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Effect of vesicle size on the heat capacity anomaly at the gel to liquid-crystalline phase transition in unilamellar vesicles of dimyristoylphosphatidylcholine

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Dynamic heat capacity has been measured near the gel to liquid-crystalline phase transition of small unilamellar vesicle (SUV) samples of dimyristoylphosphatidylcholine with a vesicle size of 30 nm. The heat capacity showed a very broad peak that is qualitatively similar to that observed in the large unilamellar vesicle (LUV) samples with a vesicle size of 100 nm reported earlier. Quantitatively, the heat capacity anomaly for SUV samples differs markedly from that for LUV samples. The half-width of the anomaly in SUV's is about 1.1 K, which is about three times that in LUV's, and the peak height in SUV's is about 30% of that in LUV's. The data on SUV and LUV samples have been analyzed in a unified manner. It has been pointed out that the curvature of the vesicle surface acts in a similar way to an external field that drives the transition from first order to critical and then to supercritical.

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I. INTRODUCTION

Phospholipid molecules are major lipid components of biomembranes. When placed in excess water, they usually form a bilayer structure as a result of the exclusion of the hydrocarbon chains by water molecules. In most cases the assembly in water takes the form of multilamellar vesicles (MLV's) with diameters ranging from several μm to several hundred nm [1]. It is also possible to obtain unilamellar vesicles made of only one sheet of bilayer by an extrusion procedure [2] or an ultrasonication technique [3]. Such unilamellar vesicles are of particular interest because (a) the vesicle radius can be as small as several tens of nm so that they are regarded as mesoscopic systems and therefore finite-size effect can be significant, and also (b) they are regarded as two dimensional, while MLV's are three dimensional.

There have been intensive theoretical efforts to describe the behaviors of such lipid bilayers, especially focusing on the gel to liquid-crystalline phase transition. Nagle [4] was the first to give a statistical theory on this line. He showed that the configurational states of the lipid chains are isomorphic to the states of a dimer model. Izuyama and Akutsu [5] extended this idea, and developed a rigorous formalism of the statistical mechanics for the model. They also showed that the finiteness of the chain length acts like an external field, and drives the transition from first order to critical, and then to supercritical. Another approach was the so-called multistate model, in which a small number q of the relatively important chain conformations are selected as the accessible chain states. This model was first proposed by Doniach for $q = 2$ [6], and generalized by Caille *et al.* [7]. Pink *et al.* [8] showed that the model gives a good description of the experimental data by choosing $q = 10$. On the other hand, as far as the authors are aware, only little theoretical effort has been made as to the effect of the vesicle-size finiteness on the transition.

Experimentally, precise measurements on unilamellar vesicles were found to be not easy especially for those with relatively small vesicle sizes because of their instability (see Refs. [9, 10], and references therein). A systematic investigation of the vesicle-size dependence of the transition is still lacking.

In a preceding paper [11], referred to as I hereafter, we measured the dynamic heat capacity of large unilamellar vesicle (LUV) samples of dimyristoylphosphatidylcholine (DMPC) with a diameter of 100 nm. It was found that the heat capacity anomaly at the gel to liquid-crystalline transition is much broader than in MLV samples [12], and shows a significant rounding over a 200 mK range. It was also found that the observed anomaly is described well when the existence of a nonzero external field was assumed, although the origin of the field was not made clear. In any case, it is quite probable that the finiteness of the vesicle size plays an important role in explaining the observed rounding.

To study the effect of the vesicle size on the transition, in the present work we have carried out dynamic heat capacity measurements on small unilamellar (SUV) samples of DMPC with a diameter of about 30 nm. Fortunately, our SUV samples prepared by an extrusion method were found to be quite stable in contrast to the SUV's made by ultrasonication. This enabled us to obtain heat capacity data with a precision which is enough for detailed quantitative analysis. The results described below show that the heat capacity anomaly depends significantly on the vesicle size. The data obtained here for SUV's are compared with the LUV data, and both data are analyzed in a unified manner. For this purpose we modify the statistical theory by Izuyama and Akutsu [5] so that we can apply it to the cases of curved bilayers. It is found that the modified theory can reproduce the size dependence of the heat capacity anomaly. In particular, we show that the curvature of the vesicle surface acts in a way similar to an external field.

