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# Low concentration of dioleoylphosphatidic acid induces an inverted hexagonal ($H_{II}$) phase transition in dipalmitoleoylphosphatidylethanolamine membranes

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**Abstract**

We have investigated the effects of anionic dioleoylphosphatidic acid (DOPA) on the structure and phase behavior of dipalmitoleoylphosphatidylethanolamine (DPOPE) membranes by small-angle X-ray scattering. The results of X-ray diffraction experiments indicate that an $L_\alpha$ to $H_{II}$ phase transition in DPOPE membranes occurred at 2.5 mol% DOPA, and above 4.0 mol% they were completely in the $H_{II}$ phase. And in the presence of 0.5 M KCl, the critical concentration of DOPA was decreased to 0.6 mol%. These results show that low concentrations of DOPA stabilize the $H_{II}$ phase rather than the $L_\alpha$ phase in DPOPE membranes. The absolute spontaneous curvature of DPOPE membrane was gradually decreased with an increase in DOPA concentrations. On the basis of these results, the $H_{II}$ phase stability in DPOPE membranes due to low DOPA concentrations is discussed by the spontaneous curvature of monolayer membrane, the packing energy of alkyl chains of the membrane and lipid packing parameter.


*Keywords:* Liquid-crystalline phase; Hexagonal II phase; Phase transition; Spontaneous curvature; Curvature energy; Packing energy of alkyl chains

## 1. Introduction

Biological membranes containing complex mixture of lipids investigated up to date adopt exclusively the liquid-crystalline lamellar phase under the physiological conditions. However, some isolated lipids from biological membranes have strong tendencies to form the nonbilayer structures in water such as inverted hexagonal ($H_{II}$) phase and cubic phases. Recently, the $H_{II}$ phase has been attracted a great deal of attention in both biological and physicochemical aspects. Because this structure has been postulated to play an important biological role in membrane fusion and a control of functions of membrane proteins [1–5]. The structures and phase stability of the $H_{II}$ phase of various kinds of phosphatidylethanolamine (PE) have been extensively studied [6–9]. As temperature increases, they undergo a gel ($L_\beta$) to $L_\alpha$ phase

*Abbreviations:* DOPA, dioleoylphosphatidic acid; DPOPE, dipalmitoleoylphosphatidylethanolamine; SAXS, small-angle X-ray scattering; $H_0$, spontaneous curvature

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transition, and after that an $L_\alpha$ to $H_{II}$ phase transition. Several substances have been shown to stabilize the $H_{II}$ phase in PE membranes such as sucrose and trehalose [10] and alkanes [11], which decrease the temperature of the $L_\alpha$ to $H_{II}$ phase transition. On the contrary, short-chain alcohols such as methanol increase the $L_\alpha$ to $H_{II}$ phase transition temperature, and stabilize the $L_\alpha$ phase in PE membranes [12].

On the other hand, the effects of anionic phospholipids, such as phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA), on the phase behaviors of PE membranes have also been investigated. Cullis and coworkers showed that PI favorably stabilizes the lipidic structure at its low (<10 mol%) concentrations and the bilayer structure at its higher levels in soya PE membrane employing $^{31}$P-NMR and freeze-fracture techniques [13]. Janes et al. [14,15] found that PS induces the increase of the temperature of the $L_\alpha$ to $H_{II}$ phase transition in palmitoyloleoylphosphatidylethanolamine (POPE) membranes from $^{31}$P-NMR spectra; whereas, PA induces a small reduction in the temperature of the $L_\alpha$ to $H_{II}$ phase transition despite the negative charge of the PA. More recently, the electrostatic control of phospholipid polymorphism has been investigated by $^{31}$P-NMR, freeze-fracture techniques, X-ray diffraction and differential scanning calorimetry. Studies showed that mixture of several cationic lipids with zwitterionic phospholipids such as strongly lamellar-preferring phosphatidylcholine and strongly nonlamellar-preferring PE form only the liquid-crystalline phase [16]. However, when mixed these cationic lipids with anionic lipids, such as PS, phosphatidylglycerol (PG), PA, a marked enhancement of the stability of nonbilayer structures occurs at the approach of the neutrality of the mean surface charge of the membrane surface. Furthermore, the nonbilayer phases of the mixture membranes can be directly regulated by changing the ratios of positive and negative charge in the membrane surface [16–18]. And the formation of the $H_{II}$ phase is more sensitive to the surface charge effect [17,18].

