

解説

Thermodynamic Investigation of Interaction between Ca Cation and Negatively Charged Phospholipid Bilayers as Studied by DSC and Isothermal Titration Calorimetry

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The interaction of a negatively charged vesicle composed of dimyristoylphosphatidylglycerol (DMPG) and Ca^{2+} was investigated by isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC). A schematic diagram of relative enthalpy (ΔH) versus temperature (t) was constructed from two types of calorimetric enthalpies obtained by ITC and DSC and was compared for the fully neutralized vesicle (Ca^{2+} -binding vesicle) and the charged vesicle (Ca^{2+} -free vesicle). It was found that the stabilization enthalpy of the DMPG vesicle due to a binding of Ca^{2+} is larger for the liquid crystal phase than for the gel phase, so that the transition enthalpy of the gel-to-liquid crystal phase, *i.e.*, the enthalpy difference between the two phases, is smaller for the Ca^{2+} -binding vesicle than for its free vesicle although the transition temperature is higher for the former than for the latter.

1. Introduction

Phospholipids are major components of biomembranes and constitute a fundamental part of their bilayer structure. There are a variety of phospholipids of differing polar headgroups such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG). PG is negatively charged in neutral pH and is a ubiquitous phospholipid in the membranes of mitochondria and chloroplast which are organs essential for the maintenance of life *in vivo*. To date, many studies have been performed for PG with a view to clarifying its functional and structural role in the biomembranes and it has become apparent that the acidic phospholipid is very sensitive to a change in environmental conditions caused by external proton and cations.¹⁻⁶⁾ For an example, when Ca^{2+} and Mg^{2+} are present at the cation/PG molar ratio $> \sim 1$, complexes are formed by a specific binding of the cations to the negative charge of PG.²⁻⁴⁾ This accompanies a drastic structural change of spherical vesicles to crystalline superstructures where the bilayers are wrapped in a cylinder. The cylindrical superstructures composed

of the complexes transform directly to the liquid crystal phase on heating at a temperature fairly higher than that of the gel-to-liquid crystal phase transition of the charged PG. In this accord, the transition enthalpy is also about threefold larger than that of the gel-to-liquid crystal phase. However, it has been reported that for the cation/PG molar ratio $< \sim 0.5$, the binding of the cations to PG is not so specific as to form the complexes observed for the higher molar ratio, so that the effect of the cations is limited to a neutralization of the negative charge of PG to a level of a protonated PG.^{2,3)} In this connection, our recent study has found that the transition temperature of the gel to the liquid crystal phase for dimyristoylphosphatidylglycerol (DMPG) increases with an increase in Ca^{2+} concentration up to a full neutralization of Ca^{2+} /DMPG molar ratio ~ 0.5 , but the transition enthalpy decreases up to the full neutralization (details are described in the text). The transition enthalpy is comparable to the difference in the enthalpy between the liquid crystal and gel phases which appear, respectively, at temperatures just above and below the transition. So, it is presumed that the enthalpy difference between the two phases is larger for the charged DMPG

(i.e., Ca^{2+} -free DMPG) than for the neutralized DMPG (i.e., Ca^{2+} -binding DMPG). To make clear this, the heat effect associated with the interaction between Ca^{2+} and DMPG was studied by isothermal titration calorimetry performed at temperatures of both the gel and the liquid crystal phase, and the relative enthalpy of Ca^{2+} -binding DMPG was evaluated, compared to Ca^{2+} -free DMPG.

2. Experimental

2.1 Material and sample preparation

1,2-Dimyrist-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DMPG, sodium salt) was purchased from Sigma Co., and was used without further purification because thin-layer chromatography of the lipid showed a single spot. A dispersion of vesicles composed of the DMPG at a lipid concentration of ~ 2 mM was prepared as follows. A lipid film was first prepared by removing chloroform from a lipid stock solution on a rotary evaporator, and then under high vacuum (10^{-4} Pa) to achieve complete removal of traces of the solvent. The dried lipid film was then suspended in distilled water and was gently vortexed at a desired temperature above the gel-to-liquid crystal phase transition. Lipid concentrations were estimated by a modified Bartlett phosphate assay.⁷⁾ The vesicle dispersion was used for titration calorimetry and DSC. In DSC experiments, to the vesicle dispersion, desired amounts of CaCl_2 aqueous solution (19.5 mM) was added at Ca^{2+} /lipid molar ratios of 0.125, 0.25, 0.375, 0.5, 0.8, and 1.0, respectively.

