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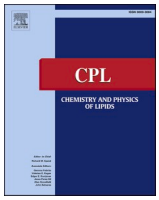

Closer look at the calorimetric lower transition in lipid bilayers

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A R T I C L E I N F O

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The thermal behavior of unilamellar vesicles has been revisited with differential scanning calorimetry to address the issue of whether it is essential to include interactions between neighboring bilayers in theories and simulations of the ripple phase. The issue focuses on the lower, *aka* pretransition, and the ripple phase that clearly exists between the lower and main transitions in multilamellar vesicles (MLV). We find anomalous thermal behavior in unilamellar vesicles (ULV) beginning at the same temperature as the lower transition in MLVs, but this feature is considerably broadened and somewhat weaker compared to the lower transition in MLVs. We ascribe this to the difficulty of packing a regular ripple pattern on small spheres. In agreement with a few reports of a ripple phase in direct images of single bilayers, we conclude that interactions between neighboring bilayers are not essential for the ripple phase in lipid bilayers.

1. Introduction

Multi-lamellar vesicles (MLVs) of saturated phospholipids, such as DPPC and DMPC, have long been known to have a ripple phase (Tardieu et al., 1973) in the temperature range between the main transition and a lower transition, usually called the pretransition. The distinction between the gel phase that exists below the lower transition and the fluid phase that exists above the main transition is well understood as due to the melting of the tilted and all-trans hydrocarbon chains in the gel phase to becoming conformationally disordered in the fluid phase above the main transition (Nagle, 1980). In contrast, even though the ripple phase is quite well characterized structurally (Akabori and Nagle, 2015), it is still not well understood theoretically regarding what brings about the breaking of the flat symmetry of the gel and fluid phases (Nagle, 2023).

By far, most theories have assumed that the ripple phase forms in a single bilayer without having to consider interactions between bilayers; various papers (Kamal et al., 2011; Mackintosh, 1997; Scott and Mccullough, 1991) point to the extensive literature. However, the structures obtained from MLVs have neighboring bilayers packed closely together with only a small water space between them. When the hydration level is increased, the unit cell changes, with a decreasing monoclinic angle (Wack and Webb, 1989). Clearly, interactions between bilayers must be considered to account for this behavior. An early paper (Cevc et al., 1981) suggested that interbilayer interactions could be essential for a ripple phase. Another theory has included them (Goldstein and Leibler, 1988), although it appears that it also predicts a ripple phase in an isolated single bilayer.

An important diagnostic for whether there is a ripple phase in lipid bilayers is whether there is a lower transition, so it is natural to consider this criterion for unilamellar vesicle (ULV) samples that are composed of many more or less spherical vesicles, each consisting of a single bilayer. Experimentally, some calorimetric studies of ULVs found a lower transition, but the enthalpic size of the transition ΔH_L was usually quite small compared to the main transition ΔH_M. Defining $R = \Delta H_L/\Delta H_M$, R is about 0.2 for MLVs. In contrast, R values for ULVs have ranged from 0.04 to 0.07 (Parente and Lentz, 1984; Biltonen and Lichtenberg, 1993; Mason et al., 1999; Heimburg, 1998) or a lower transition, but without an apparent enthalpy (Males et al., 2022). Other studies have reported no lower transition (Kreutzberger et al., 2015; Parry et al., 2010; Inoue et al., 1981). The focus of this paper is to analyze new calorimetry scans on ULVs to clarify this issue.

There are two reasons why the DSC lower transition in ULVs could be smaller than in MLVs. The first explanation is that even the best methods of forming ULVs yield a fraction of pauci-lamellar vesicles (PLVs) and only that fraction has a lower transition. However, it has been shown (Kucerka et al., 2007; Scott et al., 2019) that essentially pure ULV samples can be obtained above the main transition either by using small enough diameters (50 nm) or by using a small fraction of charged lipids (4%) in larger (100 nm) ULVs. Nevertheless, even if the original sample is pure metastable ULVs, a fraction of the sample might form more thermodynamically stable PLVs as the sample is taken through its phase

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transitions and it could be this fraction that might give the small reported ΔH_L. Let us call this the artifactual explanation for the small ratio R in the lower transition relative to what occurs in MLVs. We have attempted to remove this artifact by preparing ULV samples that are verified by x-ray scattering to have negligible PLV contamination both before and after taking them through the phase transitions.