PA has been reported to remarkably enhance the activity of diacylglycerolgalactosyltransferase at less than 5 mol% in the chloroplast thylakoid membrane, but not PG [19]. It may imply that PA has an important role on the activity of some proteins. Our previous studies showed that dioleoylphosphatidic acid (DOPA) induces a $Q^{224}$ to $Q^{229}$ and then to $L_\alpha$ phase transitions with an increase in DOPA concentration in the monoolein membranes, due to the electrostatic repulsive interactions in head groups [20]. However, the effects of PA on the structures and the phase stability of the $H_{II}$ phase in PE membrane are not well understood. In this report, we have investigated effects of PA on the phase behaviors and the phase stability of PE membranes by small-angle X-ray scattering (SAXS). As the PE membrane, we used dipalmitoleoylphosphatidylethanolamine (DPOPE) (C 16:1) membrane, which is known to be in the $L_\alpha$ phase in excess water at 20 °C [21]. We found that an $L_\alpha$ to $H_{II}$ phase transition occurred in this membrane at very low concentrations of DOPA (C 18:1). To elucidate the mechanism of the $H_{II}$ phase stability in the DPOPE membrane due to low DOPA concentrations, we also measured the structures of the DPOPE/DOPA membranes containing 16% tetradecane to gain the information of the spontaneous curvature of DPOPE/DOPA mixture membranes. On the basis of these results and our previous results [22], we have discussed the mechanism of the $H_{II}$ phase stability of the DPOPE membranes containing low concentrations of DOPA.

## 2. Materials and methods

1,2-Dipalmitoleoyl-*sn*-glycero-3-phosphatidylethanolamine (DPOPE) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid (DOPA) sodium salt were purchased from Avanti Polar Lipids. Tetradecane was purchased from Sigma Chemical Co. (St. Louis, MO). They were used without further purification. Lipid dispersions were prepared as follows: appropriate amounts of phospholipids dissolved in chloroform were dried by nitrogen stream, and then under vacuum by a rotary pump for more than 12 h. An appropriate amount of 10 mM PIPES buffer (pH 7.0) containing 0 or 0.5 M concentration of KCl was added to these dry lipids in excess water condition (final concentration of lipids was 50 mM), and the suspensions were

vortexed for approximately 30 s at room temperature (~25 °C) several times. For measurements of X-ray diffraction, pellets of the lipid suspensions after centrifugation (13 000×*g*, 30 min at 20 °C; Tomy, MR-150) were used.

To gain the information of the spontaneous curvature of DPOPE monolayer membranes containing various amounts of DOPA, we used almost the same method of Chen and Rand [23,24]. The appropriate amount of mixture lipids in chloroform was dried by nitrogen stream, and then under vacuum by rotary pump for more than 12 h. Sixteen weight percentage of tetradecane was added to the dry lipids. After 48 h incubation for equilibration, the appropriate amount of 10 mM PIPES buffer (pH 7.0) was added to this lipids/tetradecane mixture in excess solvent, and the suspension was vortexed for approximately 30 s several times. Then, it was incubated for another 48 h for equilibration. For measurement of X-ray diffraction, the precipitation of the suspensions without centrifugation was used.

X-ray diffraction experiments were performed by using Nickel filtered Cu Kα 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 (1D) position sensitive proportional counter (Rigaku, PSPC-5) [25] with camera length of 350 mm and associated with electronics (multichannel 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 thermostable holder whose stability was ±0.2 °C [26].

