Phase Diagrams and Ordering in Charged Membranes: Binary Mixtures of Charged and Neutral Lipids
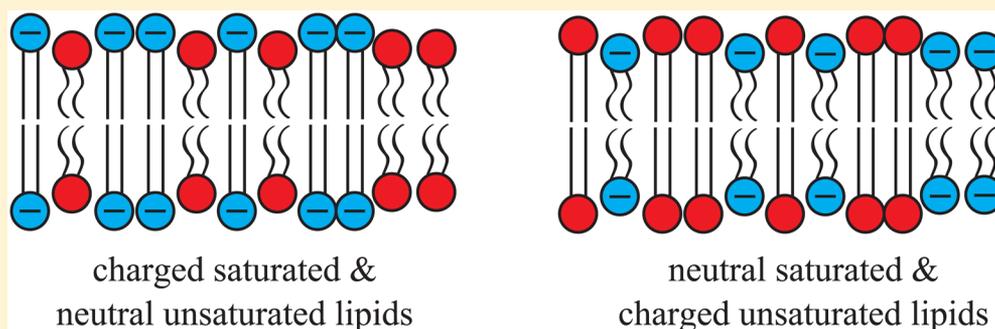
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charged saturated & neutral unsaturated lipids

neutral saturated & charged unsaturated lipids

ABSTRACT: We propose a model describing the phase behavior of two-component membranes consisting of binary mixtures of electrically charged and neutral lipids. We take into account the structural phase transition (main-transition) of the hydrocarbon chains, and investigate the interplay between this phase transition and the lateral phase separation. The presence of charged lipids significantly affects the phase behavior of the multicomponent membrane. Due to the conservation of lipid molecular volume, the main-transition temperature of charged lipids is lower than that of neutral ones. Furthermore, as compared with binary mixtures of neutral lipids, the membrane phase separation in binary mixtures of charged lipids is suppressed, in accord with recent experiments. We distinguish between two types of charged membranes: mixtures of charged saturated lipid/neutral unsaturated lipid and a second case of mixtures of neutral saturated lipid/charged unsaturated lipid. The corresponding phase behavior is calculated and shown to be very different. Finally, we discuss the effect of added salt on the phase separation and the temperature dependence of the lipid molecular area.

1. INTRODUCTION

Biomembranes are composed of various components such as phospholipids, sterols, and proteins, and are believed to have compositional heterogeneities. In multicomponent membranes, regions or domains enriched in saturated lipid and cholesterol are called "rafts", and are thought to play an important role in various biocellular functions, such as signal transduction and membrane trafficking.[1,2] Artificial bilayer membranes composed of several phospholipids and cholesterol have been used as typical model systems for biomembranes. Phase separation in such model membranes has been widely investigated in order to understand the mechanism of raft formation.[3] Considerable effort has been given to ternary systems composed of saturated lipid, unsaturated lipid, and cholesterol, where it is known that domains enriched in saturated lipid and cholesterol form a liquid-ordered phase in a matrix of an otherwise liquid-disordered phase.[4−10]

Two-component lipid bilayers consisting of saturated and unsaturated lipids also show a phase separation between solid-like (L_β) and liquid-like (L_α) phases. This phase separation can be directly visualized by fluorescence microscopy, which demonstrates that the solid-like L_β domains exhibit various anisotropic shapes.[11−14] The resulting phase diagrams of different binary mixtures have been experimentally explored in great detail,[14−18] and have been quantitatively reproduced in several theoretical works.[19−23] In particular, in models proposed by some of the present authors,[19,20,22] a coupling between the membrane composition and its internal structure was suggested in order to consider the interplay between the phase separation and the chain phase transition (main-transition). In these experimental and theoretical studies, however, the lipids were taken to be electrically neutral.
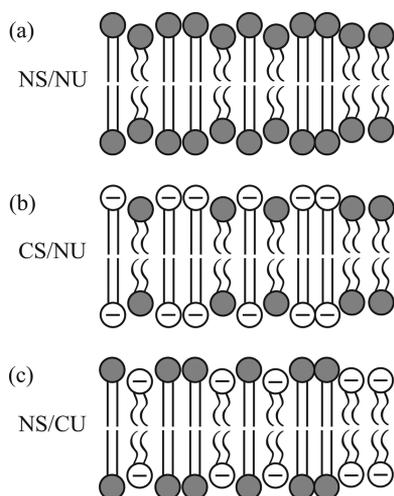
Figure 1. Schematic illustration of two-component membranes. (a) A neutral membrane consisting of a binary mixture of neutral saturated (NS) and neutral unsaturated (NU) lipids. (b) A charged membrane consisting of a binary mixture of charged saturated (CS) and neutral unsaturated (NU) lipids. (c) A charged membrane consisting of a binary mixture of neutral saturated (NS) and charged unsaturated (CU) lipids.

Since a certain amount of charged lipid is always present in organelles such as mitochondria and lysosomes,[24] electrostatic interactions are significant in biomembranes. Recently, several experiments have investigated the phase separation of multi-component lipid mixtures containing at least one type of charged phospholipid.[25−30] In binary mixtures of neutral saturated lipid and negatively charged unsaturated lipid, the phase-separated region was suppressed as compared with the case when both lipids are neutral. This phase-separated region reappears when salt (e.g., NaCl), which screens the electrostatic interactions, was added. This is a strong indication that the electrostatic interaction affects the phase behavior. Although such behavior has been qualitatively explained by phenomeno-logical models using the Poisson−Boltzmann (PB) theory,[26,31,32] the structural phase transition (the main-transition) of the membrane was not considered.

In membranes composed of binary mixtures of neutral and charged lipids, either the saturated or unsaturated lipids can be charged. Hence, it is convenient to define two types of charged membranes: (i) mixtures of charged saturated (CS) and neutral unsaturated (NU) lipids (denoted as CS/NU, see Figure 1b) and (ii) mixtures of neutral saturated (NS) and charged unsaturated (CU) lipids (denoted as NS/CU, see Figure 1c). In recent experiments by Himeno et al.,[29] it was reported that the phase separation was suppressed in the NS/CU lipid mixture as compared with the neutral membrane (NS/NU as in Figure 1a), while it was enhanced in the CS/NU case. This result indicates that the phase behavior of charged membranes is very different between these two types of charged mixtures.

Being motivated by this experimental work, we consider in this paper charged membranes, and propose a phenomeno-logical model that distinguishes between the above-mentioned two cases. In particular, we discuss the interplay between the main-transition and the lateral phase separation between the two lipid species. The main-transition of lipid chains is taken into account by extending the model of Goldstein and Leibler[33,34] to include electrostatic interactions using the PB theory.[35,36] Furthermore, we employ the constraint of constant

molecular volume of the lipids, which leads to a coupling between the electrostatic interactions and the lipid lateral phase separation. We calculate the different phase diagrams of charged membranes by changing the interaction between the two lipids and/or by changing the amount of added salt. We also explain the different combination of hydrocarbon saturation and headgroup charge (either CS/NU or NS/CU as were defined above). On the basis of the calculated phase diagrams, we discuss the main-transition temperature, and the effect of added salt in a binary mixture of charged/neutral lipids.

In the next section, we explain the model by Goldstein and Leibler, the phase separation, and the PB theory. In section 3, we show the calculated phase diagrams and investigate the effects of the interaction between the two lipids, salt concentration, and combination of the lipids (either CS/NU or NS/CU) on the phase separation. Moreover, we explore how the molecular area changes and its dependence on the added salt. Finally, we offer a qualitative discussion in section 4 and some concluding remarks in section 5.

2. MODEL

In order to calculate the phase diagrams of binary mixtures of charged lipid membranes, we consider a model that takes into account both the lipid main-transition and the lateral lipid phase separation. For simplicity, we consider a perfectly flat membrane ignoring any possible out-of-plane membrane undulations. To avoid further complexity, we do not consider any specific bilayer attributes such as asymmetric lipid composition between the two leaflets, or ionic strength difference between the electrolytes in contact with the two sides of the membrane. Therefore, our model describes the phase behavior of an isolated symmetric bilayer.

