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NaCl-dependent formation of the highly crystalline phase in sufficiently hydrated dimyristoylphosphatidylglycerol bilayers

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Abstract

We investigated the low-temperature phase behavior of dimyristoylphosphatidylglycerol (DMPG) bilayers in the presence of high concentration of NaCl (≥100 mM). Differential scanning calorimetry showed that the highly crystalline (HC) phase grew after an initial delay period when DMPG bilayers were sufficiently hydrated and incubated at 1˚C in the presence of more than 100 mM NaCl. The HC phase formation reached a plateau, the level of which depended on NaCl concentration; all the lipids were unable to be in the HC phase at the plateau stage without a quite high concentration of NaCl. Since electron microscopic observations suggested that the HC phase formed coexists with the precursor phases in a closed vesicle, elastic constrain and/or shortage of free sodium ions in the inside of the closed vesicle may prevent the complete transition into the HC phase.

1. Introduction

Dimyristoylphosphatidylglycerol (DMPG) bilayers have been extensively studied as a model for the negatively charged biomembrane (Eklund et al., 1987; Lotta et al., 1988; Kodama et al., 1993; Zhang et al., 1997; Garidel et al., 2000; Toca-Herrera et al., 2000; Riske et al., 2001; Lamy-Freund and Riske, 2003; Lewis et al., 2005). Recently, it has been reported that DMPG bilayers show a unique low-temperature phase behavior depending on environmental conditions. Under low ionic strength, the stable subgel phase forms relatively fast, especially below 0˚C, e.g., with the relaxation time of 3 min at -10˚C in the presence of 2 mM NaCl. It was suggested that these properties of the subgel phase are related to the sheet-like morphology of the DMPG bilayer (Kinoshita et al., 2008). On the other hand, under high ionic strength (in the presence of more than about 100 mM NaCl), DMPG bilayers assume vesicular structures and the stable phase at low temperature is the highly crystalline (HC) phase, which is more ordered than the subgel phase (Salonen et al., 1989; Epand et al., 1992). In this study, we focused on the process of the HC phase formation under high ionic strength.

Zhang et al. (1997) reported that when DMPG bilayers are incubated at low temperatures in the presence of 100 mM NaCl, the HC phase forms via a metastable subgel (L_c1^b) phase which directly transforms into the liquid crystalline (L_α) phase at 24.5ºC upon heating (see Table 1 in their paper). They demonstrated spectroscopically the quasicrystalline nature of the metastable subgel phase. On the other hand, Kodama et al. (1999) reported that incubation at 5˚C gives rise to two endothermic peaks in the DSC heating thermogram, corresponding to the L_1–to-ripple ($P_{\beta'}$) phase transition and the L_2–to-$P_{\beta'}$ phase. The appearance of plural metastable subgel phases has been also reported in the well-characterized diacylphosphatidylcholine (PC) bilayers (Ruocco and Shipley, 1982; Tristram-Nagle et al., 1987; Tenchov et al., 2001). The described low-temperature phase behavior of DMPG bilayers varies from paper to paper probably because the phase behavior of acidic lipid bilayers depends on environmental conditions such as lipid concentration, thermal hysteresis and ionic strength, the finer detail of which is not specified in the papers.

The presence of 100 mM or more NaCl concentration leads to the gradual formation of the HC phase via the metastable subgel phase(s) on prolonged low-temperature incubation. The formed HC phase directly transforms into the L_α phase with the transition enthalpy of 60-80 kJ/mol at about 40˚C (Salonen et al., 1989; Epand et al., 1992; Koynova, 1997; Zhang et al., 1997; Kodama et al., 1999). The transition temperature is much higher than the main ($P_{\beta'}$-L_α) transition temperature (24˚C). X-ray diffraction measurements showed that the HC phase obtained after incubation for 7 days at 4˚C has highly ordered hydrocarbon chains and headgroups, and a lamellar repeat distance

close to that in the anhydrous crystal of DMPG (Pascher et al., 1987; Garidel et al., 2001). Spectroscopic study revealed that the bilayer polar/apolar interface and/or the location of the ester carbonyl groups are more dehydrated in the HC phase than those in the metastable subgel phase (Zhang et al., 1997). Moreover, Epand et al. (1992) suggested that in the HC phase interlipid hydrogen bonds establish between glycerol groups in the DMPG polar head moiety. Formation of a crystalline phase similar to the HC phase has also been observed in diacylphosphatidylethanolamine (PE) bilayers, in which hydrogen-bonding interactions of the headgroups are believed to stabilize the low-temperature phase (Chang and Epand, 1983; Seddon et al., 1983; Wilkinson and Nagle, 1984; Boggs, 1987). The crystalline phase in DMPE bilayers directly transforms into the L_α phase at the temperature higher by 6˚C than the main transition temperature with a large transition enthalpy of 82 kJ/mol (Kodama et al., 1995).

The HC phase in DMPG bilayers has been formed under different incubation, salt conditions and lipid concentrations depending on the authors (Table 1). There is no systematic and quantitative study on the kinetics of the HC phase formation as far as we know. One of the factors that make the kinetics complicated is the dependence of the phase behavior on the sample pretreatment as seen in Table 1: Koynova (1997) reported that the HC phase formation in the presence of 150 mM NaCl completes within ~14 hours at 26-28˚C as long as DMPG bilayers are preincubated below 42˚C, and that if DMPG bilayers experience the temperature higher than 42˚C, only a fifth of the lipids are in the HC phase even after incubation at 25-27˚C for ~15 hours. Furthermore, some authors reported that there appear intermediate phases other than the L_1 and L_2 phases in the process of the HC phase formation (Koynova, 1997; Kodama et al., 1999). Under consideration of the possibility that the incubation at 42˚C, which is only a little higher than the HC-to-L_α phase transition (HC transition) temperature, results in insufficient hydration of the sample, we prepared DMPG bilayers by preincubating a sample for two hours at 65˚C with vigorous vortex mixing in this work.

In the sufficiently hydrated DMPG bilayers the well-defined stable HC phase which transformed into the L_α phase at ~40ºC was formed directly from the L_2 phase without the intermediate phases. Moreover, we found that preincubation for one to two hours (even at 65˚C) is required for complete elimination of the intermediate phases. Using the sufficiently hydrated DMPG bilayers obtained by the high temperature preincubation, we analyzed quantitatively the kinetics of the HC phase formation and its dependence on NaCl concentration. The time course of the HC formation consisted of three stages, i.e., initial delay, growth and plateau stages. Interestingly, the plateau level depended on NaCl concentration. We will discuss the mechanism of the incomplete transition in relation to the vesicular morphology of the DMPG bilayer.

Table 1. Experiments on the HC phase formation
[1] Garidel et al., 2001, [2] Koynova, 1997, [3] Epand et al., 1992, [4] Kodama et al., 1999.

