Eur Biophys J (2002) 31: 126–135
DOI 10.1007/s002490100183

ARTICLE

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Crystallization of water in multilamellar vesicles

Received: 7 May 2001 / Revised: 4 July 2001 / Accepted: 10 August 2001 / Published online: 19 December 2001

Abstract The two-step crystallization of water in multilamellar vesicles (MLVs) of phosphatidylcholines has been investigated. The main crystallization occurs near –15 °C and involves bulk water. Contrary to unilamellar vesicles, a sub-zero phase transition is observed for MLVs at –40 °C that corresponds to the crystallization of interstitial water, as proved by Fourier transform infrared absorption and differential scanning calorimetry (DSC) experiments. Furthermore, by means of the DSC method and, more specifically, using the enthalpy change values ΔH_{sub} at the sub-zero transition, the number of water molecules per 1,2-dipalmitoylpzhosphatidylcholine (DPPC) molecule giving rise to this transition has been estimated for different H_2O/DPPC molar ratios. The curve of the molecular fraction of water molecules involved in the sub-zero transition versus the H_2O/DPPC molar ratio exhibits a maximum for H_2O/DPPC equal to 27 (40% in mass of water) and tends towards zero for H_2O/DPPC ratio values approaching that of the swelling limit of the membrane. A smaller enthalpy value of the sub-zero transition is found for 1-oleoyl-2-palmitoyl-3-phosphatidylcholine (OPPC) than for DPPC. This may be explained by the decrease of interstitial water's quantity when the lipid contains an unsaturated chain. When troxerutin, a hydrophilic drug, is added to the DPPC multilayers, the decrease of ΔH_{sub} and melting enthalpy of bulk water is attributed to a decrease of the entropy of the liquid phase owing to the network of water molecules surrounding troxerutin molecules. In all cases, the experiments revealed that the sub-zero transition occurs only in the presence of excess water with respect to the swelling limit of membranes. This evidence could be, at least qualitatively, related to an increase of membrane pressure on interstitial water subsequent to bulk water crystallization.

Keywords Interstitial water · Bulk water · Phospholipid multilayers · Crystallization · Troxerutin

Introduction

Since most biological systems are present in an aqueous medium, the thermodynamics of biological processes is strongly influenced by the properties of the aqueous environment which are, themselves, related to hydrophobic, electrostatic, and hydrogen-bonding interactions. The purpose of this work is to provide some insights into the two-step crystallization of water in multilamellar vesicles (MLVs) that have been investigated by Fourier transform infrared absorption (FT-IR) and differential scanning calorimetry (DSC) experiments. When the latter method is implemented, two exothermic peaks are shown in the case of MLVs prepared with 1,2-dipalmitoylphosphatidylcholine (DPPC) and excess water (bulk water), upon cooling from the gel phase$L_{\beta'}$ to low temperature (–60 °C). The first one, near –15 °C, which we call the "first crystallization", involves the crystallization of the aqueous phase. The second one, at –40 °C, had not been yet completely

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explained, and here is referred to as the "sub-zero phase transition". It is characteristic of amphiphilic lamellar systems. On heating, no transition occurs up to the melting of ice at 0 °C.

The sub-zero phase transition was first mentioned by Chapman et al. (1967) and then by Coolbear et al. (1983). It has been more specifically studied by means of DSC by Grabielle-Madelmont and Perron (1983b). These authors argued that the sub-zero phase transition involves, at least in part, the crystallization of interstitial (confined) water. They noticed that excess water had to be present for the occurrence of this phenomenon. They also detected such a transition for a sodium soap-water system. Grünert et al. (1984) studied the sub-zero phase transition by means of DSC, dielectric response measurements, and small-angle X-ray diffractometry. They investigated the system at temperatures situated between the two phase transitions: they found the water layer (*d*) to be 9 Å thick and the hydration of the polar headgroup of the DPPC molecules to be 7 moles of water per mole of DPPC (7 H_2O/DPPC). More recently, using ^{31}P NMR, ^{2}H NMR, and DSC studies of egg sphingomyelin and phosphatidylcholine-D_2O bilayers (Wu et al.1991), this transition has been tentatively assigned to the freezing of phosphocholine headgroups, although no direct evidence is available. The authors found that, in D_2O, the sub-zero phase transition occurs at –34 °C and that water molecule motions are affected by the transition. More recently, Bronstheyn and Steponkus (1993) focused their work on the freeze-induced dehydration of DPPC by submitting samples to isothermal storage at different low temperatures. They clearly point out that ice crystals may nucleate in both the extra- and intraliposomal compartments, but they have not investigated the influence of the extraliposomal crystallization on the intraliposomal crystallization.

The proportion of bulk water relative to interstitial water depends on the type of phospholipid (anionic or zwitterionic), temperature (e.g. the phase in which the system is in), and water content. For example, the amount of interstitial water is larger in the liquid-crystalline L_α than in the gel phase $L_{\beta'}$ (Janiak et al.1979). In the gel phase $L_{\beta'}$ (we mean the usual gel phase$L_{\beta'}$ in contact with the liquid bulk water phase), the hydration of DPPC molecules is maximal at around 18 H_2O/DPPC (Janiak et al.1979; Ruocco and Shipley1982) (30% by mass of water), but another value has also been found: 11 H_2O/DPPC (Grabielle-Madelmont and Perron 1983a). The threshold for maximum hydration corresponds to the swelling limit of the membranes. For water concentrations higher than this limit, for which excess water is present, the interstitial layer thickness (*d*) of DPPC as well as its main phase transition temperature are constant (17 Å and 41 °C, respectively). In these conditions, the chemical potential of bulk water is equal to that of interstitial water (Janiak et al.1979). When the water concentration is smaller than that corresponding to the swelling limit, *d* becomes smaller. The system does not contain excess water and the main phase transition temperature increases and reaches 90 °C for anhydrous DPPC.

