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Morphological Change of Vesicle Particles Can Produce a Peculiar Stepwise Transition in Dipalmitoylphosphatidylglycerol Bilayer at High NaCl Concentration

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Complete formation of the most stable lamellar crystal (L_c2) phase was achieved for dipalmitoylphosphatidylglycerol (DPPG) bilayer membrane in 1.0 mol kg^{-1} aqueous NaCl solution. A peculiar stepwise transition was observed from the differential scanning calorimetric measurements when the L_c2 phase was completely formed. This is probably relevant to the morphological change from rod-like or cylindrical particles to spherical vesicles concomitant with the transition.

Several kinds of acidic phospholipids are known to exist in biomembranes. Phosphatidylglycerol (PG) is one of them and behaves as an anionic lipid with a negatively charged headgroup in aqueous environments around neutral pH because of the dissociation of a counter ion. Therefore, the effective molecular dimensions of the polar headgroup of the PG molecule within the bilayer membrane are dominated by the electrostatic repulsive interaction between the negatively charged polar headgroups. As a result, the structural stability of the PG bilayer membrane is markedly affected by cations in the bulk water phase.[1–3] Actually, it has been shown for a dimyristoylphosphatidylglycerol (DMPG) bilayer membrane that the temperatures of the bilayer phase transitions increase with increasing concentration of NaCl added into the aqueous phase.[4] Moreover, it has been reported that several metastable lamellar crystal (L_c) phases are formed as intermediate phases in the process of the formation of the most stable L_c phase.[5–7] In particular, it is interesting that the morphology of the vesicle particle is also changed from globular multilamellar vesicles into rod-like or cylindrical particles as the L_c phase is formed.[5,7] Similar behavior is expected to be observed also for a bilayer membrane of dipalmitoylphosphatidylglycerol (DPPG), but the formation process of the most stable L_c phase, which we refer to as L_c2 phase, for the DPPG bilayer has been overwhelmingly less studied. This is mainly due to the fact that it takes much longer for the DPPG bilayer to form the L_c2 phase compared to the DMPG bilayer. In general, long-term cold storage of the sample solution, which is referred to as thermal annealing, is needed to induce the L_c phase for lipid bilayers, and the period of the cold storage tends to be longer as the acyl-chain length becomes longer.[6]

Recently, we have started the study of the thermotropic phase behavior of bilayer membranes of acidic phospholipids including DPPG by means of high-sensitivity differential scanning calorimetry (DSC) and found a peculiar phase transition of the DPPG bilayer at a high NaCl concentration in connection with the L_c2 phase formation. In this paper, we report on the thermal characterization of this peculiar thermotropic event along with an effective procedure of the annealing treatment for the complete formation of the L_c2 phase.

Sodium salt of DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)], purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and sodium chloride (Guaranteed reagent) from Kanto Chemical Co., Inc. (Tokyo, Japan) were used without further purification. A weighed amount of DPPG powder was suspended in an appropriate volume of 1.0 mol kg^{-1} aqueous NaCl solution by sonication using a sonifier (Branson Model 3510J-DTH) at a temperature above the main-transition temperature of the DPPG bilayer for a few minutes to obtain a sample dispersion of DPPG multilamellar vesicles (DPPG concentration: 1.0 mmol kg^{-1}). The obtained dispersion was annealed by any of the following procedures: (i) the sample solution was allowed to stand at 5 °C for five days; (ii) 29 successive cycles of thermal treatments, each of which consists of the cold storage at −15 °C for 23 h, subsequently at −30 °C for an hour, and finally at 5 °C for one day, were performed on the sample solution; (iii) additional four cycles of thermal treatments, including the cold storage at −30 °C for two days and the storage at room temperature (ca. 15 °C) for ca. 14 days in one cycle, were performed subsequently after the completion of procedure (ii). Every DSC heating scan was performed using a VP-DSC (MicroCal, Northampton, MA) at a heating rate of 0.75 K min^{-1} to observe the thermotropic phase transitions of the DPPG bilayer with different thermal history. We should note that DSC thermograms obtained at a lower heating rate (0.25 K min^{-1}) were almost identical to those obtained at 0.75 K min^{-1}.

