including sugars, and the effects that favour the transition to the gel phase are thereby reduced.

An additional effect is observed when the interlamellar solution vitrifies during dehydration or cooling. Koster et al. (1994, 1996, 2000) first noted that vitrified sugar solutions depress the T_m of many dry phosphatidylcholines below T_o, the fluid-gel transition temperature of the fully hydrated lipid. Zhang and Steponkus (1996) proposed that this effect results from the mechanical resistance of the glass to the area change associated with the fluid-gel transition, and calculations of membrane tension in the presence of vitrified solutions support this idea to first order (Koster et al. 2000).

In order to exert these various effects on T_m, however, the solutes must remain between the bilayers. Large polymers that do not have strong chemical interactions with the membrane surface may be excluded from the interlamellar space during dehydration and, thus, would not limit the dehydration-induced rise in T_m, nor would vitrification of these polymers depress T_m below T_o (Bryant et al. 2001; Koster et al. 2000, 2001; Wolfe and Bryant 1999). Based on the measured effects of solutes on lipid T_m, we suggested that disaccharides having a molecular weight of 342 remained between bilayers during dehydration of multilamellar vesicles, while polymers having a molecular weight of 40,000 were excluded (Koster et al. 2000). In a related study, Suzuki et al. (1996) showed that maltodextrins up to a molecular weight of 1260 were able to fit between freeze-dried liposomes and prevent an increase in T_m; however, they did not test larger solutes.

The objective of the current study was to investigate the size-exclusion limit of polymers between membranes in a dehydrated multilamellar system. To test this, phosphatidylcholine (PC) bilayers were mixed with solutions containing linear maltodextrins with molecular weights ranging from 342 to 12,000. After the lipid-solute mixtures were dried to a range of hydrations, differential scanning calorimetry (DSC) was used to measure phospholipid phase transitions and glass transitions of the solutions, and the location of the solutes—between or excluded from the interlamellar space—was inferred from T_m. The results suggest that exclusion of linear glucose polymers begins to occur between M_w of 1000 and 5000, with the smaller solutes able to stabilize dry membranes through both osmotic effects and vitrification, while the larger solutes have very little effect on T_m.

Materials and methods

Materials

The phosphatidylcholines POPC (1-palmitoyl-2-oleoylphosphatidylcholine) and DMPC (1,2-dimyristoylphosphatidylcholine), having stated purities >99%, were obtained as solutions in chloroform from Avanti Polar Lipids (Alabaster, Ala., USA) and were used with no further purification. Glucose was purchased from Sigma (St. Louis, Mo., USA) and the solutes maltose, maltotriose, dextran 1000, dextran 5000, and dextran 12,000 were obtained from Fluka (Buchs, Switzerland). Phospholipids and solutes were mixed in 1:1 (POPC) and 2:1 (DMPC) solute:lipid weight ratios.

Methods

Samples were prepared as previously described (Koster et al. 2000) with the following modifications. Phospholipids were dried under a stream of N_2 at 40 °C, then were resuspended in solutions of water/methanol (1:1, v/v) containing the desired solute. As controls, samples of each phospholipid were also resuspended in water/methanol (1:1, v/v) without added solute. The addition of methanol to the dissolved solutes is believed to help them to distribute more evenly among the multiple bilayers created when the lipids are resuspended. To ensure that the use of methanol did not significantly affect the samples mixed with dextrans by causing their precipitation, replicate samples of DMPC were resuspended in aqueous solutions of the dextrans (1000, 5000, and 12,000) with no added methanol. No significant differences were detected between the samples prepared with and without methanol, so data for both sets of samples were combined (Figs. 3, 4, 5, 6). All suspensions were mixed by at least 10 repeated cycles of freezing in liquid N_2 and thawing at 45 °C, sonication, vortex mixing, and centrifugation.

The phospholipid-solute suspensions were dried overnight in a vacuum oven with P_2O_5 at 60 °C to remove the methanol and water, then were resuspended in purified water. After repeated mixing via freeze-thawing, sonication, and centrifugation, as described above, aliquots of the lipid-solute suspension were transferred into pre-weighed DSC volatile sample pans. Samples were incubated at 24 ± 1 °C over saturated salt solutions for periods ranging from 1 to 10 weeks to obtain a range of hydrations, as previously described (Koster et al. 2000). The pans were sealed and reweighed before calorimetry. After calorimetry, dry weights were measured by puncturing the lids of the sample pans and drying the pans for at least 16 h at 70 °C in a vacuum oven with P_2O_5. Previous studies (Koster et al. 1994) indicated that no further weight loss occurs in samples after this time under these drying conditions. It is possible that some residual water remains in the oven-dried samples, particularly those containing the maltodextrins; however, removal of this water would require elevated drying temperatures at which there is increased risk of sample decomposition. Sample hydrations were calculated based on the weights before and after oven drying and are expressed as g/g on a dry weight basis.

DSC was performed using a Perkin-Elmer DSC-7, and thermograms were analyzed using either the Pyris software or the software for the model 1020 controller provided by Perkin-Elmer. The DSC apparatus was calibrated for onset melting temperature and enthalpy using indium, with heptane used as a second tem-

perature standard. In typical experiments, samples at hydrations greater than about 0.15 g/g were cooled at 200 °C/min to −100 °C and allowed to equilibrate. The samples were then scanned at 10 °C or 20 °C/min while heating to 90 °C, cooling to −100 °C, and rewarming to 90 °C. Drier samples were heated to higher temperatures, as there was less danger that they would cause the hermetically sealed pans to rupture above 100 °C. In many cases, when the first and second heating scans differed, additional cooling and heating cycles were monitored to ensure that the thermal behaviour of the sample stabilized after the initial heating. The relatively rapid scanning rates were used to permit clear glass melting transitions to be recorded (Koster 1991; Koster et al. 1994, 2000). Although some hysteresis in measured T_m values between heating and cooling scans may result, comparisons among the data sets are not affected because all the data reported were taken from heating scans.

