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Freeze/thaw effects on lipid-bilayer vesicles investigated by differential scanning calorimetry

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Abstract

Differential scanning calorimetry (DSC) has been used to study the effects of repeated freezing and thawing on dipalmitoylphosphatidylcholine (DPPC) vesicles. Aqueous suspensions of both multilamellar vesicles (MLVs) and large unilamellar vesicles (LUVs) were cycled between −37 and 8 °C, and for each thawing event, the enthalpy of ice-melting was measured. In the case of MLVs, the enthalpy increased each time the vesicles were thawed until a steady state was attained. In contrast, the enthalpies measured for LUV suspensions were independent of the number of previous thawing events. It was concluded that MLVs in terms of freezing characteristics contain two pools of water, namely bulk water and interlamellar water. Interlamellar water does not freeze under the conditions employed in the present study, and the MLVs therefore experience freeze-induced dehydration, which is the reason for the observed increase in ice-melting enthalpy. Furthermore, the thermodynamic results suggest that the osmotic stress resulting from the freeze-induced dehydration changes the lamellarity of the MLVs.

Keywords: Freezing; Thawing; Lipid bilayer; Unilamellar vesicle; Multilamellar vesicle

1. Introduction

Lipid bilayer vesicles are spontaneously formed when amphiphilic phospholipid molecules are mixed with water. Depending on the preparation methods, these self-assembled macromolecular aggregates can exist as uni- or multilamellar lipid bilayer systems [1,2]. Lipid bilayer vesicles display a number of phase transitions of which the gel-to-fluid transition that is characterized by a disordering of the long acyl chains constituting the hydrophobic core element of the lipid bilayer, has been most intensively investigated [3–5]. Much less work has been carried out in relation to the thermotropic events that are associated with the hydrophilic head groups of the phospholipids, e.g. the hydration and interaction of the polar part of the lipids with water molecules. Several differential scanning calorimetry (DSC) studies, however, have shown the presence of two separate peaks in thermograms of multilamellar vesicles (MLV) upon freezing [6–9]. These two peaks have been ascribed to freezing of the bulk water phase and freezing of interlamellar water, respectively. The position of the bulk water peak is located at approximately −20 °C, while the interlamellar water peak is located at −45 °C. It has been suggested that the bulk water phase freezes by heterogeneous nucleation and the interlamellar water by homogeneous nucleation [6]. Heterogeneous nucleation refers to a process where the formation of the first small ice crystal, a nucleus, is catalyzed by a foreign particle. Since the extravesicular water represents one large continuous phase, the probability that it contains some foreign particle, which can act as a nucleating agent, is consequently very large [10]. Keeping in mind that only a few nuclei are involved in the crystallization of an entire liquid phase, it is not surprising that crystallization of the bulk water phase is initiated by heterogeneous nucleation.

Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; PE-PEG^{2000}, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[poly(ethylene glycol)-2000; MLV, multilamellar vesicles; LUV, large unilamellar vesicles; DSC, differential scanning calorimetry

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Interlamellar water, on the other hand, is present in a large number of small subcompartments, each having a very slim chance of containing a nucleating agent. Only a very small fraction of the interlamellar water compartment is therefore expected to freeze at temperatures above the homogeneous nucleation temperature. Moreover, the relatively narrow interlamellar space may be too small for a nucleus of larger than the critical size to develop [10]. At −40 °C the critical radius has been estimated to be 18.5 Å [10]. The interlamellar distance of DSPC MLVs is approximately 12–20 Å [11] and the value for DPPC is undoubtedly very similar. Thus, at −40 °C the critical radius for a spherical nucleus yields a nucleus with a diameter that is larger than the distance between neighboring bilayers. Obviously, interlamellar water cannot be directly compared to bulk water, as the interfacially oriented water molecules interact with the polar head groups of the phospholipids. Such interactions must be expected to affect the freezing properties, and the critical radius for nucleus formation in interlamellar water is therefore likely to be different than the aforementioned value. Indeed, it has been postulated that water molecules hydrogen bonded to the polar head groups are unfreezable [6–8,12]. Nonetheless, the distance between lamellae defines the upper limit of the nucleus size, and it is therefore conceivable that crystallization is inhibited by the fact that stable nuclei cannot form.

