

Monosaccharide composition, chain length and linkage type influence the interactions of oligosaccharides with dry phosphatidylcholine membranes

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Abstract

Sugars play an important role in the desiccation tolerance of most anhydrobiotic organisms and disaccharides have been extensively investigated for their ability to stabilize model membranes in the dry state. Much less is known about the ability of oligosaccharides to protect dry membranes. However, it has been shown that different structural families of oligosaccharides have different efficacies to interact with and protect membranes during drying. Here, we have compared three families of linear oligosaccharides (fructans, malto-oligosaccharides, manno-oligosaccharides) for their chain-length dependent lyoprotective effect on egg phosphatidylcholine liposomes. We found increased protection with chain length for the fructans, a moderate decrease in protection with chain length for malto-oligosaccharides, and a strong decrease for manno-oligosaccharides. Using Fourier-transform infrared spectroscopy and differential scanning calorimetry, we show that the degree of lyoprotection of the different sugars is closely related to their influence on the gel to liquid-crystalline phase behavior of the dry membranes and to the extent of H-bonding to different groups (C=O, P=O, choline) in the lipids. Possible structural characteristics of the different oligosaccharides that may determine the extent to which they are able to interact with and protect membranes are discussed.

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1. Introduction

Sugars are important stabilizers for biological cells during stresses such as desiccation (see [1] for a review). Since membranes are the primary sites of injury during drying and rehydration, the effects of sugars on the stability of dry membranes have been extensively investigated, mainly with the help of liposomes as convenient model systems (see [2] for a recent review). While the role and mode of action of the disaccharides sucrose (Suc) and trehalose have received considerable attention, much less is known about the function of oligosaccharides as membrane stabilizers (see [3,4] for reviews).

From research into the physiology of plant stress tolerance, raffinose family oligosaccharides (RFO; [5]) and fructosyl

oligosaccharides (fructans; [6]) have been implicated in the cellular stress responses. Data from transgenic plants over-expressing genes encoding enzymes catalyzing the synthesis of such oligosaccharides provided evidence for a protective role of RFO in plants under drought stress [7], but not under freezing stress [8]. The accumulation of fructans, on the other hand, provided increased tolerance to transgenic plants under both drought and freezing conditions [9–11].

Previous publications have shown that malto-oligosaccharides (linear oligomers of α 1–4 linked Glc; Glc–Glc_n, with n =number of monosaccharide units) decrease in their effectiveness to protect liposomes during drying with increasing chain length (degree of polymerization; DP) [12,13]. RFO (linear oligomers of Gal α 1–6 linked with a capping Suc; Glc–Fru–Gal_n) [14] and inulins (linear oligomers of Fru β 2–1 linked with a capping Glc; Glc–Fru_n) [12], on the other hand, become more effective with increasing DP. These differences between the different oligosaccharide families were, at least in part, related to the different ability of the sugars to interact with the membrane lipids in the dry state [12,14]. However, a detailed

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analysis of the DP-dependence of the possible interactions of the different oligosaccharides was still missing. In the present paper, we present such an analysis, using FTIR and DSC, to elucidate the effects of DP in three families of oligosaccharides on lyoprotection, gel to liquid–crystalline lipid phase transitions, and H-bonding patterns between sugars and lipids. The data we present clarify the differences in the interaction potential between the different oligosaccharide structures and dry lipids.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC) was purchased from Lipid Products (South Nutfield, Surrey, UK) and 1,2-dimyristoyl-D54-*sn*-glycerol-3-phosphocholine-1,1,2,2-D4-*N,N,N,N*-trimethyl-D9 (DMPC-d67) was obtained from Avanti Polar Lipids (Alabaster, AL). Carboxyfluorescein (CF) was purchased from Molecular Probes (Leiden, The Netherlands) and was purified according to the procedure described in [15]. Suc and malto-oligosaccharides were obtained from Sigma, fructo- and manno-oligosaccharides were purchased from Megazyme (Wicklow, Ireland). The purity of the sugars was at least 95%, as determined by analytical High Performance Liquid Chromatography (HPLC), as described in detail in previous publications [12,14].

2.2. Preparation of liposomes

Lipids were dried from chloroform under a stream of N_2 and stored under vacuum overnight to remove traces of solvent. Liposomes were prepared from hydrated lipids using a hand-held extruder with two layers of polycarbonate membranes with 100 nm pores ([16]; Avestin, Ottawa, Canada).

2.3. Leakage experiments

Liposomes for leakage studies were made as previously described [17]. Briefly, an appropriate amount of EPC was hydrated in 0.25 ml of 100 mM CF, 10 mM TES, 0.1 mM EDTA (pH 7.4) and 10 mg/ml of the appropriate sugar. After extrusion, the vesicles were passed through a NAP-5 column (Sephadex G-25; Pharmacia) equilibrated in TEN buffer (10 mM TES, 0.1 mM EDTA (pH 7.4), 50 mM NaCl), to remove the CF and sugar not entrapped by the vesicles. The eluted samples were then diluted with TEN to a lipid concentration of approximately 10 mg/ml. Liposomes (40 μ l) were mixed with an equal volume of concentrated solutions of sugars in TEN and 20 μ l aliquots were filled into the wells of 60-well microplates. The plates were dried in desiccators at 28 °C and 0% relative humidity for 24 h in the dark [12]. Damage to the liposomes was determined as CF leakage after rehydration, with a Fluoroskan Ascent (LabSystems, Helsinki, Finland) fluorescence microplate reader at an excitation wavelength of 444 nm and an emission wavelength of 555 nm [12].

2.4. FTIR (Fourier-transform infrared) spectroscopy

Spectra were obtained from samples containing liposomes and sugar at a mass ratio of 1:2 (mg lipid:mg sugar). Liposomes were extruded in the presence of sugars, so that the solute was present on both sides of the membranes. Samples (50 μ l) were spread on CaF_2 windows and dried as described above. A window was then fixed in a cuvette holder connected to a temperature control unit (Specac Eurotherm, Worthington, UK) placed in a vacuum chamber in the infrared beam [12]. Temperature was controlled by a liquid N_2 reservoir and an electrical heater. The temperature was measured with a thermocouple attached to the cuvette holder next to the sample. The sample was first heated to 50 °C for 20 min under vacuum to remove residual moisture the lipid had taken up during sample handling. The sample was then cooled to –50 °C and, after 30 min equilibration, the temperature was increased at a constant rate of 1 °C/min to 100 °C.

To obtain lipid melting curves and determine the T_m values, spectra with 4 cm^{-1} resolution and two co-added scans were recorded in a Perkin-Elmer GX 2000 FTIR spectrometer every minute. After normalization of absorbance and baseline correction using the Spectrum 5.0.1 software, the wavenumber of the CH_2 symmetric stretching (ν_{CH_2s}) band around 2850 cm^{-1} was determined by the automatic peak identification routine. The T_m values were estimated as the midpoints of the lipid melting curves [18]. Duplicate samples were measured for each sugar along with a freshly prepared sample containing Suc for direct comparison. There were no significant changes in the T_m of samples containing Suc prepared on different days (data not shown).