NaCl conc. (mM)	Lipid conc. (mM)	sample preparation	incubation temp./time	phase behavior (method)	reference
100	31	sonicated for 15min above 60℃	4℃ / 7 days	all lipids in the HC phase (DSC)	[1]
100	290 / 1452*	sonicated for 15 min above 60℃	4℃ / 3 months	all lipids in the HC phase (FT-IR/X-ray)	[1]
150	1.6- 2.6	heated up to 42℃ after several successive cycles of freeze-thaw (-18℃ - 20℃)	26-28℃ / 14 hours	all lipids in the HC phase (DSC)	[2]
150	1.6-2.6	cycled several times between 10-50℃ after several successive cycles of freeze-thaw (-18℃ - 20℃)	25-27℃ / 15 hours	~20% lipids in the HC phase (DSC)	[2]
150	1.6-2.6	cycled several times between 10-50℃ after several successive cycles of freeze-thaw (-18℃ - 20℃)	0-4℃ / 10 days	all lipids the HC phase (DSC)	[2]
150	0.4	voltexed at 45℃	0℃ / 3 days	almost all lipids in the HC phase (DSC)	[3]
150	14.5	voltexed at 45℃	2℃ / 50 hours	almost all lipids in the HC phase (FT-IR)	[3]
500	1-2	voltexed above the main transition temperature	5℃ / 24 hours	all lipids in the HC phase (DSC)	[4]

* lipid concentration of 290 mM for FT-IR and 1452 mM for X-ray measurements.

2. Materials and methods

2.1. Sample preparation

1,2-dimyristoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (DMPG, sodium salt) was purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. The DMPG (powder crystals) was used as it was purchased for hydration experiments. The other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

DMPG dissolved in chloroform was dried under a flow of nitrogen and then under reduced pressure overnight. The resulting lipid film was dispersed into a buffer containing 10 mM HEPES (pH 7.4) and an appropriate concentration of NaCl. The sample was incubated for two hours with intermittent vortexing at 65ºC, which is enough higher than the HC transition temperature (~40°C). The final concentration of lipid was 50 mM unless otherwise mentioned. After incubation for two

hours at 65°C, the sample gave a single spot on thin-layer chromatography.

2.2. Freeze-fracture electron microscopy

The surface structures and overall morphology of lipid bilayers were examined by freeze-fracture electron microscopy (FFEM). A few microliters of the lipid dispersion was put onto a small copper block and kept at a desired temperature for 3 minutes before rapidly frozen in nitrogen slush which was prepared just before its use by decompression in a vacuum chamber (Shotton and Servers, 1995). The quenched sample was fractured in a freeze-fracture apparatus JFD-9010 (JEOL, Tokyo, Japan). The fractured surface was shadowed with platinum-carbon at an angle of 45° and the shadowed surface was coated with carbon. The freeze-fracture replica obtained was washed with chloroform/methanol (1:1) and observed with an electron microscope JEM-1010 (JEOL, Tokyo, Japan).

When DMPG bilayers in the fluid phase were quenched, irregular ripple undulations, so-called jumbled structures, were observed on the vesicle surface as previously described (Stewart et al., 1979; Boni et al., 1981; Boni et al., 1984).

2.3. Differential scanning calorimetry (DSC)

We analyzed the kinetics of the HC phase formation using a multi-cell type differential scanning calorimeter CSC4100 (Calorimetry Science Corp., UT) with the scanning rate of 0.5°C/min. The sample was cooled down to and incubated at 1°C for an appropriate period in the DSC apparatus before the heating DSC measurement to detect the newly formed low-temperature phases in sufficiently hydrated DMPG bilayers. When the sample was reused, it was reincubated for about two hours at 70°C before DSC measurement. When the endothermic peaks of the L_2-to-$P_{\beta'}$ phase transition and the main transition were partially merged, we estimated the main transition enthalpy by fitting Gaussian or Lorenzian functions to the experimental data.

On the basis of the time course of increase in the HC transition enthalpy per mole of total added DMPG ΔH_{HC}, we calculated the initial delay periods and the growth rate for the HC phase formation. The initial delay period t_{lag} is defined as;

$$t_{lag} = \frac{t_{ND} + t_{det}}{2}, \qquad \text{Eq. (1)}$$

where t_{ND} is the maximum incubation time at 1°C during which the HC transition was not detected and t_{det} is the minimum incubation time to give a minimum positive ΔH_{HC} in the series of thermograms of the samples incubated at 1°C with an incubation time interval of two hours (150 mM and 175 mM NaCl) or 0.5 hours (>175 mM NaCl).

The relaxation time τ for the growth of the HC phase was estimated by fitting the time course of increase in the HC transition enthalpy after t_{lag} to the following equation:

$$\Delta H_{\mathrm{HC}} = \Delta H_{\mathrm{HC}}^{\mathrm{p}} \left\{ 1 - \exp\left(-\frac{t - t_{\mathrm{lag}}}{\tau} \right) \right\}, \qquad \text{Eq. (2)}$$

where $\Delta H_{\mathrm{HC}}^{\mathrm{p}}$ is the HC transition enthalpy at the plateau and t is the incubation time at 1°C.

2.4. X-ray diffraction

Small angle X-ray diffraction (SAXD) experiments were carried out at beam line BL-40B2 in SPring-8 (Sayo, Japan) and BL-15A in the High Energy Accelerator Research Organization (Tsukuba, Japan) with a monochromatized beam with a wavelength (λ) of 0.10 nm and 0.15 nm, respectively. The camera length (150-180 cm) was calibrated using cholesterol. A lipid solution was placed between kapton films kept parallel with a washer as a spacer. In the experiments at BL-15A the sample was mounted on a DSC apparatus for an optical microscope (FP 84HT-4000, Mettler-Toledo), which was used as a temperature controller (Hatta et al., 1995; Takahashi et al., 1995), and the exposure time was 1-10 min for the imaging plate BAS-MS 2025 (Fuji photo film Co., Ltd, Kanagawa, Japan). In the experiments at BL-40B2 the sample was inserted into a metal holder kept at an appropriate temperature, and the exposure time was 20 s for the CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). The X-ray diffraction pattern was linearized by integrating the intensity along the Debye-Scherrer rings. The modulus of scattering vector is defined as $s = 2\sin\theta/\lambda$ (2θ is the scattering angle).

2.5. Separation of the HC and non-HC fractions by centrifugation

When the sufficiently hydrated DMPG bilayers were incubated at 1ºC, the HC phase formation reached a plateau in an NaCl concentration-dependent manner. Thus, the HC phase coexisted with the metastable subgel phase at the plateau. We separated the HC phase fraction from the other (non-HC) phase fraction on the basis of density difference between these two fractions as follows. After reaching the plateau, the sample in the presence of 400 mM NaCl was incubated at 30ºC, the temperature between the main transition and the HC transition, so that only the non-HC fraction transforms into the L_α phase. The density of the solvent was adjusted to 1.076 g/mL by addition of an appropriate amount of deuterium oxide in the presence of 400 mM NaCl (H_2O:D_2O=40:88 v/v). The adjusted density is slightly higher than the density of the L_α phase (1.068±0.001 g/mL), which was determined by the neutral floatation method according to Nagle and Wilkinson (1978). The

incubated sample was centrifuged (19,000×g) at 30ºC for about 30 minutes (KUBOTA 1910, KUBOTA Co., Ltd., Tokyo, Japan). The HC fraction was obtained as a precipitate and the non-HC fraction as a supernatant (see Section 3-4).