T_m was recorded as the peak maximum temperature of the lipid chain-melting transition. Depending on the hydration and sample history, the phase with frozen chains may be the gel phase or one of at least two crystalline phases (e.g. Handa et al. 1985). The exact nature of this phase cannot be determined by DSC alone, and as it is not critical to the arguments presented here, for convenience we adopt widely used terminology and simply call this the gel-fluid transition. Although onset temperatures are often used to describe both lipid and glass transitions, the presence of overlapping transitions in numerous samples made it difficult to determine onset temperatures with precision. Therefore, T_g was taken as the midpoint temperature of the glass-melting transition. In a few samples, the lipid gel-to-fluid transition had more than one peak maximum; for example, at hydrations greater than about 0.15 g/g, POPC typically exhibits a double-peaked endotherm as it melts. To keep the graphs simple and because of its proximity to the onset of the endotherm, only the lower of the T_m values was graphed in these cases. In samples at hydrations where the lipid and glass melts converged, thermograms sometimes displayed multiple transitions, which are presumed to reflect heterogeneity within samples that may contain some paucilamellar and unilamellar vesicles in addition to the multilamellar arrays. For these samples, minor transitions are not displayed to keep the graphs simple. For each phospholipid, T_o designates the T_m of the pure lipid in excess water and is used as a reference.

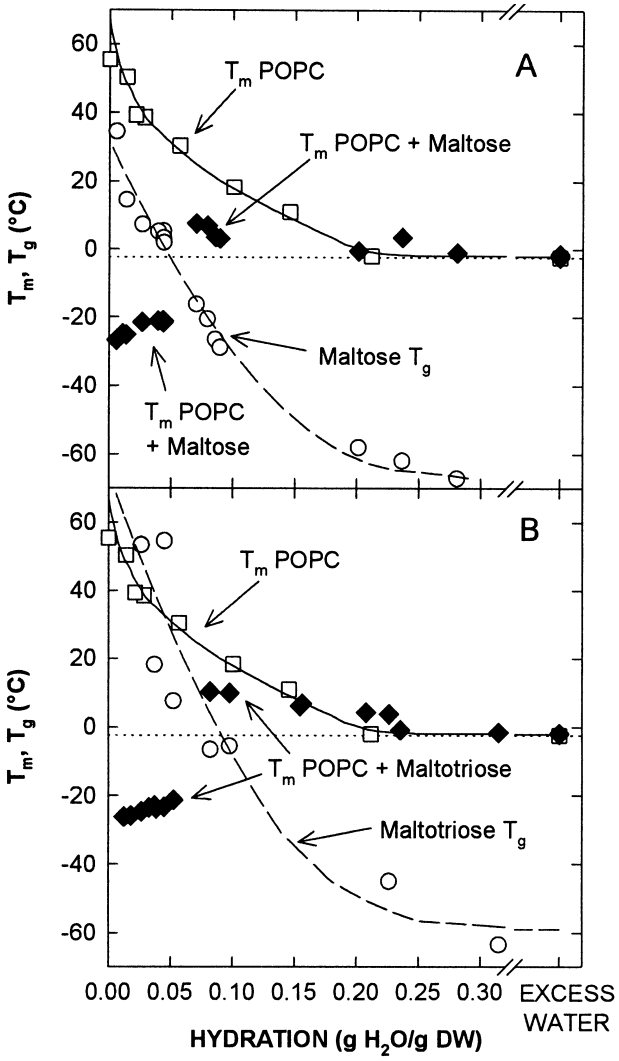

Fig. 1 Transition temperatures as a function of hydration for POPC and (**A**) maltose and (**B**) maltotriose. *Open squares* represent T_m of the lipid in the absence of sugar; the *solid line* is a guide to the eye and for simplicity is shown in subsequent graphs instead of the open squares. *Filled diamonds* represent T_m of the lipid in the presence of sugar. *Open circles* represent the midpoint T_g of the sugar-glass transition in the presence of the lipid, and the *dashed line* is an aid to the eye. The *dotted line* designates T_o, the lipid gel-to-fluid phase transition temperature at full hydration. Hydration values were calculated based on dry weights obtained after drying samples at 70 °C in vacuo with P_2O_5

Results and discussion

The phase diagrams in Figs. 1, 2, 3 and Fig. 5 show the effects of glucose polymers of increasing molecular weight on the gel-to-fluid phase transition temperatures, T_m, of POPC and DMPC at a range of water contents. In both cases, T_m of the pure phospholipid increased with dehydration, as has been previously reported (e.g. Chapman et al. 1967; Collins et al. 1990; Koster et al. 1994, 2000; Lynch and Steponkus 1989). For pure POPC (Fig. 1A, open squares), the gel-to-fluid transition temperature for the lipid in excess water (T_o) was −2.3 °C, and T_m rose to 55.5 °C in samples dried over P_2O_5 in vacuo. For pure DMPC, T_o was 26.4 °C, while dehydration progressively increased T_m to a value of 74.7 °C in samples dried over P_2O_5 (Fig. 2A, open squares). Higher values of T_m have been measured for pure unhydrated POPC (68 °C) and DMPC (87.5 °C) (Bryant et al. 1992; Handa et al. 1985; Lynch and Steponkus 1989), which suggests that the aqueous samples dried over P_2O_5 contained some residual water, as discussed in the Methods section above. The glass transition temperatures (T_g) of the solutions also increased as the solutions were dried (Figs. 1, 2, 3 and Fig. 5, open circles), as has been previously described for a variety of sugars and maltodextrins (Green and Angell 1989; Koster 1991; Koster et al. 2000; Slade and Levine 1995). Values of T_g depended both on sample hydration and on the M_w of the solute, with larger solutes generally producing higher T_g values at any given hydration. It should be noted that, in these figures, hydration is expressed in terms of total sample dry weight; therefore, the T_g values shown for the solute mixed with the lipid are lower than values of T_g would be for the pure solute if plotted on the same figure. This apparent discrepancy