Interactions between EPC and sugars were investigated by an analysis of the phosphate asymmetric stretching ($\nu P=O_{as}$) and carbonyl stretching ($\nu C=O$) vibrational modes of the lipid. Spectra at different temperatures between –50 °C and 50 °C were recorded with 2 cm^{-1} resolution and 16 co-added scans. For the $\nu C=O$ peak, deconvolution and curve fitting were performed using the peak-fitting module of OriginPro 7.0. A Pearson7 function was used for peak fitting, with only the number of peaks used as a set parameter. All other parameters were optimized by the software through an iterative process to find the best curve fit [19].

Interactions of the sugars with the choline moiety of the lipid headgroups and sugar-induced conformational changes were investigated with the deuterated lipid DMPC-d67 by analysis of the totally symmetric stretching vibration of the choline C–N bond ($\nu C-N(CD_3)_3ts$). The drying conditions were the same as described above, but due to the fact that the $\nu C-N(CD_3)_3ts$ vibrational mode appears in the spectral region below 800 cm^{-1} , samples for these measurements were spread on ZnSe windows. Because DMPC is a completely saturated lipid, the samples had to be pre-treated at high temperature before spectra acquisition to allow sugar–lipid interactions. After 20 min at 50 °C, the samples were heated to 100 °C and held there for 2 h. Samples were then cooled to –50 °C and, after a 30 min equilibration, the temperature was increased at 1 °C/min up to 100 °C. Spectra were recorded at different temperatures at a resolution of 2 cm^{-1} , using 16 co-added scans.

2.5. DSC (differential scanning calorimetry)

The thermal behavior of the samples was investigated by DSC using a Netzsch (Selb, Germany) DSC 204 as described in detail recently [19]. The analysis of the thermograms and the determination of the phase transition temperatures were performed with the Netzsch software package. DSC thermograms were obtained from samples containing EPC and sugar at a mass ratio of 1:2, with sugar present on both sides of the membranes. Samples were dried as described above. In all cases, the amount of residual H_2O was ≤ 0.02 g H_2O /g dry weight. After cooling the samples from room temperature to –60 °C at a rate of 20 °C/min, they were scanned at 5 °C/min or 20 °C/min while heating to 100 °C. This cooling and heating cycle was performed four times and data used for the analyses were taken from the heating thermograms. Duplicate samples were prepared and scanned for each sugar. T_m was recorded as the temperature at the peak maximum.

3. Results and discussion

3.1. Effects of the different oligosaccharides on lyoprotection of EPC liposomes

In the experiments presented in this paper, we have investigated the effects of three structural families of oligosaccharides on EPC membranes in the dry state. They comprise two families that have been investigated before, namely inulins (β 2–1 linked Fru with a capping Glc; [12,20,21]) and malto-oligosaccharides (α 1–4 linked Glc; [12,13]) and one family that has not been investigated previously, manno-oligosaccharides (β 1–4 linked Man). We used commercially available sugars from DP2 to DP5 for inulins, DP2 to DP7 for malto-oligosaccharides, and DP2 to DP6 for manno-oligosaccharides.

The purity of all sugars was checked by HPLC and was found to be at least 95% (data not shown).

Fig. 1 shows the effects of these sugars on liposome stability after drying and rehydration, as determined by the

loss of the encapsulated soluble fluorescent dye CF. In general, the sugars showed concentration dependent protection. The degree of protection, however, clearly depended on DP and oligosaccharide family. While the inulins showed slightly

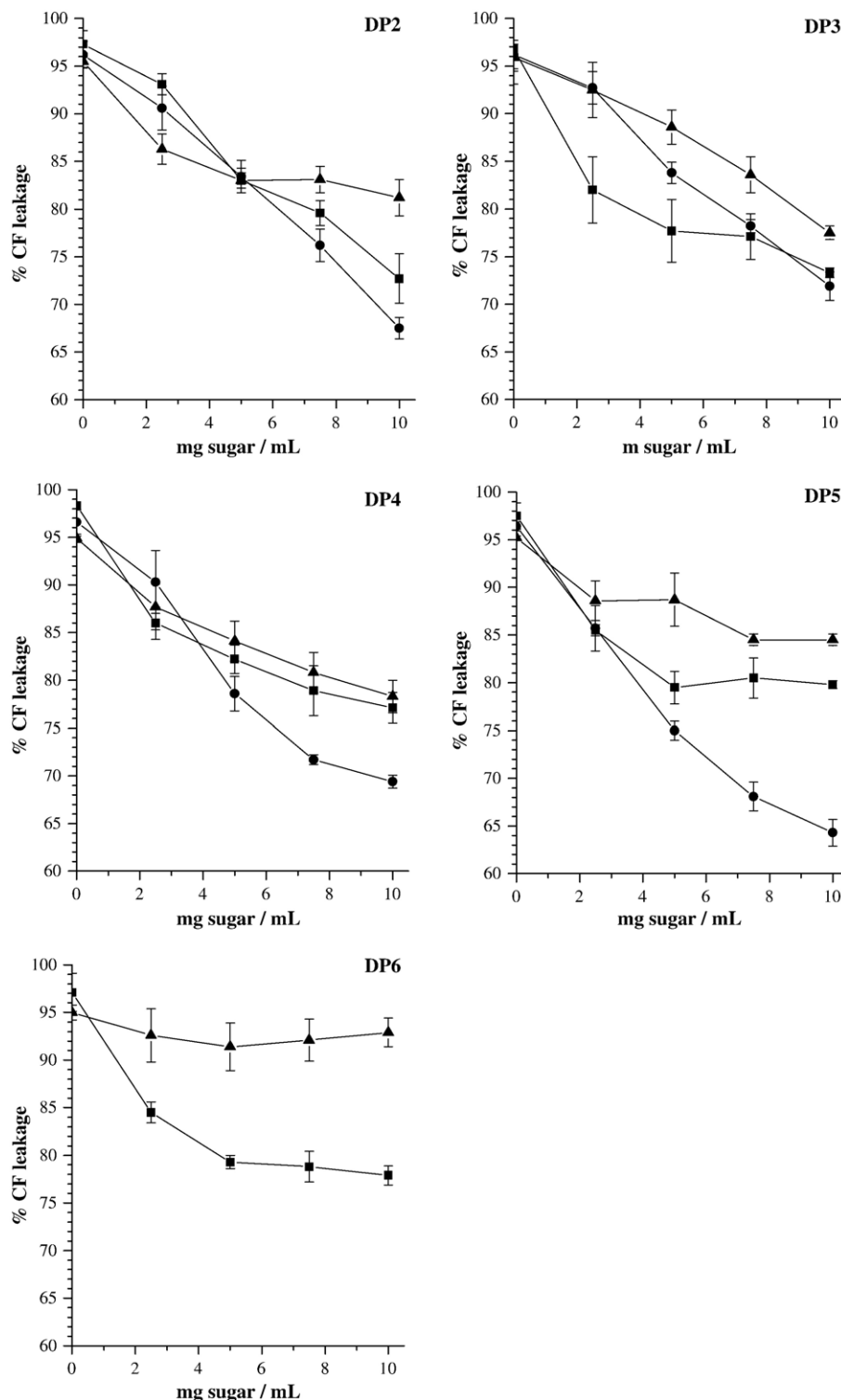


Fig. 1. Protection of large unilamellar liposomes from damage during drying by inulins (circles), malto-oligosaccharides (squares) and manno-oligosaccharides (triangles) of different degrees of polymerization (DP). Leakage of CF from the vesicles after air-drying and rehydration is shown as a function of the concentration of the sugars in the suspending medium. Data points represent means \pm S.D. from three parallel samples.