Previously, it was established that the interlamellar water of MLVs does not freeze at temperatures above the homogeneous nucleation temperature. As a result, interlamellar water does not contribute to the measured enthalpy of ice-melting in a DSC thermogram as long as the temperature is kept above −40 °C. DSC can therefore detect changes in the bulk/interlamellar water ratio, because the enthalpy of ice-melting will change accordingly. In the present study, this has been utilized to investigate freeze/thaw effects on lipid bilayer vesicles by subjecting multilamellar DPPC lipid vesicles to a freeze/thaw cycle. On the basis of the obtained thermograms, the enthalpy of ice-melting associated with each thawing event has been calculated. In order to investigate whether the enthalpy changes could be attributed to the interlamellar water, measurements on large unilamellar vesicles (LUVs) of the same concentrations were carried out. To further examine to what extent the enthalpy changes were related to the interlamellar spacing and the lamellarity of the vesicles, measurements were performed on MLVs with 5 mol% polymer PEG-lipids (PE-PEG^{2000}) [13,14].

2. Materials and methods

The lipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[poly(ethylene glycol)-2000] (PE-PEG^{2000}) were obtained from Avanti Polar Lipids, Inc. and used without further purification.

2.1. Preparation of vesicles

Appropriate amounts of DPPC were weighed out and suspended in milliQ-H_2O (Millipore, Denmark). The lipid suspensions were then placed in a 51 °C water bath for at least 90 min, during which they were vigorously shaken every 15 min. This produces MLVs. Subsequently, to create LUVs, the MLV lipid suspensions were extruded (Lipex Biomembranes, Vancouver, Canada) 10 times through two stacked 0.1-μm polycarbonate filters at 20 bar extrusion pressure, as described previously [15,16].

In the case of PE-PEG^{2000} containing vesicles, stock solutions of DPPC and PE-PEG^{2000} were made in $CHCl_3$:CH_3OH 1:1 (v/v). Appropriate amounts of these stock solutions were mixed in a test tube, and the solvent evaporated using a gentle stream of N_2, followed by drying at low pressure overnight. The dry lipid mixtures were suspended in milliQ-H_2O (Millipore) and placed at a 51 °C water bath for at least 90 min, during which they were vigorously shaken every 15 min.

2.2. DSC of freeze/thaw effects on vesicles

Differential scanning calorimetric measurements of freeze/thaw effects on vesicles were made using a CSC 4100 multi-cell differential scanning calorimeter (Calorimetry Sciences Corp., Utah, USA). The lowest temperature accessible by the scanning calorimeter was −40 °C. This is the reason why the peak corresponding to interlamellar water-freezing of the MLVs is not studied. DSC scans were obtained according to the following protocol:

Protocol 1

1. Temperature kept at 8 °C for 15 min
2. Temperature lowered to −37 °C at a scan rate of 120 °C/h
3. Temperature kept at −37 °C for 15 min
4. Temperature raised to 8 °C at a scan rate of 6 °C/h
5. Steps 1–4 repeated eight times
6. Temperature kept at room temperature for 41 h
7. Steps 1–6 repeated three or four times

At Step 4, a thermogram of ice-melting was recorded. The enthalpy of ice-melting, (ΔH), was calculated by integrating the ice-melting peak in the C_p-curve. ΔH-values are in units of J/g, which refers to the weight of the lipid–water suspension.

DSC scans were recorded for MLVs and LUVs of concentrations 2% (w/w) and 15% (w/w). Furthermore, a scan of 2% (w/w) MLVs with 5 mol% PE-PEG^{2000} incorporated was obtained. Finally, pure milliQ-H_2O was subjected to the same freeze/thaw cycle in order to check for instrumental effects and effects from the pure water phase.

In addition to the above measurements, the effect of increasing the time that the vesicles were incubated at −37 °C was investigated. For this purpose, Protocol 2,

described below, was used. The only differences between the two protocols are: in Protocol 2, the vesicles are incubated at −37 °C for a prolonged time between measurements 1 and 2, and only four measurements are recorded in each cycle.