The aqueous phase is believed to have the properties of pure water, whereas the characteristics of interstitial water are specific. In fact, the hydrogen-bond networks of polar molecules with interstitial water or bulk water are different (Alfsen and Berteaud 1976; Israelachvili 1991). Using Raman spectroscopy, the influence of the size of the water domains on the properties of confined water has been demonstrated (Lafleur et al. 1989). By means of a surface-force apparatus, the layered structure of water near membranes constituted of zwitterionic amphiphilic molecules has been established (Chapel et al. 1993). This has been revealed by discontinuities observed by compression of two bilayers, with a characteristic length of the size of the water molecule. Recent neutron diffraction measurements have shown that, at a given temperature, there is enhanced hydrogen bonding in water layers with respect to pure water, and modification of the intermolecular structure over the 2.5–8 Å range (Baker et al.1996). The present study shows also that the structure of the hydrogen-bond network seems to be influenced by the water confinement between the amphiphilic interfaces.

While water has been the subject of numerous vibrational studies (Walrafen1973) in order to better understand the structure of the liquid state and allotropic varieties of ice, fewer works have dealt with water in multilamellar systems. Lafleur et al. (1989) have compared Raman spectra of multilamellar and unilamellar vesicles and have shown that the shape of the O-H vibration band depends on the extent of the water domains. Kint et al. (1992) have recorded Raman spectra of multilamellar dispersions over a relative range of humidity. They observed that the intensity and frequency of the symmetric C-N stretching band is sensitive to the concentration of water, and noted that the headgroup conformation is influenced by the degree of hydration. By FT-IR spectroscopy, Grdadolnik et al. (1994) have performed thermotropic and lyotropic studies of multilamellar systems and have concluded that the profile of the stretching band of water can be explained in terms of a distribution of hydrogen bonds caused by multiple binding possibilities with the polar head. They also showed that no crystallization occurs for samples exhibiting a molar ratio below 9 H_2O/DPPC. Maréchal and Chamel (1996), in a FT-IR investigation of biomembranes, concluded that water molecules may be bound by one or two H-bonds.

The main interest that encouraged us to undertake this work was the study of the influence of the crystallization of bulk (extraliposomal) ice on the crystallization of interstitial (intraliposomal) supercooled water. DSC and FT-IR techniques are used for this purpose. The first goal was the decisive characterization of the sub-zero transition as crystallization of water confined in MLVs by means of two independent methods, IR spectroscopy and DSC. The second goal, which establishes the novelty of our work, was the estimation of the number of water molecules involved in this transition as a function of the content of free (non-confined) water and the study of the role played by free water on the sub-zero transition. Conversely, we point out that the so-called sub-transition obtained after sample incubation at around 0 °C (Chen et al.1980; Ruocco and Shipley1982) was outside the scope of this work. The use of FT-IR spectroscopy has been first performed in this study in order to obtain new insights about the sub-zero phase transition and, in particular, to determine which are the molecular vibrations involved in this phenomenon. Once water was recognized to be implicated in the sub-zero transition, the number of water molecules involved in this transition per one DPPC molecule has been estimated by appropriate treatment of the DSC results.

In addition to DPPC, 1-oleoyl-2-palmitoyl-3-phosphatidylcholine (OPPC) has been studied in order to observe the effect of one double bond on the above phase transition. Moreover, the influence of a highly hydrophilic molecule, troxerutin (Fig. 1), has also been investigated. Troxerutin is a flavonoid only soluble in water, ethylene glycol, or glycerol. This molecule is well known for its beneficial action on blood fluidity: it increases red blood cell deformability and decreases erythrocyte aggregation (Berthault et al.1994). Nevertheless, although troxerutin is currently used against pathologies such as venous insufficiency (Boisseau1988), the mechanism of its action at the molecular level has not been elucidated.

Materials and methods

Sample preparation

Commercial samples of 1-(*cis*-9-octadecenoyl)-2-hexadecanoyl-*sn*-glycero-3-phosphocholine (OPPC) and 1,2-hexadecanoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from Sigma (St. Louis, Mo.). As overall purity is 99% (by thin layer chromatography by Sigma) and isomer positional purity (at the glycerol backbone) is 98%, they were used without further purification.

Multilayers were prepared by dissolving the lipid powder in analytical grade chloroform from Prolabo. The solvent was evaporated at 60 °C under a nitrogen stream and the resulting dry film stored for at least 10 h in a desiccator under vacuum. Triply distilled water prepared on an Elgastat UHQ 2 system was added, and the sample vortexed extensively during 5 min at room temperature (OPPC) and beyond 45 °C (DPPC), respectively. This treatment was repeated three times. Samples prepared following this method gave the more intense sub-zero transition enthalpies. For the samples containing troxerutin, dry films of lipid were hydrated with an aqueous solution of troxerutin (at a suitable concentration of troxerutin), and vortexed as above. Water concentration will be expressed as mass percentage of water (per cent mass of water to the total mass of water and lipid) and as a molar ratio (moles of water to those of lipid) and enthalpies of transition as kJ mol^{-1} of water. For DPPC, the following concentrations have been studied: 91, 83, 80, 75, 62, 50, 40, 33, and 26% by weight of water.

Fourier transform infrared absorption

The above sample preparation was placed directly between CaF_2 windows with a pathlength of 7 or 6 µm. FT-IR experiments were carried out on a Nicolet 5DX spectrometer equipped with a TGS detector. Cooling was performed with a liquid nitrogen bath. The cooling and heating rates could not be accurately controlled but the former was around 1.5°C min^{-1} and the latter around 0.5°C min^{-1}. For each spectrum, 20 scans at a 4 cm^{-1} resolution were collected, coadded, apodized with a Happ-Genzel function, and Fourier transformed.