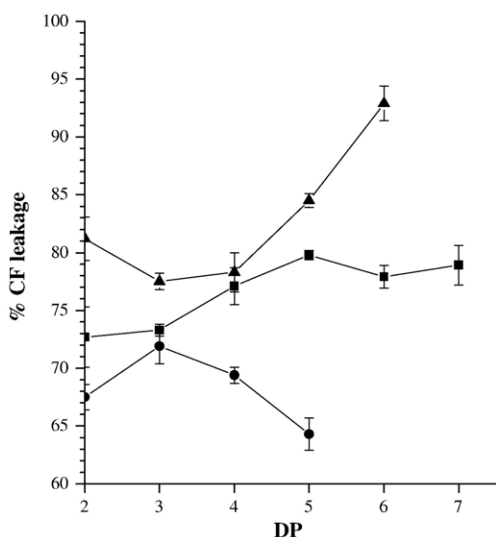


Fig. 2. Percentage of CF leakage from liposomes after air-drying and rehydration as a function of the DP of inulins (circles), malto-oligosaccharides (squares) and manno-oligosaccharides (triangles). All samples contained 10 mg sugar/ml (compare Fig. 1).

increased protection with DP, the members of the other two families became progressively less stabilizing with increasing DP (Fig. 2). This has been noted before for inulins [12] and malto-oligosaccharides [12,13]. In addition, these experiments identified manno-oligosaccharides as a family of sugars with an even stronger dependence of lyoprotection on DP. In this case, no protection was evident for DP6, while the corresponding malto-oligosaccharide still showed a clear protective effect (Figs. 1 and 2).

3.2. Effects of different oligosaccharides on lipid phase transitions in the dry state

It has been shown in several previous publications (see [2,22] for reviews) that the protection of liposomes against leakage is related to the ability of sugars to depress the gel to liquid–crystalline phase transition temperature (T_m) of the dry membranes. Suc and all investigated inulins were able to depress T_m by about 60 °C, as determined by FTIR spectroscopy (Fig. 3A). There was no significant difference between the effects of inulins of different DP on T_m (between –24 °C and –26 °C for all samples). These results differ from data published by our group earlier [12], where the depression of T_m was smaller (approximately 25 °C) and the inulins showed a small decrease in T_m with increasing DP. However, the T_m reported here for Suc has been independently confirmed by us [19] and others [22] using FTIR and DSC. The differences to our older data are probably due to changes in the sample preparation and cooling protocols. The samples in the older study contained slightly more water, as indicated by a lower wavenumber of the P=O band (1259 cm^{-1} vs. 1261 cm^{-1} ; compare Fig. 6). Also, the sample cooling by liquid nitrogen was optimized to ensure that the apparent temperature recorded by the automatic control unit and the actual sample temperature recorded by an independent thermocouple matched closely.

A comparison of the three different DP5 oligosaccharides reveals differences between the members of the different families (Fig. 3B). As reported previously [12], T_m of the low temperature transition of dry EPC was higher in the presence of the malto-oligosaccharide in comparison to the inulin of this DP (by approximately 5 °C). The lipid melting curve in the presence of the malto-oligosaccharide shows evidence of an additional transition at about 50 °C. These two transitions are also evident in the temperature dependence of the $\nu\text{CH}_2\text{s}$ vibration from the samples containing the DP5 manno-oligosaccharide (Fig. 3B). Here, however, the low temperature transition is much smaller than with the other sugars and a third transition is evident at about 70 °C. This ranking of the different sugars with regard to their effects on lipid phase behavior

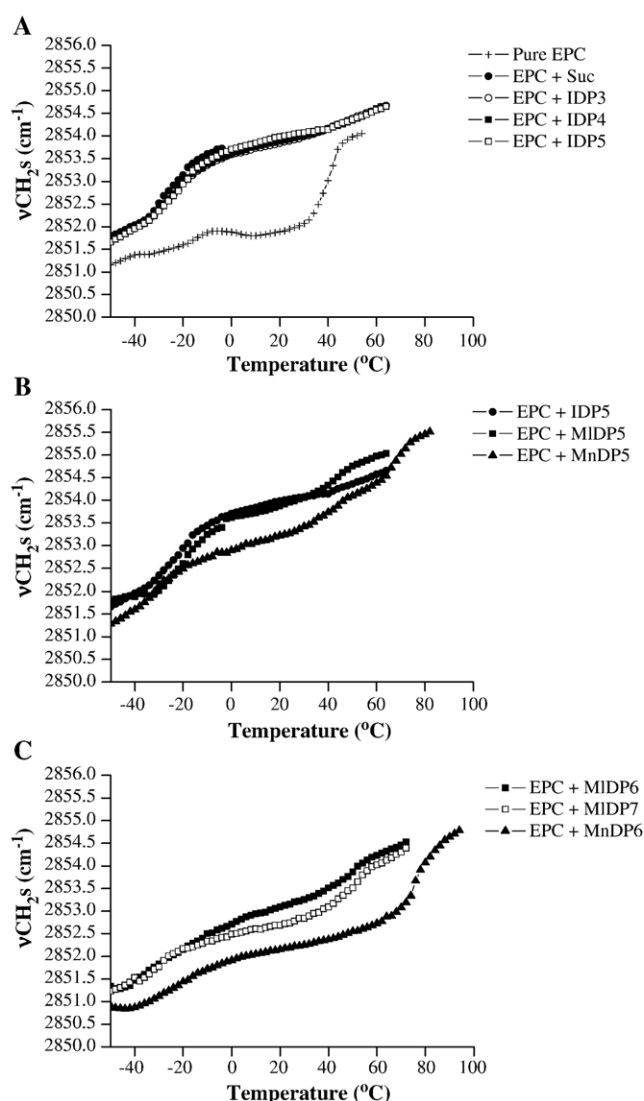


Fig. 3. Lipid melting curves of dry EPC liposomes determined by FTIR spectroscopy. The wavenumber of the symmetric CH_2 stretching peak ($\nu\text{CH}_2\text{s}$) is plotted as a function of the sample temperature. (A) Samples contained either only EPC liposomes or EPC and inulins with different DP (IDP); (B) Samples contained EPC liposomes with inulin DP5 (IDP5), malto-oligosaccharide DP5 (MIDP5), or manno-oligosaccharide DP5 (MnDP5); (C) Samples containing EPC liposomes with malto-oligosaccharides DP6 (MIDP6) or DP7 (MIDP7), or manno-oligosaccharide DP6 (MnDP6).

corresponds to the ranking of the sugars with regard to their effects on CF leakage (Fig. 2).