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II. EXPERIMENT

First, LUV's of 100 nm diameter were prepared with a similar extrusion procedure on MLV's as described in I. Two stacked polycarbonate filters (100 nm pore size, Nuclepore 110405, Costar Corp., Cambridge, MA) were used for the extrusion, employing nitrogen pressure of 20 atm. Then residual MLV's were removed by centrifugation under 20 000g for 30 min at 21 °C, which was repeated three times. After this, LUV's were again extruded using filters of 30 nm pore size (Nuclepore 110402) ten times employing 50 atm pressure, which was followed by a similar centrifugation for 10 min.

The vesicle size of the SUV's thus obtained was measured by dynamic light scattering (DLS) utilizing a Nicomp model 370VHPL submicrometer particle sizer (Pacific Scientific, MD) with an argon laser at a wavelength of 514.5 nm. The sample was diluted about ten times with the same buffer solution as used in preparing MLV's (see I). The sample temperature was kept at 23.0 °C and DLS data were collected for 10–20 min. The diameter thus obtained was 29±9 nm, where the range shows the standard deviation. These values are for the distribution in the vesicle number. In the present case of heat capacity measurement, it is more practical to evaluate the distribution weighted with the squares of the vesicle sizes, since the number of lipid molecules contained in each vesicle is proportional to the square of the vesicle radius for unilamellar vesicles. This gave a value of 35±11 nm as the effective size. We also measured the vesicle size of the LUV's used in I in the same way, which yielded 86±17 nm for the particle number distribution and 93±19 nm for the effective distribution.

The dynamic heat capacity has been measured with a heat capacity spectrometer which is basically the same as used in I. About 60 mg of the SUV sample was hermetically sealed into a sample cell made of gold. The bath temperature was changed stepwise, each step being 20 mK in the vicinity of the transition temperature, and the measurement was done after the bath temperature was stabilized within ±0.15 mK. The measuring frequency was set to 0.01 Hz.

It has been reported that SUV's prepared by ultrasonication are generally unstable (see Refs. [9, 10], and references therein). On the other hand, our 100 nm LUV's prepared by the extrusion method showed an excellent stability [11]. It has been found that the present 30 nm SUV's prepared by a similar extrusion method are also extremely stable. The measurements have been made on two SUV sample cells. For both of these, the peak height of the heat capacity anomaly at the gel to liquid-crystalline phase transition remained unchanged within 2%, and the peak position remained unchanged within 20 mK over the whole measurement period of about five weeks for one of the samples, and about three weeks for the other.

III. RESULTS

Figure 1 shows the temperature dependence of the anomalous heat capacity ΔC_p obtained on heating [Fig. 1(a)] and on cooling [Fig. 1(b)] near the gel to liquid-crystalline phase transition of a SUV sample. The concentration of the lipid was 1.89 wt % for this sample. The temperature of the heat capacity maximum is 24.40 °C, which is about 0.5 K higher than the main transition temperature of MLV's [12], and almost the same as in LUV's [11]. The heat capacity anomaly shows qualitatively similar features to that of LUV's reported in I. First, the anomalies on heating and on cooling are almost identical. Secondly, the heat capacity anomaly accompanying the transition is very broad and shows a significant rounding. In a quantitative sense, on the other hand, the present data for SUV's differ markedly from those for LUV's. In Fig. 2, the ΔC_p data are compared for the present SUV data [Fig. 2(a)] and the LUV data obtained in I [Fig. 2(b)]. The full width at half maximum in SUV's is about 1.1 K, which is about three times that in LUV's. The peak height of ΔC_p for SUV's is about 30% of that in LUV's. Thus we see that the heat capacity anomaly at this transition shows a significant vesicle-size dependence.

