Low pH Stabilizes the Inverted Hexagonal II Phase in Dipalmitoleoylphosphatidylethanolamine Membrane

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Abstract. Dipalmitoleoylphosphatidylethanolamine (DPOPE) membrane is in the L_α phase in neutral pH at 20 °C. The results of small-angle X-ray scattering (SAXS) indicate that an L_α to H_{II} phase transition in DPOPE membranes occurred at pH 1.9 in the absence of salt, and at pH 2.8 in the presence of 0.5 M KCl, at fully hydrated condition at 20 °C. The spontaneous curvature of DPOPE monolayer membrane did not change with a decrease in pH values. To elucidate the mechanism, we have investigated the effect of the cationic dioctadecyldimethylammonium (DODMA) on the structure and phase behavior of DPOPE membrane. The result shows that DODMA stabilizes the H_{II} phase rather than the L_α phase in DPOPE membrane at its low concentrations. Based on these results, the H_{II} phase stability of DPOPE membrane due to low pH is discussed in terms of the spontaneous curvature of the monolayer membrane and the packing energy of acyl chains in the membrane.

Key words: liquid-crystalline phase, inverted hexagonal phase, phase transition, low pH, spontaneous curvature

1. Introduction

In recent years, a great deal of attention has been given to the liquid-crystalline (L_α) phase/inverted hexagonal II (H_{II}) phase transition (Figure 1), because it is believed to be vital to understand the membrane fusion and the functions of membrane proteins [1–4]. The structure and phase properties of various kinds of phosphatidylethanolamine (PE) membrane have been extensively studied by a variety of methods, including differential scanning calorimetry (DSC), nuclear magnetic resonance (NMR), X-ray diffraction, Fourier-transform infrared spectroscopy (FTIR), and fluorescence [5–7]. As the temperature increases, they undergo a gel (L_β) to L_α phase transition, and then an L_α to H_{II} phase transition. Several substances have been shown to influence the phase stability and the structures of H_{II} phase of PE membrane, high concentration of NaCl [7], sugars such as sucrose and trehalose

Figure 1. Schematic representation of H_{II} (a) and L_{α} phospholipid phases (b). Lipid head groups (circles) attach with two melted hydrocarbon chains. The distance, d , is the ordinate for Figures 3–6.

[8], and alkanes [9], which decrease the temperature of the L_{α} to H_{II} phase transition, and stabilize the H_{II} phase more than the L_{α} phase; In contrast, the short-chain alcohols such as methanol [10] increase the L_{α} to H_{II} phase transition temperature, and stabilize the L_{α} phase.

The pH-titration, as a method to investigate the phase behaviors of the phospholipid membranes has been extensively used [11–14]. This method gives the opportunity to vary the surface charge density without change of the chemical structure and the size of the surface segments. It is more helpful to understand the effects of electrostatic interactions in the membrane interface on the structure and phase stability of charged lipid membranes [12–15]. The mixtures of PE with charged phospholipids have been investigated [16, 17]. These mixture membranes are very sensitive to pH and univalent and divalent ions. Ca^{2+} ions and lowering the pH tend to induce the appearance of an H_{II} phase in these mixtures. However, the effect of pH on the structures and phase behaviors of PE membrane is not understood well.

In this report, we have investigated the effect of low pH on the phase stability of PE membrane by small-angle X-ray scattering (SAXS). As the PE membrane,

we used DPOPE membrane, which is in the L_α phase in fully hydrated condition at 20 °C [18]. We found that an L_α to H_{II} phase transition occurred in this membrane at low pH. To elucidate the mechanism of the H_{II} phase stability of DPOPE membrane due to low pH, the spontaneous curvature of the DPOPE monolayer membrane and the effect of cationic dioctadecyldimethylammonium (DODMA) on DPOPE membrane have also been investigated. Based on these results, the mechanism of the L_α to H_{II} phase transition and the H_{II} phase stability in the DPOPE membrane was discussed.

2. Materials and Methods

1,2-Dipalmitoleoyl-*sn*-glycero-3-phosphatidylethanolamine (DPOPE) was purchased from Avanti Polar Lipids. DODMA bromide and tetradecane were purchased from Sigma Chemical Co. (St. Louis, MO). They were used without further purification.