Such high temperature transitions become even more pronounced at higher DP (Fig. 3C). For the malto-oligosaccharides DP6 and DP7, it can be seen that the transition at 50 °C increases in magnitude, while the low temperature transition decreases. For the manno-oligosaccharide DP6, the transition at 50 °C disappears completely and the low-temperature transition becomes very shallow, while the transition at about 70 °C is very prominent. In the case of the manno-oligosaccharides, the high temperature transitions were already observable for DP4, while they only appeared for DP5 in the presence of malto-oligosaccharides (data not shown). The high temperature transitions occur at temperatures significantly above the T_m of the pure lipid, indicating the presence of lipid domains that melt sequentially and then interact with the sugars to increase $\nu\text{CH}_2\text{s}$ above that of the pure dry lipid. Such an increase in $\nu\text{CH}_2\text{s}$ has been shown previously for various sugars (e.g. [14,19,23]).

The FTIR data described above indicate that in the presence of all inulins, of malto-oligosaccharides up to DP4 and of manno-oligosaccharides up to DP3, there is only one lipid phase transition, at a temperature around –25 °C. This could be verified by DSC measurements (Fig. 4). The transition endotherm associated with T_m was centered at 40 °C for the pure dry lipid and at about –23 °C for EPC dried in the presence of Suc, as reported previously [19]. In the presence of inulin DP5, malto-oligosaccharide DP4, or manno-oligosaccharide DP3, the same thermal behavior was evident (Fig. 4) and was also observed for the corresponding sugars of lower DP (data not shown). It should be emphasized that there was no difference in the thermal behavior of these samples between the first and subsequent heating scans (data not shown), indicating that these effects of the sugars were established spontaneously during drying at 28 °C.

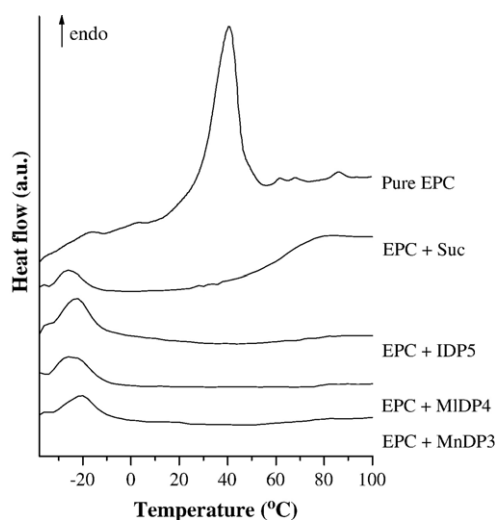


Fig. 4. DSC heating thermograms of dry EPC liposomes in the absence and presence of different sugars. The sample composition is indicated to the right of each trace. Traces have been vertically offset for greater clarity. Thermograms correspond to the second heating scan at 20 °C/min, after cooling the samples from 100 °C to –60 °C and subsequent equilibration at this temperature for 5 min.

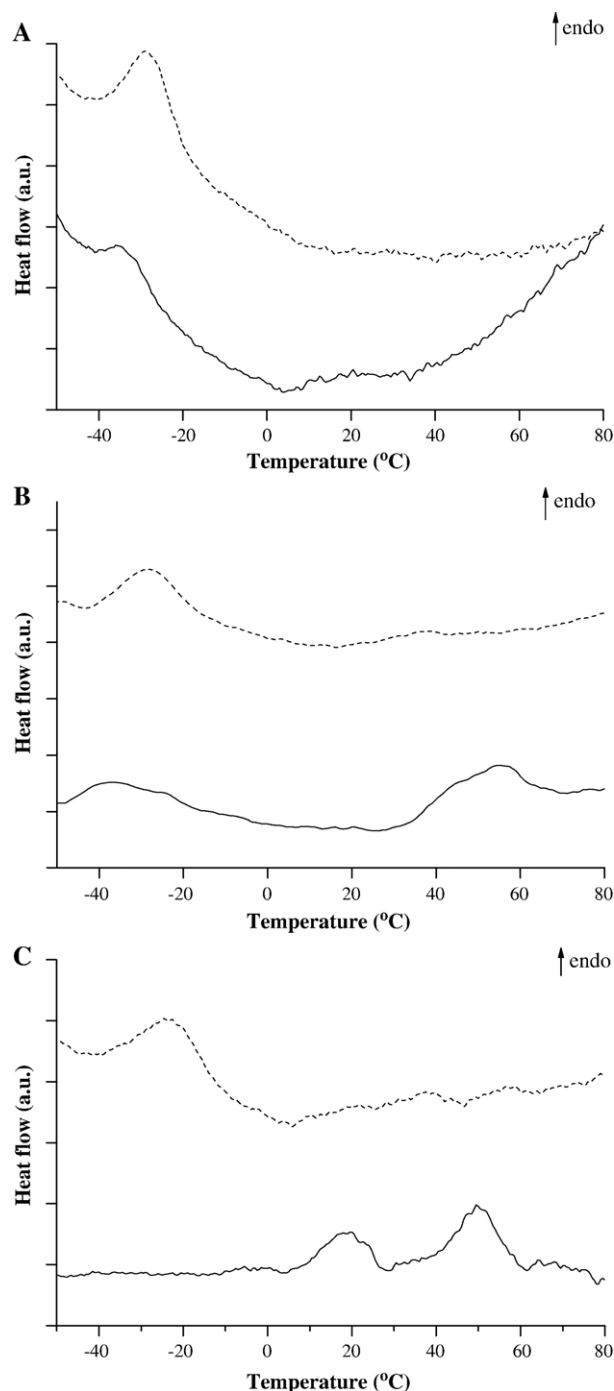


Fig. 5. DSC heating thermograms, obtained at a warming rate of 5 °C/min, of dry EPC liposomes in the presence of malto-oligosaccharide DP6 (A), malto-oligosaccharide DP7 (B), or manno-oligosaccharide DP6 (C). Solid line, first heating scan; dashed line, second heating scan. Traces have been vertically offset for greater clarity.

For the liposomes dried in the presence of higher DP malto- or manno-oligosaccharides, thermal behavior was more complex (Fig. 5). In the presence of malto-oligosaccharides of DP5 (data not shown) and DP6 (Fig. 5A), there was only a small low-temperature transition in dry EPC visible during the first heating scan. The enthalpy of this transition increased in the second heating scan, indicating improved interactions of

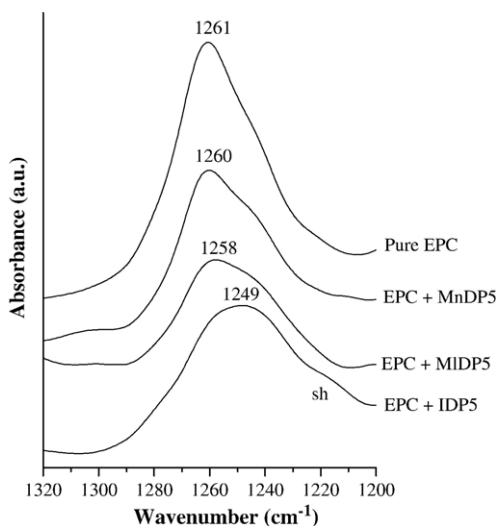


Fig. 6. FTIR spectra of the asymmetric P=O stretch region (ν POas) in dry EPC liposomes at 50 °C. The samples contained either only EPC liposomes or EPC liposomes with inulin DP5, malto-oligosaccharide DP5, or manno-oligosaccharide DP5 at a 1:2 mass ratio. Spectra have been vertically offset for greater clarity.

these oligosaccharides with the dry lipid after heating to 100 °C. This shift between the first and second heating scans was even more pronounced in the presence of the malto-oligosaccharide of DP7 (Fig. 5B), but the low-temperature transition was already visible in the first scan. Here, a clear high-temperature transition was visible in the first scan at temperatures above the T_m of the pure dry lipid. This high-temperature transition was strongly reduced in the second scan, with a concomitant increase in the enthalpy of the low-temperature transition.