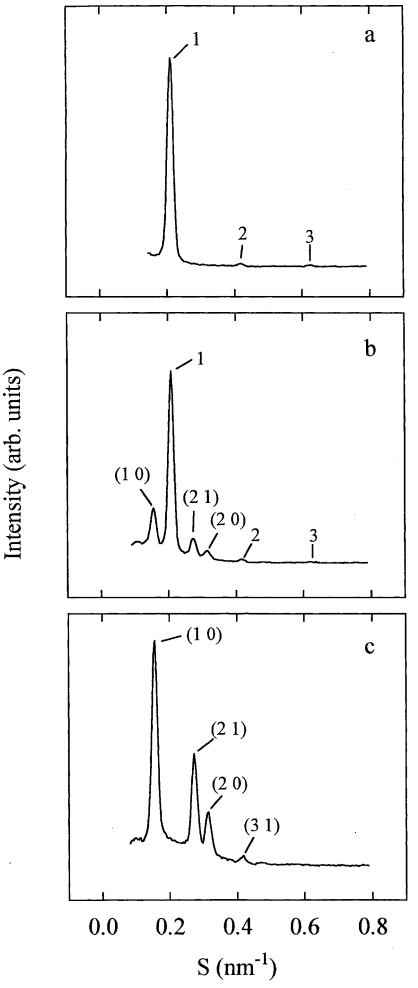


Fig. 1. X-ray diffraction profiles of DPOPE membranes containing various DOPA concentrations in 10 mM PIPES buffer (pH 7.0) at 20 °C. (a) 0 mol% DOPA; (b) 3 mol% DOPA and (c) 5 mol% DOPA.

## 3. Results

The DPOPE membrane is known to be in the liquid-crystalline phase ($L_\alpha$ phase) at 20 °C [21]. The SAXS data of the DPOPE membrane in 10 mM PIPES buffer (pH 7.0) at 20 °C showed that a set of SAXS peaks had spacings (lamellar repeat period) in the ratio of 1:2:3:…, and the spacing was 4.9 nm (Fig. 1a). At 2.5 mol% DOPA, a new set with spacings in the ratio of 1:√3:2:√7:…, which is consistent with a 2D hexagonal ($H_{II}$) phase, appeared (Fig. 1b). Above 4.0 mol% DOPA, the $L_\alpha$ peaks disappeared, and this membrane was completely in the $H_{II}$ phase (Fig. 1c). The basis vector length of the $H_{II}$ phase (center to center distance of adjacent cylinders), *d*, calculated by $d=(2/\sqrt{3})x$ (*x* is the spacing in the SAXS), was almost constant ($d=7.3$ nm) from 2.5 to 8.0 mol% DOPA (Fig. 2). Above 8.0 mol%, *d* gradually increased with an increase in DOPA concentrations, and above 10 mol% ($d=7.5$ nm) the peaks in SAXS pattern became broad, and thereby it is difficult to determine it due to the

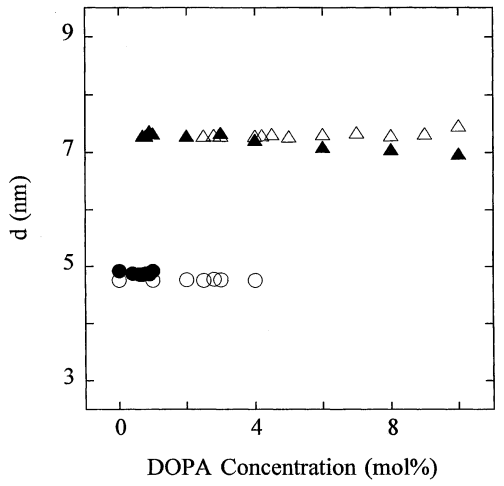


Fig. 2. Spacing or basis vector length, *d*, of DPOPE membranes containing various DOPA concentrations at 20 °C. ○ and △, in 10 mM PIPES buffer (pH 7.0); ● and ▲, in 10 mM PIPES buffer (pH 7.0) containing 0.5 M KCl. △ and ▲ show membrane in $H_{II}$ phase, ○ and ● in $L_\alpha$ phase.

electrostatic repulsive interactions in the membrane interface [20].

To consider the effects of ionic strength in the bulk phase on the structures and the phase transitions of DPOPE/DOPA mixture membranes, we have investigated effect of the presence of 0.5 M KCl in the bulk phase on these mixture membranes (Fig. 2). In the presence of 0.5 M KCl in 10 mM PIPES buffer (pH 7.0), at 0.6 mol% DOPA, a new set of SAXS peaks, which had spacings in the ratio of $1:\sqrt{3}:2:\sqrt{7}:\ldots$, was superimposed on the $L_\alpha$ peaks, showed that the $H_{II}$ phase appeared in DPOPE membranes. And above 1.0 mol%, the $L_\alpha$ peaks disappeared, and the DPOPE membranes were completely in the $H_{II}$ phase. The basis length of the $H_{II}$ phase, *d*, almost did not change until 3.0 mol% DOPA, which was 7.3 nm. And from 3.0 to 10 mol% DOPA, the *d* gradually decreased from 7.3 to 7.0 nm. The critical concentration of DOPA at which the $L_\alpha$ to $H_{II}$ phase transition occurs in presence of 0.5 M KCl is lower than that in the absence of KCl (Fig. 2).