Figure 1a shows the DSC thermograms obtained for the DPPG bilayer membrane in 1.0 mol kg^{-1} aqueous NaCl solution that was thermally treated by procedure (i). A similar procedure was adopted in the previous studies[5,7] on the thermotropic phase behavior of the DMPG bilayer. The thermogram obtained from the first heating scan (curve 1) exhibited three endothermic peaks at ca. 32, 40.7, and 42.4 °C. However, only two endothermic peaks at 40.7 and 42.4 °C were observed for the second heating scan (curve 2) which was carried out subsequently after the completion of the first scan. Therefore, the peak at the lowest temperature detected only in the first scan indicates that the DPPG bilayer membrane underwent the transition from an L_c phase to the lamellar gel ($L_{\beta'}$) phase considering that the thermal treatment of annealing is usually required to induce the L_c phase. Taking into account the previous report[6] which showed that the transition from a metastable L_c phase (i.e., L_c1 phase) to the $L_{\beta'}$ phase occurs at 30.1 °C for the DPPG bilayer in 100 mM aqueous NaCl solution containing 50 mM Tris and 10 mM EDTA, the peak at ca. 32 °C in curve 1 can reasonably be regarded as the same kind of transition. On the

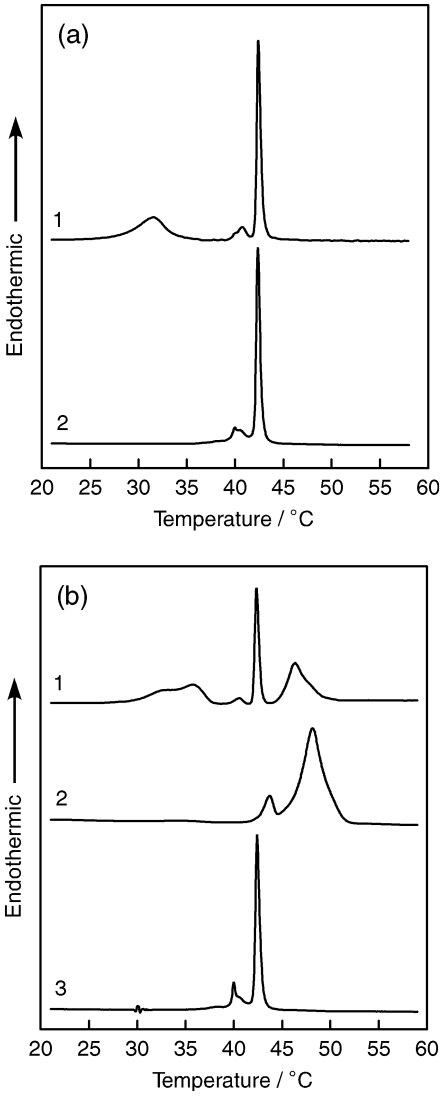

Figure 1. DSC thermograms for DPPG bilayer in 1.0 mol kg^{-1} aqueous NaCl solution. (a) First (curve 1) and second (curve 2) heating scans after annealing treatment by procedure (i). (b) First heating scan (curve 1) after annealing treatment by procedure (ii), first (curve 2) and second (curve 3) heating scans after annealing treatment by procedure (iii).

other hand, the peaks at 40.7 and 42.4 °C were observed in both the first and second heating scans; thus, they correspond to the pretransition ($L_{\beta'}$/ripple gel ($P_{\beta'}$)) and the main transition ($P_{\beta'}$/liquid crystalline (L_{α})), respectively. These transition temperatures were higher than those reported by Zhang et al.[6] This is attributable to the difference in the concentration of NaCl added.