A similar picture emerged for the manno-oligosaccharides, only that here the effects were always visible at one DP below those of the malto-oligosaccharides. EPC in the presence of manno-oligosaccharides of DP4 and DP5 (data not shown) showed the same thermal behavior as in the presence of malto-oligosaccharide DP6 (Fig. 5A). The thermogram of EPC in the presence of manno-oligosaccharide DP6 (Fig. 5C), on the other hand, corresponded closely to that in the presence of malto-oligosaccharide DP7 (Fig. 5B), but showing two high-temperature transitions in the first heating scan and no evidence for a low-temperature transition.

Taking the FTIR and DSC data on lipid phase transitions in the presence of the different sugars together, three main conclusions emerge.

First, with increasing DP, oligosaccharides are less able to depress T_m of lipids during drying. This is evident from the appearance of additional gel to liquid–crystalline phase transitions at higher temperatures. The magnitude of this DP dependence, however, clearly depends on the oligosaccharide family used, with inulins showing no dependence (up to DP5) and manno-oligosaccharides showing the biggest dependence on DP. Most of this DP dependence can be overcome by heating the samples through the high-temperature phase transitions. A significant decrease in T_m in the second heating scan in DSC experiments has been noted for various sugar and lipid

combinations in the past [24–26] and we will discuss its physical basis below.

Second, from the fact that we observed two or three lipid phase transitions during the first heating scans in samples with high DP sugars in both FTIR and DSC experiments, we conclude that in these samples different lipid domains were present, that either interacted with the sugars at the incubation temperature (28 °C), or did not. Apparently, interaction of the lipid molecules with the oligosaccharides in the dry state is rather an “all-or-none” than a gradual process. A similar observation has been made for dry EPC membranes in the presence of very low amounts of Suc [19].

Third, although we had to heat our samples for FTIR measurements to 50 °C before cooling to –30 °C to start the temperature scan, their behavior is clearly more similar to the first than to the second heating scan in the DSC. This indicates that 50 °C was not enough to induce major changes in sugar–lipid interactions and that these samples closely resemble those used for the CF leakage measurements. Such changes only occurred at temperatures above 50 °C, as seen in the second heating scans in the DSC thermograms after heating the samples to 100 °C. However, no second heating scans have been performed in our FTIR experiments to verify this.

3.3. H-bonding interactions of the oligosaccharides with EPC in the dry state

The ability of different solutes to depress the T_m of dry lipids is strongly related to their ability to H-bond to the lipid headgroups and in particular to the P=O moiety [19,23,27]. The ν P=Oas vibration is the most strongly affected vibration in FTIR spectra of dry phosphatidylcholines and shows a strong down-field shift due to H-bonding [28]. The ν P=Oas peak was located at 1261 cm^{-1} in the pure dry lipid (Fig. 6), as reported in several previous publications (see [2] for a review). It was strongly depressed by the disaccharides Suc, Mal, and Man (Fig. 7). When the ν P=Oas peaks of EPC in the presence of the

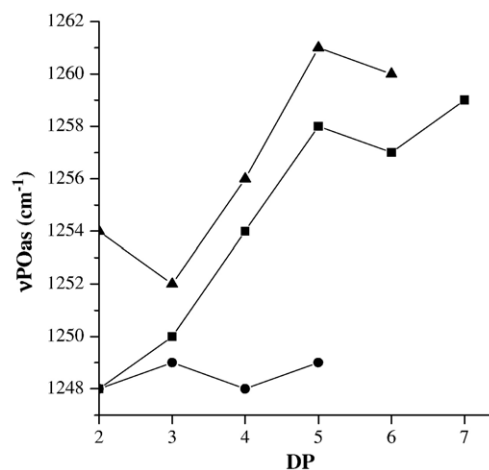


Fig. 7. Wavenumber of the asymmetric P=O stretching vibrational mode (ν P=Oas) as a function of the DP for samples containing dry EPC liposomes with inulins (circles), malto-oligosaccharides (squares) and manno-oligosaccharides (triangles).

DP5 oligosaccharides of all three families are compared (Fig. 6), significant differences become apparent. The manno-oligosaccharide had only a small effect on $\nu\text{P}=\text{Oas}$ that hardly changed the position of the peak and only broadened it somewhat on the low-field side. The malto-oligosaccharide showed a slightly stronger effect, with a peak shift by 3 cm^{-1} and more pronounced broadening on the low-field side, indicating more H-bonding. The inulin DP5 showed clearly the strongest H-bonding to the $\text{P}=\text{O}$ group of EPC, with the biggest shift in peak position and a shoulder at about 1220 to 1230 cm^{-1} , corresponding to the wavenumber of $\nu\text{P}=\text{Oas}$ in

fully hydrated EPC [29,30]. The DP-dependence of the position of the $\nu\text{P}=\text{Oas}$ peak (Fig. 7) shows clear similarity to the lipid melting curves, i.e. the appearance of high temperature transitions: increasing DP had no effect for the inulins, a strong effect for the malto-oligosaccharides, and an even stronger effect for the manno-oligosaccharides. Similar effects of the inulins and malto-oligosaccharides on $\nu\text{P}=\text{Oas}$ have been reported previously [12].

The $\text{C}=\text{O}$ groups in diacyl lipids are also potential partners for H-bonding interactions with sugars [19,29,31,32]. In the presence of water, the $\nu\text{C}=\text{O}$ peak is shifted downfield