Lipid dispersions were prepared as follows. Appropriate amount of various pH values buffer containing 0 M or 0.5 M KCl was added to dry lipids in fully hydrated condition (the final concentration of lipids was 50 mM). Then, the suspensions were vortexed for about 30 second at room temperature (~25 °C) several times. For pH 6.0 ≤ pH ≤ pH 7.0, 10 mM PIPES buffer, and for pH 2.5 < pH < pH 6.0, 20 mM citrate buffer were used. For pH 1.5 ≤ pH ≤ pH 2.5, HCl/KCl buffers were used. For measurements of X-ray diffraction, pellets of the lipid suspensions after centrifugation (13,000 × g, 30 minutes at 20 °C; Tomy, MR-150) were used. The pH values of the supernatants of the suspensions after the centrifugation were rechecked. Hydrolysis of phospholipids at low pH was not observed in our experiments. To take the information of spontaneous curvature of DPOPE monolayer membrane, we have investigated the structures of lipid membranes containing 16 wt% tetradecane. The method is the same as that described in our previous paper [19] else than the incubation for 10 hours for equilibration from the addition of the various pH values buffer to DPOPE/tetradecane.

X-ray diffraction experiments were performed by using Nickel-filtered Cu K_α X-ray (λ = 0.154 nm) from rotating anode type X-ray generator (Rigaku, Rotaflex, RU-300) at the operating condition (40 kV × 200 mA). SAXS data were recorded using a linear (one-dimensional) position sensitive proportional counter (PSPC) (Rigaku) [20] with camera length of 350 mm and associated electronics (multi-channel analyzer, etc., Rigaku). In all cases, samples were sealed in a thin-walled glass capillary tube (outer diameter 1.0 mm) and mounted in a thermostatable holder whose stability was ± 0.2 °C.

3. Results

The DPOPE membrane is in the liquid-crystalline phase (L_α phase) at 20 °C [18]. The SAXS data of DPOPE membrane in 10 mM PIPES buffer (pH 7.0) at 20 °C

Figure 2. X-ray diffraction profiles of the DPOPE membranes at 20 °C in pH 7.0 (a), pH 1.9 (b), and pH 1.8 (c), respectively.

showed that a set of SAXS peaks had spacing (lamellar repeat period) in the ratio of 1:2:3, ..., and the spacing was 4.9 nm (Figure 2(a)). WAXS patterns of this membrane consisted of a diffuse broad band around 0.46 nm. At pH 1.9, a new set had spacing in ratio of $1:\sqrt{3}:2:\sqrt{7}, \dots$, which is consistent with a two-dimensional hexagonal (H_{II}) phase, appeared (Figure 2(b)). Below pH 1.8, the L_{α} peaks disappeared, and this membrane was completely in the H_{II} phase (Figure 2(c)). The basis vector length of the H_{II} phase (center to center distance of adjacent cylinders), as shown in Figure 1(a), d , calculated by $d = (2/\sqrt{3})x$ (x is the spacing in the SAXS), was almost constant ($d = 7.7$ nm) with a decrease in pH values from pH 1.8 to pH 1.5 (Figure 3). To consider effect of ionic strength in the bulk phase on this phase transition, effect of pH on the DPOPE membrane in the presence of 0.5 M KCl has been investigated. As shown in Figure 3, an L_{α} to H_{II} phase transition in the

Figure 3. Spacing or basis vector length, d , of DPOPE membranes in various pH values at 20 °C in 0 M KCl (○, Δ), and in 0.5 M KCl (●, ▲). ○, and ● correspond to the L_α phase, Δ, and ▲ corresponds to the H_{II} phase.

DPOPE membrane occurred at pH 2.8, which is higher than that of the absence of KCl, and d , was not changed ($d = 7.5$ nm) from pH 2.8 to pH 1.5.

To elucidate the mechanism of the H_{II} phase stability in DPOPE membrane due to low pH, the dependence of the spontaneous curvature of the DPOPE membrane on pH values and the effect of DODMA on the DPOPE membrane have been investigated.