2.2 Differential scanning calorimetry

Differential scanning calorimetric experiments were performed with a Microcal MC 2 calorimeter operated under computer control. Lipid concentrations in the DSC experiments were in ranges of 1 - 2 mM with a calorimetric cell volume of 1.2 ml. A heating scanning rate of 45°C h^{-1} was used.

2.3 Isothermal titration calorimetry

Isothermal calorimetric experiments were performed with a ThermoMetric TAM (Thermal Activity Monitor) calorimeter at desired temperatures of 5, 35, and 45°C . A calorimeter perfusion cell for the exclusive use of titration system was used as a sample cell. The calorimeter was interfaced to a PC microcomputer system for automatic data collection and titration operation such as stirring and injection. The sample cell was filled with 3 ml of the DMPG vesicle dispersion at lipid

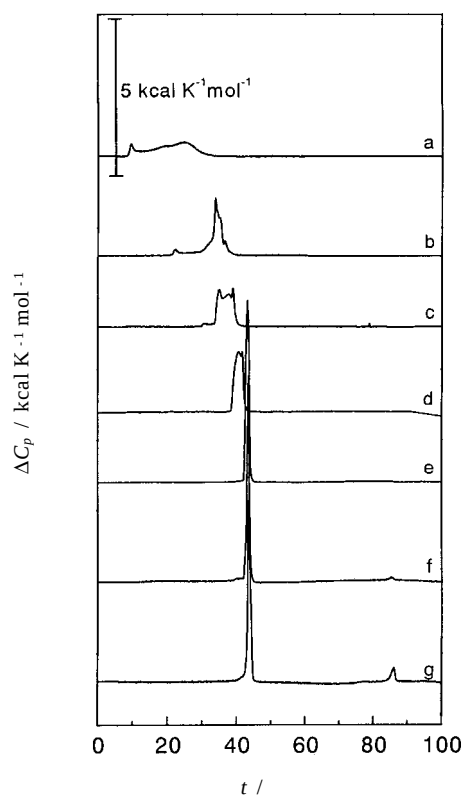


Fig.1 Thermal behavior of DMPG at varying Ca^{2+} /lipid molar ratios. Apparent, excess heat capacity (ΔC_p) per mole of lipid is plotted as a function of temperature (t). [Ca^{2+}]/[DMPG]: a, 0; b, 0.125; c, 0.25; d, 0.375; e, 0.5; f, 0.8; g, 1.0. (1 cal = 4.184 J)

concentrations of 1.9 to 1.7 mM, and the reference cell was filled with distilled water. The vesicle dispersion in the sample cell was titrated stepwise with $10\ \mu\text{l}$ of 1.95 mM CaCl_2 aqueous solution, and the differential heat effect between the sample and reference cells was measured as a function of time. Additionally, two separate titration experiments were performed, respectively, by stepwise additions of (i) $10\ \mu\text{l}$ of the CaCl_2 solution to water in the sample cell and (ii) $10\ \mu\text{l}$ of water to the vesicle dispersion, but no heat effect was observed for the dilutions of both the CaCl_2 solution and the vesicle dispersion used in the present study.

3. Results and Discussion

Fig.1 shows DSC curves for the DMPG at varying Ca^{2+} /lipid molar ratios. The transition peak of the gel

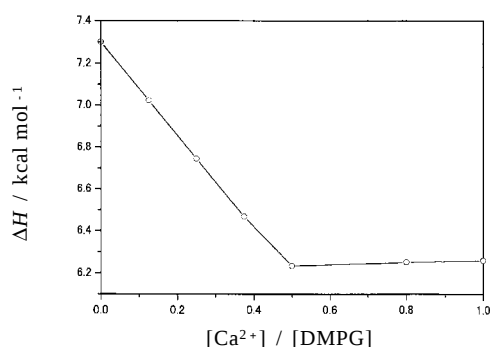


Fig. 2 Variation of the transition enthalpy (ΔH) of the gel to the liquid crystal phase for DMPG with Ca^{2+} /lipid molar ratio. (1 cal = 4.184 J)