3. Results

3.1. Hydration of DMPG powder crystals

We examined the hydration behavior of DMPG powder crystals by DSC. Fig. 1a shows the first heating scan of DMPG powder crystals in the presence of 200 mM NaCl and 10 mM HEPES buffer (pH 7.4). The thermogram has an endothermic peak corresponding to the hydration of DMPG powder crystals at 40.5°C with a large enthalpy of 70 kJ/mol (Fig. 1a). Therefore, to prepare sufficiently hydrated DMPG bilayers, we should incubate the DMPG dispersion at the temperature high enough above 40.5°C. When the DMPG assembly was incubated for two hours at 65°C, the subsequent heating scan gave rise to the gel ($L_{\beta'}$)-to-$P_{\beta'}$ phase transition (pretransition) at 14.0°C and the $P_{\beta'}$-to-L_{α} phase transition (main transition) at 24.0°C (Fig. 1b). It should be noted that the crystal-to-hydrated bilayer transition takes place at the temperature higher by 16.5°C than the main transition temperature.

We examined the effect of incubation time at 65ºC on the structural homogeneity of the sample. When DMPG bilayers were prepared by incubation of powder crystals for 10 minutes at 65ºC without vortex mixing, their SAXD pattern gave a broad peak centered at 0.21 nm^{-1} and a small peak at 0.10 nm^{-1} (arrow in Fig. 2a). Thus, the structure of DMPG bilayers was not homogeneous after the 10 minutes incubation at 65ºC. The vigorous vortex mixing of DMPG bilayers at 65ºC removed the small peak at 0.10 nm^{-1} and seemingly homogeneous DMPG bilayers were formed (Fig. 2b). However, calorimetric measurements showed that the low-temperature phase behavior of DMPG samples even after incubation for one hour at 65ºC with vortex mixing was often irreproducible. The DMPG sample prepared by incubation for two hours at 65ºC with intermittent vortex mixing showed reproducibility in the low-temperature phase behavior and was therefore thought to be sufficiently hydrated

In addition, we examined the morphology of the sufficiently hydrated DMPG assemblies by FFEM (Fig. 2c). The DMPG assembly in the presence of 200 mM NaCl was incubated for two hours at 65°C before quench. Under these conditions only vesicular structures (vesicles) were observed. Incidentally, most of the small vesicles must be unilamellar, judging from the smooth surface without steps to the adjacent bilayer. In the case steps were observed, the thickness of interbilayer space (arrows) was not uniform and seemed to be far larger than that observed in

representative PC multilamellar vesicles. Those FFEM observations are consistent with the SAXD pattern of sufficiently hydrated DMPG bilayers giving a single broad peak centered at 0.21 nm^{-1}, which indicates no correlation between adjacent DMPG bilayers (Fig. 2b).

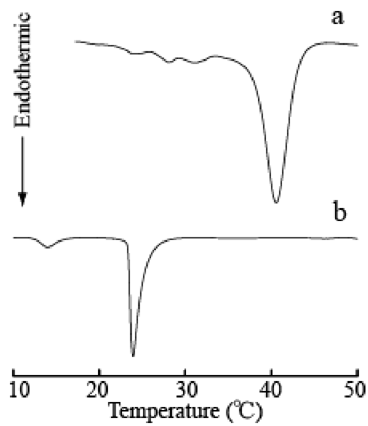
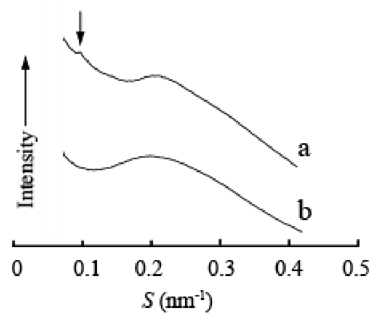


Fig.1. Hydration of the DMPG powder crystals. (a) A DSC heating thermogram of DMPG powder crystals in the presence of 10 mM HEPES buffer (pH 7.4) and 200 mM NaCl. The buffer solution kept at 4ºC was added to the powder crystals immediately before the DSC heating scan from 0ºC. The endothermic peak related to hydration of DMPG powder crystals appeared at 40.5°C. (b) A DSC heating thermogram of the DMPG bilayers preincubated for two hours at 65°C so as to be sufficiently hydrated. The main transition temperature of the sufficiently hydrated DMPG bilayer was 16.5°C lower than the hydration temperature of DMPG powder crystals. These results are consistent with previous report on hydrated DMPG bilayers (Salonen et al., 1989).



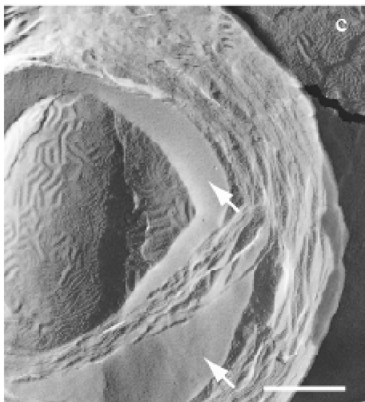


Fig. 2. Influence of preparation procedure on the structure of DMPG bilayers. SAXD patterns of DMPG bilayers in the presence of 200 mM NaCl after the incubation for 10 min at 65ºC (a) without and (b) with vortex mixing. Both pattern show a broad peak centered at 0.21 nm^{-1}. In addition to the broad peak, a small peak is seen at 0.10 nm^{-1} in (a) (arrow). Further incubation up to two hours gave almost the same SAXD pattern as in (b). (c) A freeze-fracture electron micrograph of the sufficiently hydrated DMPG bilayer in the presence of 200 mM NaCl. DMPG bilayers were kept at 65°C for two hours before quenched into nitrogen slush. Flat surfaces without jumble patterns (arrows) must correspond to interbilayer water spaces between independently behaving closed DMPG vesicles. Bar indicates 200 nm.

3-2. Kinetics of the HC phase formation

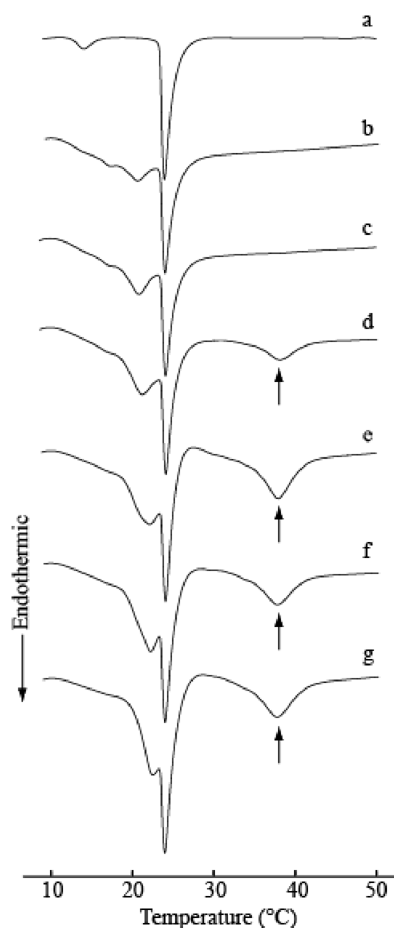


Fig. 3. DSC heating thermograms of sufficiently hydrated DMPG bilayers in the presence of 200 mM NaCl after incubation at 1°C for (a) 0, (b) 2, (c) 3, (d) 4, (e) 6, (f) 8 and (g) 10 hours. There appeared an endothermic peak corresponding to the highly crystalline (HC)-to-L_α phase transition (HC transition) at 38.2°C when the sample was incubated for four hours or more (arrows). See Fig. 4 for the detailed phase scheme.