2.1. Two-Component Neutral Membranes. On the basis of the Goldstein−Leibler model,[33,34] we first review the free energy of a single-component neutral lipid membrane, which leads to the main-transition. The main-transition involving the chain ordering and stiffening is described via a scalar order parameter

$$m = \frac{\delta - \delta_0}{\delta_0} \tag{1}$$

where δ is the actual membrane thickness and δ_0 a reference membrane thickness in the L_α phase. Notice that m represents the changes that may occur in several degrees of freedom, including the conformation of the hydrocarbon chains, their interchain correlations, molecular tilt, and positional ordering. For an isolated lipid bilayer, a Landau expansion of the stretching free energy per lipid molecule in powers of the order parameter m is given by

$$f_{st}(m) = \frac{a_2'}{2}(T - T^*)m^2 + \frac{a_3}{3}m^3 + \frac{a_4}{4}m^4 \tag{2}$$

where $a_2' > 0$, $a_3 < 0$, and $a_4 > 0$ are phenomenological coefficients. Here a_3 is taken to be negative, since the main-transition is a first-order transition,[16] while a_4 is always positive to ensure thermodynamical stability. In the above, T is the temperature and T^* is a reference temperature. The main-transition temperature is different from T^* and is denoted by T^{**}. It can be calculated from the conditions $f_{st} = 0$ and $df_{st}/dm = 0$, yielding

$$T^{**} = T^* + \frac{2(a_3)^2}{9a_2'a_4} \tag{3}$$

Note that the main-transition temperature described by eq 3 is appropriate only for neutral lipids.

Next, we describe the phase separation in membranes containing a binary mixture of neutral saturated (NS) and neutral unsaturated (NU) lipids, as shown in Figure 1a. We model the two-component membrane as an incompressible NS/NU lipid mixture containing ϕ molar fraction of NS lipid and $1 - \phi$ molar fraction of NU lipid. (Throughout this paper, ϕ denotes the molar fraction of saturated lipid.) For simplicity, we assume the same area per molecule for the two species in the L_α phase and ignore any lipid exchange with the surrounding solvent. The mixing free energy (per lipid molecule) consists of the mixing entropy and enthalpy. It can be expressed within mean-field theory as

$$f_{\text{mix}}(\phi) = k_B T[\phi \ln \phi + (1 - \phi) \ln(1 - \phi)] + \frac{J}{2}\phi(1 - \phi) \tag{4}$$

where k_B is the Boltzmann constant and $J > 0$ is an attractive interaction parameter between the lipids, which enhances the NS/NU demixing.

In general, the two lipids have different main-transition temperatures originating from different molecular parameters such as their chain length, degree of saturation, and hydrophilic headgroup. The dependence of the main-transition temperature on the composition ϕ cannot be calculated from such a phenomenological approach. Therefore, we proceed by further assuming the simplest linear interpolation of T^* as a function of ϕ

$$T^*(\phi) = \phi T_S^* + (1 - \phi)T_U^* \tag{5}$$

where T_S^* and T_U^* are the reference temperatures of pure NS and NU lipids, respectively. In general, saturated lipids have higher transition temperatures than the unsaturated ones, $T_S^{**} > T_U^{**}$, for the cases when the numbers of hydrocarbons in the lipid tails are the same. Therefore, we generally use $T_S^* > T_U^*$.

By combining eqs 2 and 4, we obtain the total free energy of a neutral NS/NU membrane

$$f_{\text{NS/NU}}(m, \phi) = f_{\text{st}}(m, \phi) + f_{\text{mix}}(\phi) \tag{6}$$

This total free energy will be minimized with respect to both m and ϕ to obtain the phase behavior of a neutral membrane.

2.2. Electrostatic Free Energy of a Charged Membrane.

In what follows, we extend the above model for the case of membranes consisting of a binary mixture of charged and neutral lipids. In order to estimate the electrostatic interactions in membranes at a fixed surface charge density (chosen to be negative throughout this paper), $\sigma < 0$, we obtain the free energy of a charged membrane on the basis of the PB theory.[35,36]

As shown in Figure 2, we consider a flat charged lipid bilayer that lies at $z = 0$. The bilayer is in contact with a monovalent salt solution located at $z > 0$. We assume that the two leaflets constituting the bilayer do not interact with each other. Within a mean-field treatment, the electric potential $\Psi(z)$ satisfies the PB equation
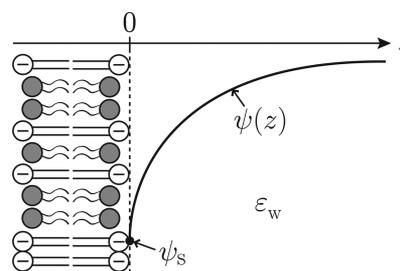
Figure 2. Schematic representation of the electrostatic potential of a two-component charged membrane consisting of charged saturated (CS) and neutral unsaturated (NU) lipids. The lipid head groups are located at the plane $z = 0$. The dimensionless electrostatic potential in solution is $\psi(z) = e\Psi/k_B T$, and $\psi_s = \psi(z = 0)$ is the surface potential at the membrane. The dielectric constant of water solution is ε_w.

$$\frac{d^2\Psi}{dz^2} = \frac{2en_b}{\varepsilon_w} \sinh \frac{e\Psi}{k_B T} \tag{7}$$

where e is the elementary charge, n_b the salt concentration in the bulk, and ε_w the dielectric constant of water. Using the dimensionless electric potential $\psi = e\Psi/k_B T$, we obtain the following dimensionless PB equation

$$\frac{d^2\psi}{dz^2} = \kappa^2 \sinh \psi \tag{8}$$

where $\kappa^{-1} = \sqrt{\varepsilon_w k_B T/2e^2 n_b}$ is the Debye screening length. Far from the membrane, the bulk electric potential should vanish, i.e., $\psi(z \to \infty) = 0$. With the use of the Gauss law, the boundary condition at the membrane ($z = 0$) is

$$\varepsilon_w \frac{d\psi}{dz}\bigg|_{z=0} = -\frac{e\sigma}{k_B T} \tag{9}$$

Under these boundary conditions, the dimensionless electric potential $\psi(z)$ can be obtained[35−37] by integrating twice eq 8

$$\psi(z) = 2 \ln \frac{1 + \Gamma \exp(-\kappa z)}{1 - \Gamma \exp(-\kappa z)} \tag{10}$$

where

$$\Gamma = \frac{\exp(\psi_s/2) - 1}{\exp(\psi_s/2) + 1} \tag{11}$$

Here $\psi_s = \psi(z = 0)$ is the dimensionless surface potential given by

$$\psi_s = 2 \sinh^{-1}\left(\frac{2\pi l_B \sigma}{e\kappa}\right) = -2 \sinh^{-1}\left(\frac{1}{b\kappa}\right) \tag{12}$$

where $l_B = e^2/4\pi\varepsilon_w k_B T$ is the Bjerrum length and $b = e/2\pi l_B|\sigma|$ is the Gouy−Chapman length.