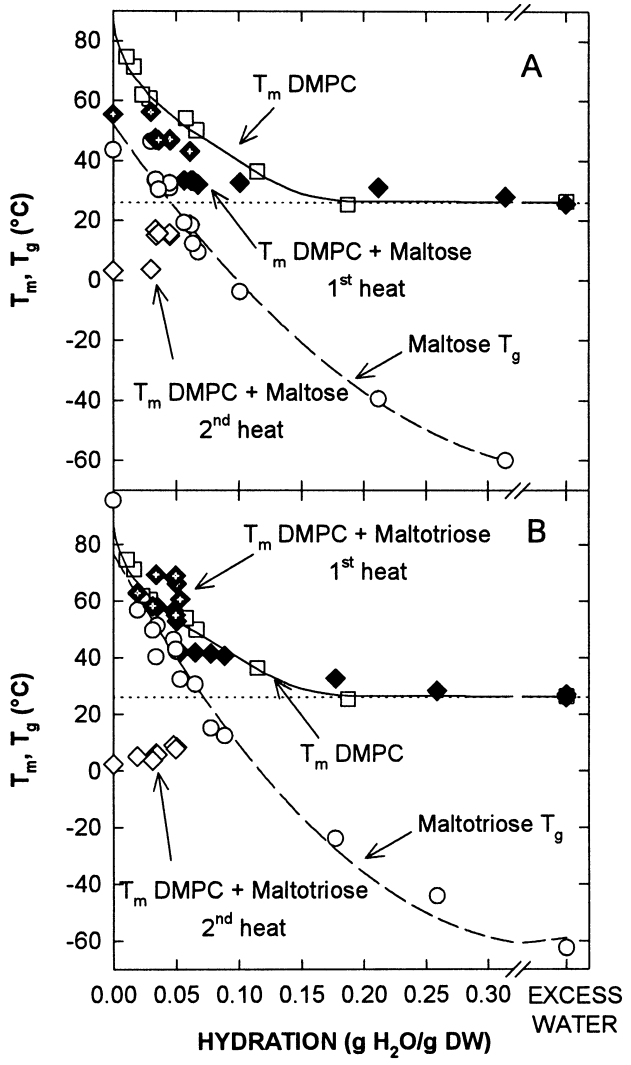

Fig. 2 Transition temperatures as a function of hydration for DMPC and (**A**) maltose and (**B**) maltotriose. *Open squares* represent T_m of the lipid in the absence of sugar; the *solid line* is a guide to the eye and for simplicity is shown in subsequent graphs instead of the open squares. *Filled diamonds* represent T_m of the lipid in the presence of sugar, taken from the first heating scan. When first and second scans differed, T_m from the first scan is shown as a *filled dotted diamond*, while *open diamonds* represent T_m of the lipid in the presence of sugar from the second and subsequent heating scans. *Open circles* represent the midpoint T_g of the sugar-glass transition in the presence of the lipid and the *dashed line* is an aid to the eye. The *dotted line* designates T_o, the lipid gel-to-fluid phase transition temperature at full hydration. Hydration values were calculated based on dry weights obtained after drying samples at 70 °C in vacuo with P_2O_5