Protocol 2

1. Temperature kept at 8 °C for 15 min
2. Temperature lowered to −37 °C at a scan rate of 120 °C/h
3. Temperature kept at −37 °C for 15 min
4. Temperature raised to 8 °C at a scan rate of 6 °C/h
5. Temperature kept at 8 °C for 15 min
6. Temperature lowered to −37 °C at a scan rate of 120 °C/h
7. Temperature kept at −37 °C for 12 h
8. Temperature raised to 8 °C at a scan rate of 6 °C/h
9. Temperature kept at 8 °C for 15 min
10. Temperature lowered to −37 °C at a scan rate of 120 °C/h
11. Temperature kept at −37 °C for 15 min
12. Temperature raised to 8 °C at a scan rate of 6 °C/h
13. Temperature kept at 8 °C for 15 min
14. Temperature lowered to −37 °C at a scan rate of 120 °C/h
15. Temperature kept at −37 °C for 15 min
16. Temperature raised to 8 °C at a scan rate of 6 °C/h
17. Temperature kept at room temperature for 41 h
18. Steps 1–16 repeated with the exception that the temperature was kept at −37 °C for 24 h instead of 12 h in Step 7.

3. Results

Multilamellar DPPC vesicles subjected to freeze/thaw Protocol 1 showed a clear trend towards larger enthalpies of ice-melting the more times the DSC scan was repeated. However, after the vesicles were allowed to remain at room temperature for 41 h, the enthalpy for the first scan returned to a lower value and once again increased with the number of times the scan was repeated. This tendency is shown in Fig. 1 for 15% (w/w) DPPC MLVs, where the enthalpy of ice-melting has been plotted as a function of the number of repetitions. Between series, the vesicles were left in the calorimeter at room temperature for 41 h. The same general trend is observed for 2% (w/w) DPPC MLVs (Fig. 2). Interestingly, for both 2% and 15% vesicles, the increase in enthalpy is more pronounced for the first series. Going from series 1 to series 4 the increase in enthalpy diminishes steadily. Only very little increase occurs in series 4 for the 2% MLVs.

In order to investigate whether the enthalpy increase could be attributed to the interlamellar water, measurements on LUVs of the same concentrations were carried

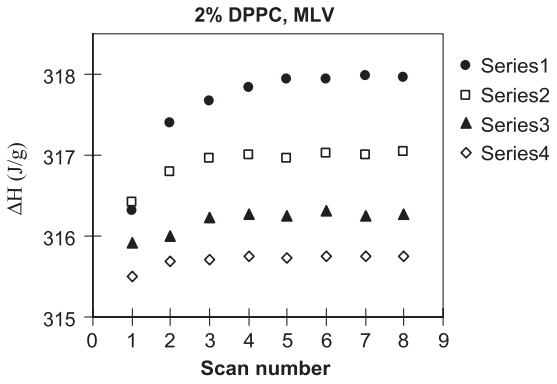

Fig. 2. Enthalpy of ice-melting for a 2% DPPC MLV-suspension as obtained from DSC. The DSC scans were performed as described in Protocol 1. The enthalpy increases with the number of repetitions until a plateau is reached. The most pronounced enthalpy increase is observed in series 1, and diminishes steadily from series 1 to series 4.

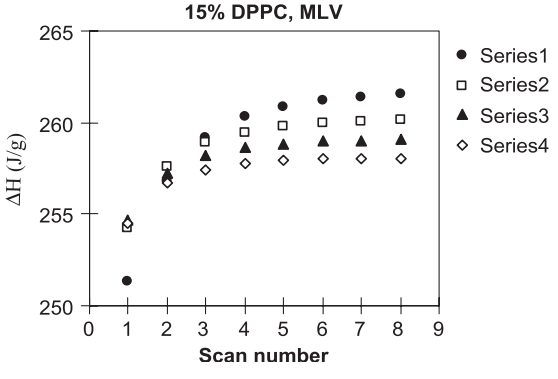

Fig. 1. Enthalpy of ice-melting for a 15% DPPC MLV-suspension as obtained from DSC. The DSC scans were performed as described in Protocol 1. The enthalpy increases with the number of repetitions until a plateau is reached. The most pronounced enthalpy increase is observed in series 1, and diminishes steadily from series 1 to series 4.