The electrostatic free energy per lipid molecule can be calculated from the charging method[37,38]

$$f_{\text{el}}(\sigma) = \frac{k_B T \Sigma}{e} \int_0^\sigma d\sigma' \psi_s(\sigma') \tag{13}$$

where Σ is the cross-sectional area per lipid. By substituting the surface potential, eq 12, into eq 13, we obtain the expression

$$f_{el}(\sigma) = \frac{k_B T \Sigma}{\pi l_B b}\left[b\kappa - \sqrt{1+(b\kappa)^2} \right.$$
$$\left. + \ln \frac{1+\sqrt{1+(b\kappa)^2}}{b\kappa} \right] \tag{14}$$

2.3. Two-Component Charged Membranes. In the case of membranes consisting of a binary mixture of charged saturated (CS) and neutral unsaturated (NU) lipids, the surface charge density, $\sigma < 0$, is related to the CS molar fraction, ϕ, by

$$\sigma = -\frac{e\phi}{\Sigma} \tag{15}$$

As illustrated in Figure 3, we further assume that the lipid molecular volume, $v = \Sigma\delta$, is conserved and stays constant at any temperature. From eq 1, the cross-sectional area Σ is related to the order parameter, m, by

$$\Sigma = \frac{\Sigma_0}{1+m} \tag{16}$$

where Σ_0 is the cross-sectional area of a neutral lipid in the L_α phase. By substituting back eqs 15 and 16 into eq 14, f_{el} can be written as

$$f_{el}(m,\phi) = 2k_B T\phi\left[\frac{1-\sqrt{1+[p\phi(1+m)]^2}}{p\phi(1+m)} \right.$$
$$\left. + \ln\left(p\phi(1+m)+\sqrt{1+[p\phi(1+m)]^2}\right) \right] \tag{17}$$

where $p = 2\pi l_B/\Sigma_0\kappa$ is a dimensionless parameter.

The total free energy of the CS/NU membrane is finally given by

$$f_{CS/NU}(m,\phi) = f_{st}(m,\phi) + f_{mix}(\phi) + f_{el}(m,\phi) \tag{18}$$

On the other hand, when the membrane consists of neutral saturated (NS) and charged unsaturated (CU) lipids, the total free energy of an NS/CU membrane becomes

$$f_{NS/CU}(m,\phi) = f_{st}(m,\phi) + f_{mix}(\phi) + f_{el}(m,1-\phi) \tag{19}$$

Note that the only change between the two cases, eqs 18 and 19, is that $\phi \leftrightarrow 1-\phi$ in the electrostatic free energy. The phase behavior of two-component charged membranes in these two cases is analyzed using either eq 18 or 19.

2.4. Equilibrium Phase Behavior. Since the electrostatic free energy described by eq 17 depends on the order parameter m, the main-transition temperature of a pure charged lipid (either CS or CU) is different from eq 3. Instead, it is given by the following conditions

$$f_{st}(m_\beta, 1) + f_{el}(m_\beta, 1) = f_{st}(m_\alpha, 1) + f_{el}(m_\alpha, 1) \tag{20}$$

$$\left.\frac{\partial(f_{st}+f_{el})}{\partial m}\right|_{m=m_\beta} = \left.\frac{\partial(f_{st}+f_{el})}{\partial m}\right|_{m=m_\alpha} = 0 \tag{21}$$

For lipid mixtures, the chemical potential μ is given by
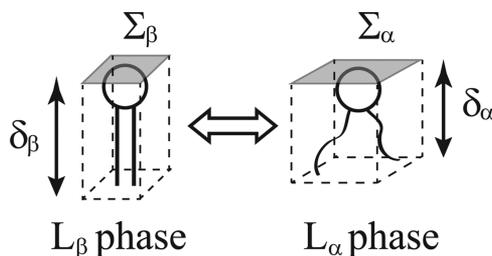
Figure 3. Schematic illustration of molecular volume conservation. The molecular area is indicated by a gray square, and the molecular volume is enclosed by the dashed lines. We assume that the molecular volume v is conserved, $v = \Sigma_\beta\delta_\beta = \Sigma_\alpha\delta_\alpha$, for all the temperatures. The larger the membrane thickness δ, the smaller the molecular area Σ.

$$\mu = \left.\frac{\partial f_{tot}}{\partial\phi}\right|_{m=m^*} \tag{22}$$

where f_{tot} is taken either from eq 6 for neutral membranes or eqs 18 and 19 for the two cases of charged membranes. The optimal order parameter, m^*, is obtained by minimizing the total free energy with respect to m, $\partial f_{tot}/\partial m|_{m=m^*} = 0$.

As the lipid molar fraction ϕ is a conserved quantity, it is convenient to introduce another thermodynamic potential given by

$$g(\phi) = f_{tot}(m=m^*, \phi) - \mu\phi \tag{23}$$

The two-phase coexistence region in the (T, ϕ) plane is calculated by using the common tangent construction to account for the constraint value of the molar fraction ϕ. In other words, the two-phase coexistence has to satisfy the conditions $g(\phi_1) = g(\phi_2)$ and $\mu(\phi_1) = \mu(\phi_2)$, where the slope of the common tangent is equal to the chemical potential. From the coexisting two points in the (T, ϕ) plane, we can also construct the phase diagram in the (T, μ) plane. The L_β phase is characterized by positive m values, while its value in the L_α phase becomes negative.
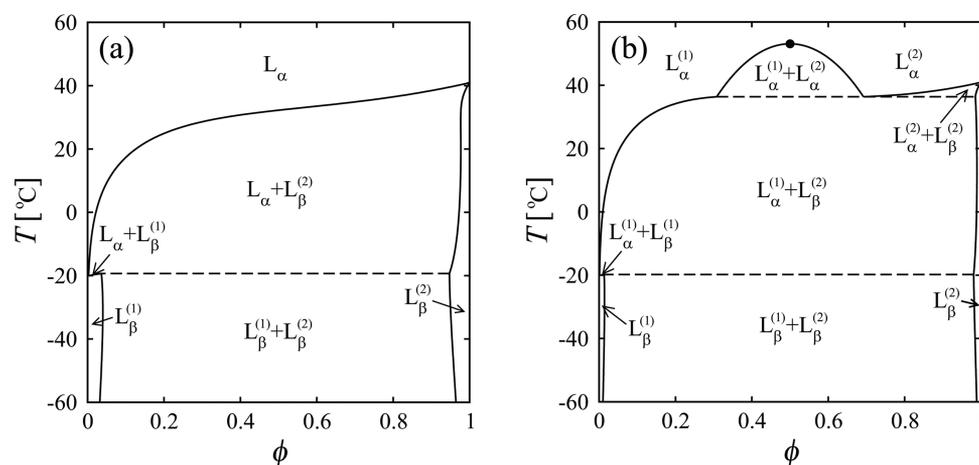
3. RESULTS

Having described our model in the previous section, we present the resulting phase diagrams and the temperature dependence of the molecular area. First, we show the phase diagrams for the neutral membrane case. This calculation essentially reproduces the theoretical results of refs 19 and 20. Then, the phase diagrams for charged membranes will be presented.

3.1. Choice of System Parameters. As examples for typical neutral saturated (NS) lipids and neutral unsaturated (NU) lipids, we consider, respectively, dipalmitoylphosphocholine (DPPC) and dioleoylphosphocholine (DOPC), whose main-transition temperature is $T_S^{**} = 41\,^\circ C$ and $T_U^{**} = -20\,^\circ C$, respectively. From eq 3, the reference temperature of NS and

Table 1. Parameter Values of the Model

parameter	value	unit
a_2'	2.4×10^{-21}	J/K (Ref 34)
a_3	-1.1×10^{-18}	J (Ref 34)
a_4	2.2×10^{-18}	J (Ref 34)
T_S^*	-10	°C
T_U^*	-71	°C
Σ_0	65	Å^2
ε_w	7.08×10^{-10}	F/m