Differential scanning calorimetry

Experiments were carried out using a DSC-10 TA (USA) calorimeter equipped with a computer-analyzer system. The different phase transition temperatures correspond to computed peak-onset temperatures. Sample masses varied between 5 and 25 mg and were sealed in aluminum pans. Each sample crucible was placed, together with an empty one used as a reference crucible, into the DSC cell purged with a dry nitrogen gas stream in order to prevent water vapor condensation during the low-temperature measurements. The cooling rate and the heating rate were 3°C min^{-1}. The accuracy for enthalpy changes is better than 5% after DSC energy calibration.

Results

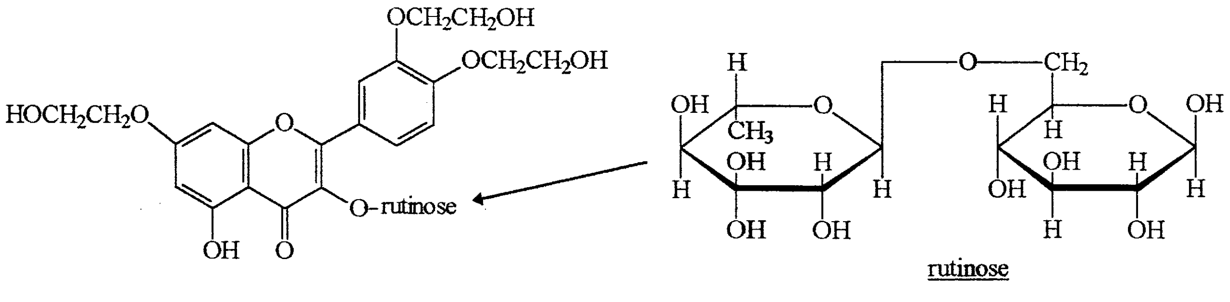
We first present and discuss FT-IR and DSC results on the DPPC-water system. Then, results on OPPC and the effects of adding troxerutin to the DPPC system will be discussed.

1-Dipalmitoyl-phosphatidylcholine

FT-IR spectroscopy

First of all, we present in Fig. 2 a comparison of the IR spectra of pure water and DPPC MLVs recorded at different temperatures. The aim is to determine the

Fig. 1 Molecular structure of troxerutin



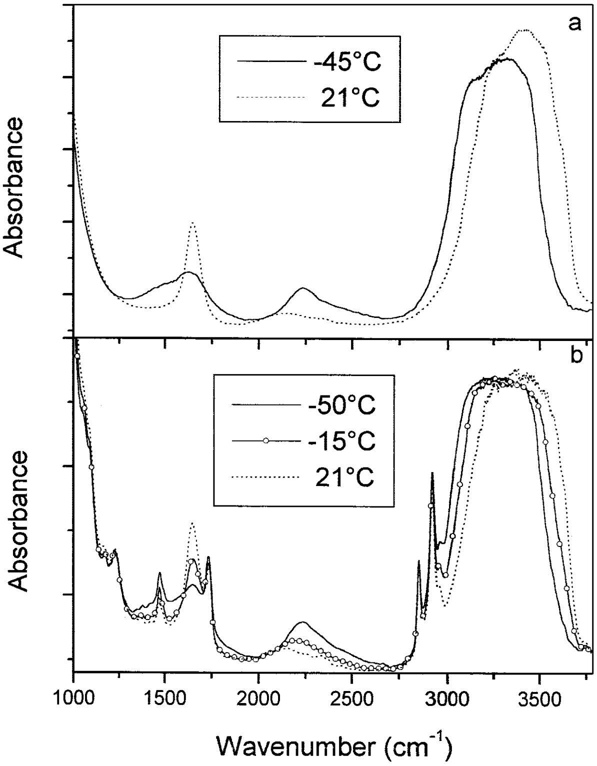


Fig. 2 Infrared absorption spectra between 1000 and 4000 cm^{-1} of: **a** pure water at 21 °C and –45 °C; **b** multilamellar DPPC (50% water by weight) at 21, –15, and –50 °C

vibrational bands affected by the phase transition, to establish synoptically the modification of the IR bands of water at the phase transitions of MLV, and to compare them to analogous spectrum modifications in pure water.

Figure 2a presents the IR spectra of pure water between 1000 and 4000 cm^{-1} recorded at 21 °C and –45 °C, e.g. in the liquid and the solid phase, respectively. Figure 2b shows IR spectra of a multilamellar dispersion of DPPC (50% by mass of water) within the same spectral region. The spectra have been recorded upon progressive cooling from room temperature to –60 °C. The different phases of the system are represented by spectra recorded at room temperature (21 °C), at –15 °C (i.e. between the first crystallization and the sub-zero phase transition), and at –50°C (i.e. below the sub-zero phase transition). The water bands are due to the O-H stretching vibrations $\nu_{\text{O-H}}$(~3370 cm^{-1}), the O-H bending vibration $\delta_{\text{O-H}}$ (near 1644 cm^{-1}), and the combination mode $\nu_{\text{L}} + \delta_{\text{O-H}}$ (around 2150 cm^{-1}), where ν_{L} is the libration mode. The main bands of DPPC are due to the symmetric and antisymmetric CH_2 stretching vibrations ν_{CH_2} of the acyl chain (2850 and 2920 cm^{-1}, respectively), the carbonyl stretching vibration $\nu_{C=O}$ (1734 cm^{-1}), the CH_2 bending vibration δ_{CH_2} (1470 cm^{-1}), and the symmetric and antisymmetric PO_2^- stretching vibrations $\nu_{PO_2^-}$ (1090 and 1220 cm^{-1}, respectively). At room temperature, frequencies, bandwidth at half height, and relative intensity of water bands in the gel phase $L_{\beta'}$ are similar to those of pure water shown in Fig. 2a, indicating that, neglecting the bound water molecules, whole water is in the liquid state at this temperature (Walrafen1973). Both Fig. 2a and b show that crystallization of water is marked by sharp discontinuities: for DPPC MLVs, the frequency of the combination mode $\nu_{\text{L}} + \delta_{\text{O-H}}$ increases abruptly from 2140 cm^{-1} to 2200 cm^{-1}, and the intensity of the $\delta_{\text{O-H}}$ mode (1644 cm^{-1}) strongly decreases. At the first crystallization, the $\nu_{\text{O-H}}$ frequency decreases from ~3370 cm^{-1} to ~3290 cm^{-1}, similar to the shift observed at the crystallization of pure water.