The above results imply that the storage at constant temperature (5 °C) for five days is not sufficient for the formation of the L_c2 phase for the DPPG bilayer, though in the DMPG bilayer the L_c2 phase is almost completely induced by the storage at 5 °C for 24 h.[5,7] We confirmed that the complete formation of the L_c2 phase was not achieved by prolonging the time of the cold storage at 5 °C up to 6 months. Then, we applied procedure (ii) to prepare the sample solution. This procedure includes thermal fluctuations between temperatures below and above the freezing point and has been applied in our previous studies[8,9] for the formation of the L_c phase for phosphatidylcholine bilayers. The result of the DSC measurement for the DPPG bilayer thus obtained is shown in Figure 1b (curve 1). Unlike for the sample from procedure (i), the DSC thermogram showed at least four endothermic peaks at ca. 36, 40.7, 42.4, and ca. 47 °C, meaning that the DPPG bilayer underwent four kinds of phase transitions on this condition. The peaks at 40.7 and 42.4 °C can be ascribed to the pre- and main transitions from the peak temperatures and areas. The peak around 36 °C exhibited a complex feature: at least two endothermic peaks overlap, as obvious from the presence of a shoulder peak at ca. 32 °C. Similar behavior was observed for the DMPG bilayer membrane.[7] It is known for several phospholipid bilayer membranes that the temperature of the subtransition tends to elevate with an increase in period or cycle of the annealing treatment, namely, with an enhancement of the stability of the L_c phase.[10] Therefore, we assigned both peaks as the $L_c1/L_{\beta'}$ phase transition. The difference in the temperatures is probably caused by the enhancement of the stability of the L_c1 phase due to the annealing treatment of procedure (ii). Actually, we confirmed that it depends on the number of the annealing cycles which of the peaks at 32 and 36 °C is dominant (data not shown).

On the other hand, the peak at ca. 47 °C is a peak that occurred newly for the sample from procedure (ii). This peak can be assigned as the L_c2/L_{α} phase transition on the basis of the literature data.[6] The procedure (ii) is still not sufficient for the complete formation of the L_c2 phase, because the endothermic peaks of the $L_c1/L_{\beta'}$, pre- and main transitions were simultaneously observed in addition to the peak of the L_c2/L_{α} transition. If the L_c2 phase is entirely formed in the DPPG bilayer, only the peak of the L_c2/L_{α} transition must be observed because the L_c2 phase is the most stable phase below the L_c2/L_{α} transition temperature.

Then we further prepared the sample solutions with the procedure (iii) by referring to the annealing method adopted by Zhang et al.[6] Curve 2 in Figure 1b represents the DSC thermogram for the DPPG bilayer obtained from procedure (iii). As expected, a large peak was observed at ca. 48 °C, which indicates that the DPPG bilayer undergoes the L_c2/L_{α} transition at that temperature. However, very interestingly, a small peak was also observed at ca. 44 °C in the thermogram. As for this small peak, there is no report, to our knowledge, that has shown such a small peak occurring a few degrees below the L_c2/L_{α} transition temperature when the sample solution of DPPG is sufficiently annealed. Also in the report by Zhang et al.,[6] the L_c2/L_{α} transition was detected as a broad but a single endothermic peak.

For this small peak, one may think that the main-transition peak still remains or that an intermediate phase is formed during the process of the conversion from the metastable L_c1 phase into the stable L_c2 phase. The former possibility can be ruled out, because the peak temperature of this small peak is slightly higher than that of the main-transition peak, and besides, no trace of the pretransition peak is discernible. We have confirmed that the main-transition temperature can be determined so precisely that the difference in temperature between this small peak and the main-transition peak can be reliably distinguished and also that a small vestige of the pretransition peak can be detected along with the small broad peak of the $L_c1/L_{\beta'}$ transition even when the

height of the main-transition peak is reduced down to almost the same height as that of the small peak at 44 °C shown in Figure 1b by the annealing treatment (procedure (ii) plus two additional cycles of thermal treatments in procedure (iii)). The latter possibility also seems unlikely, because we could so far not observe any tendency for the small peak at 44 °C to be smaller with an increase in the number of the thermal cycles in procedure (iii). If the small peak is indicative of the formation of an intermediate phase, it is expected to be progressively smaller as the number of the thermal cycles increases.

At the present stage, we cannot give a clear explanation for this small endothermic peak but currently speculate as follows. As described above, it has been shown for the DMPG bilayer that the change in the morphology of the vesicle from a spherical multilamellar vesicle to rod-like or cylindrical vesicular particles occurs in the process of the formation of the L_c phase.[5,7] A similar morphological change is expected to occur in the DPPG vesicles. In the case of such rod-like vesicular particles, the membrane curvature at both ends of the rod and edges of the lamellar stack in the rod is larger than that in its body. Since the thermal motion of the DPPG molecules within the bilayer membrane is severely restricted in the L_c2 phase, the molecular packing of DPPG is expected to be much tighter and denser in the L_c2 phase than in the other phases. This means that a smaller curvature is probably preferable to the DPPG bilayer in the L_c2 phase because a larger curvature produces a larger intermolecular spaces between adjacent lipid molecules in the membrane. Therefore, the stability of the L_c2 phase at the ends and edges is presumed to be more or less lower than the stability of the same phase in the body. Eventually, the L_c2/L_α transition of the DPPG bilayer at the ends and edges of the rod-like vesicular particles precedes the same kind of phase transition in its body.