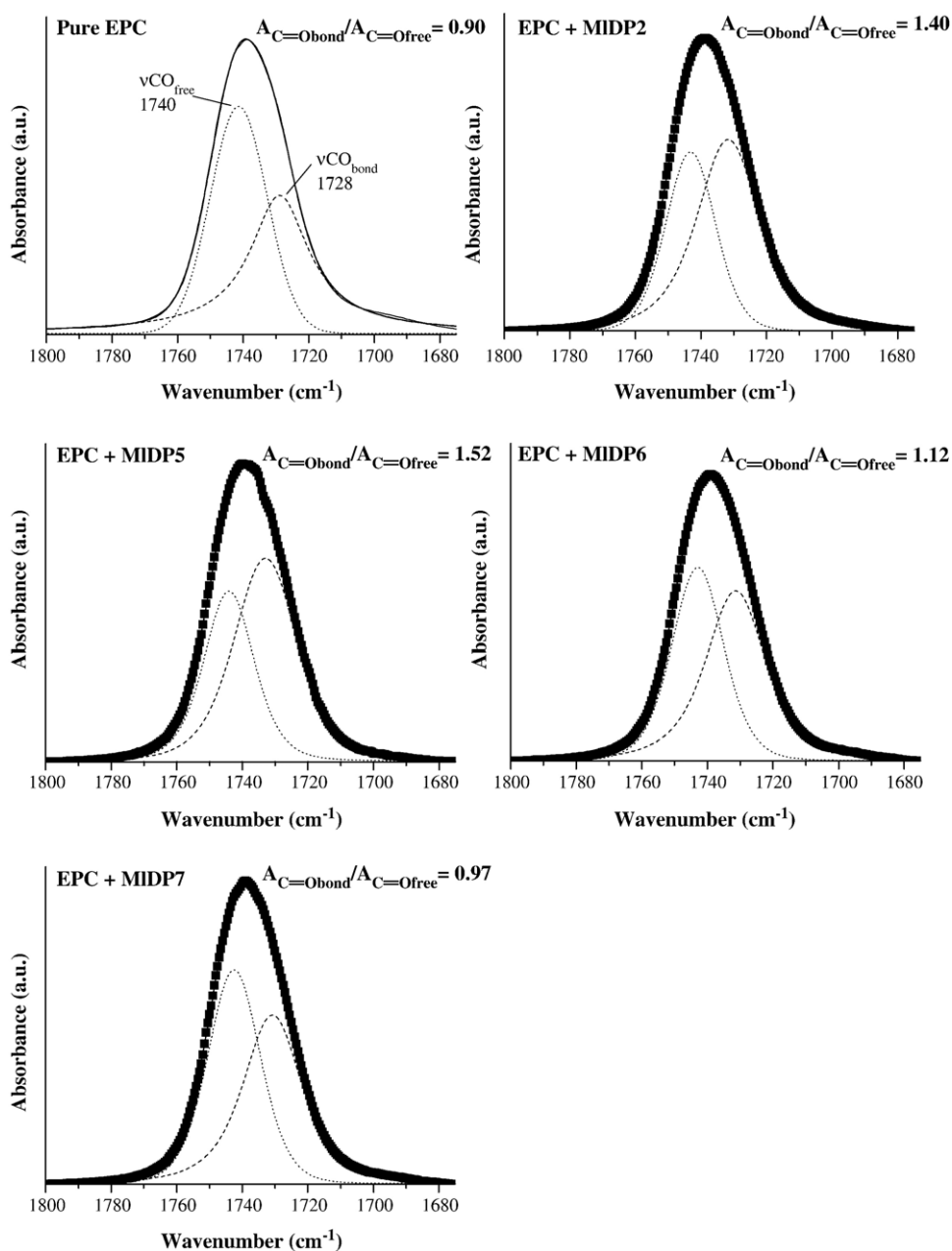


Fig. 8. Infrared spectra in the carbonyl stretching region ($\nu\text{C}=\text{O}$) of pure dry EPC liposomes and dry EPC liposomes and malto-oligosaccharides of different DP. The peaks were deconvoluted and fitted into two band components corresponding to $\nu\text{C}=\text{O}_{\text{free}}$ (short dashes, upfield peak, no H-bonding) and $\nu\text{C}=\text{O}_{\text{bond}}$ (long dashes, lowfield peak, H-bonded). The solid curve comprises both the measured and the fitted absorbance curves. The samples and the corresponding ratio $A_{\text{C}=\text{O}_{\text{bond}}} / A_{\text{C}=\text{O}_{\text{free}}}$ are indicated on top of each graph. The spectra were all recorded at 50°C .

compared to the dry membranes [28,29,33], which is due to $\text{C}=\text{O}\cdots\text{HO}$ H-bonding interactions. In the presence of sugars, however, there is no apparent shift in the $\nu\text{C}=\text{O}$ band in dry membranes [23,34]. There is evidence in the literature, however, that the $\nu\text{C}=\text{O}$ peak (located at $\approx 1739\text{ cm}^{-1}$) can be decomposed into two components. The higher wavenumber band component ($1740\text{--}1742\text{ cm}^{-1}$) has been assigned to the $\nu\text{C}=\text{O}$ of free $\text{C}=\text{O}$ groups ($\nu\text{C}=\text{O}_{\text{free}}$), while the lower wavenumber component ($\approx 1728\text{ cm}^{-1}$) has been attributed to the $\nu\text{C}=\text{O}$ vibration of H-bonded conformers ($\nu\text{C}=\text{O}_{\text{bond}}$) [35,36]. These two components can be quantified by deconvolution of the main $\nu\text{C}=\text{O}$ band [19].

Fig. 8 shows the result for pure air-dried EPC and for samples also containing malto-oligosaccharides from DP2 to DP7. The two band components $\nu\text{C}=\text{O}_{\text{free}}$ (1740 cm^{-1}) and $\nu\text{C}=\text{O}_{\text{bond}}$ (1728 cm^{-1}) changed their relative area and intensity in the presence of the low DP sugars. Assuming that the relative area of a band component is proportional to its conformer population, the relative amount of the conformers can be quantified from the deconvoluted peaks. The ratio $A_{\text{C}=\text{O}_{\text{bond}}}/A_{\text{C}=\text{O}_{\text{free}}}$, with $A_{\text{C}=\text{O}_{\text{bond}}}$ and $A_{\text{C}=\text{O}_{\text{free}}}$ the respective fitted peak areas, was 0.9 for pure EPC. This shows that the population of free $\text{C}=\text{O}$ groups was larger than the population of $\text{C}=\text{O}$ groups involved in interactions (compare [19]). In the pure lipid, these interactions probably involve residual water molecules and also choline groups of other lipid molecules, which may interact through charge-pair interactions [37]. The residual water content in all our samples was very low ($< 0.02\text{ g H}_2\text{O/g}$ dry weight), as also indicated by the position of the $\nu\text{P}=\text{O}_{\text{as}}$ peak at 1261 cm^{-1} . We would therefore assume that the ratio $A_{\text{C}=\text{O}_{\text{bond}}}/A_{\text{C}=\text{O}_{\text{free}}}$ in the pure lipid samples was mainly determined by interactions of the $\text{C}=\text{O}$ groups with choline. These interlipid interactions, however, imply that we may systematically underestimate the interactions of sugars with the $\text{C}=\text{O}$ groups, as these interactions may, at least in part, replace the former interactions between $\text{C}=\text{O}$ and choline groups.

Nevertheless, in the presence of malto-oligosaccharides between DP2 and DP5, the number of bonded $\text{C}=\text{O}$ groups increased compared to the pure lipid (Fig. 8). This means that these sugars were able to penetrate the bilayer to interact with the lipid through $\text{C}=\text{O}\cdots\text{HO}$ H-bonding. H-bonding decreased with DP6 and was virtually absent with DP7.