The sub-zero phase transition at –40 °C is also shown by FT-IR, with similar changes as those observed at the first crystallization, but to a smaller extent. The spectral changes involve essentially the water vibration bands. At –50 °C, the $\nu_{\text{L}} + \delta_{\text{O-H}}$ frequency is shifted up to 2234 cm^{-1} and the intensity of the $\delta_{\text{O-H}}$ decreases again, while the $\nu_{\text{O-H}}$ frequency is shifted down to ~3270 cm^{-1} (Fig. 2b). All the frequencies of the water bands in MLVs at –50 °C (and down to –60 °C) are very close to the corresponding frequencies of pure ice (Walrafen1973) represented in Fig. 2a, suggesting that most of the water in DPPC bilayers is crystallized when cooled down to –50 °C. This implies that a significant amount of water remains in the liquid state between the first crystallization and the sub-zero phase transition. Therefore, although the presence of lipids is necessary for the sub-zero transition to occur, they are not directly involved in the sub-zero phase transition. This finding is corroborated by the absence of discontinuities of the lipid bands ($\nu_{C=O}$, $\nu_{PO_2^-}$, ν_{CH_2}) when the transition occurs (data not shown). Upon heating from –60 °C to room temperature (data are not reported in this figure), no change occurs up to 0 °C, the fusion of water, where the frequencies and intensities of the water bands almost return to their room temperature values. These FT-IR results, revealing that the water vibrational modes show abrupt modifications near –40 °C, gave the first direct evidence that the sub-zero phase transition involves water.

To better visualize the thermotropic behavior of water, one can plot some specific spectral parameters such as band intensity or frequency. We chose the intensity of the $\delta_{\text{O-H}}$ mode since, as seen above, this band is very sensitive to sub-zero temperatures and to changes affecting the water structure. In addition, it is in the proximity of the $\nu_{C=O}$ mode that is constant within the investigated temperature range and, for this reason, may then be used as an intensity reference. Figure 3 presents the intensity ratio$I(\delta_{\text{O-H}})/I(\nu_{C=O})$ versus temperature (cooling to –60 °C and subsequent heating to room temperature) for DPPC MLVs (58% by weight of water). Upon cooling, the ratio diminishes progressively between room temperature and –20 °C. At this temperature, a sharp discontinuity is observed corresponding to the crystallization of bulk water. Then, the ratio remains constant down to –40 °C, where a smaller jump is observed which we attribute to the sub-zero phase transition. Upon heating, the intensity ratio remains

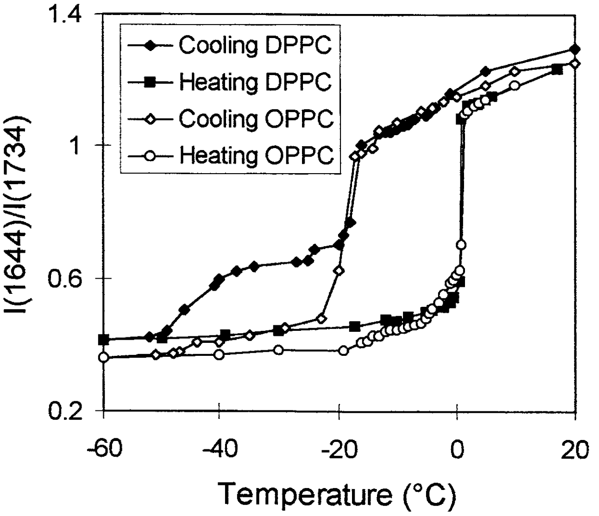


Fig. 3 Intensity ratio of $\delta_{\text{O-H}}$ and $\nu_{\text{C=O}}$ modes versus temperature for aqueous dispersions of DPPC and OPPC (58% water by weight)

nearly constant up to 0 °C, where a dramatic increase appears, corresponding to the fusion of water. Then, the value of the $I(\delta_{\text{O-H}})/I(\nu_{\text{C=O}})$ ratio returns to its initial values (e.g. the values for temperatures at which water is liquid). It should be noted that no discontinuities are observed upon heating up to 0 °C, suggesting that no phase transition occurs between –60 °C and the fusion of water.