Finally, a tentative enthalpy diagram for the various phases observed for the DPPG bilayer in 1.0 mol kg^{-1} aqueous NaCl solution is given in Figure 2. The enthalpy change (ΔH) of each phase transition was evaluated from the area of the endothermic peak in the DSC thermograms. The ΔH values of the pre- and main transitions are almost comparable to the corresponding values for the dipalmitoylphosphatidylcholine (DPPC) bilayer (4.6 and 36.4 kJ mol^{-1}),[8] indicating that those phase transitions for both bilayers thermodynamically resemble each other. In contrast, the ΔH value of the L_c2/L_α transition for the DPPG bilayer is significantly larger than that of the L_c/L_α transition for the dipalmitoylphosphatidylethanolamine bilayer (74.3 kJ mol^{-1})[9] and also than the total enthalpy change of the L_c/L_β', L_β'/P_β', and P_β'/L_α transitions for the DPPC bilayer (67.0 kJ mol^{-1}).[8] This unusual large ΔH value of the L_c2/L_α transition may be relevant to the morphological change of the vesicular particles concomitant with the phase transition, as speculated above.

In summary, the complete formation of the L_c2 phase was successfully achieved for the DPPG bilayer in 1.0 mol kg^{-1} aqueous NaCl solution. For the DPPG bilayer in the L_c2 phase obtained thus, two endothermic peaks at ca. 44 and ca. 48 °C were observed in the DSC thermogram, suggesting that the L_c2/L_α transition takes place stepwise. This stepwise transition is probably due to the morphology of the DPPG bilayer in the L_c2 phase; that is, it is presumed to have rod-like or cylindrical structure, where at least two different parts of the bilayer aggregates with different curvature coexist, and this difference in curvature will produce the difference in the stability of the L_c2 phase. This paper is probably the first report that demonstrates the calorimetric results suggesting the possibility that the morphological change can give rise to the stepwise phase transition in bilayer membranes of acidic phospholipids.

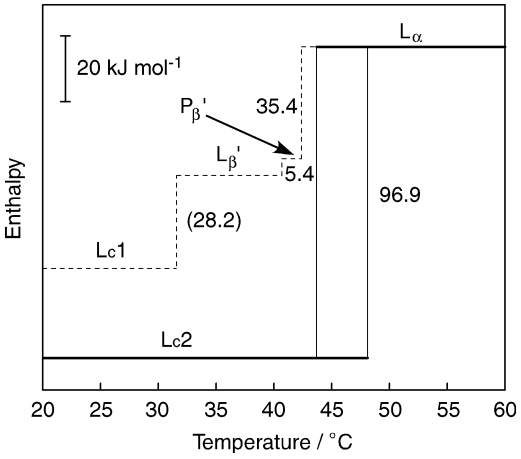

Figure 2. Tentative enthalpy versus temperature diagram for DPPG bilayer membrane in 1.0 mol kg^{-1} aqueous NaCl solution. Solid and dashed lines represent stable and metastable phases, respectively. Numerical values correspond to transition enthalpies (kJ mol^{-1}).

To ensure the validity of this speculation, further investigation, especially on detailed structure of the L_c2 phase, is necessary. As a first step for this purpose, we are now investigating the annealing process in a systematic way to find a more effective procedure for the complete formation of the L_c2 phase and have some results suggesting conditions that are preferable or required for faster formation of the L_c2 phase. As expected from this study, cold storage for a long period, which we call *static annealing*, has no significant effect on the acceleration of the L_c2-phase formation, while the thermal fluctuation by repeating freeze–thaw cycles, which we classify as *dynamic annealing*, tends to promote the formation as long as the thawing process is carried out at a relatively higher temperature below the L_c1/L_β' transition temperature. In the near future, we will report the effect of the annealing procedure on the kinetics of the formation of the L_c2 phase for the DPPG bilayer in aqueous NaCl solution.

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