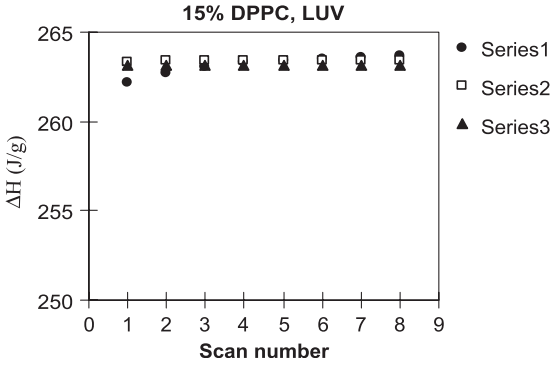

Fig. 3. Enthalpy of ice-melting for a 15% DPPC LUV-suspension as obtained from DSC. The DSC scans were performed as described in Protocol 1. The enthalpy rapidly reaches a plateau as a function of scan number.

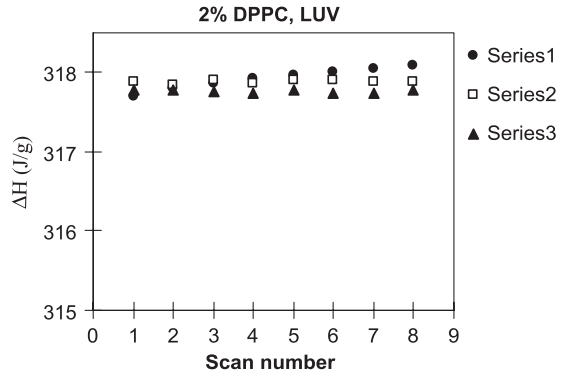

Fig. 4. Enthalpy of ice-melting for a 2% DPPC LUV-suspension obtained from DSC. The DSC scans were performed as described in Protocol 1. The enthalpy rapidly reaches a plateau as a function of scan number.

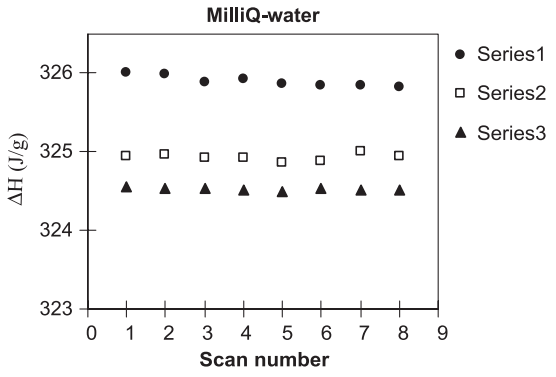

Fig. 6. Enthalpy of ice-melting for pure milliQ-H_2O obtained from DSC. The DSC scans were performed as described in Protocol 1.

out. The results are given in Figs. 3 and 4. Only three series were done for the LUVs. Clearly, the effect is much less pronounced for unilamellar vesicles. A slight increase is observed in series 1, whereas the enthalpy remains approximately constant in series 2 and 3. The reason why series 1 shows a small increase is likely related to a small amount of MLVs that are still present after the extrusion process.

Incorporation of PEG-lipids into MLVs affects the lamellarity of the lipid vesicles [13,14]. This can be used to examine if the enthalpy increase is related to interlamellar water. Measurements were performed on 2% (w/w) MLVs with 5 mol% PE-PEG^{2000}. As shown in Fig. 5, the presence of PEG also eliminates the increase in enthalpy. The enthalpy of pure milliQ-H_2O subjected to the same cycle as the vesicles is presented in Fig. 6. As expected, no enthalpy increase is observed within the individual series. Between series, however, the enthalpies decrease, which suggest that some effect coming from either the bulk water or the instrument causes the enthalpy to decrease as the series are repeated. With this in mind, it is not unlikely that all three series in Fig. 6, in reality, lie on top of each other. Furthermore, it is not unlikely that series 2–4 in Figs. 1 and 2 might reach a plateau at the same value if it was possible to correct for this tendency.