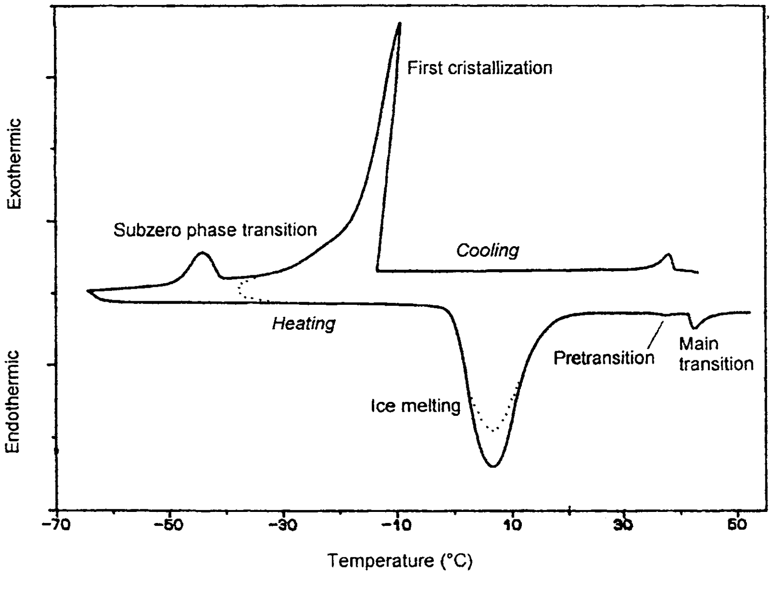
Differential scanning calorimetry

Figure 4 represents a complete cycle between + 50 °C and –60 °C obtained by DSC for an aqueous dispersion of DPPC (83% water by by mass). All the phase transitions are visible: the main water crystallization near –15 °C, the sub-zero phase transition at –40 °C, the melting of ice at 0 °C, as well as the pre-transition and the main transition of DPPC at 35 °C and 41 °C, respectively. The crystallization exotherm leans to the right because of the sudden and very energetic release of heat that is associated with the crystallization of supercooled water. The strong exothermal effect involves a transitory temperature increase which perturbs the programmed cooling scan to an extent which depends on the cooling scan rate: the lower the rate, the greater the perturbation on the thermogram. The same effect is also observed on pure water thermograms.

It is known (Chen et al.1980; Ruocco and Shipley 1982) that a sub-transition, induced by sample incubation at 0 °C, would have occurred at 18 °C on heating with an enthalpy variation largely higher than those of the pre-transition (at 35 °C) and the main transition (at 41 °C). However, this transition was not observed in this experimental work since it was not induced to occur. As a matter of fact, our DSC thermograms as well as FT-IR spectra reveal no extra transition between the melting peak of ice and the very small one due to the pre-transition at 35 °C. The thermogram of Fig. 4 leads one to conclude that, if the pre-transition and main transitions are not affected by this cyclic thermal treatment, the system must be in the gel phase $L_{\beta'}$ just after the melting of ice. Consequently, this indicates that the reverse of the sub-zero phase transition is necessarily hidden by the melting of ice on heating. Since the melting of ice is not preceded by any endothermic event upon heating, it appears that the reverse of the sub-zero transition involves also the fusion of some water molecules. Like FT-IR, DSC analysis reveals that water is directly involved in the sub-zero transition.

This argument is confirmed by another result. Let us call $\Delta H'_{\text{f}}$ the enthalpy of melting of ice, and ΔH_{sub} the enthalpy of the sub-zero phase transition. If the sample is cooled to –30 °C (i.e. just before the occurrence of the

Fig. 4 DSC thermogram (cooling and heating) between –60 °C and + 50 °C for an aqueous system of DPPC (83% water by weight). The *dottedline* is the thermogram obtained when the sample is cooled only to –30 °C and then heated



sub-zero phase transition), melting of ice occurs with a smaller enthalpy $\Delta H_f < \Delta H'_f$, as we can see with the dotted line on Fig. 3. In fact, we found that $\Delta H'_f \approx \Delta H_f + \Delta H_{sub}$, which confirms the preceding assumption and proves that ΔH_{sub} should not change much with varying temperature. So, ΔH_f represents the melting of water not confined in the membranes and, hence, not involved in the sub-zero phenomenon.

The values of $\Delta H'_f$, ΔH_f, and ΔH_{sub}, obtained for different water concentrations, are given in Table 1 for DPPC MLVs made with H_2O. Comparison between the calculated value $\Delta H_f^{cal} = \Delta H'_f - \Delta H_{sub}$ and the experimental one ΔH_f (obtained by cooling down to –30 °C) is given. For most of the samples there is a very good agreement between the calculated and experimental values. Furthermore, as no modification altered the IR vibrations of DPPC at –15 °C and –40 °C, one could neglect the interactions between lipids and water. Consequently, the enthalpy of the reversible sub-zero phase transition ΔH_{sub} was assumed to be equal to the crystallization enthalpy of water at the sub-zero transition temperature. With this assumption it was then possible to evaluate both the number of water molecules involved in the sub-zero phase transition and that of the molecules which melt at 0 °C. Upon cooling to –60 °C, the melting enthalpy allows one to calculate the number of water molecules (n_{nc}) which do not crystallize at least down to –60 °C:

$$n_{nc} = \left(1 - \frac{\Delta H'_f}{\Delta H_w}\right) c \tag{1}$$

where ΔH_w is the enthalpy of melting of ice ($5.98\ \text{kJ mol}^{-1}$) and c is the ratio of the number of water molecules to that of DPPC molecules. n_{nc} keeps a nearly constant value around 8–10 H_2O/DPPC molar ratio for each sample except at high water content, in agreement with Grdadolnik et al. (1994).

When the sample is cooled only to –30 °C, melting allows one to calculate the number of water molecules which are not confined in the membranes (n_f), and, consequently, the number of the interstitial molecules (n_{sub}) which are involved in the sub-zero phase transition (second crystallization). For the former, we have:

$$n_f = \frac{\Delta H_f}{\Delta H_w} c \tag{2}$$

For the latter, one obtains:

$$n_{sub} = \left[\left(1 - \frac{\Delta H_f}{\Delta H_w}\right) c\right] - n_{nc} \tag{3}$$

These values are reported in Table 1 . The mean value of n_{sub} in the range from the 27 H_2O/DPPC molar ratio up to pure water is around 9. The number of water molecules involved in the sub-zero transition decreases sharply only when water content further decreases (20 H_2O/DPPC) and approaches the swelling limit of the membranes. On the other hand, n_f and its molecular fraction n_f/c decrease as the water content decreases.

During DSC experiments we have noticed that the onsets of the melting peaks of water, when the sub-zero transition was forced to occur on cooling the samples to –60 °C, were systematically recorded at temperatures 0.5–1 °C lower than those measured when the same samples were initially cooled only to –30 °C (in this case, only the first crystallization occurred). This evidence may suggest that, during interstitial crystallization, water (and then ice) remains confined within intralamellar compartments instead of being expelled. On heating, melting of extralamellar (bulk) water is probably just preceded or triggered by that of water retained by the lipid membrane, so that no splitting of the melting peak is involved.