To gain information of spontaneous curvature of phospholipid membrane, it is required to allow lipid membrane in the H_{II} phase to express the spontaneous curvature, H_0 . For this purpose, we added 16 wt% tetradecane into the DPOPE membrane, because tetradecane can fill the interstitial region of the H_{II} phase and relax the acyl chain packing stress, and therefore allow the DPOPE membranes in the H_{II} phase [19, 22, 23]. As shown in Figure 4, the DPOPE membranes containing 16 wt% tetradecane were in the H_{II} phase in various pH values at fully hydrated condition, and the basis vector length, d , of the DPOPE/tetradecane membranes did not change ($d = 7.8$ nm) with a decrease in pH values (Figure 4).

As described in the above section, the DPOPE membrane in 10 mM PIPES buffer (pH 7.0) at 20 °C is in the L_α phase. At 2.0 mol% DODMA, a new set of SAXS peak was superimposed on the L_α peaks, which had spacing in the ratio of $1:\sqrt{3}:2:\sqrt{7}, \dots$, appeared. It is consistent with a two-dimensional hexagonal (H_{II}) phase. Above 3.8 mol% DODMA, the L_α phase disappeared, and the DODMA/DPOPE membranes were completely in the H_{II} phase (Figure 5). Hence, DODMA induced an L_α to H_{II} phase transition in the DPOPE membrane at 2.0 mol%. The basis vector length of the H_{II} phase, d , was almost constant ($d = 7.6$ nm) with an increase in DODMA concentration from 2.0 to 8.0 mol% (Figure 5).

Figure 4. Basis vector length, d , of DPOPE/tetradecane membranes in various pH values at 20 °C. All the membranes were in the H_{II} phase.

Figure 5. Spacing or basis vector length, d , of DPOPE membranes containing various concentrations of DODMA in 10 mM PIPES buffer (pH 7.0) at 20 °C. ● and ▲ correspond to the L_{α} phase and H_{II} phase, respectively.

To obtain the dependence of spontaneous curvature of the DPOPE/DODMA membranes, we also added 16 wt% tetradecane into the DPOPE/DODMA mixture membranes. As shown in Figure 6, all DPOPE/DODMA/tetradecane membranes were also in the H_{II} phase in 10 mM PIPES buffer (pH 7.0) at fully hydrated condition, and the basis vector length, d , of the DPOPE/DODMA/tetradecane membranes almost did not change ($d = 7.8$ nm) with an increase in DODMA concentration (Figure 6).

Figure 6. Basis vector length, d , of DODMA/DPOPE/tetradecane membranes with various DODMA concentrations in 10 mM PIPES buffer (pH 7.0) at 20 °C. All the membranes were in the H_{II} phase.

4. Discussion

The results of Figure 3 clearly show that low pH stabilizes the H_{II} phase rather than the L_α phase in the DPOPE membrane. To elucidate the mechanism of this phase stability, we use the conception of the spontaneous (or intrinsic) curvature of a single monolayer membrane. The spontaneous curvature, H_0 , can be expressed as $H_0 = 1/R_0$, where R_0 is the radius of spontaneous curvature [21, 22]. Lipid monolayer membranes, in the H_{II} phase in fully hydrated condition, have a curvature close to the spontaneous curvature to minimize the curvature energy. To allow the lipid membranes in the H_{II} phase in fully hydrated condition to express the spontaneous curvature, the addition of alkanes such as decane and tetradecane to the membrane is required. Because they fill the interstitial region of the H_{II} phase and relax the alkyl chain packing stress [22–24]. On the other hand, the basis vector length of the H_{II} phase, d , can also be expressed as a sum of the radius to the neutral plane (or pivotal plane or neutral surface), R_{pp} , and the distance between the bilayer midplane and the neutral plane, ξ , i.e., $d = 2 (R_{pp} + \xi)$ [23, 25, 26, 27]. As described above, in fully hydrated condition, the DPOPE membrane containing 16 wt% tetradecane in the H_{II} phase has a spontaneous curvature, H_0 , and thereby, $R_{pp} \approx R_0$, since the change in ξ is assumed to be small. Thus, the result shown in Figure 4 indicates that the spontaneous curvature of the DPOPE membrane do not change with a decrease in pH values. And the result shown in Figure 6 indicates that the spontaneous curvature of the DPOPE/DODMA membranes almost do not change with an increase in DODMA concentration.