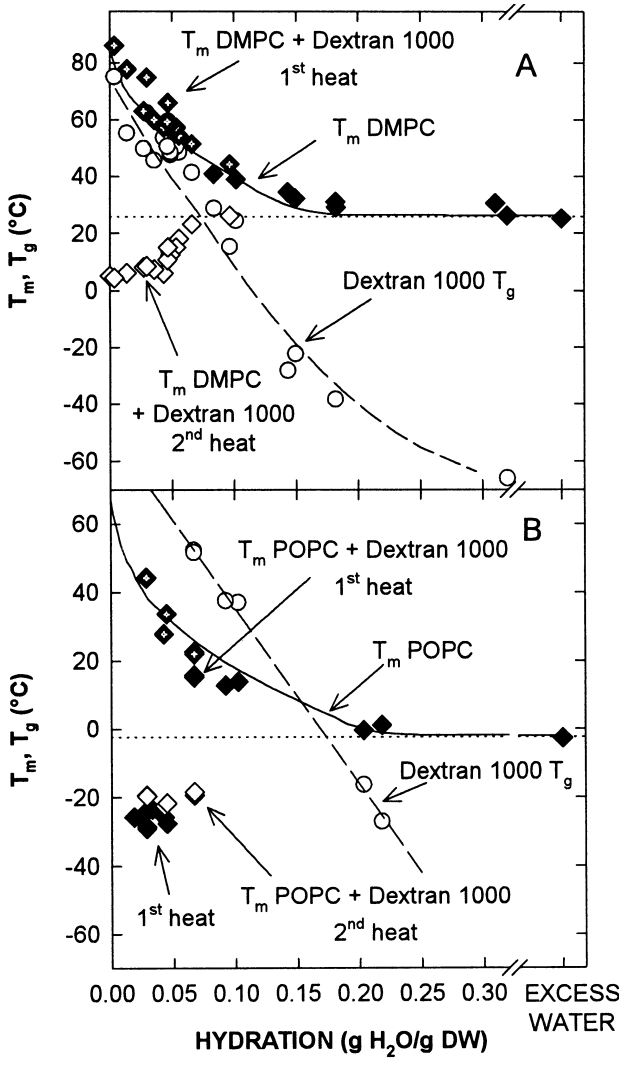

Fig. 3 Transition temperatures as a function of hydration for dextran 1000 with (**A**) DMPC and (**B**) POPC. For both lipids, the *solid line* designates the T_m of the pure lipid. *Filled diamonds* represent T_m of the lipid in the presence of sugar, taken from the first heating scan. When first and second scans differed, T_m from the first scan is shown as a *filled dotted diamond*, while *open diamonds* represent T_m of the lipid in the presence of sugar from the second and subsequent heating scans. *Open circles* represent the midpoint T_g of the sugar-glass transition in the presence of the lipid and the *dashed line* is an aid to the eye. The *dotted line* designates T_o, the lipid gel-to-fluid phase transition temperature at full hydration. Hydration values were calculated based on dry weights obtained after drying samples at 70 °C in vacuo with P_2O_5

results because, on a weight basis, the maltodextrins hydrate to a greater extent than do the lipids.

The presence of maltodextrins near the lipid multilayers during dehydration had several effects on T_m, as shown in Figs. 1, 2, 3, 4, 5, 6. These effects depended on the size (M_w) of the solute, whether the solution vitrified at a temperature above the gel-to-fluid transition temperature of the lipid, and whether the lipid was in the fluid or gel phase during dehydration. We address these different effects in turn.

The effect of small solutes on T_m

The presence of the small solutes maltose (dp = 2, M_w = 342) and maltotriose (dp = 3, M_w = 504) limited the dehydration-induced increase in the T_m of the phosphatidylcholines. POPC was in the fluid phase during dehydration and demonstrates clearly the principal effects of small solutes on the phase behaviour of membranes, as described by the HFE (Bryant et al. 2001; Koster 2001; Koster et al. 2000; Wolfe and Bryant 1999). This can be seen for POPC in the presence of maltose

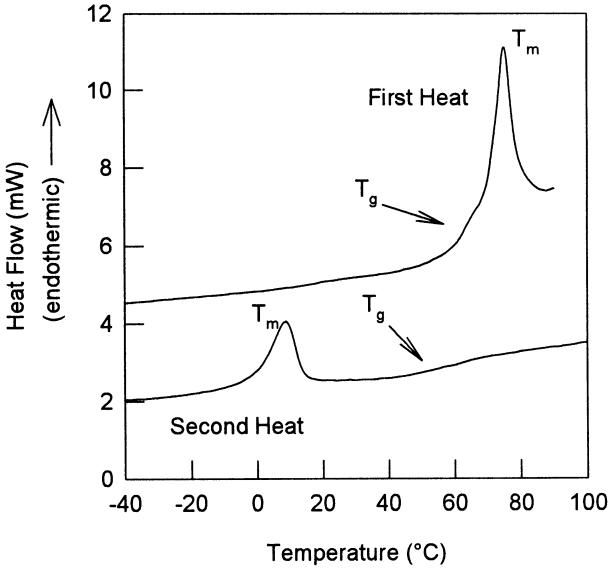

Fig. 4 DSC thermogram showing the effects of a vitrified dextran 1000 solution on the T_m of dehydrated DMPC. The first heating scan shows a broad endotherm between about 60 °C and 85 °C, which appears to contain the glass melting transition (the endothermic shift in the baseline, T_g) followed by the peak that signifies the melting of the lipid (T_m). Immediate cooling of the molten sample caused the solution to vitrify while the lipid was still in the fluid phase and led to the depression of T_m to 6 °C, as seen in the second heating scan. T_g in the second heating scan is about 10 °C lower than it was during the first scan. Subsequent scans of the sample during the same day did not differ from the second heating scan; however, longer periods of annealing sometimes led to the reappearance of the high-temperature peak seen in the first scan and the disappearance of the low-temperature peak