Figure 5 is a plot of the molecular fraction n_{sub}/c of the water molecules involved in the sub-zero phase transition versus water content expressed in terms of c,

Table 1 Thermodynamic results concerning the crystallization of water and the sub-zero transition. $\Delta H'_f$: enthalpy variation at the ice melting; this thermal event corresponds to the sum of the enthalpy change associated with the melting of non-confined water (bulk phase) and that associated with the melting of interstitial water. ΔH_{sub}: enthalpy variation at the crystallization of interstitial water (sub-zero transition). ΔH_f^{cal}: calculated enthalpy change at melting of non-confined water by difference between experimental $\Delta H'_f$ and ΔH_{sub} values, the latter being taken as absolute value. ΔH_f: experimental enthalpy change at melting of non-confined water; n_{nc}: number of water molecules per one DPPC molecule which do not crystallize even at temperatures slightly lower than the sub-zero transition; n_{sub}: number of interstitial water molecules per one of DPPC molecule involved in the sub-zero transition; n_f: number of non-confined water molecules per one DPPC molecule

Water content		$\Delta H'_f$ cooling temp.: –60 °C (kJ mol^{-1})	ΔH_{sub} (kJ mol^{-1})	ΔH_f^{cal} (kJ mol^{-1})	ΔH_f cooling temp.: –30 °C (kJ mol^{-1})	n_{nc}	n_{sub}	n_f
(wt%)	H_2O/DPPC							
91	413	5.98	0.23	5.77	5.82	2	8	403
83	200	5.69	0.35	5.36	5.56	1	12	187
80	163	5.94	0.43	5.52	5.56	1	10	152
75	122	5.44	0.69	4.73	4.94	9	13	101
62	66	5.15	0.85	4.31	4.44	9	8	49
50	41	4.56	1.12	3.43	3.39	10	6	25
40	27	5.56	2.16	3.39	3.72	2	8	17
33	20	3.60	0.86	2.72	2.68	8	3	9
26	14	3.18	0.60	2.59	2.47	7	1	6

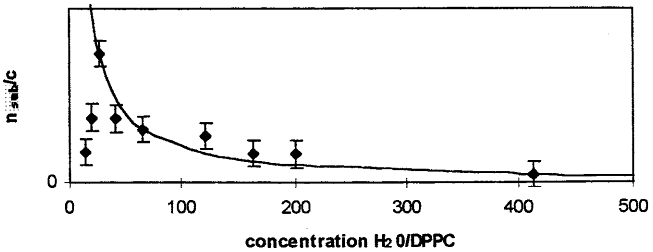


Fig. 5 Variation of the molar fraction of the interstitial water molecules involved in the sub-zero transition (n_{sub}/c) versus the whole water molecules (c) expressed as the ratio of the number of water molecules to one DPPC molecule. The *line* represents the molar fraction, for the same samples, if 8 H_2O/DPPC are implicated

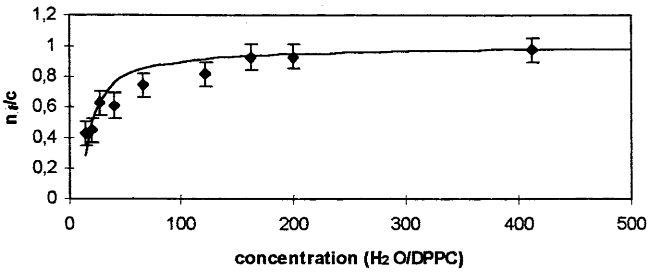


Fig. 6 Variation of the molar fraction of the melting water molecules (n_f/c) not confined in the membranes versus the whole water molecules (c). The *line* represents the molar fraction, for the same samples, if the whole water except 10 H_2O/DPPC would melt at 0 °C

the H_2O/DPPC molar ratio. This curve shows a maximum for 27 H_2O/DPPC (40% of water mass) and n_{sub}/c tends towards zero for high and low water concentrations. As n_{sub} is nearly constant at least for high water concentrations (Table 1), comparison between the experimental values and a calculation obtained for n_c = constant is shown in Fig. 5. The full line represents then_{sub}/c curve that one should find if 8 H_2O/DPPC moles were crystallizing at –40 °C. The agreement between this estimation and the experimental values is rather satisfactory and suggests that the number of water molecules involved in the sub-zero phase transition is nearly constant, except for low water contents. This number has to correspond to the difference between the number of interstitial water molecules and that of the water molecules that do not crystallize. The latter should be that of the water molecules forming DPPC hydrates, as already suggested by Grünert et al. (1984).

It is not surprising to notice that the maximum of the curve ofn_{sub}/c versus H_2O/DPPC molar ratio occurs for a ratio value at which excess bulk water is present. Besides, for the sample whose water mass fractions exceed 0.2 the temperature of the main DPPC phase transition remains constant (41 °C). It is then confirmed that the presence of bulk water is a necessary condition for the sub-zero phase transition to occur.

Figure 6 represents a plot of the molecular fraction n_f/c of the water molecules not confined in the membranes versus H_2O/DPPC molar ratio. The full line corresponds to the normalized difference between the number of whole water molecules and that of the confined water molecules (10 H_2O/DPPC). As in Fig. 5, some discrepancies appear between this estimation and the experimental results for low water content, in particular for the 27 H_2O/DPPC molar ratio value for which the value ofn_f/c computed from experimental enthalpy results is sensibly higher. This must be related to the fact that, for this water concentration, only two water molecules with respect to one DPPC molecule are expected not to crystallize.