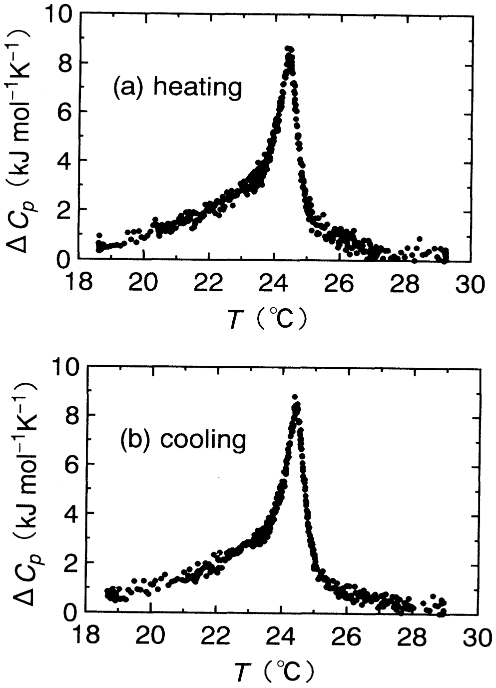

FIG. 1. Temperature dependence of the anomalous heat capacity ΔC_p of SUV sample of DMPC obtained (a) in a heating run and (b) in a cooling run.

The transition enthalpy obtained by integrating the present heat capacity anomaly is 19±2 kJ/mol. This almost agrees with the value 22 kJ/mol for LUV's in I, and also with the static transition enthalpy obtained by differential scanning calorimetry (DSC): 18.9–26.4 kJ/mol [13–16]. This fact ensures that the frequency of the present measurement is low enough to obtain the quasistatic value of the heat capacity.

The present data differ from those reported earlier for

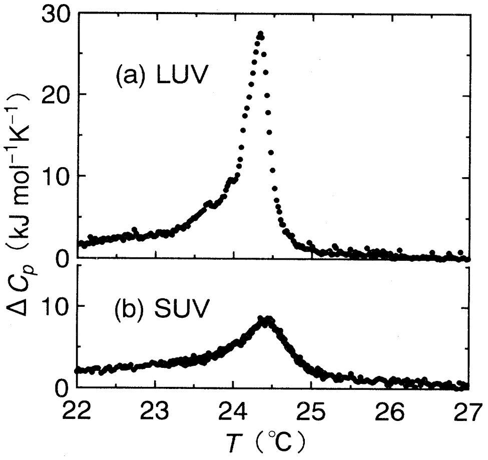

FIG. 2. Comparison of the anomalous heat capacity ΔC_p of (a) LUV's and (b) SUV's of DMPC.

SUV's of almost the same vesicle size but prepared by ultrasonication. It has been reported that such sonicated SUV's show transition temperatures about 5 K lower than in MLV's [9, 10, 17, 18], while the transition temperature of the present SUV's almost coincides with that for MLV's, or, to be more precise, is even slightly higher than that. Further, the heat capacity anomaly obtained on sonicated SUV's had a half-width of 3.1–3.6 K [9], which is about three times wider than our result. Our opinion is that the SUV's prepared by ultrasonication have considerably different physical properties from those obtained by the extrusion method. Because the sonicated SUV's are unstable, it is natural to expect that they are in a metastable state. Based on the fact that the enthalpy change at the gel to liquid-crystalline phase is smaller in sonicated SUV's than in LUV's and MLV's, Kodama *et al.* concluded that the SUV's at a gel phase temperature exist in an enthalpically higher state than LUV's and MLV's [10]. On the other hand, the excellent stability of our SUV's made by extrusion indicates that they are in the stable state and therefore show properties which are intrinsic to the unilamellar vesicles. The fact that the transition enthalpy for our SUV's agrees well with that for MLV's also supports this idea.

IV. DATA ANALYSIS

In this section we try to explain the size dependence of the heat capacity anomaly described in the last section with a model theory for lipid bilayers. Since all theories mentioned in Sec. I are for flat bilayers, they need some modification in application to the case of finite-size vesicles. Pink *et al.*'s ten-state model [8] can be treated beyond the mean-field approximation and therefore is expected to give a precise description of the transition. However, we have to rely on computer simulations in that case because of its mathematical complexity. Here we prefer to use the statistical theory by Izuyama and Akutsu (I-A) [5] for the present purpose, because their theory is tractable on one hand, and expected to be moderately accurate on the other. Both the ten-state theory and the I-A theory take into account the intermediate states between the fully ordered and the fully disordered states. In the former, they are counted as eight intermediate states, while the I-A theory sums up these states in a rigorous way. Apparently, the I-A theory has the drawback that it eventually comes to a mean-field approximation. However, as we will discuss later, the effect of critical fluctuations turns out to be practically negligible in the present case, if we use the I-A theory as the starting point.