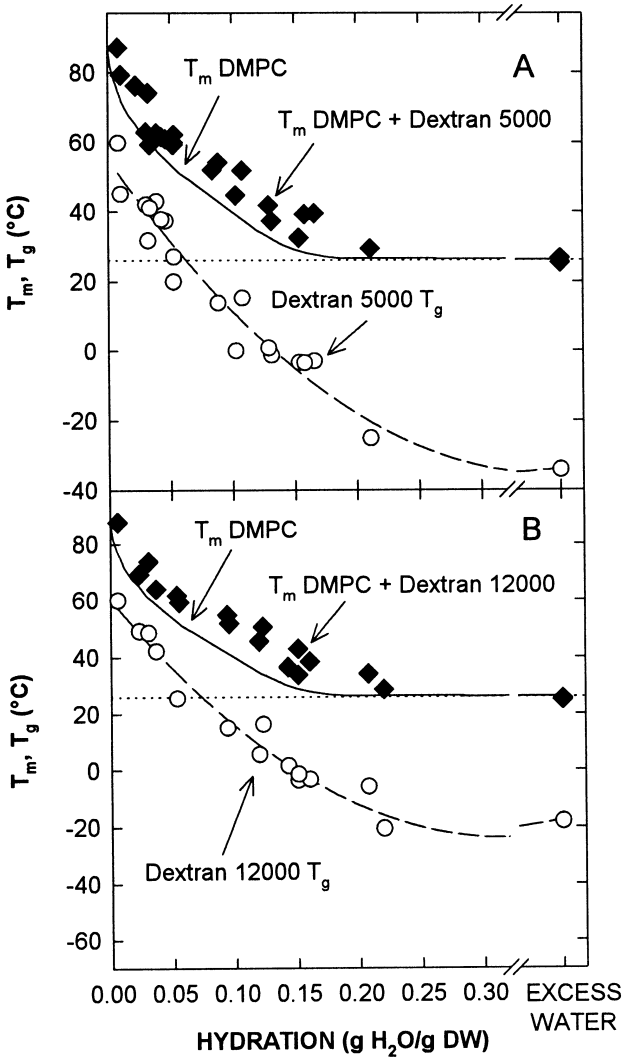

Fig. 5 Transition temperatures as a function of hydration for DMPC and (**A**) dextran 5000 and (**B**) dextran 12,000. The *solid line* designates the T_m of the pure lipid, and *filled diamonds* represent T_m of the lipid in the presence of sugar, taken from the first heating scan. *Open circles* represent the midpoint T_g of the sugar-glass transition in the presence of the lipid and the *dashed line* is an aid to the eye. The *dotted line* designates T_o, the lipid gel-to-fluid phase transition temperature at full hydration. Hydration values were calculated based on dry weights obtained after drying samples at 70 °C in vacuo with P_2O_5

(Fig. 1A) or maltotriose (Fig. 1B). Moderate dehydration (to water contents greater than about 0.05 g/g) in the presence of the sugars led to a small elevation in T_m (filled diamonds) above T_o for the fully hydrated lipid. In these samples, T_g of the sugar solution (open circles, dashed line) was less than T_o of the lipid. The extent of the increase in T_m in the presence of the sugars was less than that for pure POPC (open squares, solid line) dried to similar water contents. This effect can be ascribed to the nonspecific osmotic and volumetric effects of small solutes that remain between the bilayers during dehydration and limit the close approach of the hydrophilic membrane surfaces (Bryant and Wolfe 1992). When the surfaces are kept apart during drying, compressive stress within the membranes does not increase significantly, and T_m does not rise much above T_o (dotted lines in Figs. 1, 2, 3 and Fig. 5). This agrees with the behaviour observed for a range of lipids and small solutes (Koster et al. 1994, 1996, 2000; Zhang and Steponkus 1995).

At lower water contents, T_g of the solution increased above T_o[1] during dehydration, and the T_m of POPC was therefore depressed to approximately 20 °C below T_o (Fig. 1A and B, filled diamonds). This effect is observed when the interlamellar solution vitrifies at the surface of a fluid-phase membrane, and is believed to result from the mechanical resistance of the solid glass to the fluid-gel phase transition (Koster et al. 2000; Zhang and Steponkus 1996). During the transition from the fluid to the gel phase, the surface area of the membrane must decrease; however, the presence of the glass at the surface resists this contraction. The membrane must therefore be cooled to a temperature below T_o until the tendency of the lipid to contract is sufficient to overcome the resistance of the glass. Effectively, the presence of the vitrified solution at the membrane surface exerts a ten-

[1]Note that we use T_o as an unambiguous reference point. However, it is likely that the extrapolated T_m of the lipid-sugar mixture at that hydration is the critical temperature, and that T_g must be above this temperature for the glassy solution to exert an effect on the lipids

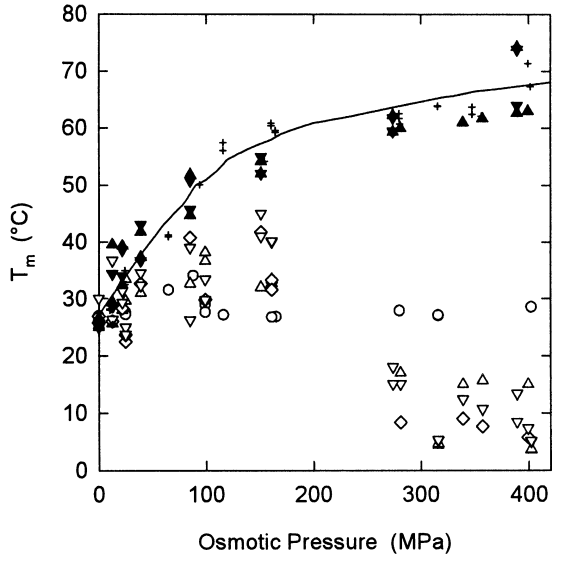

Fig. 6 Fluid-gel transition temperatures as a function of osmotic pressure for pure DMPC (*plus signs*), DMPC dried with glucose (*circles*), maltose (*up open triangles*), maltotriose (*diamonds*), dextran 1000 (*down open triangles*), dextran 5000 (*up filled triangles*), and dextran 12,000 (*down filled triangles*). The *solid line* represents the T_m of pure DMPC and is shown as an aid to the eye. For those samples in which T_m was depressed by the presence of the glass during second heating scans, only the depressed values of T_m are shown. Data for DMPC dried in the presence or absence of the solutes at 37 °C are also shown on this graph, and are consistent with the other data

sion in the bilayer that depresses T_m below the value for the lipid in excess water (Koster et al. 2000). As previously observed (Koster et al. 2000), the depression of T_m that results from glass formation at the surface of a fluid phase membrane did not exhibit significant hysteresis; T_m was depressed to a value below T_o on the first and subsequent scans (Fig. 1A and B).