In order to confirm our assignment of the sub-zero phase transition, we performed some specific experiments (data not shown). First, the thermal cycle protocol used above was applied to DPPC-H_2O unilamellar vesicles (90% H_2O) of 100 nm diameter, obtained by extrusion from MLVs. This system does not contain interstitial water. Entrapped water in the interior of the liposome forms large macroscopic domains and may not be considered as confined water. DSC thermograms do not shown any transition after the crystallization of water on cooling to –60 °C. Thus, this confirms that interstitial water of MLVs is at the origin of the sub-zero phase transition. Secondly, experiments performed with D_2O instead of H_2O confirm some results of Wu et al. (1991), i.e. that the sub-zero phase transition occurs at –34 °C instead of at –40 °C (our experiments give –32 °C). This difference can be related to the higher melting temperature of D_2O with respect to that of H_2O. As a consequence, it seems that the nature of the solvent strongly influences the sub-zero transition temperature.

Oleoyl-palmitoyl-phosphatidylcholine

FT-IR spectroscopy

Whereas DPPC is a symmetric fully saturated phospholipid (two palmitoyl chains), OPPC is an asymmetric phospholipid with the same choline headgroup, but with one mono-unsaturated chain (oleoyl chain) at the *sn*-1 position and one saturated chain (palmitoyl chain) at the *sn*-2 position. It seemed interesting to observe the effect of unsaturation on the water behavior at low temperature. The intensity ratio $I(\delta_{\text{O-H}})/I(\nu_{\text{C=O}})$ for OPPC MLVs (58% weight of water) is plotted versus temperature on Fig. 3. It is compared with the same temperature profile obtained for DPPC at the same water concentration. As for DPPC, upon cooling, the ratio diminishes slightly between room temperature and –20 °C. At the first crystallization, a sharp discontinuity is also observed, although the decrease of the intensity of the $\delta_{\text{O-H}}$ vibration is larger for OPPC. Then, the ratio remains constant down to the sub-zero phase transition, where a small discontinuity appears. In the case of OPPC, the phenomenon is almost perceptible. Below –50 °C, the ratio remains constant. These results suggest that, in the case of OPPC, more water molecules are involved in the first crystallization and less in the sub-zero phenomenon than in the case of DPPC.

Differential scanning calorimetry

By means of DSC, differences between the enthalpies of the transitions of OPPC and DPPC for the same mass fraction of water in the sample (0.83) have been determined. While ΔH_{sub} is smaller for OPPC (0.15 kJ mol^{-1} of water for the former instead of 0.42 kJ mol^{-1} for the latter), ΔH_{f} is higher (5.96 kJ mol^{-1} for OPPC instead of 5.58 kJ mol^{-1} for DPPC). Thus, less water takes part in the sub-zero phase transition, and more in the first crystallization. This is in agreement with the results obtained by FT-IR spectroscopy.

Effect of troxerutin

Adding troxerutin to model membranes may be relevant for two reasons: it may allow us to better understand the sub-zero phenomenon, and a better knowledge of troxerutin action can be acquired. Effectively, previous results obtained by DSC and FT-IR (unpublished data) have shown that troxerutin has no effect on both the pre-transition and the main phase transition of DPPC for troxerutin-to-DPPC molar ratios ranging from 0.05 to 1 that were investigated. More precisely, no modifications of the different transition temperatures or enthalpies were observed by DSC. This may be surprising if one considers that the pre-transition, for instance, is very sensitive to external molecules and disappears for low amounts of guest molecules. These results prove that troxerutin molecules are located in the aqueous phase and/or in interstitial water, without interaction with the lipid molecules. This assumption is consistent with the IR results, which showed that no absorption frequency ($\nu_{\mathrm{C=O}}$, $\nu_{\mathrm{PO_2^-}}$, $\nu_{\mathrm{CH_2}}$) is altered by troxerutin. Thus it seemed interesting to investigate the effect of the hydrophilic molecules on the phase transitions in which water is involved.

In preparing the sample, the troxerutin mass fraction was varied, whereas that of water with respect to DPPC was kept constant. The DSC results are given in Table 2. When troxerutin is incorporated in the bilayers, ΔH_{sub} and ΔH_{f} decrease. Nevertheless, no trend in the variation of ΔH_{sub} is noticed as a function of water content. Conversely, ΔH_{f} decreases as the troxerutin content increases, suggesting that troxerutin molecules are also located within the interstitial water. These variations of ΔH_{sub} and ΔH_{f} are consistent with the idea of the formation of a water molecules "network" surrounding the troxerutin molecules in the liquid phase, resulting in a decrease of the entropy of this phase and, consequently, of the melting enthalpy change.

Concerning experiments on troxerutin-containing samples, we can conclude that the same observed effects (decreasing of ΔH_{f} and ΔH_{sub} with respect to troxerutin-free samples) should be expected for other hydrophilic compounds that are able, like troxerutin, to form hydrogen bonds with water molecules. In fact, our thermodynamic and spectroscopic results show that interactions of troxerutin at the molecular level occur with water molecules both in extraliposomal and intraliposomal solutions. Conversely, no apparent interaction occurs between troxerutin molecules and lipid chains of DPPC even if troxerutin is certainly present also in intraliposomal water, as proved by its influence on the sub-zero transition enthalpy. In our opinion, the study of the behavior of a drug, such as troxerutin, when it is inserted in a model system, such as the DPPC-water system, can represent an important step in localizing its widespread mechanism of action in cellular membranes. To give an example, Berthault et al. (1994) reported that erythrocyte aggregation is strongly decreased when vitamin E and troxerutin are both present and they found that vitamin E was mainly situated in lipophilic parts of red blood cells while troxerutin was ascertained to be located in hydrophilic regions. These results appear to be strictly conforming to the behavior of troxerutin in the DPPC-water system.