Fig. 3 shows the DSC heating thermograms of the sufficiently hydrated DMPG vesicles after the incubation at 1˚C for 0 to 10 hours in the presence of 200 mM NaCl. There appeared the pretransition at 14.0˚C (3 kJ/mol) and the main transition at 24.0˚C (25 kJ/mol) in the absence of the incubation at 1˚C (Fig. 3a). When the samples were incubated for two and three hours at 1°C, there observed two peaks corresponding to the L_1-to-$P_{\beta'}$ phase transition at 16.8˚C and the L_2-to-$P_{\beta'}$ phase transition at 21.2˚C in addition to the main transition (Figs. 3b and c). Here, we used the notation of L_1 and L_2 for the new phases formed after the prolonged incubation at 1˚C according to Kodama et al. (1999). When the sample was incubated at 1˚C for four hours or more, the subsequent heating thermogram showed the L_2-to-$P_{\beta'}$ phase transition, the main transition and the transition with a constant transition temperature of 38.2˚C (arrows in Fig. 3d-g), which corresponds to the highly

crystalline (HC)-to-L_α phase transition (Salonen et al., 1989). Thus, the transition from the L_2 phase to the HC phase gradually proceeded without intermediate states during incubation at 1˚C over four hours.

For the help of understanding the relationship between the endothermic peaks in the heating thermogram and the appearance of the L_1, L_2 and HC phases, we illustrate the phase scheme of DMPG vesicles in the presence of 200 mM NaCl using Fig. 3d as an example (Fig. 4). Here, we call the transition at 38.2˚C the HC transition for convenience and focus on its behavior because it is a well-defined transition with a constant transition temperature and characterizes the low-temperature phase behavior of the sufficiently hydrated DMPG vesicles.

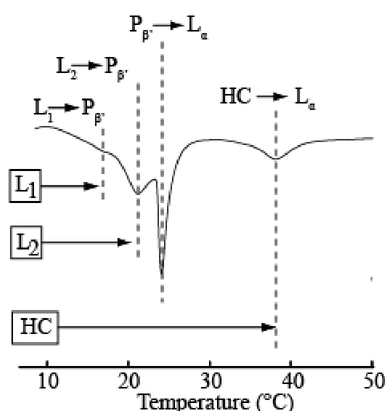


Fig. 4. Illustration of the relationship of the L_1, L_2 and HC phases formation with the DSC heating thermogram in Fig. 3d. When DMPG bilayers are incubated at 1°C, the $L_{\beta'}$ phase transforms into the HC phase via the metastable (L_1 and L_2) phases. When the incubated sample is heated up, the L_1 and L_2 phases transform into the $P_{\beta'}$ phase at 16.8°C and 21.2°C, respectively, and subsequently the $P_{\beta'}$ phase into the L_α phase at 24.0ºC. On the other hand, the HC phase transforms directly into the L_α phase at 38.2°C.

First, we examined the kinetics of the HC phase formation by plotting the HC transition enthalpy as a function of the incubation time at 1˚C (Fig. 5). The main transition enthalpy, which should reflect the amount of the remaining L_2 phase, was also plotted because the main transition was sharper than the L_2-to-$P_{\beta'}$ phase transition and easy to be separated from the other transitions by curve-fitting. The obtained profiles of their time course are nearly mirror symmetric each other, indicating that the HC phase grows at the expense of the L_2 phase. The time course of change in the HC transition enthalpy consisted of three stages, (1) the initial delay, (2) the growth and (3) the plateau stages. The $L_{\beta'}$ phase transformed into the L_2 phase via the L_1 phase during the initial delay period. After the onset of the HC phase formation, the HC transition enthalpy increased monotonously until reaching a plateau value of about 22 kJ/mol, which is much less than the hydration enthalpy obtained in Fig. 1a. After the incubation for 10 hours at 1ºC (plateau stage), 32% of all DMPG molecules were in the HC phase as the main transition enthalpy was reduced from

25 to 17 kJ/mol. Considering that 32% of DMPG molecules contributed to the HC transition enthalpy of 22 kJ/mol after the incubation, we calculated the enthalpy difference between the HC phase and the L_α phase to be 69 kJ/mol, which is nearly equal to the hydration enthalpy of DMPG powder crystals obtained in Fig. 1a. In order to check the accuracy of the main transition enthalpy estimated by deconvolution of the superposed endothermic peaks with symmetric functions, we measured it in an alternative way. A DMPG sample was heated up to 32ºC (above the L_2-to-$P_{\beta'}$ transition temperature) after the low-temperature incubation for eight hours at 1ºC so that only the main transition and the HC transition appears in the subsequent heating scan from 0ºC. Thus, we could estimate the main transition enthalpy without the curve fitting, and calculate the enthalpy difference between the HC phase and the L_α phase to be 70 kJ/mol, which is close to that obtained above (69 kJ/mol).

The plateau stage was stable at least 12 hours (data not shown). Thus, the transition from the L_2 phase to the HC phase at 1˚C seemed to proceed in a simple two-state manner though it ceased halfway.

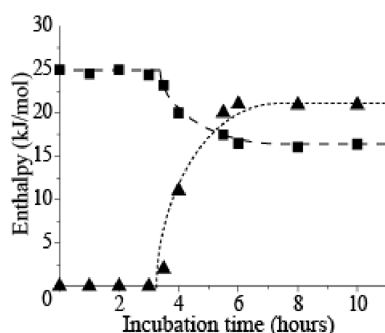


Fig. 5. Growth of the HC phase at 1°C. The transition enthalpies of the main transition (■) and the HC transition (▲) were estimated from the heating thermograms in Fig. 3 and plotted as a function of the incubation time at 1°C. Lines are guide for eyes.

3-3. Dependence of the HC phase formation on NaCl concentration

We examined the influence of NaCl concentration on the HC phase formation in the sufficiently hydrated DMPG vesicles. Fig. 6 shows the DSC heating thermograms of DMPG bilayers after the incubation for four hours at 1ºC in the presence of 150-500 mM NaCl. In the presence of 150 mM NaCl the DSC thermogram showed only the L_2-to-$P_{\beta'}$ phase transition at 20.8°C and the main transition at 24.0°C; no transition peak was detected above the main transition temperature (Fig. 6a). Thus, four hours' incubation at 1°C is too short for DMPG vesicles in the presence of 150 mM NaCl to form the HC phase. On the other hand, DMPG vesicles in the presence of 200 mM NaCl or

more exhibited the HC transition at 38-40ºC (arrows in Fig. 6b-e) in addition to the L_2-to-$P_{\beta'}$ phase transition and the main transition. The higher the NaCl concentration was, the slightly higher the HC transition temperature was though it was constant throughout the transition. The HC transition enthalpy increased as NaCl concentration increased, suggesting that higher concentration of NaCl is more favorable for the HC phase formation.