The free energy obtained in the I-A calculation is given as

$$G = G^{\text{chain}} + G^{\text{elas}} , \tag{1}$$

where

$$G^{\text{chain}} = \epsilon\,\rho + k_{\text{B}}T\left[\frac{\rho\ln\rho}{M} - (\ln 4)\rho + \frac{\pi}{2}\,\rho^2 + \frac{\pi^2}{12}\,\rho^3 + \frac{5\pi^3}{192}\,\rho^4\right] \tag{2}$$

is the contribution from the cooperative conformation of hydrocarbon chains, and

$$G^{\text{elas}} = \frac{1}{2}\,\omega\,Q^2 + P\,(1+Q)^2 \tag{3}$$

is the elastic energy coming from the dilatation in the spacing of the lipid molecules [5]. Here, ρ is the "disorder" parameter which becomes zero in the completely ordered state, Q is the normalized increase in the chain spacing from the value in the ground state for zero pressure, $P = 0$, and M represents the chain length. The excitation energy ϵ diminishes as Q increases because the steric hindrance of the CH$_2$ groups against the chain bending is reduced, and therefore we have

$$\epsilon = \epsilon_0 - \alpha\,Q , \tag{4}$$

with positive constants ϵ_0 and α.

The above free energy is for a flat bilayer. We therefore modify it here for the application to the case of unilamellar vesicles with finite surface curvature. Before starting this, some words should be added as to the definition of Q. In a curved layer, the interchain distance depends on the position along the chain as described below, so that it should be made clear at which position Q is measured. Here we choose to define that Q is measured at the position of the head groups. This is because Eq. (3) describes the elastic energy, which mainly comes from the interactions between the head groups.

Let us consider here the case of the outer layer. Because of the finite curvature, the spacing at the position of the chains is smaller than that measured at the position of the head group, as seen in Fig. 3(b). Therefore Eq. (4) should be modified as follows. The interchain spacing at the position of the head is given as $1 + Q$ from the above definition. Then the interchain spacing at a position with a distance x from the head is given

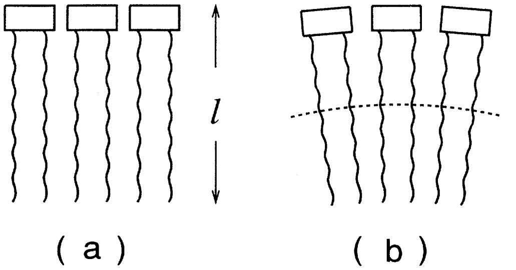

FIG. 3. Comparison of (a) a flat lipid layer and (b) a curved lipid layer. In (b), dashed line shows the position where the cross-sectional area of the lipid molecule is the same for the two cases.

as $(1+Q)(1-2x/D)$ where D is the vesicle diameter, and thus the increase from the ground state is given by $Q'=(1+Q)(1-2x/D)-1$. This Q' is to be inserted in place of Q in Eq. (4). As a result, we find that

$$\epsilon=\left[\epsilon_0+\frac{2x}{D}\alpha\right]-\left[\alpha-\frac{2x}{D}\alpha\right]Q \tag{5}$$

$$=(\epsilon_0+\Delta\alpha)-(\alpha-\Delta\alpha)Q\ , \tag{6}$$

where $\Delta\alpha\equiv 2\alpha x/D$, which is proportional to the curvature. It was assumed in the I-A theory that the excitation energy does not depend on the position within the chain. Since the x dependence of ϵ is weak, we can assume that the "effective" excitation energy is given by Eq. (6) using some appropriate value for x. The simplest way is to choose $x=l/2$, where l is the thickness of the monolayer, or half of the bilayer thickness (see Fig. 3). With $D=100$ nm, $l=2.2$ nm [19], and $\alpha=1125$ K (the same value as used in Ref. [5]), we obtain $\Delta\alpha\sim 25$ K. The flexibility of the chains probably reduces this estimation. Indeed, we can imagine that the chain tries to reduce the increase in the excitation energy ϵ by enhancing the disorderedness of those parts where the increase is relatively small.