Figure 4. Phase diagrams of a neutral NS/NU membrane as a function of the molar fraction, ϕ, of the NS lipids, and temperature, T, reproducing the theoretical results of refs 19 and 20. The attractive interaction parameter J is chosen to be 1.2×10^{-20} J in part a and 1.8×10^{-20} J in part b. The other parameters are listed in Table 1 and are mentioned in the text. The critical point located at $(\phi_c, T_c) = (0.5, 53\ °C)$ in part b is indicated by a filled circle. The horizontal dashed lines at $T = -19.2\ °C$ in part a and $T = -19.7\ °C$ and $T = 36.4\ °C$ in part b indicate a three-phase coexistence (triple point).
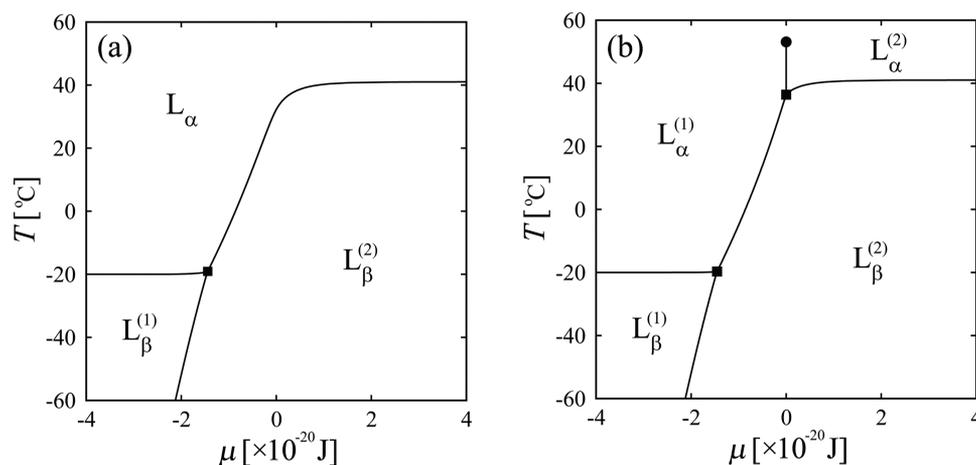
Figure 5. Phase diagrams of a neutral NS/NU membrane as a function of the chemical potential, μ, of the NS lipids and temperature T. The attractive interaction parameter J is chosen to be 1.2×10^{-20} J in part a and 1.8×10^{-20} J in part b. The other parameters are listed in Table 1 and mentioned in the text. The critical point located at $(\mu_c, T_c) = (0\ J, 53\ °C)$ in part b is indicated by a filled circle. The filled squares located at $(\mu, T) = (-1.44 \times 10^{-20}\ J, -19.2\ °C)$ in part a and $(-1.45 \times 10^{-20}\ J, -19.7\ °C)$ and $(0\ J, 36.4\ °C)$ in part b indicate the triple points.

NU lipids is set as $T_S^* = -10\ °C$ and $T_U^* = -71\ °C$, respectively. The molecular area of the neutral lipid in the L_α phase is fixed to $\Sigma_0 = 65\ Å^2$. The interaction parameter is $J = 1.2 \times 10^{-20}$ or 1.8×10^{-20} J as considered before,[19,20,22] and the bulk salt concentration is chosen either as $n_b = 1$ M or 10 mM. All the parameters and physical constants used in our calculations are summarized in Table 1.

3.2. Two-Component Neutral Membranes. For the NS/NU membrane, the total free energy is given in eq 6. As mentioned before, the NS lipid (whose molar fraction is ϕ) has a higher main-transition temperature than that of the NU lipid. Following the previous theoretical studies,[19,20] we reproduce in Figures 4 and 5 the phase diagrams of two-component NS/NU neutral membranes in the (T, ϕ) and (T, μ) planes, respectively. Figures 4a and 5a are calculated for $J = 1.2 \times 10^{-20}$ J, while Figures 4b and 5b are obtained for $J = 1.8 \times 10^{-20}$ J. The main-transition temperature of NS ($\phi = 1$) and NU ($\phi = 0$) lipids is $T_S^{**} = 41\ °C$ and $T_U^{**} = -20\ °C$, respectively. As shown in Figure 4a, we obtain three two-phase coexistence regions denoted by $L_\alpha + L_\beta^{(1)}$, $L_\alpha + L_\beta^{(2)}$, and $L_\beta^{(1)} +$

$L_\beta^{(2)}$, and in Figure 4b, there are five two-phase coexistence regions indicated by $L_\alpha^{(1)} + L_\alpha^{(2)}$, $L_\alpha^{(1)} + L_\beta^{(1)}$, $L_\alpha^{(1)} + L_\beta^{(2)}$, $L_\alpha^{(2)} + L_\beta^{(2)}$, and $L_\beta^{(1)} + L_\beta^{(2)}$. As discussed before,[19,20] the phase-separated region is more extended when the interaction parameter J is larger.

3.3. Two-Component Charged Membranes. Next, we calculate the phase diagrams for two-component charged membranes. We first consider the charged saturated/neutral unsaturated (CS/NU) (Figure 1b) lipid mixtures whose total free energy is given by eq 18. In Figures 6a and 7a, we show the calculated phase diagrams for $J = 1.2 \times 10^{-20}$ J and $n_b = 1$ M, recalling that ϕ is the molar fraction of the CS lipids. The main-transition temperature of the CS lipid is calculated numerically from eqs 20 and 21, yielding $T_S^{**} \simeq 29\ °C$. On the other hand, the main-transition temperature of the NU lipid is $T_U^{**} = -20\ °C$, as before. In this phase diagram, there is only one coexistence region between the L_α and L_β phases.

In Figures 6b and 7b, we obtain the phase diagrams for $J = 1.8 \times 10^{-20}$ J, while the other parameters are the same as those in Figures 6a and 7a. The main-transition temperatures of the
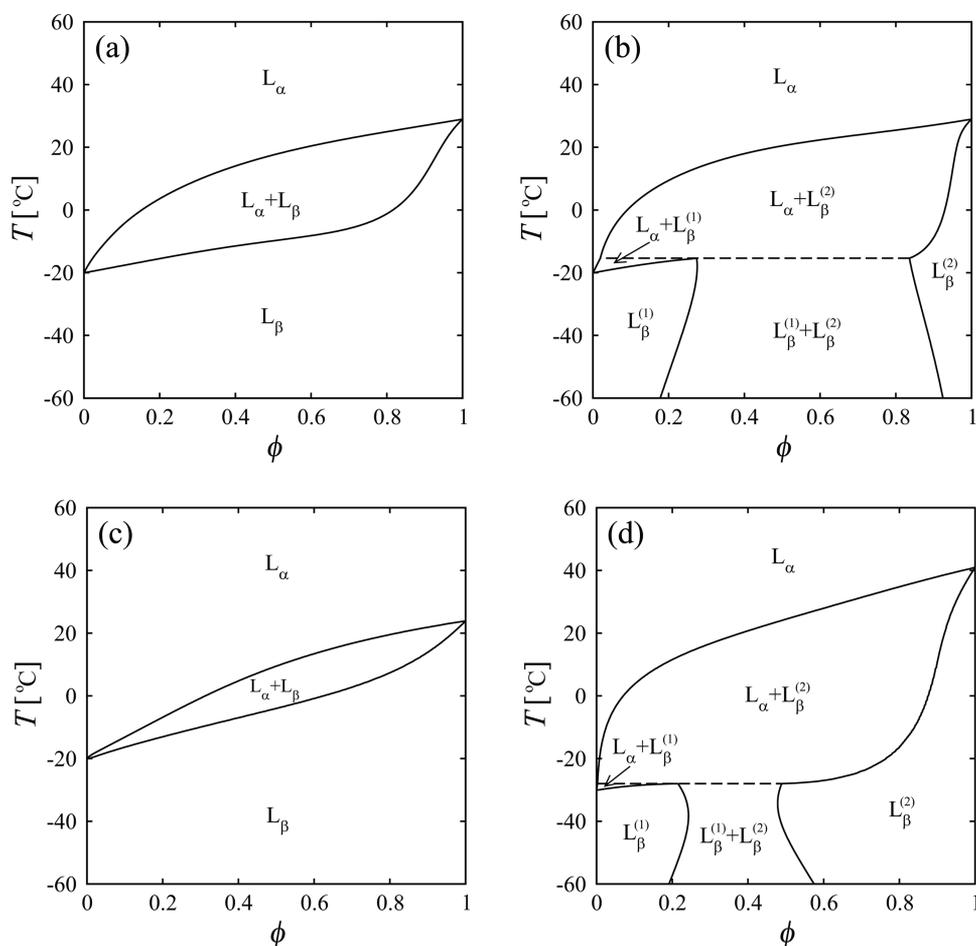
Figure 6. Phase diagrams of two-component charged membranes as a function of the molar fraction, ϕ, of saturated lipid (either CS or NS) and temperature T. (a) CS/NU membrane with $J = 1.2 \times 10^{-20}$ J and $n_b = 1$ M; (b) CS/NU membrane with $J = 1.8 \times 10^{-20}$ J and $n_b = 1$ M; (c) CS/NU membrane with $J = 1.2 \times 10^{-20}$ J and $n_b = 10$ mM; and (d) NS/CU membrane with $J = 1.2 \times 10^{-20}$ J and $n_b = 1$ M. The other parameters are listed in Table 1 and are mentioned in the text. The horizontal dashed lines located at $T = -15.3$ °C in part b and $T = -28.0$ °C in part d indicate the three-phase coexistence (triple point).