Fig. 9 shows the ratio of $A_{\text{C}=\text{O}_{\text{bond}}}/A_{\text{C}=\text{O}_{\text{free}}}$ as a function of DP at different temperatures. There was an increase in $A_{\text{C}=\text{O}_{\text{bond}}}/A_{\text{C}=\text{O}_{\text{free}}}$ with temperature for all DPs, indicating an increase in the $\text{C}=\text{O}_{\text{bond}}$ conformer population with temperature, which may be related to a temperature dependent increase in the spacing of the lipids. This, however, was not related to the lipid T_m , although the accompanying lamellar extension might be expected to facilitate the access of sugar molecules to the interfacial region of the bilayer. We have recently reported this lack of an effect of the phase transition on interactions of Suc with the $\text{C}=\text{O}$ moiety of dry EPC [19] and extend it here to oligosaccharides.

Interestingly, the DP-dependence of H-bonding to the $\text{C}=\text{O}$ groups was the same for all three families of oligosaccharides.

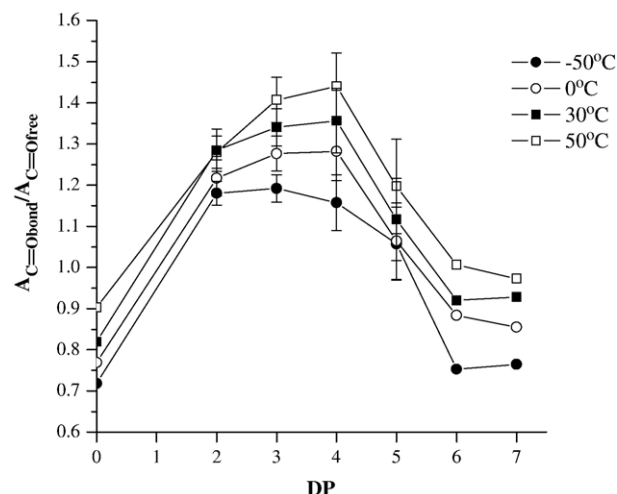


Fig. 9. Average ratio $A_{\text{C}=\text{O}_{\text{bond}}}/A_{\text{C}=\text{O}_{\text{free}}}$ (fitted peak areas of the two $\nu\text{C}=\text{O}$ band components identified in Fig. 8) in dry EPC as a function of the DP of the oligosaccharides at different temperatures. From DP2 to DP5 the values represent the averages and S.E.M. obtained for the three sugar families. For DP6 they represent the average of the $A_{\text{C}=\text{O}_{\text{bond}}}/A_{\text{C}=\text{O}_{\text{free}}}$ of the malto- and manno-oligosaccharides, and for DP7, the value was obtained for the malto-oligosaccharide only.

Therefore, Fig. 9 presents the appropriate averages for each DP. It is apparent that H-bonding is extensive up to DP4 and then strongly decreases, until it is virtually absent in the presence of DP6 and DP7 oligosaccharides. This is clearly different to the pattern observed for H-bonding of the sugars to the $\text{P}=\text{O}$ groups that was much more dependent on the chemical structure of the sugars. This indicates that penetration into the membrane to the level of the $\text{C}=\text{O}$ groups is largely limited by steric factors related to molecular size and not to specific structural factors.

The choline group is the most exposed part of the phosphatidylcholine molecule and vibrational bands of this group have been shown to shift in response to the presence of sugars in dry liposome preparations [19,29]. However, the investigated choline bands overlapped with vibrations from the sugars and could therefore not be identified in the presence of the amounts of sugar used in the present study. We have therefore used a deuterated lipid (DMPC-d67), where the choline vibrations are shifted away from the sugar vibrations. In addition, we have used the $\nu\text{C}-\text{N}(\text{CD}_3)_3$ vibrational mode that had not been used to analyse sugar–membrane interactions before. The band assignment was taken from [38,39]. This band can be clearly identified in the deuterated lipid, even in the presence of a 2:1 sugar:lipid mass ratio (Fig. 10). However, since no deuterated EPC or POPC was commercially available, we used DMPC-d67. DMPC is a completely saturated lipid with a T_m in the dry state of 89°C [29].

It has been shown previously, that the lipid bilayer has to be heated above T_m to allow the maximum effect of sugars on membrane phase properties and optimal interactions between sugars and membranes [24–26]. When dry DMPC-d67 in the presence of Suc was heated to 100°C , we observed a time dependent decrease in $\nu\text{P}=\text{O}_{\text{as}}$, indicating increased H-

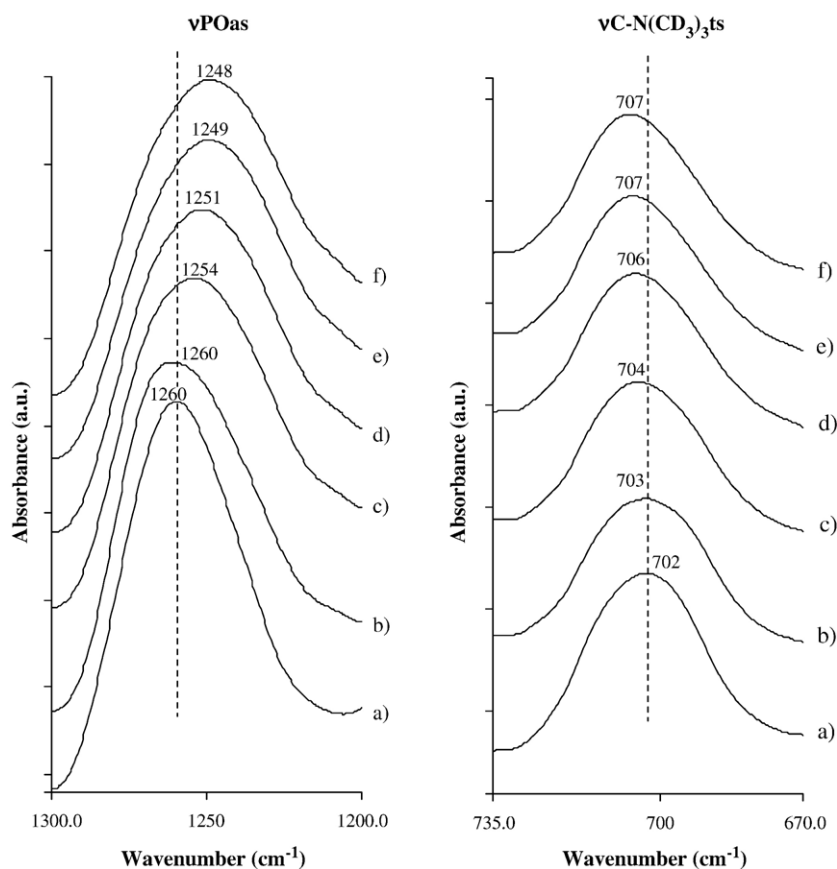


Fig. 10. Infrared spectra in the asymmetric $P=O$ stretch region ($\nu P=O_{as}$, left side) and in the totally symmetric stretching region of the choline $C-N$ bond ($\nu C-N(CD_3)_3ts$, right side) of dry DMPC-d67 liposomes in the presence of sucrose at a 1:2 lipid:sugar mass ratio. (a) 50 °C; (b) 100 °C after 30 min; (c) 100 °C after 50 min; (d) 100 °C after 70 min; (e) 100 °C after 120 min; (f) 100 °C after cooling to -50 °C and heating at 1 °C/min. Spectra have been vertically offset for greater clarity.