In general, the difference of the chemical potential of the phospholipid membrane in the H_{II} phase (μ^{HII}) and in the bilayer liquid crystalline (L_α) phase (μ^{bil}), $\Delta\mu$, can be expressed as a sum of the curvature elastic energy ($\Delta\mu_{\text{curv}}$) and the

acyl chain packing energy ($\Delta\mu_{\text{ch}}$) [25, 28]. The curvature energy can be expressed as [29, 30]

$$\mu_{\text{curv}} = 1/2(N_A\kappa A_0)(H_w - H_0)^2 \quad (1)$$

where N_A is Avogadro's number, κ is the elastic bending modulus, A_0 is the optimal surface area of the membrane, H_w is the curvature of the lipid monolayer, and H_0 is the spontaneous curvature of the lipid monolayer. As described above, in fully hydrated condition, the membrane in the H_{II} phase has a curvature close to the spontaneous curvature to minimize the curvature energy, and thereby, $H_w \approx H_0$, and since, in the L_α phase, $H_w = 0$,

$$\Delta\mu_{\text{curv}} = -1/2(N_A\kappa A_0^{\text{bil}})H_0^2 \quad (2)$$

where A_0^{bil} is the optimal surface area of the L_α phase. Equation (2) shows that $\Delta\mu_{\text{curv}}$ is always negative ($\Delta\mu_{\text{curv}} < 0$), and thereby, is an important factor stabilizing the H_{II} phase. On the other hand, in the H_{II} phase, acyl chains of lipids have to extend to different lengths to fill the interstitial hydrocarbon region, which decreases the entropy of chains and thereby increases the free energy of the membrane [28]. Therefore, this packing energy of the acyl chains unstabilizes the H_{II} phase, and thus, $\Delta\mu_{\text{ch}}$ is always positive ($\Delta\mu_{\text{ch}} > 0$). In most cases, phase transitions between the H_{II} and the L_α phases are determined by the interplay of these two factors, $\Delta\mu_{\text{curv}}$ and $\Delta\mu_{\text{ch}}$.

As analyzed above, the spontaneous curvature, H_0 , of the DPOPE membranes did not change with pH values. From Eq. (2), we know that the $\Delta\mu_{\text{curv}}$ did not change with pH values. Therefore, the $\Delta\mu_{\text{ch}}$ is the only factor to determine an L_α to H_{II} phase transition due to low pH. Figure 5 clearly shows that DODMA also induced an L_α to H_{II} phase transition in the DPOPE membrane at its low concentrations. And the spontaneous curvature of the DPOPE/DODMA mixture membranes almost did not change with an increase in DODMA concentration, as shown in Figure 6. It is well known that DODMA that has a positive charge in neutral pH induces the conformational change of the head group of phosphatidylcholine [31]. For PE membrane in water, their head groups almost parallel to the membrane surface [32]. Therefore, it can be reasonably assumed that low DODMA concentration in the DPOPE membrane changes the conformation of the head groups of electrically neutral PE membranes. This may result from an electrostatic attraction between the positive charges of the amino groups (N^+) of DODMA and the negative charges of the phosphate groups (O^-) of the neighboring DPOPE. This conformational change increases the length of acyl chains of DPOPE neighboring DODMA, and the longer length than the average length of acyl chains of DPOPE produced by a little amount of DODMA fill the interstitial hydrocarbon region in the H_{II} phase, this decreases the packing energy of the acyl chains ($\Delta\mu_{\text{ch}}$) in the H_{II} phase. Therefore, at a critical concentration of DODMA, the L_α to H_{II} phase transition occurs.

From the analysis above, we can propose a model for the mechanism of an L_α to H_{II} phase transition induced by low pH in the DPOPE membrane. Similar to DODMA, the protons (H^+) can bind the negative charges of the phosphate groups (O^-) of DPOPE, and this electrostatic attractive interaction between protons and

the negative charges of the phosphate groups (O^-) of DPOPE induces the extension of acyl chains of a small amount of DPOPE. A very small percentage of PE lipids with longer acyl chains fills the interstitial hydrocarbon region in the H_{II} phase membrane. This decreases the packing energy of the acyl chains in the H_{II} phase, and thereby decreases $\Delta\mu_{ch}$ in the DPOPE membrane. Therefore, at the critical pH, an L _{α} to H_{II} phase transition in DPOPE membrane occurs.