The second explanation for a small R in ULVs is intrinsic and not artifactual. The repeat spacing of ripples is 15 nm in the plane of the bilayer. That makes it difficult to arrange a uniform array of ripples on a sphere with a diameter of only 50 nm or 100 nm. This high curvature would make any ripples disordered and incomplete compared to the ripple phase in MLVs in which the bilayers have much larger radii of curvature on average.[1] One way to overcome this intrinsic curvature effect is to make larger ULVs. It would not apply to GUVs (Giant Unilamellar Vesicles) which have radii similar to MLVs. Importantly, there is one report that briefly noted that GUVs (and also ULVs) did not have a calorimetric pretransition (Kreutzberger et al., 2015). However, calorimetry of GUVs necessarily has very little lipid compared to water, and the lower transition was not a focus of that study. We have focused on smaller ULVs (50 nm and 100 nm) which allow for a higher concentration of lipid and a better view of small DSC features.

It should also be noted that direct imaging of the ripple phase has generally been performed on MLVs (Woodward and Zasadzinski, 1996). However, it was strongly suggested in an early study on mixed vesicles that single walled vesicles also have ripples (Copeland and Mcconnell, 1980), and (Vinson et al., 1991) showed an image of ripples in a DPPC sample that the paper suggested was probably a single bilayer. Perhaps the most direct evidence that there is a ripple phase in single bilayers comes from atomic force microscopy studies of bilayers adsorbed to a solid substrate that focused on a region that was considered to have had only one bilayer on top of a monolayer (Kaasgaard et al., 2003). That system appears to be rather like the "floating bilayer" that was studied by diffraction (Fragneto et al., 2001) which showed an intervening phase, although it was not identified as a ripple phase in that paper. Also, interactions between bilayers have been thought to be far too small to contribute substantially to transition enthalpies (Nagle, 1980) and that remains the case using more recent values of the interaction parameters (Petrache et al., 1998) as is shown in supplementary material SM-1. While the studies mentioned in this paragraph support single bilayers having a ripple phase, they do not shed light on the DSC results.

2. Materials and methods

Synthetic DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) and DPPG (1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (sodium salt)) were purchased from Avanti Polar Lipids (Alabaster, AL). Purity was affirmed by thin layer chromatography and by the sharpness of the main transition (0.1° HWHM). Organic solvents were high-performance liquid chromatography grade from Aldrich (Milwaukee, WI).

Samples had nominal concentrations of 7.5 mg lipid in 1 ml of water. Higher concentrations were not useful because there would not be enough water between individual vesicles. For mixtures, dry DPPC and DPPG were first dissolved in chloroform, followed by evaporation of the organic solvent. Both mixed and single component samples were dispersed in water with temperature cycling three times between room temperature and more than 10 degrees above the main transition temperature.

Unilamellar vesicles were made using an Avanti (Alabaster, AL) mini-extruder. The sample was subjected to 25 passes above the main transition temperature with filter diameters of 50 nm for our smaller ULV samples and 100 nm for our larger ULV samples. Some samples were lightly centrifuged for 10 min at 500 g (International Clinical Centrifuge model CL) to reduce non-ULV material. Final lipid content of studied samples was obtained by weighing the sample dried under vacuum for one day and by redispersing the lipid in deuterated chloroform for ^{31}P analysis using the NEO™ 500 MHz NMR (Bruker, Berlin, Germany).

Differential scanning calorimetry (DSC) was performed using an MC-2 (Microcal, Amherst, MA) in heating mode at 15 °C/hour, from room temperature (~ 23 °C) to between 50 and 60 °C. Backgrounds were drawn such that when the subtracted data were normalized, C_P matched previous carefully determined values for MLVs in the gel and fluid single phase regions at the lowest and highest temperatures (Wilkinson and Nagle, 1982). The procedure is illustrated in SM-2 and water backgrounds are shown in SM-3.

X-ray scattering was performed on a Xeuss 3.0 (Xenocs, Holyoke, MA) diffractometer with a Rigaku (The Woodlands, TX, USA) rotating anode source and an Eiger (Dectris AG, Baden-Daettwil, Switzerland) 1 M detector. Samples were robotically loaded into the same capillary that was used for the water background, which was then subtracted. The result was then multiplied by a q^2 Lorentz factor to obtain the square of the form factors shown in the figures. Sample exposure time was limited to 10 min which results in negligible radiation damage.

Volume measurements were performed using a DMA 5000 M densimeter (Anton Paar, Ashland, Virginia) with the protocol of (Hallinen et al., 2012).

3. Results

Fig. 1 shows our specific heat results using a scale that emphasizes the lower (pre) transition and the behavior of C_P in the single phase regions. The MLV samples exhibited the usual lower and main transitions. The main transition is very sharp with a 0.1° half width at half maximum. The main transitions of both ULV samples are considerably broadened. The MLV lower transition at T_L near 36 °C is quite pronounced. In contrast, there is only an enhanced C_P near T_L for ULVs of both sizes. For MLVs, C_P in the ripple phase is considerably higher than in the gel and fluid phases and that is even more so for ULVs above T_L as well as below T_L.