Finally, to study the effect of incubation time at −37 °C, a measurement of 10% (w/w) DPPC MLVs treated according to freeze/thaw Protocol 2 was carried out. Briefly, fewer measurements were made in each series; in series 1 the vesicles were incubated at −37 °C for 12 h between measurements 1 and 2; in series 2 the vesicles were incubated at −37 °C for 24 h between measurements 1 and 2. Otherwise, the protocol was the same as previously. The results, which are given in Fig. 7, clearly show that a large enthalpy results from increasing the incubation time (scan no. 2), whereupon the enthalpy shows no clear signs of a further increase. Hence, it appears that more water freezes when the vesicle suspension is incubated below the freezing temperature for a prolonged time.

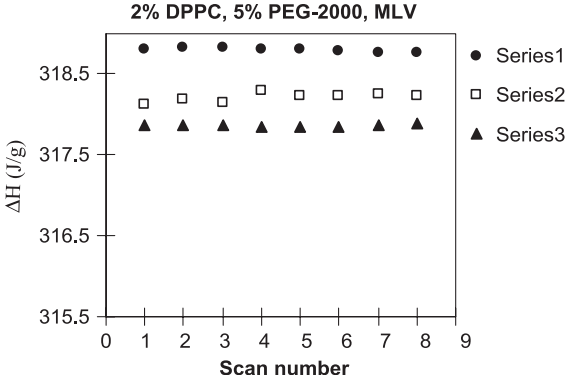

Fig. 5. Enthalpy of ice-melting for a 2% DPPC MLV-suspension containing 5% PE-PEG^{2000} obtained from DSC. The DSC scans were performed as described in Protocol 1. The enthalpy rapidly reaches a plateau as a function of scan number.

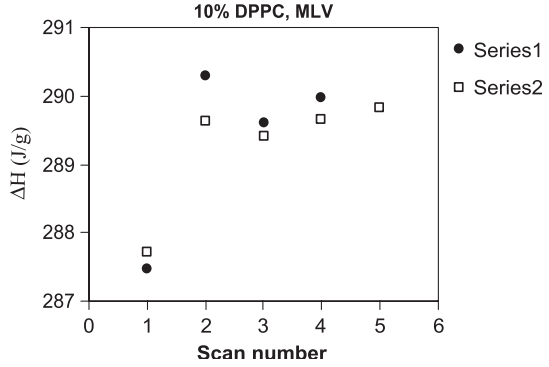

Fig. 7. Enthalpy of ice-melting for a 10% (w/w) DPPC MLV-suspension obtained from DSC. The DSC scans were performed as described in Protocol 2. The suspension was incubated at −37 °C between measurements 1 and 2.

4. Discussion

It is a well-known fact that ice is the stable form of water at temperatures below 0 °C (at standard pressure). However, it is also known that is possible to cool water to temperatures well below 0 °C before ice is formed. The crystallization of water requires the formation of a nucleus onto which water molecules can condense [10]. The formation of such a nucleus is associated with an energy barrier, and it is this barrier that accounts for the supercooling ability. When a nucleus is formed in supercooled water, two counteracting energy contributions are of importance. The energy release (per unit volume), ΔG_v related to the difference in chemical potential between ice and supercooled water promotes nucleation, whereas the energy (per unit area), γ required to form an ice/water boundary opposes nucleation [10,17]. This energy requirement is related to the fact that the atoms at the interface lack the maximum coordination available to the atoms at the center of the crystal. For a spherical nucleus of radius r, the net free energy of nucleation, ΔG_n is:

$$\Delta G_n = \frac{4}{3}\pi r^3 \Delta G_v + 4\pi r^2 \gamma \quad (1)$$

As γ is positive and ΔG_v is negative, ΔG_n is positive for small values of r, and a nucleus is only stable when a certain volume to surface area ratio is exceeded. Growth of a nucleus is however energetically favored at somewhat smaller volume to surface area ratios, namely when the differential of the above expression for ΔG_n is negative. The critical radius, r^* at which growth becomes favored can be obtained by equating the differential of ΔG_n to zero, leading to:

$$r^* = -\frac{2\gamma}{\Delta G_v} \quad (2)$$

By substituting r^* into the expression for ΔG_n, the corresponding energy barrier becomes:

$$\Delta G_n{}^* = \frac{16\pi\gamma^3}{3(\Delta G_v)^2} \quad (3)$$

The meanings of r^* and $\Delta G_n{}^*$ are visualized in Fig. 8, which shows ΔG_n as a function of r. Whenever a nucleus having a radius larger than r^* is formed, liquid water will condense onto the nucleus and it will quickly grow in size. With increased supercooling, ΔG_v becomes significantly more negative, whereas γ is relatively insensitive to temperature variations. Both the critical radius and the energy barrier thus decrease with decreasing temperature. Nucleation can take place by two fundamentally different processes called homogeneous- and heterogeneous nucleation.