Another aspect of the surface-curvature effect is the expanding force the head group experiences from the repulsion between the chains. Because of this, the pressure P should be replaced by $P-\Delta P$, where ΔP is again proportional to the curvature. A rough estimation of the magnitude of ΔP is made as follows. Let us approximate the lipid molecule as an elastically uniform cylinder. If we bend a flat layer as shown in Fig. 3(a) into a curved layer as Fig. 3(b), the cross-sectional area of the head part expands and that of the tail part contracts, while it remains unchanged somewhere between them, at the center in the first approximation, which is marked as a dashed line in the figure. Then the spacing of the head groups becomes $(D/2)/(D/2-l/2)\sim 1+l/D$ times larger compared with that for the flat surface. This means that the head group experiences an extra pressure to cause a strain of $\Delta Q\sim l/D$. On the other hand, from Eqs. (1)–(4) we have

$$Q=\frac{\alpha\rho-2P}{\omega+2P}\ . \tag{7}$$

Therefore we find that the extra pressure amounts to $\Delta P\sim(1/2)(\omega+2P)\Delta Q\sim(\omega+2P)l/(2D)$. If we use the same values as above for D and l, and $P=20$ K and $\omega=985$ K which have been assumed in the calculation in Ref. [5], we obtain $\Delta P\sim 11$ K. This seems rather large because it is almost the same as P itself. However, this is clearly an overestimation and the flexibility of the chains reduces ΔP considerably.

The above considerations made for the outer layer can be applied for the case of the inner layer by just reversing the signs of the corrections. As a result, the values of ρ and Q become generally different for the outer and inner layers. We therefore write them ρ^+ and Q^+ for the outer and ρ^- and Q^- for the inner layer. Attention should also be paid to the fact that Q^+ and Q^- are not independent in curved bilayers. This situation is essentially different from the case of flat bilayers. Denoting the vesicle radius (at the position of the head) and chain length as $R^\pm$ and $l^\pm$ for the outer and inner layers, we have

$$R^+=R^-+l^-+l^+\ . \tag{8}$$

A similar relation holds between the increments ΔR^+, etc., measured from the ground state, $P=0$ and $\rho=0$,

$$\Delta R^+=\Delta R^-+\Delta l^-+\Delta l^+\ . \tag{9}$$

Noting that from the definition we have $Q^+=\Delta R^+/R_0^+$, with R_0^+ being the value of R^+ for the ground state, etc., we obtain

$$Q^+=\frac{R_0^-}{R_0^+}Q^-+\frac{\Delta l^+}{R_0^+}+\frac{\Delta l^-}{R_0^+}\ . \tag{10}$$

Since it is expected in the first approximation that $\Delta l^\pm$ are proportional to $\rho^\pm$, we can rewrite Eq. (10) as

$$Q^+=\mu Q^--\lambda(\rho^++\rho^-)\ , \tag{11}$$

where $\mu\equiv R_0^-/R_0^+$ and λ is a constant inversely proportional to the vesicle radius. The value of λ is roughly estimated as follows. According to Janiak *et al.* [19], the bilayer thickness decreases about 0.8 nm in the transition region. On the other hand, the distance between the nearest hydrocarbon chains is 0.425 nm below the transition and 0.450 nm above the transition [20]. Therefore the change of Q in the transition range is about 0.06, and the change of ρ comes out to be about 0.06 from Eq. (7). Both these data are for MLV's of DMPC, but we can expect that the values are not so different in the case of DMPC LUV's. Thus we obtain $\lambda\sim 0.13$ for 100 nm LUV's with $R=50$ nm.

The total free energy per molecule is given as

$$G^{\text{total}}=\frac{N^+}{N^++N^-}G^{\text{outer}}+\frac{N^-}{N^++N^-}G^{\text{inner}}\ , \tag{12}$$

where N^+ and N^- are the number of molecules in the outer and inner layers, and G^{outer} is given by Eqs. (1)–(3) and (6) using ρ^+ and Q^+, and G^{inner} using ρ^- and Q^-. Since it is expected that $N^-/N^+=\mu^2$, we obtain

$$G^{\text{total}} = \frac{1}{1+\mu^2} G^{\text{outer}} + \frac{\mu^2}{1+\mu^2} G^{\text{inner}} \,. \tag{13}$$

Then G^{total} is minimized with respect to $\rho^{\pm}$ and $Q^{\pm}$ under the constraint Eq. (11).