Fig. 2 shows the enthalpy H obtained by integrating C_P in Fig. 1 and adding a constant to the integral for the ULV samples so that their enthalpies nearly equal the MLV enthalpy in the fluid phase above the main transition. There are clearly defined main transitions for all three

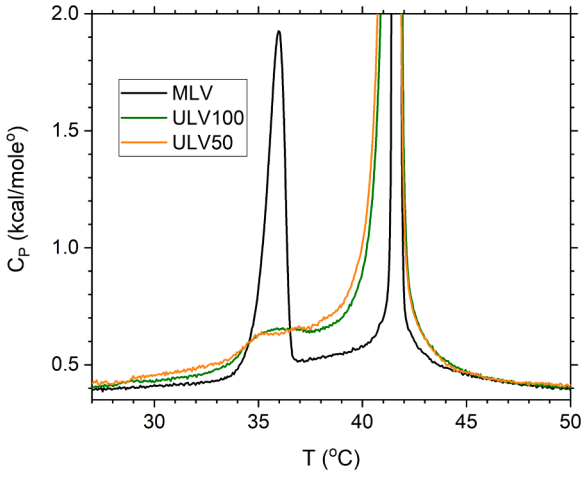

Fig. 1. Background subtracted and normalized specific heat C_P versus temperature. The maximum C_P of the MLV main transition was 40 kcal/mole/deg. Fig. S3 shows the full C_P range.

[1] This packing problem on spheres also pertains to the DPPC gel phase in which the lipids are tilted with respect to the bilayer normal. This effect may be less significant because the separation between chains is only 0.5 nm, although it might be more because the tighter packing of hydrocarbon chains could be more affected by curvature than in the ripple phase.

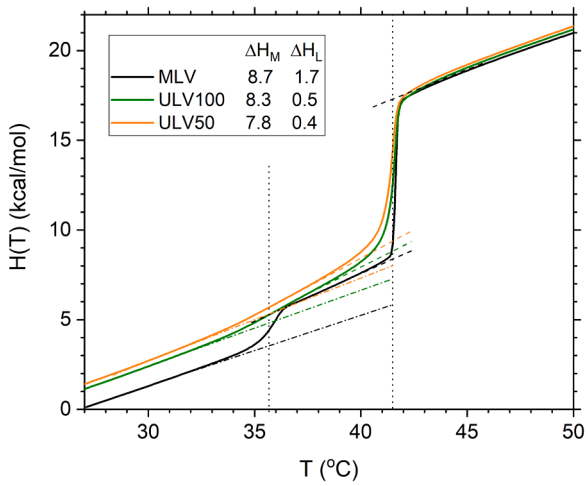

Fig. 2. Enthalpy H(T) relative to MLV at T = 27 °C obtained by integrating the data in Fig. 1 and adding constants to the ULV data to achieve near overlap above the main transition. As described in the text, the various dashed and dash-dotted baselines enable extraction of the enthalpies of transition shown in the legend. The vertical dashed lines show the midpoints of the lower transition at T_L = 35.7 °C and the main transition at T_M = 41.4 °C.

systems. The values of the main transition enthalpies ΔH_M shown in the legend are the differences between the intersection of the vertical dotted line at T_M = 41.4 °C with the single upper dashed baseline and with the similar intersections with the three lower dashed baselines that differ for the three systems. The ULV main transition enthalpies are somewhat smaller than the MLV transition enthalpy. For the lower transition, dash-dotted baselines are drawn from the gel phase in Fig. 2. The enthalpy of the lower transition ΔH_L is defined to be the difference between the intersections at T_L = 36 °C of the ripple phase dashed line and the gel phase dash-dotted line. Results for ΔH_L are shown in the legend to Fig. 2.

Most of the preceding results are qualitatively quite robust for different DSC scans. However, different background subtractions have had to be made for different samples and even for repeated scans on the same sample. An example is shown in Fig. S1. We now mention particular features in Figs. 1 and 2 that therefore are not robust, but that are nevertheless consistent with the shown data. In Fig. 1, the result that C_P is nearly the same at T = 27 °C and 50 °C for the three samples is due to the way we did background subtractions. The background for the MLV sample was chosen so that the temperature dependence of C_P in the gel and fluid phases agrees well with previous MLV data (Wilkinson and Nagle, 1982), and then the higher slopes below T_L for the ULV samples and the larger C_P between T_L and T_M follow robustly. Turning to Fig. 2, the enthalpies of all three samples were fixed to nearly the same value at T = 50 °C. Then, the enthalpy at 27 °C depended upon how much lipid was in each sample; our 5% uncertainty in the concentration for the 100 nm ULV sample could make its $H^{ULV100}(T)$ enthalpy curve nearly overlap the $H^{ULV50}(T)$ curve.