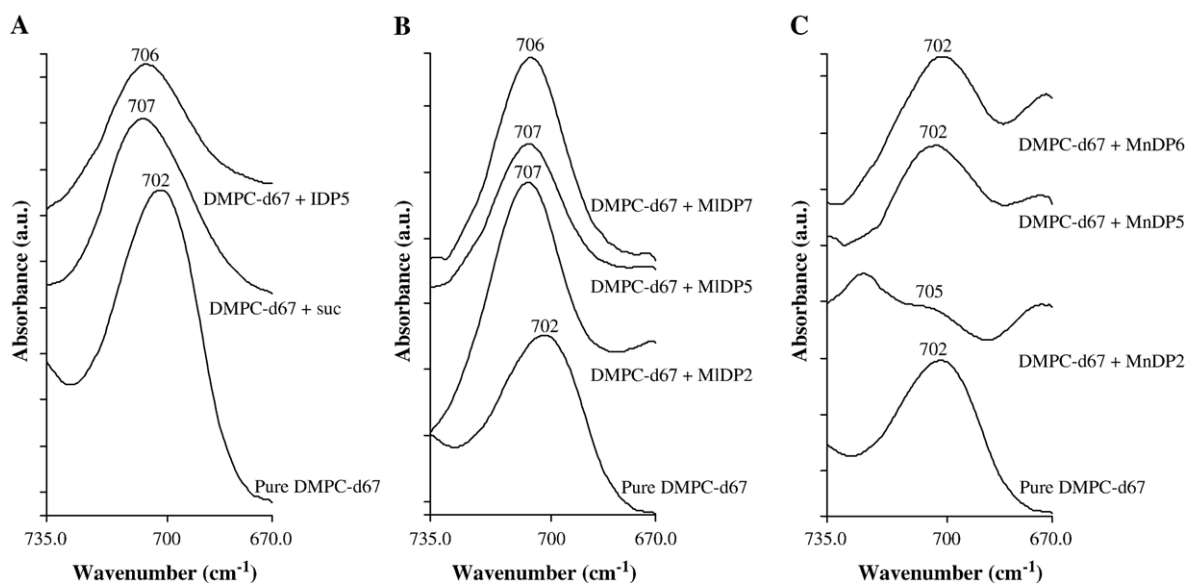


Fig. 11. Infrared spectra in the totally symmetric stretching region of the choline $C-N$ bond ($\nu C-N(CD_3)_3ts$) of pure dry completely deuterated DMPC (DMPC-d67) and of dry DMPC-d67 liposomes in the presence of (A) inulins, (B) malto-oligosaccharides (C) manno-oligosaccharides. Spectra were registered at 50 °C after cooling the samples to -50 °C, equilibration at this temperature during 30 min and warming at 1 °C/min. Samples containing lipid and sugar were incubated for 2 h at 100 °C before cooling (compare Fig. 10). Spectra have been vertically offset for greater clarity.

bonding of the sugar to the lipid P=O groups (Fig. 10). Once this interaction was established, the samples could be cooled down to -50°C and still showed the same degree of interaction after a temperature scan to 100°C (Fig. 10, compare e) and f) in the left panel). In the same samples, the $\nu\text{C}-\text{N}(\text{CD}_3)_3$ ts band showed a similar behavior (Fig. 10, right panel), but here we observed a wavenumber increase by about 5 cm^{-1} . An upshift has also been reported for $\nu\text{CN}(\text{C}-\text{H}_3)_3$ as in EPC in the presence of low amounts of Suc [19] and after hydration [40–42]. These results indicate that the sugars interact with the choline groups of dry phosphatidylcholine. The shifts in the choline band positions could be the result of conformational changes in the choline group because of the intercalation of sugar molecules between the choline and phosphate groups due to H-bonding to P=O, or because of conformational transitions due to hydrocarbon chain melting [34]. *Ab initio* calculations [41,43], however, indicated the possibility of H-bonding to choline methyl groups, as discussed in detail recently [19].

The data presented in Fig. 10 show that the shift in the $\nu\text{C}-\text{N}(\text{CD}_3)_3$ ts band accurately reflects the interaction of the sugar molecules with the lipid headgroups. Therefore, we have used this vibration to further characterize the differences in sugar–membrane interactions between the different oligosaccharide families (Fig. 11). For both the inulins and the malto-oligosaccharides, the magnitude of the peak shift in the $\nu\text{C}-\text{N}(\text{CD}_3)_3$ ts band in comparison to the pure lipid was not influenced by the DP of the sugars. In the presence of manno-oligosaccharides, however, $\nu\text{C}-\text{N}(\text{CD}_3)_3$ ts was already back to the peak position of the pure lipid in the presence of DP3 (data only shown for DP5 and DP6), indicating reduced interactions of these sugars with the lipid headgroups also at the level of the choline.

4. Conclusions

Collectively, our data indicate that the three families of oligosaccharides have different propensities to interact with and stabilize liposomes in the dry state. These differences are not very pronounced up to DP3, but become more obvious at the higher DPs. They are most pronounced at the level of H-bonding to the P=O and choline groups and not apparent at the level of the C=O groups, where all oligosaccharides show reduced interactions with increasing DP. At this least exposed site of the lipids, steric factors probably dominate all other possible contributions. The differences in H-bonding to the P=O groups between the different sugars are mirrored in the complexity of the phase behavior of the dry liposome preparations and in the degree of protection against CF leakage the different oligosaccharides can afford.

An interesting, but still unresolved, question is why different oligosaccharides of the same DP show such dramatically different abilities to interact with membranes in the dry state. There is some indication in the literature that structural flexibility may be the crucial factor that distinguishes the different oligosaccharide families. The main difference between

inulins and malto- and manno-oligosaccharides is that inulins are mainly (except for one terminal Glc unit) oligofructoses composed of structurally rather flexible furanose rings [44,45], while the other two families are composed of more rigid pyranose Glc and Man rings [44]. Therefore, the higher flexibility of the furanose rings may counterbalance the negative steric effects of increasing DP in inulins, leading to an independence of inulin–membrane interactions from size. In the more rigid oligosaccharides, on the other hand, the negative steric effects are dominating the size dependence of these interactions.

This leaves open the question why different pyranose-based oligosaccharides behave differently and the available evidence suggests a significant contribution of linkage type to the structural flexibility of such sugars. Of the pyranose-based oligosaccharides, RFO show the highest degree of interaction with dry lipids [14]. RFO are 1–6 linked carbohydrates and it has been shown recently that this linkage type affords the oligosaccharides additional flexibility compared to 1–4 linked oligosaccharides [46]. This is related to the fact that 1–6 linkages involve three dihedral angles, while 1–4 and 1–3 linkages only involve two dihedral angles, contributing different amounts of structural flexibility [47]. This is reflected in the fact that 1–6 linked sugars show a strong influence of H-bonding to water molecules on their conformation [48], implying the ability to adapt their conformation also to optimize H-bonding to lipid molecules in the absence of water. Similarly, the differences in the effects of malto- and manno-oligosaccharides may be related to the higher flexibility of the α -glycosidic linkage compared to the β -glycosidic linkage [49]. Clearly, more research is needed to understand the details of the structural aspects of sugar–lipid interactions.

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