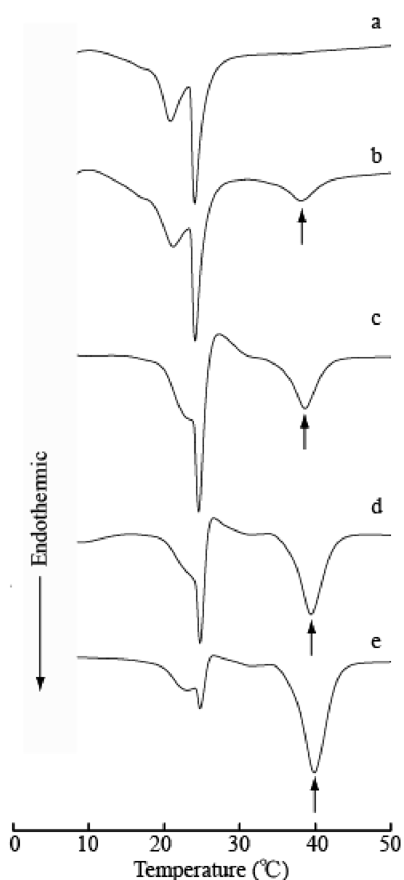


Fig. 6. DSC thermograms showing dependence of the HC phase formation on NaCl concentration. DMPG bilayers were incubated for four hours at 1°C in the presence of (a) 150, (b) 200, (c) 300, (d) 400 and (e) 500 mM NaCl. In the presence of 200 mM NaCl or more, there appeared the endothermic peak corresponding to the HC transition in their subsequent heating scan (arrows).

To quantitatively evaluate the dependence of the kinetics of the HC phase formation on NaCl concentration, we plotted the HC transition enthalpy as a function of the incubation time at 1°C for each NaCl concentration (Fig. 7). In the presence of high concentration of NaCl (≥150 mM), the time course of change in the HC transition enthalpy consisted of three stages as described above. Dependence of the parameter characterizing each stage on NaCl concentration is shown in Fig. 8. The initial delay period t_{lag} decreased as NaCl concentration increased (Fig. 8a). In the second

growth stage, the HC transition enthalpy seemed to increase in a simple two-state manner in case the growth was observed. In contrast to the initial delay period, the relaxation time τ for the HC phase formation in the second stage was almost insensitive to NaCl concentration (Fig. 8b). In the third stage, the HC transition enthalpy at the plateau ΔH^{p}_{HC} decreased as NaCl concentration decreased (Fig. 8c). Extrapolation of the data to ΔH^{p}_{HC} =0 suggests that there is a threshold concentration of NaCl for the onset of the HC phase formation at about 100 mM. Actually, DMPG vesicles in the presence of 100 mM NaCl did not show the HC transition even after the incubation for five days at 1°C (data not shown).

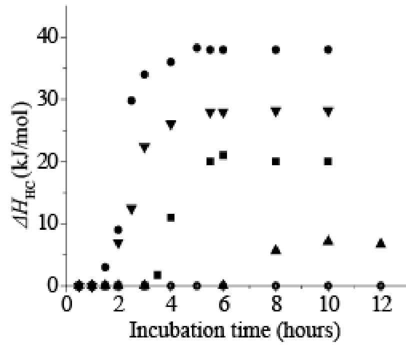


Fig. 7. The time course of increase in the HC transition enthalpy ΔH_{HC}. NaCl concentrations are (○) 100, (▲) 150, (■) 200, (▼) 300 and (●) 400 mM. The transition enthalpies were calculated from DSC heating thermograms after incubation at 1°C for appropriate periods.

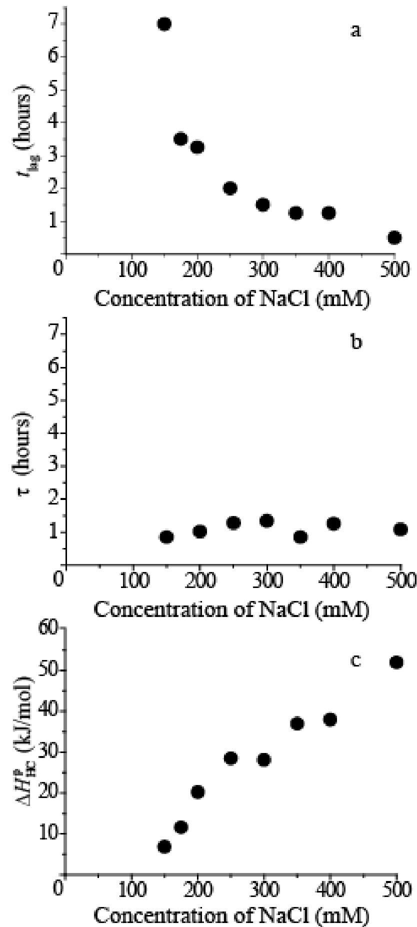


Fig. 8. Dependence of (a) the initial delay period t_{lag}, (b) the relaxation time τ and (c) the plateau value of the HC transition enthalpy ΔH^{p}_{HC} on NaCl concentration. The value of t_{lag} was estimated by Eq. (1). The relaxation time was calculated by fitting Eq. (2) to the data in Fig. 7 ($t > t_{lag}$). The value of ΔH^{p}_{HC} is the average of the transition enthalpies in the plateau region.

3-4. Separation of the HC fraction from the non-HC fraction

Figs. 7 and 8c show that the HC phase growth reaches a plateau before the L_2 phase entirely transforms into the HC phase, and the plateau level depends on NaCl concentration. What causes the suppression of further growth of the HC phase? In order to get the insight into the suppression mechanism we examined in what manner the grown HC phase exists in the DMPG bilayer vesicle in the plateau stage by FFEM (Fig. 9). In order to make the domains in the HC phase easily discernible we controlled the thermal history of the sample so that the lipids in the phase other than the HC phase transform into the $P_{\beta'}$ phase (see the figure legend for detail). As a result, the flat domains in the HC phase are clearly seen laterally connected to the $P_{\beta'}$ phase (arrows in Fig. 9). Thus, the HC phase seems to grow keeping the vesicular structure intact and coexist in a vesicle with the precursor phases in the plateau stage.

Fig. 9. A freeze-fracture electron micrograph showing coexistence of the HC phase with the non-HC phase in a DMPG vesicle. DMPG bilayers were incubated for 12 hours at 1ºC in the presence of 200 mM so that about 32% of lipids are in the HC phase (see Fig. 5). The incubated sample was heated up to 30ºC and then cooled down to 18ºC before quenched into nitrogen slush. The flat domains in the HC phase (arrows) are clearly seen laterally connected to the rest, which transformed into the ripple phase after those temperature treatments. Bar indicates 200 nm.

To scrutinize the effect of coexistence of these phases on the appearance of the plateau stage, we tried to break down the vesicle structure and separate the fraction in the HC phase (HC fraction) from the fraction which still remains to be in the L_2 phase even in the plateau stage (non-HC fraction). The separation was based on the difference in density between these two fractions. We

centrifuged the sample in the plateau stage at 30°C to enhance the density difference (see Materials and methods for detail). Figs. 10a and b show the DSC heating thermograms of the precipitate and the supernatant obtained after the centrifugation, respectively. The supernatant gave a thermogram similar to that of the sample without low-temperature incubation and the precipitate exhibited predominantly the HC transition. Thus, the HC fraction (precipitate) was successfully separated from the non-HC fraction (supernatant), indicating that layers of rigid HC domains tore off the non-HC matrix in the fluid phase at 30°C to induce collapse of vesicles during the centrifugation.

Released from the constraints imposed by coexistence of the HC phase, the separated non-HC fraction was able to restart the HC phase formation (Fig. 10c). When the non-HC fraction was re-incubated for four hours at 1°C, there appeared the HC transition at 39.6°C together with the main and L_2-to-$P_{\beta'}$ phase transitions.