We raised the possibility in the introduction that ULVs might fuse into pauci-lamellar vesicles upon going through the transitions and that might give rise to small lower transitions. If that were the case, then one would expect that a repeated DSC scan would have a larger lower transition than the first scan. However, repeated DSC scans gave essentially the same results.

Furthermore, multilamellar contamination of nominally unilamellar samples was checked with low angle x-ray scattering. Scattering from unilamellar vesicles follows the form factor squared, which is a smooth function. In contrast, scattering from MLVs of DPPC has sharp lamellar peaks located at integer orders h times 0.1 $Å^{-1}$. Fig. 3 shows scattering from a 100 nm extruded sample after light centrifugation that is consistent with only ULVs. In contrast, scattering from a sample before centrifugation had a small first order (h=1) peak near 0.1 $Å^{-1}$ and a

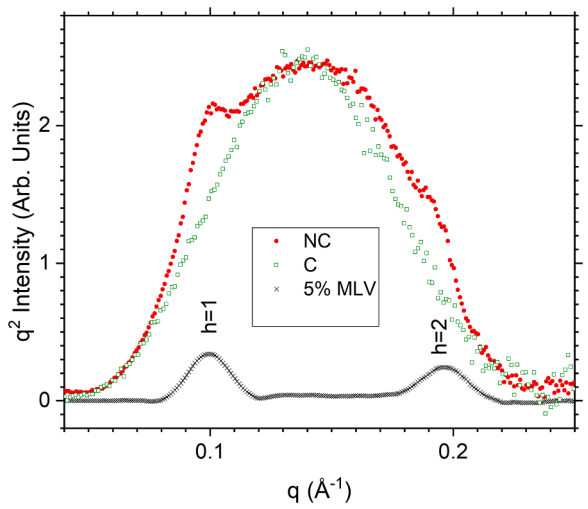

Fig. 3. X-ray scattering from ULVs before centrifugation (NC, red filled circles) and after centrifugation (C, green open squares). The black crosses show 5% of the scattering from MLVs.

shoulder near 0.2 $Å^{-1}$, consistent with contributions from multilamellar scattering. The MLV curve shown is scaled to about 5% of the scattering from the same amount of lipid as was in the ULV samples. The sizes of these scaled down peaks is comparable to the difference between the scattering from the two ULV samples, consistent with the sample before centrifugation having about 5% MLV contamination. Light centrifugation was employed for the samples in the DSC scans in Fig. 1.

A different way to avoid multilamellar contamination mixes the charged lipid DPPG with DPPC thereby repelling the stacking of bilayers, as has been shown previously (Kucerka et al., 2007; Scott et al., 2019; Males et al., 2022) and as we have verified for our samples. As has been shown (Zhang et al., 1997), DPPG has the same overall thermodynamic behavior of DPPC when buffer and salt are added. However, pure DPPG in low salt has different and quite complicated phase transition behavior (Lamy-Freund and Riske, 2003), as we have also found, and this persists even when only 5% or 10% DPPG was added to DPPC. Interestingly, when these samples were extruded, the thermodynamic phase behavior simplified to what is shown in Fig. 4. Even though the transition temperatures are not the same for different vesicle sizes, the lower transition was more pronounced for the larger vesicles. Curiously, while the main transition occurred near 41° C for the smaller vesicles, it was smaller for the larger vesicles and even smaller at 32° C for MLVs

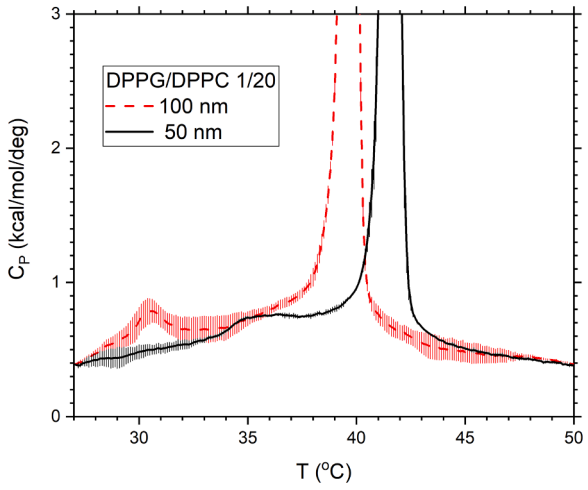

Fig. 4. DSC scans with uncertainties from repeated scans for mixtures of DPPG and DPPC in 1:20 molar ratios.