To allow lipid membrane in the $H_{II}$ phase to express the spontaneous curvature, $H_0$, 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 [24,27,28]. To get the information of the dependence of the spontaneous curvature of the DPOPE monolayer membranes on DOPA concentrations, we added 16 wt.% tetradecane into DPOPE/DOPA membranes. In this condition, all DPOPE/DOPA mixture membranes were in the $H_{II}$ phase. As shown in Fig. 3, the basis vector length, *d*, of the DPOPE/DOPA/tetradecane membranes in 10 mM PIPES buffer (pH 7.0) in excess water condition almost did not change until 4.0 mol% DOPA ($d=7.8$ nm), and then gradually increased from 7.8 to 8.4 nm, with an increase in DOPA concentration from 4.0 to 10 mol%.

## 4. Discussion

The results of X-ray diffraction experiments in Fig. 2 clearly show that low DOPA concentration stabilizes the $H_{II}$ phase rather than the $L_\alpha$ phase in DPOPE membranes, and that the presence of 0.5 M KCl decreases the critical concentration of DOPA for an $L_\alpha$ to $H_{II}$ phase transition. To elucidate the mechanism, we consider the chemical potential of the phospholipid membrane in the

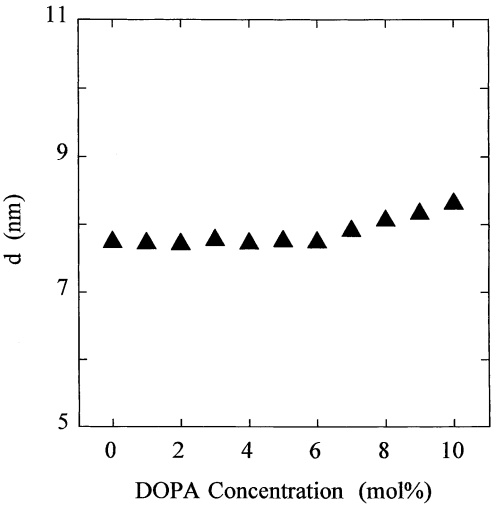


Fig. 3. Dependence of basis vector length, *d*, of DPOPE/DOPA/tetradecane membranes on various DOPA concentrations in 10 mM PIPES buffer (pH 7.0) at 20 °C. All membranes were in $H_{II}$ phase.

$H_{II}$ phase ($\mu^{H_{II}}$) and the bilayer liquid-crystalline ($L_\alpha$) phase ($\mu^{bil}$).

The difference of the chemical potential of the phospholipid membrane in the $H_{II}$ phase and in the $L_\alpha$ phase, $\Delta\mu$, can be expressed as follows [29,30]:

$$\Delta\mu = \mu^{H_{II}} - \mu^{bil} = (\mu^{H_{II}}_{curv} - \mu^{bil}_{curv}) + (\mu^{H_{II}}_{ch} - \mu^{bil}_{ch}) = \Delta\mu_{curv} + \Delta\mu_{ch} \quad (1)$$

where $\Delta\mu_{curv}$ is a term due to the curvature elastic energy of the membrane and $\Delta\mu_{ch}$ is a term due to the energy of interstitial chain packing of the membranes. In general, the curvature elastic energy of membrane, $\mu_{curv}$, in Eq. (1) can be expressed as [30,31]

$$\mu_{curv} = 2\kappa\langle H - H_0\rangle^2 + \kappa_G\langle K\rangle \quad (2)$$