By contrast, DMPC was in the gel phase during dehydration. At hydrations greater than approximately 0.05 g/g, T_g for the maltose and maltotriose solutions (Fig. 2, open circles) was lower than T_o, and the presence of the sugars between the bilayers limited the rise of T_m (filled diamonds) during dehydration, as described above for POPC. However, at lower water contents, T_g for these solutions was greater than T_o for DMPC, and somewhat different behaviours were observed. Specifically, a hysteresis was observed in that the apparent T_m of DMPC differed between first and subsequent scans in the DSC. During the first heating scan, T_m of the lipid was elevated above T_g of the solution (Fig. 2, filled dotted diamonds), while T_m in subsequent scans was depressed to a value below T_o (Fig. 2, open diamonds). The hysteresis observed in these samples resulted from vitrification of the interlamellar solution at the surface of gel phase membranes (Koster et al. 2000; Zhang and Steponkus 1996). In order to undergo the transition from the gel to the fluid phase, the surface area of phospholipids must expand; however, the presence of the glass mechanically resists this expansion. During heating of the sample, once the glass melted, the lipid was free to expand, and T_m (Fig. 2, filled dotted diamonds) was thus elevated above T_g. During subsequent cooling in the DSC, the solution vitrified while the lipids were still in the fluid phase, and as a result, T_m was depressed to a value below T_o (Fig. 2, open diamonds).

The same hysteresis of T_m was observed by Suzuki et al. (1996) after freeze-drying DPPC (dipalmitoylphosphatidylcholine) with maltodextrins ranging from glucose through maltoheptaose (dp = 7; M_w = 1280). In their study, T_m of the dried lipid in the absence of solute was 105 °C. After drying in the presence of glucose (dp = 1; M_w = 182), T_m was 40.5 °C (a value equal to the T_o for DPPC) in first and subsequent scans. However, after freeze-drying in the presence of the larger maltodextrins, the hysteresis of T_m was observed. The DPPC T_m was elevated to temperatures ranging from 65 °C to 82 °C during the first heating scan, while subsequent heating scans showed T_m depressed to about 27 °C (Fig. 4 of Suzuki et al. 1996). These data agree with those published by Koster et al. (2000) and those shown in Figs. 2 and 3 for gel phase lipids dried with glass-forming solutions. The effects reported by Suzuki et al. (1996) may be explained as follows: the T_m of DPPC dried with glucose was equal to T_o because the osmotic and volumetric properties of the solute limited the close approach of the liposomes. No depression of T_m below T_o occurred because T_g of dried glucose is less than T_o of the lipid. For the larger solutes, however, T_g of the dried solutions is equal to or greater than the T_o of DPPC. After drying in the gel phase, T_m of DPPC was elevated by the presence of the glass. After heating through T_g on the first scan, the second scan shows a depression of T_m below T_o.

The effect of increasing solute size on T_m

The phase transition data for the phosphatidylcholines dried with maltose and maltotriose suggest that sufficient quantities of these small solutes remained between the bilayers to affect the hydration force between membrane surfaces during dehydration. When the lipids were mixed and dried with larger glucose polymers, however, different effects were observed (Figs. 3 and 5), suggesting that the larger polymers are partially or completely excluded from the interlamellar space during dehydration. Figure 3 shows the behaviour of DMPC and POPC dried with dextran 1000 (dp = 5–6). In samples at water contents greater than approximately 0.08 g/g for DMPC and 0.17 g/g for POPC, T_g of the solution was less than T_o of the lipid, and single lipid transitions were observed in the DSC scans. At lower sample hydrations, where T_g was greater than T_o, DMPC dehydrated with dextran 1000 (Fig. 3A) displayed a hysteresis of the lipid T_m similar to that observed for DMPC dried with the smaller sugars. During the first heating scan there was one large melting endo-

therm, spanning about 20 °C, that appeared to begin with a step-like endothermic glass melt, followed by a peak that was interpreted as the lipid melt (Fig. 4). The temperatures of these endotherms increased with decreasing hydration. During subsequent heating scans, the largest lipid melting endotherms were depressed to about 6 °C (Fig. 3A, open diamonds, Fig. 4), with some samples having additional smaller lipid melts at higher temperatures, presumably resulting from heterogeneity within the sample. The elevation of T_m during the first heating scan, followed by its depression in subsequent heating scans, suggests that the dextran 1000 solution vitrified between the DMPC bilayers during dehydration.

For POPC, which was dehydrated while in the fluid phase, there is evidence that dextran 1000 was partially excluded from the interlamellar space during dehydration. In dry samples (< 0.1 g/g) in which T_g was greater than T_o, the first heating scans sometimes had two apparent lipid melting endotherms: one at or slightly below the T_m for pure POPC at the same hydration (Fig. 3B, filled dotted diamonds), and one depressed about 20 °C below T_o (Fig. 3B, filled diamonds). This heterogeneity suggests that some of the POPC had excluded dextran 1000 during dehydration, while some of the lipid was still affected by the presence of the glassy dextran solution at the membrane surface. During heating in the DSC, the samples remixed, and the lipid T_m was depressed by about 20 °C below T_o in second and subsequent heating scans (Fig. 3B, open diamonds). The depression of T_m is consistent with previous observations of POPC in the presence of vitrified solutions of small sugars (Koster et al. 1994, 1996, 2000).

For those samples dried with dextran 1000 such that T_m was depressed below T_o (Fig. 3), DSC carried out after further incubation of 2–20 days at 22 °C revealed a gradual disappearance of the low-temperature T_m and reappearance of the high-temperature T_m, particularly for the DMPC samples (data not shown). This implies that the samples underwent a slow relaxation, during which the lipids presumably reverted to the gel phase, which is more stable at this temperature. A similar effect has been noted for gel phase DPPC incubated with trehalose and raffinose (Crowe et al. 1996), which were vitrified at the low water contents reported. Crowe et al. (1996) reported that the T_m of the dehydrated lipid in samples mixed with the sugars was initially elevated above T_o. After heating the samples, T_m was depressed to a value below T_o; however, further incubation at a temperature at which the lipid was in the gel phase caused the lipid T_m to rise back to its initial value. Thus, although the glassy state can confer mechanical stability to systems, it is a metastable state, and slow relaxations can occur. From the limited data available, the rates of these membrane relaxations seem to depend upon the viscosity of the glassy or rubbery solution near the membrane surface, which in turn depends upon the solution's composition, its hydration, and the incubation temperature.