Homogeneous nucleation refers to a process, taking place within the pure liquid itself. In liquid water, there will be continuous fluctuations in density and structure due to thermal motion [17]. Occasionally, these fluctuations will form a cluster of water molecules resembling the structure of an ice crystal and if such a cluster is larger than critical size, crystallization is initiated. Under most practical conditions, crystal growth is rapid compared to nucleation, so that only a few nuclei are involved in freezing of bulk water [10]. The rate of nucleation is strongly dependent on temperature, and homogeneous nucleation is consequently a very well-defined event. Around −40 °C, nucleation rate increases dramatically with decreasing temperature and pure water virtually always freezes near this temperature [10,17,18].

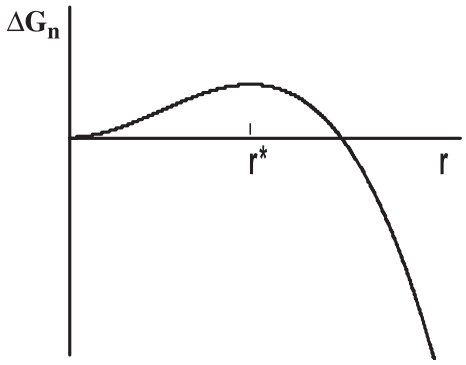

Fig. 8. Free energy of nucleation as a function of nucleus size. When r^* is exceeded, growth becomes favored and the nucleus will quickly grow to a size where it is stable, i.e. $\Delta G_n < 0$.

Homogeneous nucleation is a very rare event that only occurs in extremely pure water. In most instances, foreign particles or dissolved molecules, which can aid the formation of a cluster of critical size, will be present [10,17]. Nucleus formation catalyzed by a foreign particle is termed *heterogeneous nucleation*. Effective nucleating agents lower the energy barrier involved in nucleation by having an interfacial free energy with ice that is smaller than the ice/water interfacial free energy [17]. An ice-like cluster, which is bounded on one side by the nucleating agent and on the other by water, will have a lower free energy than a cluster surrounded entirely by water. This minimizes ΔG_n, r^*, and $\Delta G_n{}^*$, which makes heterogeneous nucleation take place at significantly less supercooling than does homogeneous nucleation. As previously mentioned, the interlamellar water of MLVs does not freeze at temperatures above the homogeneous nucleation temperature, which is approximately −40 °C.

In the present study, the vesicles were kept at temperatures between −37 and 8 °C. Consequently, interlamellar water never freezes and a situation arises where the bulk water phase is frozen and the interlamellar water is unfrozen. Such a situation results in dehydration of the vesicles, as interlamellar water molecules will diffuse through the bilayer, whereas virtually no diffusion in the other direction takes place [6]. In each cycle of freeze/thaw Protocol 1, the vesicles spend far more time below 0 °C than they spend above 0 °C. As a result, interlamellar water will have more time to diffuse out of the vesicles at subzero temperatures than the extravesicular water has to diffuse back into the

vesicles at temperatures above 0 °C. Each cycle therefore drives more water into the extravesicular phase until equilibrium between efflux and influx is reached. This is exactly what Figs. 1 and 2 show. Between series, the 41 h that the vesicles are incubated at 25 °C is sufficient time for water to re-enter the vesicles, which explains why each new series starts at a lower enthalpy value.

