Phase separation of a mixture of charged and neutral lipids on a giant vesicle induced by small cations

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Abstract

Phase separation of giant vesicles composed of neutral saturated lipid, negatively charged unsaturated lipid, and cholesterol, is observed at different calcium concentrations. Confocal microscopy provides the information where the phase separation becomes distinct as the calcium concentration is increased. The negatively charged lipid domains tend to bud toward the interior of the vesicle. This budding is assumed to be due to an increase in the osmotic pressure, in cooperation with the spontaneous curvature change in the outer leaflet of the bilayer caused by the adsorption of calcium ions and charge screening effect. We interpret the effect of small cations on the phase separation based on the theoretical model with the Poisson-Boltzmann equation.

1. Introduction

Lipid bilayers consisting of saturated lipids, unsaturated lipids, and cholesterol (Chol) have attracted significant attention as a model biomembrane system. Below the phase separation temperature, this model membrane exhibits phase separation between the saturated lipid and Chol-rich region and the unsaturated lipid-rich region. Domain formation accompanied by phase

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separation has been referred to as the "raft model" [1]. Since it is believed that raft domains are related to various cell functions, for example, signal transduction and cooperative membrane trafficking, it is important to determine the mechanism of domain formation in relation to the biological functions. Moreover, raft formation is not only important on a biocellular level, but is also on a fundamental physical aspect providing a specific example of two-dimensional phase separation. Many experimental and theoretical studies have been carried out to explore various phenomena related to the phase separation in this model membrane, such as the domain morphology, the budding of domains, the growth dynamics of domains, or the periodic structure. In particular, Langmuir-Blodgett films and giant lipid vesicles have been actively used for such experiments, because domain formation in a mesoscopic length scale can be directly observed using fluorescence microscopy [2, 3, 4, 5, 6]. Theoretical models have been proposed to explain the experimental results [7].

Most of these studies have examined the temperature-dependent phase separation in systems consisting of only electrically neutral phospholipids. However, a few studies have reported the phase separation in ternary systems including some charged phospholipids, although biomembranes have several types of charged phospholipids, which have, for example, phosphatidylserine (PS) or phosphatidylglycerol (PG) head groups. Moreover, highly expected biological molecules that have electric charges (e.g. DNA, protein, or salt) exhibit significant effect to the phase separation in charged membranes through electrostatic interactions.

Previous experimental research has reported that the aggregation of charged lipids in binary charged membranes is induced by the addition of some electrolyte [8, 9, 10]. In these studies, the aggregation of negatively charged lipids by the addition of electrolytes has been argued, when both of the lipids have unsaturated hydrocarbon chains and exhibit a disordered phase. However, mesoscopic phase separation between the ordered and disordered phases does not occur in these binary systems; therefore, the relation between this aggregation and the mesoscopic phase separation is not considered. On the other hand, when the mixed lipids exhibit phase separation between the charged disordered (ordered) and neutral ordered (disordered) phases, it is expected that electrostatic Coulombic repulsion between charged lipid molecules is expected to compete against domain formation by phase separation. This competition is not yet fully understood, and thus, a new physical phenomenon may arise from the competition.

In this letter, we report on the phase behavior of ternary charged membranes consisting of neutral saturated lipids (1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPC), negatively charged unsaturated lipids (1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt); DOPS), and cholesterol (Chol), with and without electrolyte, observed using confocal laser scanning microscopy. A small cation species, i.e., calcium ions, is used as one of the electrolytes. In addition, we present a theoretical model to explain the phase behavior with and without the small cations.

2. Materials and methods

Giant vesicles composed of ternary phospholipids DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine, Wako), DOPS (1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt), Avanti Polar Lipids) and Chol (Sigma-Aldrich) were prepared. The chain melting temperatures of DPPC and DOPS are 41 and -11 °C, respectively. *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2,-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (NBD-PE, triethylammonium salt, Molecular Probes) and rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (rhodamine DHPE, triethylammonium salt, Molecular Probes) were used as fluorescent probes. When these lipids exhibited phase separation, NBD-PE was localized in the liquid-ordered (L_o) phase (DPPC and Chol rich) and displayed green fluorescence at 536 nm. Rhodamine DHPE was localized in the liquid-disordered (L_d) and negatively charged phase (DOPS rich) and displayed red fluorescence at 581 nm. Giant vesicles were prepared by the natural swelling method [11, 12] from dry lipid films according to the following process. Firstly, 10 mM lipid and Chol were dissolved in chloroform/methanol (=2:1) so that the total volume became 10 μL, and 2 μL of each of the 0.1 mM fluorescent probes dissolved in the same organic solvent were added. The lipid solutions were dried in air and then placed under vacuum overnight to form completely dry thin lipid films. The films were then hydrated with 100 μL ultrapure water (MilliQ, specific resistance ≥ 18 MΩ) at 60 °C for over 4 h. The lipid solutions were mixed with calcium chloride solutions so that the final calcium chloride concentrations were 0.05 and 0.1 mM, respectively. The final total lipid concentration was 0.9 mM. The mixed solutions were observed using confocal laser scanning microscopes (Nikon A1, Nikon and LSM 510, Carl Zeiss) at room temperature ($\sim 22 \pm 2$ °C).