to liquid crystal phases at the molar ratio of zero extends over temperatures of 8 to 35 °C, but it becomes sharper with an increase in Ca^{2+} concentration up to the molar ratio of ~0.5. Simultaneously, the transition temperature is shifted to higher temperatures and finally to a limiting temperature of 43 °C observed over the molar ratios of 0.5 to 1.0. The limiting transition temperature is nearly the same as that observed for a fully protonated DMPG previously reported in the literature.¹⁾ Furthermore, the convergence into the fixed transition peak is accompanied by the disappearance of the peak due to the pretransition of the $L_{\beta'}$ gel-to- $P_{\beta'}$ gel phase, showing a change of tilted acyl chains ($L_{\beta'}$ gel phase) to nontilted ones (L_{β} gel phase) characteristic of a closer chain packing.⁴⁾ These facts indicate that a full neutralization of the negatively charged vesicle by Ca^{2+} is attained at the molar ratio of ~0.5 and the fully neutralized vesicle is characterized by the limiting, fixed transition peak at 43 °C. For a DSC curve at the molar ratio of 1 (Fig. 1g), a trace due to the transition of the crystalline phase composed of Ca^{2+} -DMPG complexes to the liquid crystal phase is observed at around 85 °C.^{2,3)} By electron microscopic observations, both the charged vesicle and the fully neutralized vesicle are shown to be a single lamellar, but the size in a diameter is a little larger for the former (100 - 150 nm) than the latter (50 - 100 nm).

Fig. 2 shows a variation of the transition enthalpy (ΔH_T) of the gel to the liquid crystal phase with Ca^{2+} /DMPG molar ratio. The transition enthalpy for the charged vesicle ($[\text{Ca}^{2+}]/[\text{DMPG}] = 0$), 7.3 ± 0.1 kcal per mole of lipid, is given by the sum of the individual

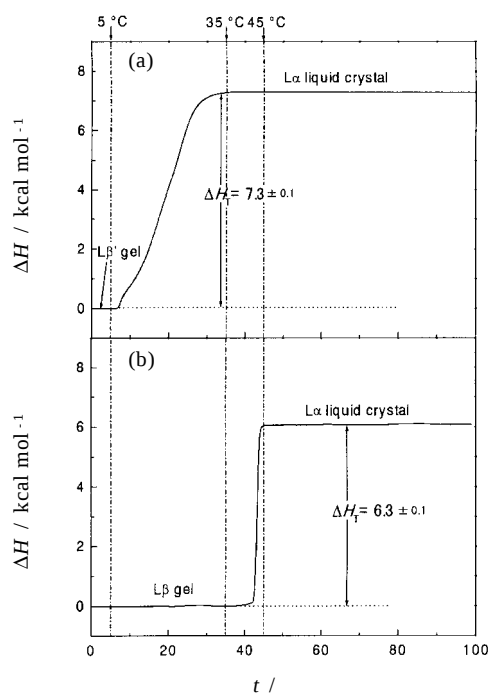


Fig. 3 Schematic diagrams of relative enthalpy (ΔH) per mole of DMPG versus temperature (t) for (a) Ca^{2+} -free and its binding DMPG vesicles. ΔH_T shows the transition enthalpy of the gel to the liquid crystal phase. Dashed lines indicate the temperatures, at which calorimetric titration experiments were performed, respectively. (1 cal = 4.184 J)

transition enthalpies of the $L_{\beta'}$ gel-to- $P_{\beta'}$ gel and the following $P_{\beta'}$ gel-to-liquid crystal phase and is reduced to a limiting value of 6.3 ± 0.1 kcal per mole of lipid for the fully neutralized vesicle observed at the molar ratios higher than 0.5. Thus, the transition enthalpy of the L_{β} gel to the liquid crystal phase for the neutralized vesicle is smaller by 1 kcal mol⁻¹ than the enthalpy of the $L_{\beta'}$ gel to the liquid crystal phase for the charged vesicle, although the transition temperature is higher for the former than for the latter. The transition enthalpy is comparable to the enthalpy difference between the liquid crystal and gel phases which appear, respectively, at temperatures just above and below the transition temperature.^{6,8)} From this viewpoint, the enthalpies of both the gel and the liquid crystal phase of the fully neutralized vesicle were investigated from isothermal titration calorimetry, compared to the corresponding