Figs. 3 and 4 show that unilamellar vesicles do not exhibit the same tendency. At first glance, this is not surprising because the enclosed volume of 100 nm vesicles is sufficiently large for stable nuclei to form. However, an estimate of the probability of homogeneous nucleation inside 100 nm vesicles makes it evident that crystallization of intraliposomal water is much more likely to take place by heterogeneous nucleation. Franks et al. [19] have evaluated the natural logarithm of the nucleation rate to be 31.07 s^{-1} m^{-3} at −39 °C. The volume of 100 nm vesicles is 5.2×10^{-22} m^3 meaning that 1.6×10^{-8} nuclei form inside a single vesicle per second. In other words, at −39 °C homogeneous nucleation only occurs every 6.2×10^7 s or once in 2 years. At −37 °C, the average waiting time would be far longer. This means that ice formation inside the vesicles must occur by heterogeneous nucleation. It has been argued that point defects in the crystal structure of effective nucleating agents are responsible for their nucleating ability [10]. If this is the case, one could speculate if imperfections such as grain boundaries or sites of hydrophobic mismatch in a bilayer membrane could act in the same way. Prolonging the incubation time between scans 1 and 2 resulted in a large enthalpy increase from scan 1 to scan 2, while subsequent scans showed no further increase (see Fig. 7). This is consistent with the diffusion hypothesis, as the interlamellar water now has plenty of time to leave the vesicles prior to the second scan.

Incorporation of 5% PE-PEG^{2000} also abolished the increase in enthalpy. Kenworthy et al. [13] have measured the lamellar repeat period of PEGylated vesicles by low angle X-ray diffraction. At concentrations higher than 3% PE-PEG^{2000} they obtained results indicating that the vesicles were present either as LUVs or as MLVs with large fluid spaces between bilayers. Consequently, the ability of the lipopolymers to abolish the enthalpy increase further supports the interpretation that the increase is coming from the interlamellar water flux.

It has been argued that repeated freezing and thawing prior to extrusion produces vesicles that are more unilamellar in nature than the vesicles obtained by extrusion alone [14]. A possible explanation for this observation may be found by comparing the individual series in Figs. 1 and 2. As the series are repeated, the difference in enthalpy between the first and the last scan decreases steadily. This difference is a measure of the amount of water confined to the small interlamellar spaces, and it therefore seems that the lamellarity shifts towards a more unilamellar nature. But how can this finding be rationalized? Multilamellar vesicles experience large osmotic stresses as a result of the freeze-induced dehydration discussed above. As a consequence of these stresses, MLVs are probably more prone to undergo structural changes than are LUVs. In a freeze/thaw step, LUVs (or MLVs with large fluid spaces between lamellae) are therefore preserved, whereas MLVs change structure. A vesicle suspension will thus become progressively more unilamellar as a freeze/thaw cycle proceeds. Experimental support for the argument that freeze-induced dehydration can result in structural changes is found in the literature. Talsma et al. [9] have found that vesicles incubated at −25 °C grow in size as a result of freezing, while vesicles stored at −50 or −70 °C do not. Both −50 and −70 °C lie below the homogeneous nucleation temperature where interlamellar water as well as extraliposomal water freezes. Hence, only the vesicles stored at −25 °C experience osmotic stresses, which might explain the fact that these vesicles are the only ones growing in size.

5. Conclusion

The DSC results show that MLVs contain two pools of water, interlamellar water and bulk water, that have different freezing characteristics. The interlamellar water can be supercooled to approximately −40 °C before it freezes by homogeneous nucleation. Interlamellar water is present in a large number of small subcompartments that each have a very low probability of containing an impurity which can promote heterogeneous nucleation. Furthermore, the interlamellar spacing of the MLVs might be too small for stable nuclei to form. Interlamellar water is therefore present as liquid water in the temperature range employed (−37 to +8 °C) and does not contribute to the enthalpy of melting. In contrast, the bulk water freezes by heterogeneous nucleation at significantly less supercooling. Bulk water is present as one large continuous phase, which undoubtedly contains some impurities that can act as nucleating agents. Consequently, the bulk water phase freezes during the freeze/thaw cycle and the enthalpy of ice-melting is therefore a measure of the amount of bulk water present. The results further suggest that during the time when the bulk water phase is frozen, and the interlamellar water is liquid, the vesicles experience an osmotic stress that results in a dehydration and a net diffusion of water molecules to the bulk water phase. The enthalpy increase observed for the MLVs is therefore a manifestation of this diffusion. The fact that the enthalpy increase becomes less pronounced as the freeze/thaw cycle is repeated indicates that structural changes towards a less multilamellar structure results from the freeze-induced dehydration that the MLVs experience.

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