where $\kappa$ is the elastic bending modulus, $K$ is the Gaussian curvature, $\kappa_G$ is the Gaussian curvature modulus and $\langle\ \rangle$ means the average value, $H$ is the curvature of membrane. $H_0$ is the spontaneous (intrinsic) curvature of a single monolayer membrane [8,27,30]. In excess water condition, the membrane in the $H_{II}$ phase has a curvature that is close to the spontaneous curvature to minimize the curvature energy, thereby, $H \approx H_0$. In hexagonal phase, $K=0$, and in bilayer phase, $H=K=0$, since we can deduce that

$$\Delta\mu_{curv} \approx -2\kappa H_0^2\ (<0) \quad (3)$$

Eq. (3) indicates that $\Delta\mu_{curv}$ is always negative ($\Delta\mu_{curv}<0$), and therefore, is an important factor stabilizing the $H_{II}$ phase. In contrast, in the $H_{II}$ phase, alkyl chains of lipids have to fill the interstitial hydrocarbon region, which reduces the entropy of chains, and thereby, the free energy of the membrane increases [29]. Therefore, this packing energy of the alkyl chains unstabilizes the $H_{II}$ phase, and thus $\Delta\mu_{ch}$ is always positive ($\Delta\mu_{ch}>0$). The $L_\alpha$ to $H_{II}$ phase transition is determined by the interplay of these two factors, $\Delta\mu_{curv}$ and $\Delta\mu_{ch}$.

To take the information of the spontaneous curvature of lipid membrane, the addition of alkanes such as decane and tetradecane to the membrane is required. Because alkanes fill the interstitial region of the $H_{II}$ phase and relax the alkyl chain packing stress [24,27,28]. Under this condition, the curvature of the monolayer membrane in the $H_{II}$ phase is very close to the spontaneous curvature. On the other hand, in the $H_{II}$ phase, the basis vector length, $d$, can be expressed as the sum of the radius of the water tube, $R_w$, and the thickness of the monolayer membrane, $d_1$, i.e. $d=2(R_w+d_1)$ [27,32]. In excess water condition, DPOPE membrane containing 16 wt.% tetradecane has a curvature of $H_0$ to minimize the curvature free energy, i.e. $R_w=R_0$, where $R_0$ is the radius of the spontaneous curvature ($H_0$). Because $d_1$ is assumed to be constant, the change in $d$ in the $H_{II}$ phase is attributed to the change in $R_w$. Therefore, the results in Fig. 3 indicate that the absolute spontaneous curvature of the DPOPE/DOPA monolayer membranes almost does not change below 4.0 mol% DOPA, and then gradually decreased with an increase in DOPA concentration. According to Eq. (3), the curvature free energy of DPOPE membrane was increased with an increase in DOPA concentration, and thereby, the effect of DOPA on the $\Delta\mu_{curv}$ unfavorably stabilizes the $H_{II}$ phase in DPOPE membranes.

As described above, the longer alkyl chains of DOPA (C18) than that of DPOPE (C16) can fill the interstitial hydrocarbon region, and then decreases the packing energy of the alkyl chains of the DPOPE membrane in the $H_{II}$ phase. The results in our previous paper [22] showed that DOPE induces an $L_\alpha$ to $H_{II}$ phase transition in DPOPE membrane which occurred at 12 mol% concentration in 10 mM PIPES buffer (pH 7.4) at 20 °C, between 12 and 26 mol%, the $L_\alpha$ phase and $H_{II}$ phase are of coexistence, and above 26 mol% the DPOPE membrane is completely in the $H_{II}$ phase. And the spontaneous curvature of DPOPE/DOPE membrane almost does not change with an increase in DOPE concentrations. These results clearly indicate that the longer alkyl chains of DOPA than that of DPOPE are not of dominant factor to induce an $L_\alpha$ to $H_{II}$ phase transition in DPOPE membranes at low DOPA concentrations, despite of it favorably stabilizes the $H_{II}$ phase in DPOPE membranes. What is the mechanism of an

$L_\alpha$ to $H_{II}$ phase transition in DPOPE membranes induced by low DOPA concentrations?