The larger solutes dextran 5000 and dextran 12,000 had little effect on the phase behaviour of dehydrated DMPC, as shown in Fig. 5. Neither solute prevented the rise of T_m with dehydration; values for the lipid dried with the solutes (filled diamonds) were slightly greater than those for the pure dehydrated lipid at the same overall sample hydration (solid line)[2]. When the samples were heated and cooled in the DSC, a small (about 3–8 °C) decrease was seen in the lipid T_m (data not shown); however, this decrease did not lower T_m below that of the pure lipid. These data suggest that the larger polymers were completely excluded from the interlamellar space during dehydration and, therefore, did not directly limit the close approach of the membranes. Similar results were obtained for POPC dehydrated with dextran 5000 and dextran 12,000 (data not shown). As mentioned above, the T_g of dextrans 5000 and 12,000 dried with DMPC (Fig. 5, open circles) were lower than the T_g of the pure polymer (data not shown) at the same sample hydration, suggesting that the dextrans were preferentially hydrated over the lipids (on a weight basis) in these samples. The lowering of the lipid T_m during the second heating scan may result from the movement of some of the water from the dextran solutions into the lipid multilayers during melting of the sample.

Size-exclusion limit for polymers between dehydrated membranes

According to the HFE, solutes must remain between hydrophilic membrane surfaces in order to directly affect the hydration force between those surfaces (Bryant et al. 2001; Koster et al. 2000; Wolfe and Bryant 1999). Large solutes that are excluded from the narrow space between dehydrated membranes can indirectly affect the phase behaviour of the lipids by sequestering water and osmotically dehydrating the membranes. This well-known effect is the basis of the osmotic stress technique for dehydrating macromolecules and liposomes (Rand and Parsegian 1989). Previously, we demonstrated the osmotic dehydration of phosphatidylcholine multilayers by dextran 40,000 (3:1, w/w), which caused the elevation of the lipid T_m by about 5 °C in comparison to that of the pure lipid incubated at the same osmotic pressure (Koster et al. 2000). By contrast, solutes that remain between the bilayers during dehydration cause a reduction in the lipid T_m in comparison to that of the pure lipid at the same osmotic pressure. This is illustrated in Fig. 6, which shows the measured T_m for DMPC with each of the solutes as functions of osmotic pressure. This

[2]Note that the total dry weight contains 2 g of dextran per g DMPC. If we were to assume that water is evenly distributed throughout the sample, and graphed the data on the basis of water content per lipid, the T_m values for the DMPC dried with dextran would shift to the left and be similar to those of the pure lipid. However, we do not know the distribution of water, and the evidence, in fact, suggests that it is not evenly distributed; therefore, we graphed the data on their original hydration basis

graph shows that T_m of lipids dried with solutes of $M_w \leq 1000$ (open symbols) are lower than the T_m of the pure lipid at the same osmotic pressure (plus symbols), which suggests that these solutes were able to fit between dehydrated bilayers and hinder their close approach. The extent to which these solutes (glucose, maltose, maltotriose, and dextran 1000) limited the rise in lipid T_m during dehydration is more or less inversely related to the solute M_w. Because the samples were prepared at the same weight ratio of solute to lipid, the smaller solutes were present at larger molar ratios to the lipid, and thus would have a correspondingly greater osmotic effect (Bryant and Wolfe 1992; Koster et al. 2000).

By contrast, the larger polymers dextran 5000 and 12,000 did not fit between the dehydrated bilayers, even after repeated heating and cooling of the samples. As a result, T_m of the lipid dried with dextrans 5000 and 12,000 (Fig. 6, filled symbols) was roughly equal to that of the pure lipid (Fig. 6, plus symbols). Unlike the larger polymers studied previously (Koster et al. 2000), dextrans 5000 and 12,000 did not cause any significant osmotic dehydration of the membranes. In a related study using fructans, fructose polymers synthesized by many plant species during periods of drought, Hincha et al. (2000) reported that polymers with an average dp = 15 and average M_w between 1600 and 5000 were able to limit the dehydration-induced increase in the T_m of phosphatidylcholine membranes. By contrast, the much larger hydroxyethylstarch ($M_w = 200{,}000$) tested caused a slight increase in T_m over that of the pure dehydrated lipid (Hincha et al. 2000). This increase in T_m can be explained as a consequence of osmotic dehydration of the lipid by the excluded polymer, as used in the osmotic stress technique (Rand and Parsegian 1989).

In the present study, the apparent size-exclusion limit for maltodextrins between dehydrated phosphatidylcholine membranes is between 1000 and 5000 M_w which corresponds to a range between dp 6 and 27. These results agree with previous findings that polymers of glucose with dp = 7 (Suzuki et al. 1996) and fructose with average dp = 15 (Hincha et al. 2000) limited the dehydration-induced rise in phospholipid T_m, as described above. Interestingly, the size-exclusion range we established in this study encompasses the minimum linear chain length at which entanglement of dextrans has been reported to occur: dp≈18 and $M_n = 3000$ (Levine and Slade 1986). Intermolecular entanglement of the dextrans occurs when the polymers are sufficiently long and concentrated that they form supermolecular arrays. Meyuhas et al. (1996) reported that the entanglement of dextrans is an important factor leading to the aggregation of phosphatidylcholine liposomes, a phenomenon which is driven by the exclusion of polymers from the spaces between liposomes.