two lipids are unchanged, because the conditions in eqs 20 and 21 are independent of the interaction parameter J. In this case, there are three two-phase coexistence regions denoted by $L_\alpha + L_\beta^{(1)}$, $L_\alpha + L_\beta^{(2)}$, and $L_\beta^{(1)} + L_\beta^{(2)}$.

In order to further explore the effect of electrostatic interaction in CS/NU mixtures, we looked at the case of lower salinity. Figures 6c and 7c are the phase diagrams corresponding to the low salt case of $n_b = 10$ mM. The other parameters are the same as those in Figures 6a and 7a. The main-transition temperature of the CS lipid is now $T_S^{**} \simeq 23$ °C, while that of the NU lipid is unchanged ($T_U^{**} = -20$ °C). In this case, there is only one two-phase coexistence region between the L_α and L_β phases.

Finally, we show in Figures 6d and 7d the phase diagrams for the neutral saturated/charged unsaturated (NS/CU) lipid mixtures whose total free energy is given by eq 19. Notice that the molar fraction ϕ refers here to that of the NS lipids. The other parameters, J and n_b, are the same as those in Figures 6a and 7a. The main-transition temperature of the NS lipid is $T_S^{**} = 41$ °C, and that of the CU lipid is decreased to $T_U^{**} \simeq -30$ °C. There are three two-phase coexistence regions denoted by $L_\alpha + L_\beta^{(1)}$, $L_\alpha + L_\beta^{(2)}$, and $L_\beta^{(1)} + L_\beta^{(2)}$.

The phase behavior of two-component membranes depends on various quantities such as the interaction parameter J, the salt concentration n_b, and whether the lipid is charged or

neutral. Due to the lipid volume conservation, the main-transition temperature of the charged lipid (either CS or CU) is reduced as compared with that of the neutral counterpart. These findings will be further discussed in section 4.

3.4. Molecular Area in Charged Membranes. In Figure 8, we show the temperature dependence of the molecular area, Σ, which is calculated from the optimal order parameter, m^*, and eq 16. For the CS/NU mixture, the molar fraction ϕ of the CS lipid is varied, and the salt concentration is either $n_b = 1$ M (high salt) or 10 mM (low salt), as shown in Figure 8a and b, respectively. In Figure 8c, we show the molecular area of the NS/CU mixture for $n_b = 1$ M. Since Σ is independent of the interaction parameter J, the calculation in Figure 8a has been used in generating Figure 6a and b. Similarly, the calculated Σ values shown in Figure 8b and c were used to obtain Figure 6c and d, respectively. In order to see the change of the molecular area clearly, we ignore here the multiphase coexistence and look at only the minimum of the total free energy, f_{tot}.

In Figure 8a and b, for a pure NU membrane ($\phi = 0$), Σ gradually increases when the temperature is increased approaching the main-transition temperature. As mentioned in section 2, the molecular area discontinuously jumps to a larger value as appropriate for the L_α phase at $T_U^{**} = -20$ °C because the main-transition is first-order. In the L_α phase of the
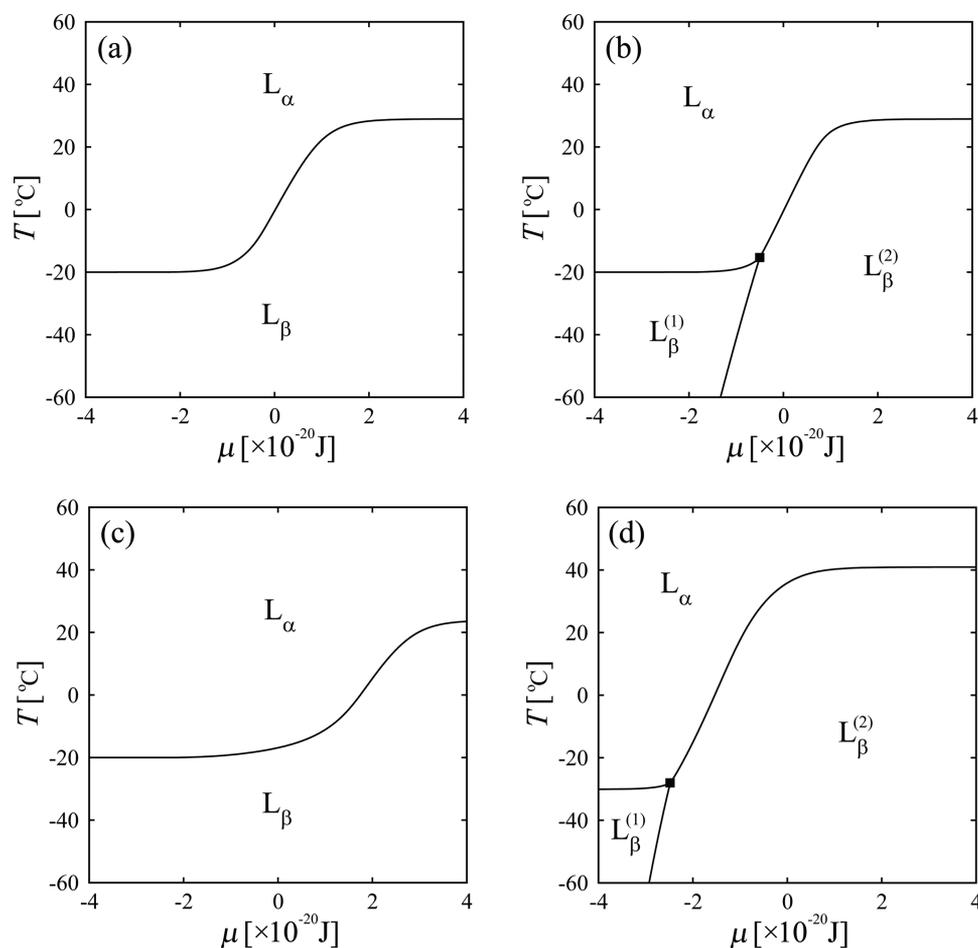
Figure 7. Phase diagrams of two-component charged membranes as a function of the chemical potential, μ, of saturated lipid (either CS or NS) and temperature T. (a) CS/NU membrane with $J = 1.2 \times 10^{-20}$ J and $n_b = 1$ M; (b) CS/NU membrane with $J = 1.8 \times 10^{-20}$ J and $n_b = 1$ M; (c) CS/NU membrane with $J = 1.2 \times 10^{-20}$ J and $n_b = 10$ mM; and (d) NS/CU membrane with $J = 1.2 \times 10^{-20}$ J and $n_b = 1$ M. The other parameters are listed in Table 1 and are mentioned in the text. The filled squares located at $(\mu, T) = (-0.50 \times 10^{-20}$ J, -15.3 °C) in part b and $(-2.48 \times 10^{-20}$ J, -28.0 °C) in part d indicate the triple points.
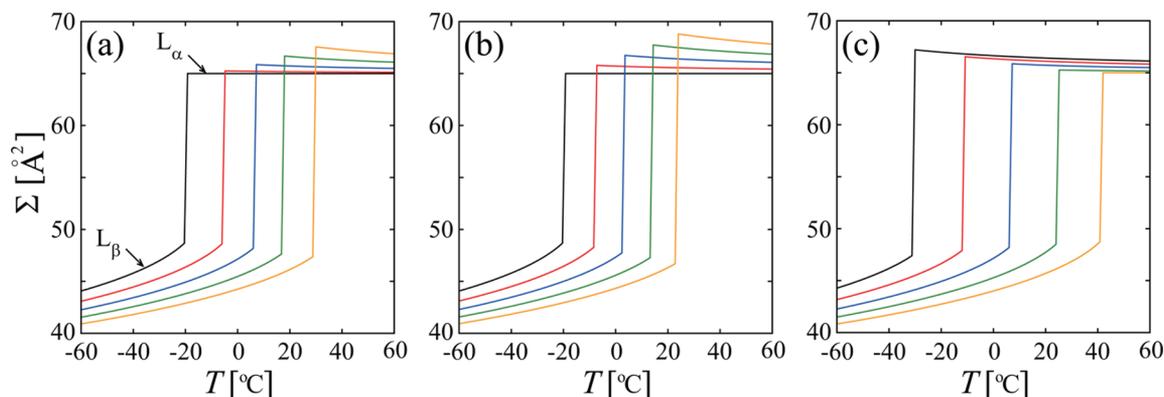
Figure 8. Plots of molecular area Σ as a function of the temperature T. (a) CS/NU lipid membrane with $n_b = 1$ M, (b) CS/NU lipid membrane with $n_b = 10$ mM, and (c) NS/CU lipid membrane with $n_b = 1$ M. Black, red, blue, green, and orange lines correspond, respectively, to $\phi = 0$, 0.25, 0.5, 0.75, and 1.