In the presence of a high concentration of KCl, the binding of protons is much enhanced, because the high concentration of salt screens the positive surface charge of the membrane produced by the binding protons [33]. This can reasonably explain that the presence of 0.5 M KCl increases the critical pH for the L _{α} to H_{II} phase transition (Figure 3).

References

- Colotto, A., Martin, I., Ruyschaert, J.M., Sen, A., Hui, S.W. and Epand, R.P.: Structural study of the interaction between the SIV fusion peptide and model membranes, *Biochemistry* **35** (1996), 980–989.
- Colotto, A. and Epand, R.P.: Structural study of the relationship between the rate of membrane fusion and the ability of the fusion peptide of influenza virus to perturb bilayer, *Biochemistry* **36** (1997), 7644–7651.
- Rietveld, A.G., Koorengevel, M.C. and de Kruijff, B.: Non-bilayer lipids are required for efficient protein transport across the plasma membrane of *Escherichia coli*, *EMBO J.* **14** (1995), 5506–5513.
- Kuzmin, P.I., Zimmerberg, J., Chizmadzhev, Y.A. and Cohen, F.S.: A quantitative model for membrane fusion based on low energy intermediates, *Proc. Natl. Acad. Sci. USA* **98** (2001), 7235–7240.
- Koynova, R. and Caffrey, M.: Phases and phase transitions of the hydrated phosphatidylethanolamines, *Chem. Phys. Lipids* **69** (1994), 1–34.
- Toombes, G.E.S., Finnefrock, A.C., Tate, M.W. and Gruner, S.M.: Determination of L _{α} –H_{II} phase transition temperature for 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine, *Biophys. J.* **82** (2002), 2504–2510.
- Seddon, J.M., Cevc, G. and Marsh, D.: Calorimetric studies of the gel-fluid (L _{β} –L _{α}) and lamellar-inverted hexagonal (L _{α} –H_{II}) phase transitions in dialkyl- and diacylphosphatidylethanolamines, *Biochemistry* **22** (1983), 1280–1289.
- Koynova, R.D., Tenchov, B.G. and Quinn, P.J.: Sugars favour formation of hexagonal (H_{II}) phase at the expense of lamellar liquid-crystalline phase in hydrated phosphatidylethanolamines, *Biochim. Biophys. Acta* **980** (1989), 377–380.
- Kirk, G.L. and Gruner, S.M.: Lyotropic effects of alkanes and headgroup composition on the L _{α} –H_{II} lipid liquid crystal phase transition: Hydrocarbon packing versus intrinsic curvature, *J. Physique (Paris)* **46** (1985), 761–769.
- Veiro, J.A., Khalifah, R.G. and Rowe, E.S.: The polymorphic phase behavior of dielaidoylphosphatidylethanolamine. Effect of *n*-alkanols, *Biochim. Biophys. Acta* **979** (1989), 251–256.
- Furuike, S., Levadny, V.G., Li, S.J., and Yamazaki, M.: Low pH induces an interdigitated gel to bilayer gel phase transition in dihexadecylphosphatidylcholine membrane, *Biophys. J.* **77** (1999), 2015–2023.
- Träuble, H., Teubner, M., Woolley, P. and Eibl, H.: Electrostatic interactions at charged lipid membranes. 1. Effects of pH and univalent cations on membrane structure, *Biophys. Chem.* **4** (1976), 319–342.