(not shown) and the transition was somewhat irregular. These and other curious results led us to discontinue phase transition studies on no salt DPPG/DPPC mixtures. Nevertheless, the X-ray intensities in the accessible range from 0.05 to 0.25 $Å^{-1}$ were essentially the same for MLV and for ULV extruded with 50 nm and with 100 nm filters, so it could be that such mixtures are helpful to avoid MLV contamination in fluid phase studies.

4. Discussion

Our DSC data in Fig. 1 confirm many results in the literature that the DSC lower transition is considerably modified and reduced in unilamellar vesicles (ULVs). Instead of the well-defined MLV lower transition peak, we describe the DSC data from ULVs as a greatly broadened lower transition that retains a fraction of the MLV lower transition enthalpy as shown in Fig. 2. We ascribe the smaller transition enthalpy in ULV to the difficulty of packing well defined, highly ordered, ripples in spherical vesicles that have radii only a few times larger than the 15 nm repeat period (Katsaras et al., 2000) of DPPC ripples. This interpretation supports the use of theories and simulations that only consider single bilayers. One might be concerned about comparing the theoretical enthalpies of the phase transitions with those measured in MLVs if interbilayer interactions are strong. However, MLVs are fully hydrated with a cushion of water between bilayers and previous estimates of the interbilayer forces (Petrache et al., 1998) indicate little contribution to ΔH_L from them compared to the measured values (SM-1).

Regarding our methodology, we have taken care to avoid MLV contamination in our ULV samples. We cannot see MLV contamination in the X-ray data for the 50 nm vesicles nor in our 100 nm centrifuged samples. Turning to the enthalpies shown in Fig. 2, integrating C_P in Fig. 1 over a limited range of temperature can, of course, only be relative to some arbitrary value. In Fig. 2 we have pinned the MLV enthalpy to zero at T = 27 °C. For ULV samples we then added different constant values to their enthalpies to make them have the same enthalpy as MLV in the fluid phase. While the assumption that the enthalpies should be the same in the fluid phase is unlikely to be exactly correct, that is much more likely than that they would have the same enthalpy at T = 27 °C because the packing of conformationally disordered lipids is less affected by local curvature than the packing of lipids in the gel or ripple phases. Equivalence of packing in the fluid phase is supported by the good agreement of bilayer structure of ULVs and bilayer stacks in the fluid phase (Kucerka et al., 2007). A correction to our assumption would be in the direction of raising the enthalpy curves for ULVs in Fig. 2, thereby further increasing the differences in enthalpy between ULVs and MLVs in the gel phase. The values of H in Fig. 2 indicate that ULVs have higher enthalpy in the gel phase, and therefore, higher energy because the PV term is negligible at atmospheric pressure. This is to be expected as curvature makes it more difficult to pack all-trans straight hydrocarbon chains together on a sphere and the ensuing curvature disorder raises the gel phase energy in ULVs compared to MLVs. These possibilities have been explored with volume measurements which don't require an arbitrary additive constant. As shown in Fig. S5, the volume at 25 °C for 100 nm ULV was about 10 $Å^3$ larger than for MLVs but there was relatively little difference in the fluid phase, thereby supporting our choice of additive constants in Fig. 2.

We had originally hoped that adding charged DPPG to DPPC would enable even better comparisons because it would remove concern over pauci-lamellar contamination. Of course, the charges on DPPG also prevent the formation of MLVs in low salt, so one can only compare different size vesicles. Surprisingly, the transition temperatures are different for the two vesicle sizes. Nevertheless, our results in Fig. 4 for the 50 nm vesicles are similar to those with no DPPG while the larger 100 nm DPPC/DPPG vesicles have a larger and better separated lower transition. This also supports the hypothesis that the smaller lower transition in vesicles is due to the difficulty of packing a ripple pattern on small spheres.

5. Conclusions

We find that there is a broad lower DSC transition in ULVs near the lower transition temperature of MLVs. We ascribe the relative weakness and broadening of this transition to the difficulty of packing a regular ripple pattern on small vesicles. We therefore conclude that interactions between neighboring bilayers are not essential for theories and simulations of lipid bilayer phase behavior and the ripple phase.

CRediT authorship contribution statement

Nagle John F.: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft. **Korono Sophia A.:** Data curation, Formal analysis, Methodology, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.chemphyslip.2023.105366.

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