NU membrane, Σ takes a constant value, $\Sigma_0 = 65$ Å^2, because the order parameter m^* always vanishes there.

In the presence of the CS lipid ($\phi > 0$), Σ also increases and exhibits a discontinuous jump at the main-transition temperature. At the transition temperature, as compared with the neutral membranes, Σ for the charged membranes is slightly smaller for the L_β phase but larger for the L_α phase. This

tendency can be clearly seen for higher molar fractions of CS lipid ($\phi \approx 1$) or for lower salt concentrations (Figure 8b). As the temperature is further increased from the main-transition temperature, T^{**}, Σ in the L_α phase asymptotically approaches the constant value, $\Sigma_0 = 65$ Å^2.

The temperature dependence of Σ for the NS/CU mixture is shown in Figure 8c. The molecular area first increases in the L_β

phase and then jumps discontinuously at the main-transition temperature. This molecular area expansion is larger for higher molar fraction of the CU lipid ($\phi \approx 0$). In the next section, we shall discuss in more detail the effect of the lipid volume conservation on the molecular area.

4. DISCUSSION

Several points merit further discussion. First, we discuss the effect of the interaction parameter J on the phase behavior. As shown in Figures 4 and 6 for neutral and charged membranes, respectively, the phase-separated region becomes larger as J increases. In particular, we find different coexistence regions such as liquid−liquid ($L_\alpha^{(1)} + L_\alpha^{(2)}$) in Figure 4b and solid−solid ($L_\beta^{(1)} + L_\beta^{(2)}$) in Figure 6b. These coexistence regions do not appear for smaller J value as in Figures 4a and 6a. Such a dependence on J was also reported in previous theoretical works.[19,20]

As seen in Figure 6, the main-transition temperature, T^{**}, is reduced for a single-component charged lipid membrane. The transition temperature of NS lipid is T_S^{**} = 41 °C, while it is decreased to $T_S^{**} \simeq 29$ °C for CS lipid as in Figure 6a and b, or to $T_S^{**} \simeq 23$ °C as in Figure 6c. The electrostatic repulsion between the charged lipid molecules tends to expand the molecular area, resulting in a decrease of the membrane thickness, while keeping the molecular volume $v = \Sigma\delta$ constant (see Figure 3). Hence, the molecular volume conservation enhances the phase transition toward the L_α phase, and the main-transition temperature is decreased.[39] For a single-component unsaturated lipid, the reduction of the transition temperature from T_U^{**} = −20 °C (for NU) to −30 °C (for CU) can also be explained by the volume conservation constraint (see Figure 6d).

Next, we comment on the effect of the electrostatic interaction on the extension of the phase-separated regions. We have presented the phase behavior of a neutral NS/NU membrane (Figure 4a), a charged CS/NU membrane with a high salt concentration (Figure 6a), and a charged CS/NU membrane with a low salt concentration (Figure 6c). The phase separation takes place below $T = 41$ °C in Figure 4a, between $T = 29$ °C and −20 °C in Figure 6a, and between $T = 23$ °C and −20 °C in Figure 6c. As the electrostatic interaction strength is increased in the order of Figures 4a, 6a, and 6c, the phase-separated region becomes narrower. For the NS/CU membrane in Figure 6d, the phase coexistence occurs below $T = 41$ °C. Although this temperature range coincides with that of neutral NS/NU membranes as in Figure 4a, the phase-coexistence region in charged membranes (Figure 6d) is narrower than that in neutral membranes (Figure 4a). Hence, the phase separation is generally suppressed in charged membranes as compared with neutral ones.

Some experiments[25−29] reported that the two-phase coexistence region for charged membranes (containing mainly CU lipid) is narrower than that for neutral membranes, and the phase separation in these membranes can be enhanced again by adding a salt. Compared with two-component neutral membranes (Figure 4a), the phase-separated region of an NS/CU membrane (Figure 6d) becomes narrower due to the electrostatic interactions. This is indeed consistent with experiments. Previous theoretical models for charged membranes[26,31,32] have considered only the contributions from the phase separation and the electrostatic interactions. Although these works also reported the suppression of the phase separation in charged membranes, the main-transition was

not taken into account. In the present work, we have included this phase transition in order to fully describe the phase behavior of two-component charged membranes. The advantage of our model is that it describes the different phase behavior, distinguishing CS/NU and NS/CU lipid mixtures.

As mentioned in section 1, there are two types of two-component charged membranes: CS/NU and NS/CU lipid mixtures. These two cases are presented in Figure 6a and d, which manifest that the phase-separated region in the former case is narrower than that in the latter one. This is because the difference in the main-transition temperature between the saturated and unsaturated lipids is larger in the NS/CU case (Figure 6d). We note again that the reduction of the transition temperature for charged lipids is induced by the molecular volume conservation.

In general, the larger the difference in the transition temperatures, the stronger the segregation, and the phase separation is enhanced. Himeno et al.[29] reported that the phase behaviors of CS/NU and NS/CU lipid mixtures are quite different. In particular, they obtained the liquidus lines and compared the phase separations in CS/NU and NS/CU mixtures with that in an NS/NU mixture. According to their results, the phase separation is enhanced for a CS/NU mixture, whereas it is suppressed for an NS/CU mixture. Although it is difficult to compare directly this experiment with our theory, our model clearly distinguishes between the two different types of charged membranes, and the trends agree with experiments. In the previous experimental work,[29] it was considered that there is a counterion mediated attraction between charged saturated lipids, which overcomes the electrostatic repulsion between the charged head groups. For simplicity, this effect was not considered in the present study. However, it will be of interest to consider such added interactions in future theoretical studies in order to make a direct comparison with the experimental results.