3. Experimental results

Figure 1 shows a typical image of a giant vesicle observed with the confocal microscope and the phase diagram of the ternary system without addition of calcium chloride. The red filled circles in the phase diagram denote the two-phase region, while the red and black filled circles denote the boundary state. Only a few phase separated vesicles are observed at the boundary state. The black filled circles indicate the homogeneous phase, and the cross marks denote the region where the formed vesicles are not stable. The dashed ellipsoid indicates the approximate phase separation region in the case of the neutral lipid system (DPPC/DOPC/Chol) [5]. Phase separation hardly occurs in the charged membranes compared with the neutral membranes, as shown in Fig. 1. In the homogeneous phase, the red and green fluorescent probes are homogeneously dispersed, as shown in Fig. 1(a) for DPPC/DOPS/Chol=4:4:2 (molar ratio). It is assumed that phase separation is depressed for the charged membrane, due to the large energy loss caused by the high concentration of negatively charged lipids, even when the temperature is below the phase separation temperature. On the other hand, phase separation is sometimes observed in the DPPC and DOPS binary system without Chol (see the base of the phase diagram in Fig. 1, such as for DPPC/DOPS/Chol=3:7:0). Such phase separation in a binary system has been reported for several similar systems [13]. In general, solid-like domains are formed in a binary system without Chol, and these domains have anisotropic shapes [14]. An anisotropically shaped domain is shown in Fig. 1(b). This domain is observed as a black area, because both fluorescent probes are excluded from this highly-ordered solid domain. In addition, no stable vesicles are formed for a low DOPS content. This is considered to be caused by the weak Coulombic repulsion, which is insufficient to cause peeling-off of the membrane from the dry lipid film to form vesicles during the hydration process.

Images of the giant vesicles and the phase diagrams after the addition of calcium chloride are shown in Fig. 2. Phase separation occurs due to the addition of calcium, especially around the composition DPPC/DOPS/Chol=4:4:2. Moreover, the phase separation region becomes larger as the calcium concentration is increased from 0.05 to 0.1 mM. It is known that calcium ions tend to bind strongly to PS head groups [15]. Decreasing of Coulombic repulsion between DOPS lipids by the bound calcium ions is considered to reduce the energy loss due to aggregation of the DOPS lipids, and results in the for-

mation of stable phase separated domains. As the calcium concentration is increased, this effect becomes significant. The phase separation region in the case of higher calcium concentration is expected to come close to that for a neutral lipid system, as shown by the dashed ellipsoid in Fig. 1. This tendency is obvious for a calcium ion concentration in the order of 0.1 mM, while the giant vesicles are ruptured for higher calcium chloride concentrations [15].

For the phase-separated vesicles formed by the addition of calcium ions, not only lateral phase separation, as in Fig. 2(a), which is usually the case for phase separation of neutral phospholipids, but also specific phase separation behavior is often observed, where the DOPS-rich region enter the inner volume of the vesicle, as shown in Fig. 2(b). It is considered that phase-separated DOPS domains bud toward the interior of the vesicle. Such budding vesicles have been observed much more often than laterally separated vesicles in our measurements. Even in the neutral lipid system, similar internal budding occurs by control of the volume or surface area of the vesicle by the addition of sugar or a surfactant [16, 17, 18, 19]. Two important effects are assumed from the selective budding of DOPS rich domains toward the vesicle interiors: the acquisition of a necessary excess area for vesicle deformation due to the osmotic pressure, and the change in the spontaneous curvature of the DOPS domains in an outer leaflet of the bilayer due to the adsorption of calcium ions on the DOPS lipids. Firstly, a salt concentration difference is generated across the membrane by the addition of calcium chloride. Therefore, the membrane can obtain the excess area required for deformation, because the water goes out of the vesicle due to the osmotic pressure. Subsequently, the binding of calcium ions on DOPS lipids together with the increase in the screening effect reduces the area occupied by PS head groups, and the spontaneous curvature of the DOPS domains in the outer leaflets with adsorbed calcium ions decreases (here we define positive curvature as the membrane curving towards the outside of the vesicle). Therefore, the DOPS rich domains bud toward the interior of a vesicle.