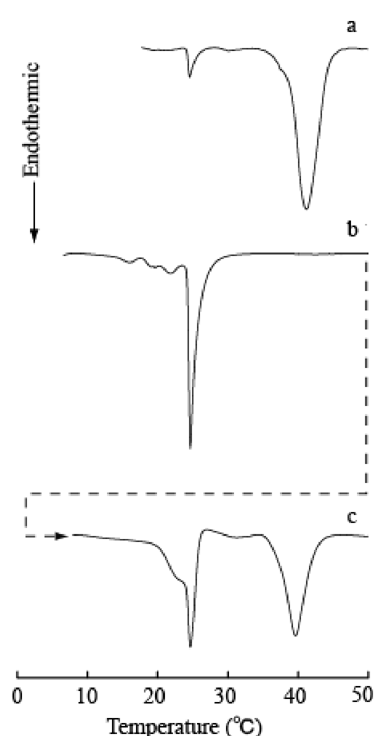


Fig. 10. DSC heating thermograms of (a) the HC fraction and (b) the non-HC fraction obtained by centrifugation as described in Materials and methods after incubation for six hours at 1 ºC in the presence of 400 mM NaCl. Under these conditions the HC phase formation is in the plateau stage and 54% of lipids are in the HC phase (see Fig. 8c). The heating thermogram of the precipitation (HC fraction) after the centrifugation shows the HC transition at 41.1ºC. In contrast, that of the supernatant (non-HC fraction) shows the main transition at 24.6ºC but not the HC transition. Note that in the presence of 400 mM NaCl small amounts of the L_1 and L_2 phases form during the temperature scan in the DSC apparatus. (c) The heating thermogram of the non-HC fraction after reincubation for four hours at 1ºC. Note that an endothermic peak corresponding to the HC transition is seen.

4. Discussion

4-1. Low-temperature phase behavior of sufficiently *hydrated DMPG vesicles*

We carefully prepared 'sufficiently hydrated DMPG bilayers' and examined the kinetics of the HC phase formation. FFEM observation and SAXD measurements revealed that the sufficiently hydrated DMPG bilayers assume closed vesicular structures with the interbilayer space of indefinite thickness (Fig. 2). The phase behavior of the sufficiently hydrated DMPG vesicles under low-temperature incubation had characteristics similar to that described previously by Koynova (1997) and Kodama et al. (1999), except for the absence of intermediate phases which transform into the L_α phase in the temperature range between the main transition and the HC transition. Our results suggest that the appearance of intermediate metastable phases between the L_2 and the HC phases in the previous works was caused by insufficient hydration in the sample preparation; probably, their preincubation temperatures were not enough higher than the crystal-to-L_α phase transition temperature of about 40˚C and their preincubation time was too short to eliminate the last traces of structural remnants from crystal aggregates. In addition to the absence of the intermediated phases, the sufficiently hydrated DMPG vesicles exhibited a well-defined HC transition with a constant transition temperature of 38.2˚C (200 mM NaCl), and allowed us to analyze the detail of the kinetics of the HC phase formation.

A series of DSC measurements after appropriate periods of incubation at 1˚C revealed that the formation process of the HC phase in the presence of more than 100 mM NaCl consists of three stages; (1) initial delay, (2) growth and (3) plateau stages. The initial delay stage seems to be related to the occupation of the whole DMPG bilayers by the L_2 phase. Actually, the L_2-to-$P_{\beta'}$ phase transition enthalpy increased gradually during the first stage and reached at its maximum just before the growth stage (Fig. 3). In the growth stage, the HC transition enthalpy increased monotonously as the incubation time at 1˚C increased. A single exponential function was well fitted to the time course of increase in the transition enthalpy. This is consistent with the fact that no intermediate phase was detected by DSC between the L_2 phase and the HC phase. Unexpectedly, in the third stage, the plateau value of the HC transition enthalpy $\Delta H_{\mathrm{HC}}^{\mathrm{p}}$ was far smaller than that expected from the enthalpy difference between the L_α and HC phases unless NaCl concentration was quite high. We infer that the closed vesicular structure of sufficiently hydrated DMPG bilayers plays a key role in preventing a part of the L_2 phase from transforming into the HC phase (see Section 4-3). We also examined the dependence of the HC phase formation on NaCl concentration. The initial delay period and the HC transition enthalpy at the plateau stage depended on NaCl concentration, while the growth rate in the second stage was almost insensitive to NaCl concentration (Fig. 8).

Here, we discuss the subgel-to-HC phase transition on the basis of the phase scheme proposed by Kodama et al. (1999). However, there is another possibility that there exists only one metastable

subgel (L_c1^b) phase as suggested by spectroscopic experiments (Zhang et al., 1997); if it is the case, the apparent plural endothermic peaks come from the transitions of the domains with different sizes (see Lewis et al., 2005) and/or exothermic transitions corresponding to the HC phase growth which may occur at temperatures higher than the incubation temperature (Zhang et al., 1997). As there have been no systematic structural studies, we cannot conclude what kind of and how many subgel phases form during low-temperature incubation of DMPG bilayers. Hence, we adopted the phase sequence of $L_1 \rightarrow L_2 \rightarrow$HC for convenience in discussion because whether the HC phase forms from the L_2 phase or the L_c1^b phase does not affect the discussion below.

4-2. Nucleation and growth in the L_2-to-HC phase transition

Formation of the L_1 phase and the L_1–to-L_2 phase transition proceed during the initial delay period in a NaCl concentration-dependent manner (Kodama et al., 1999). These low-temperature phases may be structurally similar to the well-characterized subgel phase in diacylphosphatidylcholines; they have characteristics of crystal-like but two-dimensional molecular ordering in a single bilayer. As the crystal-like tight packing can be achieved by depression of the electrostatic repulsion between negatively charged DMPG molecules, screening effect of Na^+ ions may promote the formation of these precursor phases and consequently reduce the initial delay period in the HC phase formation in DMPG vesicles.

As for the onset of the nucleation in the HC phase formation there seems to be a threshold structure because the initial delay period in the HC phase formation was able to be defined definitely and relatively long comparing with the subsequent growth period (Figs. 8a and b). As mentioned above, it was not until almost all the lipids transformed into the L_2 phase that the nucleation of the HC phase initiated. Therefore, we speculate that the nucleation may require fairly large domain of the L_2 phase and the nucleus for the HC structure may be large in lateral size and/or three-dimensional. In fact, the mutually isolated bilayers in the L_2 phase were stacked up into the three-dimensional crystal-like structure of the HC phase in the process of the L_2-to-HC phase transition (see below). Furthermore, Na^+ ion binding to DMPG molecules in the L_2 phase may contribute to the formation of tightly packed nuclei because it reduces the intermolecular and interbilayer repulsions. Association constant of the Na^+ ion with the tightly packed PG headgroup in the L_2 phase may be relatively high as it is suggested to increase as effective size of the PG headgroup decreases (Riske et al., 1997).

We infer that at least three processes are involved in the L_2-to-HC phase transition; (1) dehydration, (2) formation of hydrogen bond networks among PG headgroups and (3) Na^+ ion binding to negatively charged PG headgroups.