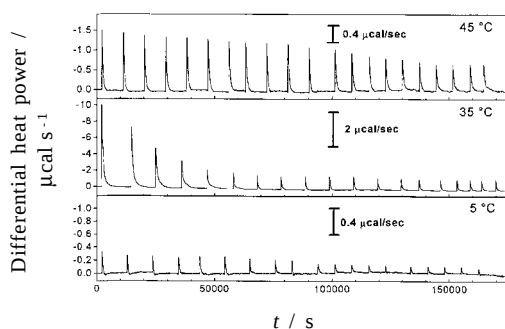


Fig.4 Calorimetric titration curves at three different temperatures of 5, 35, and 45 indicated in Fig.3. The differential heat power in $\mu\text{cal/s}$ ($1 \mu\text{cal/s} = 4.184 \mu\text{J/s} = 4.184 \mu\text{W}$) is shown as a function of time (t), for stepwise injections of $10 \mu\text{l}$ of 1.95 mM CaCl_2 solution to vesicle suspensions ranging in DMPG concentration from 1.9 to 1.7 mM in an initial volume of 3 ml .

enthalpies of the charged vesicle.

The titration experiments were performed at temperatures of 5, 35, and 45, respectively, which were selected by reference to schematic diagrams of relative enthalpy (ΔH) versus temperature (t) curves for the charged and neutralized vesicles shown in Fig.3. Thus, as shown in this figure, the temperatures 5 and 35 are those just below and above the phase transition for the charged vesicle, respectively, and the temperature 45 is that just above the phase transition for the neutralized vesicle. In these experiments, $10 \mu\text{l}$ of 1.95 mM CaCl_2 aqueous solution was injected stepwise to the DMPG vesicle suspension, for which lipid concentrations range from 1.7 to 1.9 mM and an initial volume is 3 ml , and the stepwise injections were continued until the heat signals observed for the last three or four injections stayed constant. Fig.4 shows calorimetric titration curves at the three different temperatures. In this figure, the differential heat power is shown as a function of time. The heat signals observed after each injection are all exothermic for the three curves, but the height of the signals observed up to at least the first six injections is extremely different for these curves. The observed heat signals consist of plural heat effects, namely, the heat of binding of Ca^{2+} to the negatively charged DMPG and the heat associated with conformational changes in the lipid molecule induced by the Ca^{2+} binding. Thus, by reference to Fig.3, it is

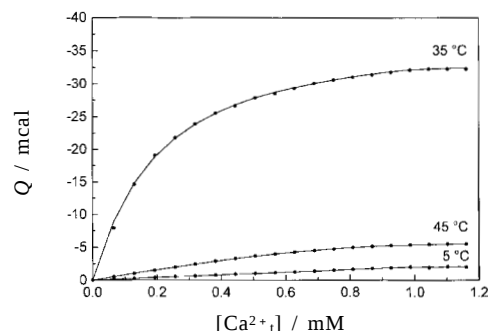


Fig.5 Plots of the cumulative heats (Q) at three different temperatures as a function of the total Ca^{2+} concentration ($[\text{Ca}^{2+}]_t$). The cumulative heats are given by the sum of individual heats released up to the indicated total concentration of Ca^{2+} . Solid lines are the best fitted curves of Eq.(3) to the experimental data. ($1 \text{ cal} = 4.184 \text{ J}$)

seen that the heat signals observed at 5 contain the heat effect due to a change in the chain arrangement from the L_{β}' gel to the L_{β} gel phase and the much larger heat signals at 35 compared with other temperatures are due to a large contribution of the transition enthalpy of the L_{α} liquid crystal to the L_{β} gel phase.

Fig.5 shows a cumulative heat, Q , for the binding reaction between Ca^{2+} and the negatively charged DMPG vesicle at the three different temperatures as a function of the total concentration of Ca^{2+} , $[L]_t$, which varies with the number of injections. The cumulative heat was calculated by adding successively individual heats released for each injection up to the indicated total Ca^{2+} concentration. The individual heats were determined by integration of each heat signal shown in the titration curves of Fig.4 and then subtraction of the average of individual integral heats for the constant signals observed for the last four injections. Other corrections were not made, since no heat effect was observed for dilutions of both the CaCl_2 solution and the DMPG vesicle dispersion used in the present study. The experimental heat data in Fig.5 were analyzed by applying Eq.(1) for a ligand binding to a macromolecule (or macromolecular assembly) possessing one set (sort) of independent ligand-binding sites, previously developed by E. Freire *et al.*^{9,10)}