In our experiments, the competitive behavior between Coulombic repulsion and phase separation is revealed. Under calcium-free conditions, the Coulombic repulsion between DOPS molecules dominates the phase behavior and mesoscopic phase separation is inhibited. As the calcium concentration is increased, the Coulombic repulsion becomes weak and comparable to the attractive potential that causes the mesoscopic phase separation as the result of cooperative effect of direct binding to PS head group and increase on the screening. Finally, the charged membrane exhibits mesoscopic phase

separation when the attractive potential becomes predominant.

4. Model

Based on the experimental results, the expansion of the phase separation region in the phase diagram by the addition of small cations is explained using a mean-field phenomenological model. The theoretical model by May et al. suggested that phase separation in mixed membranes consisting of charged and neutral lipids is induced by the adsorption of a macro-ion, such as a charged protein [20, 21, 22]. We discuss the effect of small cations such as calcium ions to the charged membrane by applying this model to our experimental system.

For simplicity, we make several assumptions. The bulk salt is dealt with as a symmetrical monovalent salt (e.g. NaCl). Calcium chloride is used as the salt in this work, and this is not a symmetrical monovalent salt. However, there are no essential differences between the monovalent and divalent salts with respect to qualitative calculations, and this difference is adjusted by the Debye screening length, which is a controllable parameter in the model. In addition, a flat isolated membrane composed of two species of lipids is assumed. Although the membrane consists of three components in the experiment, it is reasonable to regard it as a simple binary mixture, because Chol is localized in the DPPC-rich domain [23]. In addition, the diameter of a giant vesicle is in the order of μm, while the thickness of the bilayer is approximately 5 nm; therefore, the membrane can be considered to be almost flat.

The mole fraction of negatively charged lipid is denoted by ϕ and the free energy is given by

$$F = [\phi \ln \phi + (1-\phi)\ln(1-\phi) + \chi\phi(1-\phi)] + f_{\mathrm{el}}(\phi). \tag{1}$$

All electrostatic interactions in the system are included in the last term f_{el}, while the other terms indicate the free energy of a bare neutral membrane. χ is a repulsive interaction parameter between the charged and neutral lipids that enhances lipid-lipid demixing, and this parameter is related to the temperature T as $\chi \sim 1/T$. When there are no electrostatic interactions in the system ($f_{\mathrm{el}} = 0$), i.e., the membranes are composed of only neutral lipids, then the binodal line in the (ϕ, χ) plane is described by the dot-dashed line in Fig. 3, where the critical point denoted by the filled black circle is located

at $(\phi, \chi) = (0.5, 2)$. The entropic term in this free energy assumes that the lipid molecules are freely dispersed in the membrane, and this corresponds to the liquid phase. Therefore, this formulation does not indicate the behavior of the solid phase which appears in the absence of cholesterol.

The expression for $f_{\rm el}$ is given under the framework of the Poisson-Boltzmann theory [24]

$$f_{\rm el}(\phi) = 2\phi \left[\frac{1-q}{p} + \ln(p+q) \right], \tag{2}$$

where $q^2 = p^2 + 1$ and $p = p_0\phi$. $p_0 = 2\pi l_{\rm B} l_{\rm D}/a$ is constant, where a is the cross-sectional area of the lipid. For simplicity, we assume that the two types of lipids have the same cross-sectional area a. $l_{\rm B}$ is the Bjerrum length, and $l_{\rm D}$ is the Debye screening length. The surface charge density σ, which is used as one of the boundary conditions in the calculation of $f_{\rm el}$, is expressed by $\sigma = -e\phi/a$, where e is the elementary charge. The phase diagram in the (ϕ, χ) plane is obtained as shown in Fig. 3. In this calculation, $l_{\rm B} = 7\,\text{Å}$, and $a = 65\,\text{Å}^2$ are fixed. The two binodal lines are indicated by solid and dashed lines, which correspond to different Debye lengths of $l_{\rm D} = 50$ and $5\,\text{Å}$, respectively. The solid line corresponds to the case of the negatively charged membrane before addition of the salt ($l_{\rm D} = 50\,\text{Å}$). On the other hand, the reduction of the Debye screening length ($l_{\rm D} = 5\,\text{Å}$) due to addition of the salt results in the binodal line indicated by the dashed line. The phase diagram shows that the phase separation region for the case of the charged membrane without the salt becomes narrower than that for the neutral membrane. Addition of the salt to the charged membrane causes the two-phase region to enlarge and approach that of the neutral membrane. Since the experiments were performed at constant temperature, the phase separation behavior must be considered at a constant χ in the phase diagram. When χ is fixed to approximately 3.7 (see Fig. 3), the charged membrane without salt (solid line) exhibits a homogeneous phase. On the other hand, phase separation occurs in the charged membrane after addition of the salt (dashed line). Moreover, the phase separation region of the neutral membrane (dot-dashed line) is larger than that of the charged membrane with the salt (dashed line). Therefore, even if the temperature is constant, phase separation is induced by decreasing the Debye length. These results are qualitatively consistent with our experimental results, where the phase separation behavior depends on the concentration of added salt.