At first, the involvement of extensive dehydration in the L_2-to-HC phase transition was examined by SAXD experiments (Fig. 11). DMPG vesicles in the L_2 phase consisted of mutually independent single bilayers because they gave a broad peak centered at 0.23 nm^{-1} (4.35 nm) (Fig. 11a), which reflects the thickness of a single bilayer (Riske et al., 2001). In contrast, the SAXD profile of the HC phase (Fig. 11b), which was obtained after a separation treatment described in section 3-4, showed a sharp reflection from a multilamellar system with the lamellar spacing of 4.76 nm (0.21 nm^{-1}) as described previously for the sample obtained by incubation for 7 days at 4°C (Garidel et al., 2001). Thus, the notable interbilayer water loss, dehydration, may take place in the L_2-to-HC phase transition.

Secondly, establishment of the interheadgroup hydrogen bonding in the process of the HC phase formation was suggested by Epand et al. (1992). In addition to the hydrogen bond other intermolecular interactions such as the coordination bond existing in the DMPG crystal may be involved as the HC phase has similar structural features to the crystal (Pascher et al., 1987; Garidel et al., 2001). These intermolecular interactions as well as the hydration state should contribute to the HC transition temperature higher than the main transition temperature (Cevc and Marsh, 1987; Tobochnik et al., 1995).

Thirdly, Na^+ ion binding to negatively charged PG headgroups may be involved in the transformation from the L_2 phase to the HC phase because suppression of electrostatic repulsion should be required to induce the tightly-packed and stacked-lamellar structure of the HC phase. Since only very few data are available on the ion binding to the low-temperature phases, further study is needed to clarify the ion-lipid interactions in the L_2 phase.

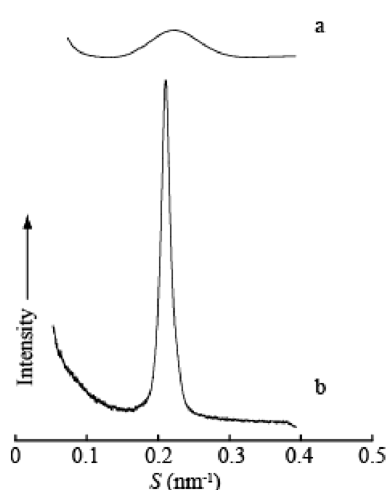


Fig. 11. Dehydration of the DMPG bilayer accompanying with the HC phase formation as shown by small angle X-ray diffraction (SAXD) measurements. (a) A SAXD profile of DMPG bilayers in the presence of 175 mM NaCl at 5ºC after incubation for four hours at 1°C. We checked by DSC that most of the DMPG bilayers were in the L_2 phase under these conditions (data not shown). There appeared a broad peak centered at 0.23 nm^{-1} (4.35 nm). (b) A SAXD profile of the HC phase fraction separated by centrifugation (see Materials and methods). The sample was kept at 5ºC. A sharp peak seen at 0.21 nm^{-1} (4.76 nm) remained until the sample was heated above the HC transition temperature (data not shown).

4-3. NaCl concentration-dependent suppression of the HC phase growth

Figs. 7 and 8c show that HC phase growth reaches a plateau before the L_2 phase entirely transforms into the HC phase, and the plateau level depends on NaCl concentration. Fig. 10c shows that the removal of the HC phase restarts the HC phase formation even after the increase in ΔH_{HC} reaches a plateau. Why does the formed HC domain suppress the additional transformation of the non-HC phase into the HC phase? Fig. 9 shows that the HC phase seems to grow keeping the vesicular structure intact, suggesting a possibility that some conflicts arise in the inside of the closed vesicle.

One of the candidates is reduction of free Na^+ ions in the inside of the vesicle because they are incorporated into the HC phase as it grows. In fact the main transition temperature in cooling was slightly higher in the separated HC fraction than in the non-HC fraction (data not shown). These results suggest higher NaCl concentration in the former fraction because increase in salt concentration leads to increase in the main transition temperature in DMPG bilayers (Salonen et al., 1989). If the HC phase formation requires the binding of Na^+ ions to DMPG molecules, the reduction of free Na^+ ions in the inside of the vesicle may suppress further growth of the HC phase and the plateau level must be NaCl concentration-dependent. Moreover, the restart of the HC phase growth in the separated non-HC fraction (Fig. 10c) can be explained by influx of Na^+ ions into vesicles breaking during centrifugation.

Another candidate for the origin of the conflict is elastic strain imposed to the closed vesicle by the growth of the flat domain in the HC phase. FFEM observation (Fig. 9) suggested neither the breaking down of the vesicle structure, which may be necessary to complete the HC phase formation, nor the formation of crinkled surface, which is adopted to release the elastic stress in the subgel phase formation (Meyer et al., 1998; 2000). It is likely that the release of the elastic stress is more difficult in the HC phase formation than in the subgel phase formation because the HC phase has crystal-like three-dimensional order in the molecular packing in contrast to the two-dimensional order in the subgel phase. Though the elastic strain could explain the incompletion of the HC phase formation and the restart of the HC phase growth in the separated non-HC fraction, it may fall short in the explanation of the NaCl concentration-dependence.

Finally we checked the effect of the incubation temperature on the plateau level because lower-temperature incubation accelerates the formation of quasicrystalline phases (Zhang et al. 1997). The formation of HC phase at -5ºC in DMPG bilayers in the presence of 400 mM NaCl showed similar kinetical characteristics to those at 1ºC: the plateau enthalpy ΔH_{HC}^{P} (41 kJ/mol at

-5ºC and 38 kJ/mol at 1ºC) and the relaxation time τ (0.9 hours at -5ºC and 1.3 hours at 1ºC), were weakly sensitive to the incubation temperature though the initial delay period at -5ºC (t_{lag}<0.5 hours) was clearly shorter than that at 1ºC (~one hour). However, temperature dependence of the kinetics may be much more complicated as Zhang et al. (1997) suggested that the proliferation dominantly occurs at the temperature lower by 10-20ºC than the main transition temperature while the nucleation proceeds fast at lower temperature. Further systematic study is required to fully understand the temperature effect on the process of the HC phase formation.

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References

Boggs J.M., 1987. Lipid intermolecular hydrogen bonding: Influence on structural organization and membrane function. Biochim. Biophys. Acta 906, 353-404.

Boni, L.T., Stewart, T.P., Alderfer, J.L., Hui, S.W., 1981. Lipid-polyethylene glycol interactions: II. Formation of defects in bilayers. J. Membrane Biol. 62, 71-77.

Boni, L.T., Stewart, T.P., Hui, S.W., 1984. Alterations in phospholipid polymorphism by polyethylene glycol. J. Membrane. Biol. 80, 91-104.

Chan, H., Epand, R.M., 1983. The existence of highly ordered phase in fully hydrated dilauroylphosphatidylethanolamine. Biochim. Biophys. Acta 728, 319-324.

Cevc, G., Marsh, D., 1987. Phospholipid bilayers, physical principles and models. Wiley and Sons, New York, pp. 232-268.

Eklund, K.K., Virtanen, J.A., Vuori, K., Patrikainen, J., Kinnunen, P.K.J., 1987. Role of the polar head group stereoconfiguration in the cation-induced aggregation of dimyristoylphosphatidylglycerol vesicles. Biochemistry 26, 7542-7545.