13. Jähnig, F., Harlos, K., Vogel, H. and H. Eible.: Electrostatic interactions at charged lipid membranes. Electrostatically induced tilt, *Biochemistry* **18** (1979), 1459–1468.
14. Cevc, G., Watts, A. and Marsh, D.: Titration of the phase transition of phosphatidylserine bilayer membranes. Effects of pH, surface electrostatics, ion binding, and head group hydration, *Biochemistry* **20** (1981), 4955–4965.
15. Watts, A., Harlos, K. and Marsh, D.: Charged-induced tilt in ordered-phase phosphatidylglycerol bilayers. Evidence from X-ray diffraction, *Biochim. Biophys. Acta* **645** (1981), 91–96.
16. Li, X., Schick, M.: Theory of lipid polymorphism: application to phosphatidylethanolamine and phosphatidylserine, *Biophys. J.* **78** (2000), 34–46.
17. Nayar, R., Schmid, S.L., Hope, M.J. and Cullis, P.R.: Structural preferences of phosphatidylinositol and phosphatidylinositol-phosphatidylethanolamine model membranes. Influence of Ca^{2+} and Mg^{2+} , *Biochim. Biophys. Acta* **688** (1982), 169–176.
18. Kinoshita, K., Li, S.J. and Yamazaki, M.: The mechanism of the stabilization of the hexagonal II (H_{II}) phase in phosphatidylethanolamine membranes in the presence of low concentrations of dimethyl sulfoxide, *Eur. Biophys. J.* **30** (2001), 207–220.
19. Li, S.J., Yamashita, Y. and Yamazaki, M.: Effect of electrostatic interactions on phase stability of cubic phases of membranes of monoolein/dioleoylphosphatidic acid mixtures, *Biophys. J.* **81** (2001), 983–993.
20. Glatter, O. and Kratky, O.: *Small Angle X-ray Scattering*, Academic Press, San Diego, CA 1982.
21. Helfrich, W.: Elastic properties of lipid bilayers: Theory and possible experiments, *Z. Naturforsch* **28c** (1973), 693–703.
22. Gruner, S.M.: Intrinsic curvature hypothesis for biomembrane lipid composition: a role for non-bilayer lipids, *Proc. Natl. Acad. Sci. USA* **82** (1985), 3665–3669.
23. Rand, R.P., Fuller, N.L., Gruner, S.M. and Parsegian, V.A.: Membrane curvature, lipid segregation, and structural transition for phospholipids under dual-solvent stress, *Biochemistry* **29** (1990), 76–87.
24. Chen, Z. and Rand, R.P.: Comparative study of the effects of several *n*-alkanes on phospholipid hexagonal phases, *Biophys. J.* **74** (1998), 944–952.
25. Gruner, S.M.: Stability of lyotropic phases with curved interfaces, *J. Phys. Chem.* **93** (1989), 7562–7570.
26. Turner, D.C., Wanf, Z.-G., Gruner, S.M., Mannock, D.A. and McElhaney, R.M.: Structural study of the inverted cubic phases of di-dodecyl alkyl β -D-glucopyranosyl-*rac*-glycerol, *J. Phys. II France* **2** (1992), 2039–2063.
27. Templar, R.H., Seddon, J.M. and Warrender, N.A.: Measuring the elastic parameters for inverse bicontinuous cubic phases, *Biophys. Chem.* **49** (1994), 1–12.
28. Andersson, S., Hyde, S.T., Larsson, K. and Lidin, S.: Minimal surfaces and structures: From inorganic and metal crystals to cell membranes and biopolymers, *Chem. Rev.* **88** (1988), 221–242.
29. Kirk, G.L., Gruner, S.M. and Stein, D.L.: A thermodynamic model of the lamellar to inverse hexagonal phase transition of lipid membrane–water systems, *Biochemistry* **23** (1984), 1093–1102.
30. Marsh, D.: Intrinsic curvature in normal and inverted lipid structures and in membranes, *Biophys. J.* **70** (1996), 2248–2255.
31. Seelig, J., Macdonald, P.M. and Scherer, P.G.: Phospholipid head groups as sensors of electric charge in membranes, *Biochemistry* **26** (1987), 7535–7541.
32. Brown, M.F. and Seelig, J.: Influence of cholesterol on the polar region of phosphatidylcholine and phosphatidylethanolamine bilayers, *Biochemistry* **17** (1978), 381–384.
33. Akutsu, H. and Seelig, J.: Interaction of metal ions with phosphatidylcholine bilayer membranes, *Biochemistry* **20** (1981), 7366–7373.