Discussion

In this paper, the sub-zero transition was established as the crystallization of interstitial water in MLVs by means of two independent experimental methods, while the nature of this transition was argued by Bronstheyn and Steponkus (1993) on the basis of only calorimetric measurements. Moreover, the method we have used to determine the portion of water molecules involved in the sub-zero transition, as well as that of supercooled non-crystallizing water, is different from that proposed by the aforementioned authors to calculate non-freezing water and appears to be of simple applicability since it is

Table 2 Enthalpies of the sub-zero transition and water melting (cooled to –60 °C) for different troxerutin-containing samples. Water content is constant with respect to the DDPC content

Concentration (mol% trox/mol DPPC)	Water content (wt%)		ΔH_{sub} (kJ mol^{-1})	$\Delta H'_{\mathrm{f}}$ (kJ mol^{-1})
	With respect to the total mass	With respect to DPPC		
10%	82	83	0.14	5.86
28%	78	83	0.09	5.82
50%	71	83	0.11	5.48

uniquely founded upon comparison between enthalpy changes occurring at the sub-zero transition and at the ice melting. By further referring to the Bronstheyn and Steponkus (1993) paper, the coupling of DSC and FT-IR spectroscopy led us to exclude the occurrence, in our experiments, of premelting phenomena in the DPPC-water samples. In fact, no endothermal deviation or peak was recorded by DSC before the ice melting peak at 0 °C; on the other hand, FT-IR spectra showed no significant variation of the ice absorption bands before melting occurred. This evidence is indirectly corroborated by results obtained, like ours, in DSC pans (Wilson et al.1999).

Infrared results give direct evidence that water is involved in the sub-zero phase transition. Conversely, the phospholipid molecules do not seem to take part in it since their vibrational spectra are affected neither in the polar region nor in the hydrophobic one. These findings are in contradiction to those of Wu et al. (1991). Nevertheless, their experiments show that water molecule movement is altered by the sub-zero phase transition. Moreover, the fact that the sub-zero phase transition temperature is higher with D_2O than with H_2O strongly suggests that water is directly involved, since it is known that the different strength of the bond between D_2O or H_2O molecules is at the origin of the difference of their melting points. It may be the same for the crystallization of confined water (or deuterated water). Phosphocholine headgroups may be less sensitive to the change of H_2O by D_2O, and if these groups were directly at the origin of the sub-zero phase transition, the modification of the transition temperature could be less than observed.

We have unambiguously demonstrated that interstitial ice crystal nucleation is triggered by the presence of extraliposomal ice and that a sample composition exists for which this phenomenon is even accentuated (30 wt% water). It seems meaningful to underline that for this composition the amount of non-freezing water (2 moles of water per mole of DPPC) is lower than the values found by Bronstheyn and Steponkus (1993) for samples containing less water when submitted to freeze-induced dehydration. These arguments should show that this work provides complementary information on the DPPC-water system with respect to former results.

The evidence that the enthalpy of the sub-zero transition is smaller for OPPC than for DPPC is in agreement with the smaller jump of the intensity of the $\delta_{\text{O-H}}$ vibration at –40°C. These differences may be explained by a lower hydration of the OPPC polar headgroups in the gel phase, as suggested by study of the $\nu_{\text{C=O}}$ vibration (Lefevre1997). This vibration is not modified all along the thermotropic cycle, except at the main phase transition. This is in agreement with the common hypothesis that the most linked water molecules form H-bonds with the phosphate group. The carbonyl groups are easier to dehydrate in the case of OPPC than in that of DPPC.

FT-IR data support the assumption that the crystallization of water is a two-step phenomenon. Calorimetric results confirm this assumption and suggest that the first crystallization involves the bulk phase, while the sub-zero transition is concerned with the crystallization of interstitial water, except for 8–10 H_2O/DPPC molecules which do not crystallize and seem to be involved in the formation of DPPC hydrates. At first sight, this crystallization delay might be attributed to the properties of interstitial water. On the other hand, the first crystallization could be taken into account also for the sub-zero transition to be explained. As the derivative $(\partial P/\partial T) < 0$ on the crystallization line of water, the volume expansion of bulk water when it crystallizes could increase the pressure exerted by the multilamellar membrane on confined water. In this way, the crystallization delay of interstitial water could, at least qualitatively, be explained. Moreover, this argument would be in agreement with the sharp decrease of the interstitial layer at the first crystallization found by Grünert et al. (1984). Nevertheless, from a quantitative point of view, to observe a transition temperature 25 K lower than that of the first crystallization, though concerned with supercooled water crystallization, would require pressure of several hundreds of bars, which is not realistic. So, we limit ourselves to suggest that interstitial water is much more structured than bulk water and needs lower temperatures to crystallize.

Conclusions

In this study, it has been unambiguously shown that interstitial water is involved in the sub-zero phase transition. FT-IR and DSC results lead us to conclude that bulk water crystallizes at –15 °C, and that interstitial water, except for those water molecules which are the most linked to the polar headgroups of the phospholipids, crystallizes at the sub-zero phase transition which occurs at –40 °C.

Although lipid chains are not involved in these crystallizations, the presence of non-hydrated C = O groups has probably to be related to the presence of a double bond in the OPPC chain. This would imply the decrease of the thickness of the interstitial layer with respect to that of the saturated lipid DPPC and, as a consequence, the decrease of the interstitial water content. This argument is supported by the fact that ΔH_{sub} is smaller in the case of aqueous dispersions of OPPC multilayers than for the DPPC ones for the same mass fraction of water.

When troxerutin is added to water dispersions of DPPC bilayers, the decrease of ΔH_{sub} and ΔH_{f} is explained by admitting that melting entropy decreases because of the formation of a "network" of water molecules around the troxerutin molecules in the bulk and interstitial aqueous phases, respectively.

Acknowledgements One of the authors (M.P.) would thank the Universidad Autonoma Metropolitana Iztapalapa and the Consejo Nacional de Ciencias y Tecnologias (CONACYT) of Mexico.

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