Epand, R.M., Gabel, B., Epand, R.F., Sen, A., Hui, S.W., Muga, A., Surewicz, W.K., 1992. Formation of a new stable phase of phosphatidylglycerols. Biophys. J. 63, 327-332.

Garidel, P., Forster, G., Richter, W., Kunst, B.H., Rapp, G., Blume, A., 2000. 1,

2-dimyristoyl-3-phosphoglycerol (DMPG) divalent cation complexes: an X-ray scattering and freeze-fracture electron microscopy study. Phys. Chem. Chem. Phys. 2, 4537-4544.

Garidel, P., Richter, W., Rapp, G., Blume, A., 2001. Structural and morphological investigations of the formation of quasi-crystalline phases of 1,2-dimyristoyl-3-phosphoglycerol (DMPG). Phys. Chem. Chem. Phys. 3, 1504-1513.

Hatta, I., Takahashi, H., Matsuoka, S., Amemiya, Y., 1995. Simultaneous X-ray diffraction and differential scanning calorimetry in the study of phase transition. Thermochim. Acta 253, 149-154.

Kinoshita, M., Kato, S., Takahashi, H., 2008. Effect of bilayer morphology on the subgel phase formation. Chem. Phys. Lipids 151, 30-40.

Kodama, M., Miyata, T., Yokoyama, T., 1993. Crystalline cylindrical structures of Na^+-bound dimyristoylphosphatidylglycerol as revealed by microcalorimetry and electron microscopy. Biochim. Biophys. Acta 1168, 243-248.

Kodama, M., Inoue, H., Tsuchida, Y., 1995. The behavior of water molecules associated with structural changes in phosphatidylethanolamine assembly. Thermochim. Acta 266, 373-384.

Kodama, M., Aoki, H., Miyata, T., 1999. Effect of Na^+ concentration on the subgel phases of negatively charged phosphatidylglycerol. Biophys. Chem. 79, 205-217.

Koynova, R., 1997. Liquid crystalline phase metastability of phosphatidylglycerols. Chem. Phys. Lipids 89, 67-73.

Lamy-Freund, M.T., Riske, K.A., 2003. The peculiar thermo-structural behavior of anionic lipid DMPG. Chem. Phys. Lipids 122, 19-32.

Lewis, R.N.A.H., Zhang, Y.P., McElhaney, R.N., 2005. Calorimetric and spectroscopic studies of the phase behavior and organization of lipid bilayer model membranes composed of binary mixtures of dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol. Biochim, Biophys. Acta 1668, 203-214.

Lotta, T.I., Salonen, I.S., Virtanen, J.A., Eklund, K.K., Kinnunen, P.K.J., 1988. Fourier transform infrared study of fully hydrated dimyristoylphosphatidylglycerol. Effects of Na^+ on the *sn*-1' and *sn*-3' headgroup stereoisomer. Biochemistry 27, 8158-8169.

Meyer, H.W., Westermann, M., Stumpf, M., Richter, W., Ulrich, A.S., Hoischen, C., 1998. Minimal radius of curvature of lipid bilayers in the gel phase state corresponds to the dimension of biomembrane structure 'caveolae'. J. Struct. Biol. 124, 77-87.

Meyer, H.W., Semmler, K., Rettig, W., Pohle, W., Ulrich, A.S., Grage, S., Selle, C., Quinn, P.J., 2000. Hydration of DMPC and DPPC at 4°C produces a novel subgel phase with convex-concave bilayer curvatures. Chem. Phys. Lipids 105, 149-166.

Nagle, J.F., Wilkinson, D.A., 1978. Lecithin bilayers-density measurements and molecular interactions. Biophys. J. 32, 159-175.

Pascher, I., Sundell, S., Harlos, K., Eibl, H., 1987. Conformation and packing properties of membrane lipids: the crystal structure of sodium dimyristoylphosphatidylglycerol. Biochim. Biophys. Acta 896, 77-88.

Riske, A.K., Politi, M.J., Reed, W.F., Lamy-Freund, M.T., 1997. Temperature and ionic strength dependent light scattering of DMPG dispersions. Chem. Phys. Lipids 89, 31-44.

Riske, A.K., Amaral, L. Q., Lamy-Freund, M.T., 2001. Thermal transition of DMPG bilayers in aqueous solution; SAXS structural studies. Biochim. Biophys. Acta 1551, 297-308.

Ruocco, M.J., Shipley, G.G., 1982. Characterization of the subtransition of hydrated dipalmitoylphosphatidylcholine bilayers. Kinetic, hydration and structural study. Biochim. Biophys. Acta 691, 309-320.

Salonen, I.S., Eklund, K.K., Virtanen, J.A., Kinnunen, P.K.J., 1989. Comparison of the effects of NaCl on the thermotropic behavior of *sn*-1' and *sn*-3' stereoisomer of 1, 2-dimyristoyl-*sn*-phosphatidylglycerol. Biochim. Biophys. Acta 982, 205-215.

Seddon, J.M., Harlos, K., Marsh, D., 1983. Metastability and polymorphism in the gel and fluid bilayer phases of dilauroylphosphatidylethanolamine. J. Biol. Chem. 258, 3850-3854.

Shotton, D.M., Servers N.J., 1995. An introduction to freeze fracture and deep etching. In: Shotton, D.M., Servers N.J. (Eds.), Rapid freezing, freeze fracture, and deep etching. Wiley, New York, pp.1-31.

Stewart, T.P., Hui, S.W., Portis, A.R., Jr., Papahadjopoulos, D., 1979. Complex phase mixing of phosphatidylcholine and phosphatidylserine in multilamellar membrane vesicles. Biochim. Biophys. Acta 556, 1-16.

Takahashi, H., Matsuoka, S., Amemiya, Y. and Hatta, I., 1995. Simultaneous differential scanning calorimetry and time-resolved X-ray diffraction of lipid-water system. 1. Relationship between chain melting and endothermic heat at the main transition of a dipalmitoylphosphatidylcholine-water system. Chem. Phys. Lipids 76, 115-121.

Tenchov, B., Koynova, R., Rapp, G., 2001. New ordered metastable phases between the gel and subgel phases in hydrated phospholipids. Biophys. J. 80, 1873-1890.

Tobochnik, J., Zuckermann, M.J., Zhang, Z., 1995. Simulation of hydrogen bonding and hydration in pure lipid bilayers. Phys. Rev. E 51, 6204-6211.

Toca-Herrera, J.L., Krustev, R., Miiller, H.-J., Möhwald, H., 2000. Effect of the charged lipid DMPG on the thickness and contact angle of foam films. J. Phys. Chem. 104, 5486-5491.

Tristram-Nagle, S., Wiener, M.C., Yung, C.P., Nagle, J.F., 1987. Kinetics of the subtransition in dipalmitoylphosphatidylcholine. Biochemistry. 26, 4288-4294.

Wilkinson, D., Nagle, J., 1984. Metastability in the phase behavior of dimyristoylphosphatidylethanolamine bilayers. Biochemistry 23, 1538-1541.

Zhang, Y.P., Lewis, R.N.A.H., McElhaney, R.N., 1997. Calorimetric and spectroscopic studies of

the thermotropic phase behavior of the *n*-saturated 1,2-diacylphosphatidylglycerols. Biophys. J. 72, 779-793.