Exclusion of solutes from the interlamellar space depends on the molecular volume of the solute (Koster et al. 2000, Wolfe and Bryant 1999). The separation between multilamellar phosphatidylcholine membranes in excess water is about 2–3 nm, while that between dehydrated bilayers may be 1 nm or less (Rand and Parsegian 1989). Linear dextrans in solution form helices with six glucose residues and an average length of approximately 0.8 nm per turn (Wangsakan et al. 2001). Thus, dextran 1000 is the smallest of the polymers used in this study that would be able to form a helix, containing a single coil that should still be able to fit between dehydrated bilayers. Dextran 5000, with an average of 27 glucose residues, should form helices about 3.6 nm in length. It is reasonable to expect that dextrans of this size would be excluded from interlamellar spaces during dehydration. Thus, the large molecular volume of the dextrans with $M_w \geq 5000$, in combination with the likelihood of intermolecular entanglement of these polymers, easily explains their exclusion from the interlamellar region, and their inability to significantly affect the phase behaviour of the membranes during dehydration.

If these larger polymers are excluded from between multilayers, where do they go? One possibility is that they are excluded into bulk external domains containing only solute and water. However, if the samples were well mixed to start with, this scenario is unlikely at low hydration. An alternative possibility is shown schematically in Fig. 7, a cartoon showing the differences between a small non-excluded solute (Fig. 7A) and a larger excluded solute (Fig. 7B). If the solutes are small (Fig. 7A) they will mostly remain between the membranes, keeping the membranes apart and allowing them to remain in the fluid phase during dehydration. At low hydration there may be some exclusion into domains, but there will always be a concentration between the membranes.

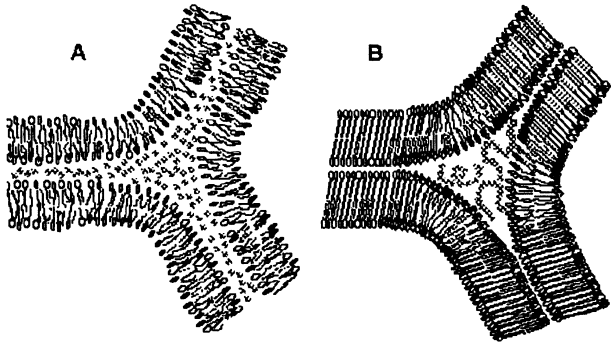

Fig. 7A, B Cartoon showing schematically how exclusion of large solutes might occur during dehydration. **A** shows a region of dehydrated membranes with small solutes at a temperature just above T_o. Many of the solutes remain between the bilayers, maintaining the membrane separation, and keeping the membranes in the fluid phase. It is possible that some solute is excluded as shown, but the protective effects of the small solutes will persist as long as some remain between the membranes. **B** shows a similar region for a larger solute that is excluded from between the membranes during dehydration. Such solutes would be concentrated into small volumes so that the most of the membrane surfaces are not separated by solutes. Consequently, the membrane separation is reduced, the compressive membrane stress is increased, and the lipids may undergo a transition to the gel phase

Once solutes reach a certain size (Fig. 7B), they will begin to be excluded into small domains between the interstices of lamellar regions. The extent of the exclusion will be determined by the size of the solutes and the sample hydration. For the maltodextrins studied here, molecular weights larger than 5000 were completely excluded during drying. Solutes smaller than these may be incorporated between the membranes, where their osmotic and volumetric properties, and their tendency to vitrify, can stabilize the membranes during dehydration. Polymers have been observed to induce a range of behaviours in liposomes which are consistent with this model, such as aggregation (e.g., Meyuhas et al. 1996) and fusion (Suzuki et al. 1996). Conversely, some polymers have also been observed to stabilize freeze-dried liposomes (Hincha et al. 2000). Whether solutes are excluded, and thus what effect they have, depends also on how they are made; for example, we would not expect significant exclusion during freeze-drying after quench freezing in liquid N_2, as the liposomes would not be brought into close proximity in the liquid state.

The model we propose is consistent with the experimental results for samples dehydrated at room temperature. However, thermal analysis does not provide direct evidence of the location of the solutes (in common with most other techniques used in this field). Clearly the evidence for exclusion, while strong, is still circumstantial: it explains the available data, but is not conclusive. Future use of more direct techniques, such as NMR, will help clarify the extent to which solutes are excluded from the interlamellar space.

Conclusions

In this paper, we have presented data showing the effects increasing solute size has on membrane phase transitions. By keeping the solutes chemically similar, the effects of size can be distinguished from any effects caused by chemical differences. These studies of the effects of linear glucose polymers of increasing size show that:

1. Maltodextrins up to $M_w = 1000$ could insert between dehydrated phospholipid bilayers and affect the lipid phase behaviour.
2. When the solution vitrified at temperatures below the fluid-gel transition, the effect was apparent as a limit to the rise in T_m normally brought about by dehydration. This effect is due to the osmotic and volumetric effects of non-excluded solutes (Bryant and Wolfe 1992).
3 When the solution vitrified at temperatures above the fluid-gel transition, the effect was apparent as a depression of T_m by about 20 °C below T_o. If vitrification occurred between gel phase bilayers, T_m was first elevated by the presence of the glass, and then depressed after the samples had been heated so that the glass melted and reformed between fluid phase bilayers. These effects are due to the presence of a glass between membranes (Koster et al. 2000).
4. Both effects disappeared when the solutes became large enough that they were excluded from the interlamellar spaces during dehydration. For the maltodextrins used here, this occurred between M_w of 1000 and 5000.

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