$$Q = nV [M_t] \Delta H_B \frac{K[L]}{1 + K[L]} \quad (1)$$

where Q is the cumulative heat, V is the reaction volume,

$[M_t]$ is the total concentration of macromolecule M , n is the number of independent and equivalent binding sites possessed by a macromolecule (*i.e.*, the molar number of binding sites per mole of macromolecule), ΔH_B is the enthalpy of binding reaction for 1 mole of ligand which binds to 1 mole of the binding site, $[L]$ is the concentration of free ligand, and K is the binding constant.

In Eq.(1), $V[M_t]$ gives the total molar number of macromolecules actually present in the sample cell, so that $nV[M_t]$ is the total molar number of the binding sites. Accordingly, for $n > 1$, there are plural independent and equivalent binding sites in the number of n for each macromolecule, indicating bindings of n ligands to a macromolecule. However, for $n < 1$, one binding site is made up of the combination of n macromolecules, indicating a binding of a ligand to n macromolecules. For an example, for $n = 0.5$ ($= 1/2$), two macromolecules constitute one binding site, indicating a contribution of each macromolecule to the binding site is a half ($= 1/2$). This is the case for a ligand-binding to a lipid assembly such as used in the present study. Therefore, n gives a stoichiometric binding ratio of ligand to macromolecule in a binding saturation.

On the other hand, a correlation between the cumulative heat (Q) and the enthalpy of binding reaction (ΔH_B) per mole of ligand is given the equation,^{9,10)}

$$Q = V\Delta H_B[L_b] \quad (2)$$

where $[L_b]$ is the concentration of bound ligand and is equal to $[L_t] - [L]$ (where $[L_t]$ is the total concentration of ligand). So, by using that $[L] = [L_t] - Q/V\Delta H_B$, Eq.(1) is replaced by

$$Q = \frac{\{(1 + nK[M_t] + K[L_t]) - [(1 + nK[M_t] + K[L_t])^2 - 4nK^2[M_t][L_t]]^{1/2}\}}{2K / V\Delta H_B} \quad (3)$$

Eq.(3) expresses Q in terms of the total concentration of ligand ($[L_t]$), so that the equation is applicable to the experimental data shown in Fig.5. So, the unknown values of n , K , and ΔH_B in Eq.(3) were estimated by fitting of Eq.(3) to the experimental data using a computer program due to a nonlinear least-squares procedure. The best fitted curves to the experimental data are shown by solid lines in Fig.5 and resultant values of n , K_{app} , and ΔH_B per mole of Ca^{2+} at the three different temperatures are summarized in Table 1. A value of n

Table 1 A summary of the number of binding sites (n), an apparent binding constant (K_{app}), and two enthalpies (ΔH_B) of binding reaction given for mole of Ca^{2+} and mole of DMPG, respectively. These values were evaluated from the best fitted curves for three different temperatures shown in Fig.5. (1 cal = 4.184 J)

t	n	K_{app} M^{-1}	ΔH_B kcal (mol of Ca^{2+}) $^{-1}$	ΔH_B kcal (mol of DMPG) $^{-1}$
5	0.449	4.9×10^4	- 0.67	- 0.3
35	0.036	0.8×10^3	- 207	- 7.5
45	0.385	1.8×10^4	- 2.86	- 1.1

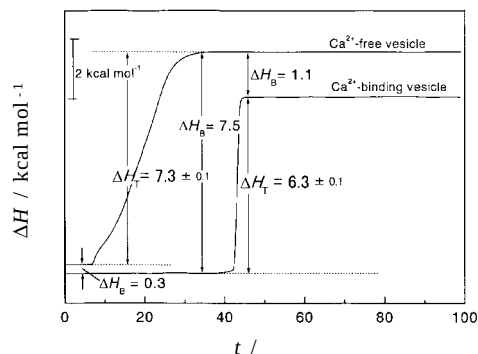


Fig.6 Comparison of schematic diagrams of relative enthalpy (ΔH) versus temperature (t) between Ca^{2+} -free and its binding DMPG vesicles. In this figure, separate diagrams shown in Fig.3 are combined. ΔH_T and ΔH_B were obtained by DSC and calorimetric titration experiments, respectively. (1 cal = 4.184 J)