Figure 4 shows an example of the calculated temperature dependence of ρ^+ for $D = \infty$, and several finite values of D. The parameter values are listed in the first four lines in Table I. In the I-A theory the transition can be first order, critical, and also supercritical depending on the choice of the parameter values [5]. The parameters used in obtaining Fig. 4 are such that they give a first-order transition for the infinite-size vesicle, which is indicated as a clear jump in ρ^+. It is seen that the transition changes into critical and then supercritical with decreasing D. Thus the finiteness of the vesicle size has quite a similar effect to that of an external field.

We looked for parameter values which reproduce both the LUV (D = 93 nm) and SUV (D = 35 nm) data, and an example is shown in Fig. 5. The ratio of the calculated peak height and the half-width for LUV's and SUV's roughly agrees with the experimentally observed values. The parameter values are listed in the last two lines in Table I. These values have been chosen in the following way. The same values were used as in the original calculation on dipalmitoylphosphatidylcholine (DPPC) by I-A [5] for α, ω, and P because they are expected to have quite similar values also in DMPC, while ϵ_0 has been adjusted to reproduce the observed transition temperature in DMPC. The factor μ has been approximated as $1 - 4l/D$ with l = 2.2 nm. Other parameters have been adjusted rather freely. It should be noted, however, that all these values are reasonable compared with the order-of-magnitude estimations described above. With the parameter values used in the results shown in Fig. 5, a supercritical behavior is expected for the infinite-radius case. We note that this fact does not contradict the first-order nature of the transition in MLV's. It is possible that the inter layer interaction drastically changes the character of the transition. Further, it is likely that the unilamellar vesicles can be regarded as two-dimensional systems, while MLV's are clearly three dimensional.

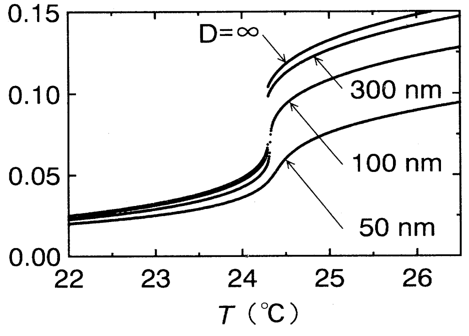

FIG. 4. Temperature dependence of the disorder parameter ρ^+ calculated with the modified Izuyama-Akutsu theory for the cases of $D = \infty$, 300 nm, 100 nm, and 50 nm. The parameter values used in the calculations are listed in the first four lines in Table I.

TABLE I. Parameter values for the calculation with a modified Izuyama-Akutsu theory. Units for ϵ_0, $\Delta\alpha$, and ΔP are K, and that for D is nm. Other parameters are set to α = 1125 K, ω = 985 K, and P = 20 K.

D	ϵ_0	M	$\Delta\alpha$	ΔP	λ
∞	407	23	0	0	0
300	407	23	0.3	0.3	0.03
100	407	23	1.0	1.0	0.10
50	407	23	2.0	2.0	0.20
93	408	21.5	0.8	1.1	0.07
35	408	21.5	2.1	2.9	0.19

V. DISCUSSION

In the last section we showed that the finiteness of the vesicle size has a similar effect to that of an external field, and that the rounding of the observed heat capacity data is explained by this effect. On the other hand, since the vesicle sizes of the LUV and SUV samples are small enough to be regarded as semimicroscopic, it is also possible in principle that the rounding of the observed heat capacity peak is caused by the finite-size effect in the usual sense, i.e., the one which becomes visible when the correlation length grows to a magnitude comparable to the system size [21]. In the present case, however, such a possibility is unlikely for the following reason. In order that the divergence of the correlation length is sufficient, the system should be close enough to the critical point. But this can happen only by an accidental coincidence, since the transition can also be weakly first order or supercritical. According to a Monte Carlo simulation based on the ten-state Pink *et al.* model, the average cluster size does show a distinct increase toward the transition temperature [22]. However, the maximum value of the cluster size at the transition is about 40 molecules in DMPC, DPPC,