Geometric packing properties have been considered an important factor for polymorphism and structures of phospholipid membranes [8,33,34]. Israelachvili has determined the structure of phospholipid aggregates in terms of a critical packing parameter ($v/a_0 l_c$) of the alkyl chains of the phospholipids, where $v$ represents the volume of the alkyl chains per molecule, $l_c$ the critical chain length, and $a_0$ the optimal surface area per molecule at the hydrocarbon/water interface, where 'optimal' denotes that the area corresponds to the local free energy minimum. When $v/a_0 l_c \approx 1$, lamellar structures such as $L_\alpha$ and $L_\beta$ phases are formed, whereas inverted curved structures such as $H_{II}$ phase and inverted micelle while $v/a_0 l_c > 1$. Marsh [8] proposed a new geometric packing parameter ($V/Al$) of the whole phospholipid molecule, where $V$ represents the volume of the entire lipid molecular, $l$ its length and $A$ is the area of the lipid group at the lipid–water interface. This theory showed that the spontaneous (intrinsic) radius of curvature of lipid monolayer can be expressed by this new packing parameter, and a decrease in packing parameter may promote the $L_\alpha$–$H_{II}$ phase transition temperature. Janes et al. [14,15] found that introduction of 5 mol% DOPA into 95 mol% POPE small decreases the temperature of $L_\alpha$–$H_{II}$ phase transition in POPE membrane from $^{31}$P-NMR spectra, and explained this result by the theory of Israelachvili's lipid packing parameter, i.e. DOPA with a small head group decreases the average optimal surface area, $a_0$, and increases the packing parameter. Cullis et al. [18] also found that the mixture of DOPA with a cationic lipid forms the inverted nonbilayer structures when the surface charge of the mixture membrane approaches zero by regulating pH. Lewis and McElhaney [16] showed that palmitoyloleoylphosphatidic acid mixed with dioleoyloxytrimethylaminopropane (molar ratio of 1:1) stabilizes the $H_{II}$ phase in the mixture membrane. They have concluded that the tendency of several anionic phospholipids to form nonbilayer phases decreases in the order PA > CL (cardiolipin) > PG > PS. This order is also the order of increasing phospholipid head group size.

In our case, Fig. 3 clearly showed that the radius of spontaneous curvature increases with an increase in DOPA concentrations in DPOPE membranes. According to the theory of Marsh's lipid packing parameter, we can reasonably assume that the introduction of DOPA into DPOPE membrane decreases the critical packing parameter resulting from the increase of the average optimal surface area at lipid–water surface, due to the electrostatic repulsive interaction between head groups. On the other hand, DOPA has a relatively small head group, and the inverted cone shape of alkyl chains, i.e. $v/a_0 l_c > 1$. When incorporation of DOPA into DPOPE membranes at its low concentrations, the alkyl chains of the inverted cone shape of DOPA can fill the interstitial hydrocarbon region in the $H_{II}$ phase membrane. This can decrease the packing energy of alkyl chains in the $H_{II}$ phase, and thereby decreases $\Delta\mu_{ch}$. At critical concentration of DOPA, an $L_\alpha$ to $H_{II}$ phase transition occurs. In brief, DOPA induced $L_\alpha$ to $H_{II}$ phase transition not by decreasing the elastic curvature energy of monolayer membrane, but by the change in the chain packing energy at the interstitial region of the $H_{II}$ phase. Addition of alkanes such as tetradecane to the DPOPE membranes induced an $L_\alpha$ to $H_{II}$ phase transition [21], indicating that the decrease in packing energy of alkyl chains in the interstitial region of the $H_{II}$ phase induced this transition. It also supports our explanation. The addition of anionic lipids such as PA and PG to PE bilayer membranes often induces the formation of an inverted cubic phase at their higher concentrations. In our case, at higher concentrations of DOPA, the inverted cubic phases may be formed. However, the peaks in SAXS pattern became broad at higher concentrations of DOPA, due to the electrostatic repulsive interactions between head groups, and we could not determine whether the inverted cubic phases occurred.

In the presence of a high concentration of KCl, the screening of the surface charges of the membranes decreases the electrostatic repulsive interactions between head groups of lipids. Hence, we can reasonably postulate that this leads to a decrease in the average optimal surface area, and then increases the packing parameter. As analyzed above, this should decrease the curvature elastic

energy of the membrane ($\Delta\mu_{curv}$). Therefore, the critical concentration of DOPA for an $L_\alpha$ to $H_{II}$ phase transition in the DPOPE membrane is decreased obviously.

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