($= 0.449$) at 5 gives a reasonable saturation stoichiometry corresponding to 1 Ca^{2+} for every 2.2 DMPG molecules, similarly to $n = 0.385$ (1 Ca^{2+} for every 2.6 DMPG molecules) at 45.⁹⁾ In Table 1, the apparent binding constant, K_{app} , is presented¹¹⁾ since it is impossible to separate a pure quantity of the intrinsic binding enthalpy in the present system from the experimental total heat of ΔH_B (mol of Ca^{2+}). By reference to Eq.(1), it is recognized that the total heat released up to a binding saturation is given by the product $nV[M_t]\Delta H_B$ (mol of Ca^{2+}) and so ΔH_B per mole of DMPG is calculated from $nV[M_t]\Delta H_B$ (mol of Ca^{2+})/ $V[M_t]$, *i.e.*, $n\Delta H_B$ (mol of Ca^{2+}). Values of ΔH_B (mol of DMPG) are added to Table 1 and a fairly large value of ΔH_B (mol of DMPG) is observed at 35 where the transition of

the liquid crystal to L_β gel phases is induced by the binding of Ca^{2+} .

The ΔH_B (mol of DMPG) values in **Table 1** were used to evaluate the enthalpy level of Ca^{2+} -binding neutralized vesicle relative to Ca^{2+} -free charged vesicle. In **Fig. 6**, the schematic diagram of relative enthalpy versus temperature for the Ca^{2+} -binding vesicle is shown in comparison with the Ca^{2+} -free vesicle, *i.e.*, the separate diagrams shown in **Fig. 3(a)** and **(b)** are combined in this figure. The schematic diagram accounts well for a reasonable relationship between two types of calorimetric enthalpies, ΔH_B and ΔH_T , obtained by titration calorimetry and DSC, respectively. Thus, $7.5 \text{ kcal mol}^{-1}$ for ΔH_B at 35°C is nearly equal to the sum of $1.1 \text{ kcal mol}^{-1}$ for ΔH_B at 45°C and $6.3 \text{ kcal mol}^{-1}$ for ΔH_T of the Ca^{2+} -binding vesicle, and is also nearly equal to the sum of $0.3 \text{ kcal mol}^{-1}$ for ΔH_B at 5°C and $7.3 \text{ kcal mol}^{-1}$ for ΔH_T of the Ca^{2+} -free vesicle. By assuming that a purely operational enthalpy of binding between Ca^{2+} and DMPG is the same for all the temperatures tested, the Ca^{2+} -binding vesicle is found to be enthalpically lower than its free vesicle, suggesting that the van der Waals interaction energy calculated from the chain-chain separation^{6,9)} is greater for the former as a result of a closer lateral packing of the DMPG molecules, compared with the latter. However, the stabilization enthalpy, mainly due to the van der Waals interaction of the hydrocarbon chains is larger for the liquid crystal phase ($1.1 \text{ kcal mol}^{-1}$) than for the gel phase ($0.3 \text{ kcal mol}^{-1}$), so that the transition enthalpy, *i.e.*, the enthalpy difference between the two phases, is smaller for the Ca^{2+} -binding vesicle ($6.3 \text{ kcal mol}^{-1}$) than for the Ca^{2+} -free vesicle ($7.3 \text{ kcal mol}^{-1}$).

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要 旨

ジミリスチルフォスファチジルグリセロール (DMPG) から構成された負電荷を有するベシクルと Ca^{2+} カチオンとの相互作用を恒温滴定型熱量測定 (ITC) と示差走査熱量測定 (DSC) から検討した。この2種の熱測定から得られたエンタルピー量を基にして、負に帯電したベシクル (Ca^{2+} -free ベシクル) と Ca^{2+} によって十分に中和されたベシクル (Ca^{2+} -binding ベシクル) に対する相対的エンタルピー vs. 温度の模式図を作製し、両ベシクル間で比較した。本研究においては、 Ca^{2+} の結合に基づく DMPG ベシクルの安定化エンタルピーはゲル相よりも液晶相の方が大きいこと、これが起因して、ゲル - 液晶相転移温度は Ca^{2+} -binding ベシクルの方がより高いのにも関わらず、相転移エンタルピーは Ca^{2+} -free ベシクルの方がより大きくなることが明らかにされた。

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