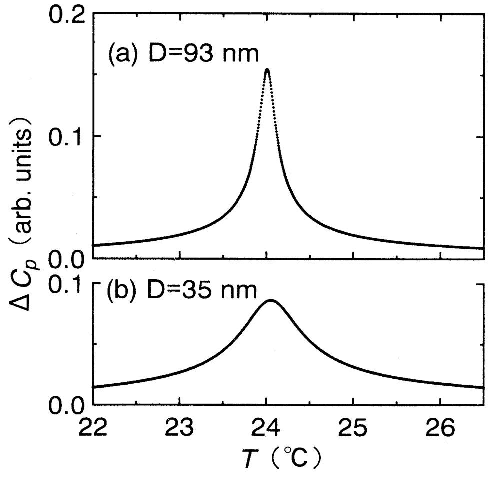

FIG. 5. Temperature dependence of the anomalous heat capacity calculated with the modified Izuyama-Akutsu theory, for the cases of (a) D = 93 nm LUV's and (b) D = 35 nm SUV's. The parameter values used in the calculations are listed in the last two lines in Table I.

and distearylphosphatidylcholine (DSPC), and therefore we can say the divergence is not so significant, even when we take into account the somewhat limited resolution of the calculation. At the same time, the calculated values of cluster size coincide well for system sizes of 200, 800, and 5000 molecules in the case of DPPC [23]. Since our 30 nm SUV has about 10 000 molecules in each vesicle, it is expected from the above results that the correlation length is still much shorter than the vesicle sizes of our LUV's and SUV's. We also note that the increase in some response functions does not necessarily imply the breakdown of the mean-field approximation. As pointed out by Pink *et al.* [24], the heat capacity diverges with a critical exponent equal to that of the susceptibility γ in the present case, and therefore can be quite siginificant even within the mean-field approximation. Indeed, the I-A theory predicts the divergence of the heat capacity with an exponent of 2/3 at the critical point [25]. These results are consistent with the recent theoretical prediction for layered systems [26]. The situation described above, together with the fact that the finiteness of the vesicle size acts as an effective field and drives the transition further away from the critical point, justifies in practice the neglect of the critical fluctuation. It is also worth mentioning that the I-A theory is expected to be accurate in the sense that the excluded volume interactions have been considered rigorously, while they have been included in the Pink *et al.* model in a rather rough manner assuming the chains as cylindrical rods. A direct verification of these ideas will be made by carrying out Monte Carlo simulations with the Pink *et al.* model for curved bilayers, and comparing the results with those obtained here with the modified I-A theory.

A common consequence of the I-A theory and Pink *et al.* model is the existence of a critical point which terminates a first-order phase transition line [27]. Our experimental results for LUV's and SUV's clearly show supercritical nature, and support such a theoretical expectation. In the present system, it is noticed that the experimentally accessible constant pressure path is not parallel to the coexistence line. Because of this, we now feel that the analysis with the parametric equation used in I [11, 28, 29] does not give a meaningful estimation of the critical indices, since it was assumed there that the field remains constant throughout the measurement, which implies that the experimental path is parallel to the coexistence line. Nevertheless, the analysis is still quite valuable for comparing the data which are apparently independent. In fact, we could successfully find the similarity between our LUV data and those on hexatic liquid crystal [11].

Besides the critical fluctuation, there are a few degrees of freedom which have not been taken into account in the Izuyama-Akutsu theory, such as the tilt of the chains from the layer normal and the ripple modulation. Both these are present below the transition in the case of MLV's. If such additional degrees of freedom also exist in LUV's, they may enhance the heat capacity anomaly below the transition to some extent. Although detailed structure data of LUV's and SUV's are still missing, it is quite likely that the tilt is also present in these cases. In this respect study on the transition in phosphatidylethanolamines is expected to provide useful information because the tilting is absent in those systems, and such measurements are planned in the near future. On the other hand, the existence of the ripple structure in unilamellar vesicles with diameters $\lesssim$ 100 nm is rather doubtful, since the period of the ripple structure observed in MLV's is around 12–14 nm [30], which seems too large to be realized in the case of such relatively small unilamellar vesicles. The absence of the pretransition in the result on SUV's reported earlier supports this idea [9, 10, 17, 18]. We also note here that our freeze-fracture electron microscopy showed no indication of the ripple structure in our 100 nm LUV sample. If the gel phase is a hexatic phase as mentioned in our previous work [11], the hexatic order also should be taken into consideration. Detailed studies of the structure in unilamellar vesicles will be very helpful.

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