We further comment now on the discontinuous jump of the molecular area at the main-transition temperature. For a pure NS lipid membrane ($\phi = 1$ in Figure 8c), the molecular area jumps from $\Sigma = 48.7$ to 65 Å^2 at T_S^{**} = 41 °C. For a pure CS lipid membrane ($\phi = 1$ in Figure 8a or b), on the other hand, it varies from $\Sigma = 47.4$ to 67.6 Å^2 at T_S^{**} = 29 °C in Figure 8a and from $\Sigma = 46.9$ to 68.8 Å^2 at T_S^{**} = 23 °C in Figure 8b. This means that the main-transition of a CS lipid occurs when the temperature is lower and the molecular area is smaller, as compared with an NS lipid. This behavior can be explained by the electrostatic repulsion between the charged head groups (see Figure 3). In general, the main-transition is induced when the attraction between the lipids becomes weaker. For CS lipids, the electrostatic repulsion between the lipids acts in addition to the attraction. Hence, the main-transition occurs at a lower temperature and smaller molecular area. Moreover, the molecular area of a CS lipid in the L_α phase is larger than that of an NS lipid ($\Sigma = 65$ Å^2) due to the electrostatic repulsion. The increase in the molecular area at the main-transition is more enhanced when the molar fraction of charged lipid is higher.

For higher temperatures, the molecular expansion in two-component charged membranes is suppressed. In the presence of charged lipid, Σ in the L_α phase asymptotically approaches Σ_0 as the temperature is further increased. We recall here that the total free energy of CS/NU or NS/CU membranes is given by eq 18 or 19. Taking into account the temperature dependence of $p = 2\pi l_B/\Sigma_0\kappa = e/\Sigma_0\sqrt{8n_b\varepsilon_w k_B T}$, we note

that the relative contribution of the electrostatic free energy vanishes in the high temperature limit. Namely, $f_{el}/(f_{st} + f_{mix}) \to 0$ as $T \to \infty$. This means that the electrostatic interaction becomes irrelevant with respect to the non-electrostatic free energies in the high temperature limit, and $\Sigma \to \Sigma_0$.

In neutral membranes, the optimal order parameter m^* vanishes in the L_α phase. On the other hand, in charged membranes, m^* in the L_α phase becomes negative due to molecular volume conservation. Since the order parameter represents the membrane thickness, negative m means an expansion of the molecular area, as shown in Figure 3. In order to understand why m^* becomes negative, we expand the electrostatic free energy f_{el} of eq 17 in powers of m

$$\frac{f_{el}(m, \phi)}{k_B T} \approx 2\phi\left[\frac{1 - \sqrt{1 + (p\phi)^2}}{p\phi}\right.$$
$$\left. + \ln\left(p\phi + \sqrt{1 + (p\phi)^2}\right)\right] - \frac{2\left(1 - \sqrt{1 + (p\phi)^2}\right)}{p}m$$
$$- \frac{\left(1 - \sqrt{1 + (p\phi)^2}\right)^2}{p\sqrt{1 + (p\phi)^2}}m^2 \tag{24}$$

The order parameter m is determined by minimizing the total free energy, eq 18, which includes eq 24. The latter equation is minimized for nonzero m due to the linear term in m because $\sqrt{1 + (p\phi)^2} > 1$. Since the stretching free energy f_{st} in eq 2 does not include any linear term in m, the total free energy is minimized for a negative $m^* < 0$. As mentioned above, the electrostatic interaction becomes negligible at higher temperatures. Therefore, m^* asymptotically vanishes when the temperature is increased.

In this study, we have used the Poisson–Boltzmann theory to obtain the electrostatic potential for symmetric monovalent salt. Such a mean-field treatment would break down when ion–ion correlations become significant. For example, when multivalent ions are present or a crowding state of charged lipids occurs, it would be important to include ion–ion correlations beyond the mean-field theory. This point will be addressed in a future study. Furthermore, modulated phases in neutral lipid membranes were discussed theoretically by taking into account both the lipid main transition and the spatial dependence of the lipid composition.[22] In the case of a charged lipid membrane, the formation of modulated phases was predicted within the Debye–Hückel approximation without considering the lipid main transition.[40] However, the occurrence of such a microphase separation has not yet been reported experimentally for charged bilayer membranes using optical microscopes.[25−30] This is because the characteristic wavelength of the microphase separation is considered to be of the order of nanometers.[41] Further theoretical and experimental studies are needed in order to investigate the possibility of such microphase separations in charged lipid membranes.

5. CONCLUSION

In this work, we have considered phase transitions and separations in binary mixtures of charged membranes. Taking into account the chain main-transition, we have calculated the phase diagrams of two-component charged and neutral lipids.

In particular, we have considered a coupling effect between the order parameter and the lipid composition through the molecular volume conservation. We showed that the phase separation in charged membranes is suppressed as compared with the neutral case. Furthermore, as the salt concentration is increased because of screening, the phase separation in charged membranes is enhanced again, signaling the importance of electrostatic interaction.

We have focused on the combination of the lipid hydrocarbon saturation and the charge on the headgroup (either CS/NU or NS/CU). The phase behavior of these two types of binary mixtures of charged/neutral lipids is very different, and is in agreement with recent experiments.[29] We also find that the molecular area of charged/neutral mixtures is smaller in the L_β phase and larger in the L_α, as compared with neutral mixtures. In other words, the discontinuous jump of the molecular area for charged/neutral mixtures is larger than that for the neutral ones. This tendency is clearly seen when the charged lipid fraction is large and/or the salt concentration is low, and can be understood by the electrostatic repulsion between the charged head groups. In general, the phase behavior is significantly influenced by the presence of the charged lipid.

Our model describes the phase behavior of binary mixtures of charged/neutral lipids. In most of the recent experimental studies,[25−29] however, the phase separation has been observed in ternary charged lipid mixtures containing cholesterol. Since these studies are motivated by the formation of raft domains enriched in cholesterol, lipid mixtures containing cholesterol are also important from the biological point of view. Putzel and Schick proposed a model[42] for the phase separation in neutral membranes consisting of saturated lipids, unsaturated lipids, and cholesterol. In the future, it will be of interest to consider a model for ternary charged lipid membranes, which will take into account explicitly the specific role of cholesterol, in addition to the effect of charges in lipid mixtures.

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Notes
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■ REFERENCES

(1) Simons, K.; Ikonen, E. Functional Rafts in Cell Membranes. *Nature* **1997**, *387*, 569−572.

(2) Simons, K.; Sampaio, J. L. Membrane Organization and Lipid Rafts. *Cold Spring Harbor Perspect. Biol.* **2011**, *3*, a004697.

(3) Komura, S.; Andelman, D. Physical Aspects of Heterogeneities in Multi-Component Lipid Membranes. *Adv. Colloid Interface Sci.* **2014**, *208*, 34−46.

(4) Veatch, S. L.; Keller, S. L. Organization in Lipid Membranes Containing Cholesterol. *Phys. Rev. Lett.* **2002**, *89*, 268101.

(5) Veatch, S. L.; Keller, S. L. Separation of Liquid Phases in Giant Vesicles of Ternary Mixtures of Phospholipids and Cholesterol. *Biophys. J.* **2003**, *85*, 3074−3083.

(6) Baumgart, T.; Hess, S. T.; Webb, W. W. Imaging Coexisting Fluid Domains in Biomembrane Models Coupling Curvature and Line Tension. *Nature* **2003**, *425*, 821−824.

(7) Heberle, F. A.; Feigenson, G. W. Phase Separation in Lipid Membranes. *Cold Spring Harbor Perspect. Biol.* **2011**, *3*, a004630.

(8) Hamada, T.; Sugimoto, R.; Nagasaki, T.; Takagi, M. Photochemical Control of Membrane Raft Organization. *Soft Matter* **2011**, *7*, 220−224.

(9) Hamada, T.; Kishimoto, Y.; Nagasaki, T.; Takagi, M. Lateral Phase Separation in Tense Membranes. *Soft Matter* **2011**, *7*, 9061−9068.

(10) Shimokawa, N.; Nagata, M.; Takagi, M. Physical Properties of the Hybrid Lipid POPC on Micrometer-Sized Domains in Mixed Lipid Membranes. *Phys. Chem. Chem. Phys.* **2015**, *17*, 20882−20888.

(11) Bagatolli, L. A.; Gratton, E. Two Photon Fluorescence Microscopy of Coexisting Lipid Domains in Giant Unilamellar Vesicles of Binary Phospholipid Mixtures. *Biophys. J.* **2000**, *78*, 290−305.

(12) Beales, P. A.; Gordon, V. D.; Zhao, Z.; Egelhaaf, S. U.; Poon, W. C. K. Solid-Like Domains in Fluid Membranes. *J. Phys.: Condens. Matter* **2005**, *17*, S3341−S3346.

(13) Chen, D.; Santore, M. M. Large Effect of Membrane Tension on the Fluid-Solid Phase Transitions of Two-Component Phosphatidylcholine Vesicles. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 179−184.

(14) Chen, D.; Santore, M. M. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)-Rich Domain Formation in Binary Phospholipid Vesicle Membranes: Two-Dimensional Nucleation and Growth. *Langmuir* **2014**, *30*, 9484−9493.

(15) Wu, S. H.; McConnell, H. M. Phase Separation in Phospholipid Membranes. *Biochemistry* **1975**, *14*, 847−854.

(16) Luna, E. J.; McConnell, M. Lateral Phase Separations in Binary Mixtures of Phospholipids Having Different Charges and Different Crystalline Structures. *Biochim. Biophys. Acta, Biomembr.* **1977**, *470*, 303−316.

(17) Arnold, K.; Lösche, A.; Gawrisch, K. ^{31}P-NMR Investigations of Phase Separation in Phosphatidylcholine/Phosphatidylethanolamine Mixtures. *Biochim. Biophys. Acta, Biomembr.* **1981**, *645*, 143−148.

(18) Blume, A.; Wittebort, R. J.; Das Gupta, S. K.; Griffin, R. G. Phase Equilibria, Molecular Conformation, and Dynamics in Phosphatidylcholine/Phosphatidylethanolamine Bilayers. *Biochemistry* **1982**, *21*, 6243−6253.

(19) Komura, S.; Shirotori, H.; Olmsted, P. D.; Andelman, D. Lateral Phase Separation in Mixtures of Lipids and Cholesterol Systems. *Europhys. Lett.* **2004**, *67*, 321−327.

(20) Komura, S.; Shirotori, H.; Olmsted, P. D. Phase Behavior of Three-Component Lipid Mixtures. *J. Phys.: Condens. Matter* **2005**, *17*, S2951−S2956.

(21) Elliott, R.; Szleifer, I.; Schick, M. Phase Diagram of a Ternary Mixture of Cholesterol and Saturated and Unsaturated Lipids Calculated from a Microscopic Model. *Phys. Rev. Lett.* **2006**, *96*, 098101.

(22) Shimokawa, N.; Komura, S.; Andelman, D. The Phase Behavior of Mixed Lipid Membranes in the Presence of the Rippled Phase. *Eur. Phys. J. E: Soft Matter Biol. Phys.* **2008**, *26*, 197−204.

(23) Yamamoto, T.; Brewster, R.; Safran, S. A. Chain Ordering of Hybrid Lipids Can Stabilize Domains in Saturated/Hybrid/Cholesterol Lipid Membranes. *Eur. Phys. Lett.* **2010**, *91*, 28002.

(24) Alberts, B.; Johnson, A.; Walter, P.; Lewis, J.; Raff, M. *Molecular Biology of the Cell*; Garland Science: New York, 2008.

(25) Vequi-Suplicy, C. C.; Riske, K. A.; Knorr, R. L.; Dimova, R. Vesicles with Charged Domains. *Biochim. Biophys. Acta, Biomembr.* **2010**, *1798*, 1338−1347.

(26) Shimokawa, N.; Hishida, M.; Seto, H.; Yoshikawa, K. Phase Separation of a Mixture of Charged and Neutral Lipids on a Giant Vesicle Induced by Small Cations. *Chem. Phys. Lett.* **2010**, *496*, 59−63.

(27) Blosser, M. C.; Starr, J. B.; Turtle, C. W.; Ashcraft, J.; Keller, S. L. Minimal Effect of Lipid Charge on Membrane Miscibility Phase Behavior in Three Ternary Systems. *Biophys. J.* **2013**, *104*, 2629−2638.

(28) Pataraia, S.; Liu, Y.; Lipowsky, R.; Dimova, R. Effect of Sytochrome c on the Phase Behavior of Charged Multicomponent Lipid Membranes. *Biochim. Biophys. Acta, Biomembr.* **2014**, *1838*, 2036−2045.

(29) Himeno, H.; Shimokawa, N.; Komura, S.; Andelman, D.; Hamada, T.; Takagi, M. Charge-Induced Phase Separation in Lipid Membranes. *Soft Matter* **2014**, *10*, 7959−7967.

(30) Himeno, H.; Ito, H.; Higuchi, Y.; Hamada, T.; Shimokawa, N.; Takagi, M. Coupling between Pore Formation and Phase Separation in Charged Lipid Membranes. *Phys. Rev. E* **2015**, *92*, 062713.

(31) May, S.; Harries, D.; Ben-Shaul, A. Macroion-Induced Compositional Instability of Binary Fluid Membranes. *Phys. Rev. Lett.* **2002**, *89*, 268102.

(32) Shimokawa, N.; Komura, S.; Andelman, D. Charged Bilayer Membranes in Asymmetric Ionic Solutions: Phase Diagrams and Critical Behavior. *Phys. Rev. E* **2011**, *84*, 031919.

(33) Goldstein, R. E.; Leibler, S. Model for Lamellar Phases of Interacting Lipid Membranes. *Phys. Rev. Lett.* **1988**, *61*, 2213−2216.

(34) Goldstein, R. E.; Leibler, S. Structural Phase Transitions of Interacting Membranes. *Phys. Rev. A: At., Mol., Opt. Phys.* **1989**, *40*, 1025−1035.

(35) Andelman, D. Electrostatic Properties of Membranes: the Poisson-Boltzmann Theory. In *Handbook of Biological Physics: Structure and Dynamics of Membranes*; Lipowsky, R., Sackmann, E., Eds.; Elsevier: Amsterdam, The Netherlands, 1995; Vol. *1B*, pp 603−642.

(36) Andelman, D. Introduction to Electrostatics in Soft and Biological Matter. In *Soft Condensed Matter Physics in Molecular and Cell Biology*; Poon, W. C. K., Andelman, D., Eds.; Taylor & Francis: New York, 2006; pp 97−122.

(37) Evans, D. F.; Wennerström, H. *The Colloidal Domain: Where Physics, Chemistry, Biology and Technology Meet*; John Wiley: New York, 1999.

(38) Verwey, E. J. W.; Overbeek, J. Th. G. *Theory of the Stability of Lyophobic Colloids*; Dover Publication: New York, 1999.

(39) Jähnig, F. Electrostatic Free Energy and Shift of the Phase Transition for Charged Lipid Membranes. *Biophys. Chem.* **1976**, *4*, 309−318.

(40) Guttman, G. D.; Andelman, D. Electrostatic Interactions in Two-Component Membranes. *J. Phys. II* **1993**, *3*, 1411−1425.

(41) Okamoto, R.; Shimokawa, N.; Komura, S. Nano-Domain Formation in Charged Membranes: Beyond the Debye-Hückel Approximation. *Europhys. Lett.* **2016**, *114*, 28002.

(42) Garbès Putzel, G.; Schick, M. Phenomenological Model and Phase Behavior of Saturated and Unsaturated Lipids and Cholesterol. *Biophys. J.* **2008**, *95*, 4756−4762.