The theoretical model suggests that the phase behavior in charged mem-

branes is dominated by the Debye screening length. In order to simplify the calculations, monovalent cations are assumed as the added salt instead of the divalent cations used in the experiment. However, the difference between monovalent and divalent cations corresponds approximately to the difference in the Debye length; a longer Debye length corresponds to the condition with monovalent cations, while a shorter Debye length is equivalent to that with divalent cations at the same concentration. The results of our model suggest that a minute amount of trivalent cations could effectively induce phase separation, which corresponds to the condition of a very short Debye length. Further studies to examine such expectations would be of value; however, a large amount of monovalent cations are required for phase separation, under which conditions the giant vesicles are destabilized due to high osmolarity.

5. Discussion

Recently, Vequi-Suplicy et al. showed similar results in the system composed of DOPG, eSM, and Chol [25]. They mentioned that the phase separation temperature was increased by addition of calcium ion. This result is consistent with ours, which shows that the region of phase separation in the phase diagram expands at higher calcium concentration. Thus, this behavior is not a specific phenomenon in our system but is more universal in ternary charged membranes.

Electron spin resonance (ESR) or deuterium nuclear magnetic resonance (NMR) spectroscopic measurements performed in previous studies indicate that the PS lipid has a phase transition from the disordered phase to the ordered phase by adsorption of calcium ions [8, 26]. This phase transition induces phase separation of the ordered-phase PS and disordered-phase PC lipids [8]. In contrast, this transition prevents phase separation in our system, because DOPS tends to mix with DPPS if DOPS forms an ordered phase. Our experimental results suggest that electrostatic interaction is stronger than the specific properties of calcium for phase separation behavior. Moreover, calcium ions reduce the absolute value of the surface charge density due to the strong binding on the head group of PS lipids [27]. In a realistic case, the binding effect should be considered in the theoretical model, although our model using the framework of Poisson-Boltzmann does not include this effect, i.e. we control the phase behavior only by changing the Debye length and assume that the surface charge density $\sigma = -e\phi/a$ is constant. However, the decrease in the surface charge density have qualitatively the same effect

as the decrease in the Debye length, since both effects reduce the surface potential $\Psi = -2\mathrm{arcsinh}(p_0\phi)$.

The existence of a nanometer-size domain was reported by atomic force microscopy (AFM) in a membrane composed of DPPC and DOPS with calcium chloride [28]. It is probable that the homogeneous phases in our experiment have nanometer-size domains, because confocal microscopy cannot observe such domains. In order to confirm the existence of these nanometer-size domains, careful experiments by means of AFM or small-angle neutron scattering (SANS) are necessary in future.

We consider the change in the spontaneous curvature of the outer leaflet of the bilayer as one of the reasons for the budding of DOPS domains. The calcium cations bound to the charged head groups not only decrease the Coulombic repulsion but also undergo the entropy gain caused by water molecules released from the hydration shell of the calcium cations and dehydration of the lipid membrane [15]. Thus, the decrease of the spontaneous curvature by the binding of calcium cations is reasonable. On the other hand, the internal budding phenomenon has not reported in similar mixtures consisted of DOPC/DOPS with peptides or DOPG/eSM/Chol with calcium chloride. [10, 25]. Therefore, our results suggest that the budding phenomenon is dominated by the change in the spontaneous curvature induced by the strong calcium binding to the PS headgroup and screening effect. Additionally, the line energy of the domain boundary is important to understand the budding behavior [7]. In contrast to previous studies, large line energy is evidenced in our experiment, which is ascribed to the difference in the ordering between the hydrocarbon chains of each separated phases. Furthermore, we have encountered some difficulties in corroborating the budding process by confocal microscopy; the buds are too small to observe in detail and keep continuous focusing due to fluctuations and water flow.

6. Conclusion

Mesoscopic phase separation of giant unilamellar vesicles composed of DPPC, DOPS, and Chol has been examined in the present study. The phase separation region in the phase diagram is enlarged as the calcium chloride concentration is increased. This phase separation is caused by the decrease in the Coulombic repulsion by the addition of calcium ion, which can be interpreted as the cooperative effect of direct calcium binding and increase

in the screening effect. The DOPS domains are found to bud toward the interior of a vesicle; it is attributed to the increase in the osmotic pressure and the change in the spontaneous curvature of the outer leaflet of the bilayer induced by the addition of calcium chloride. Thus, we consider that the phase separation and the budding phenomenon arise from the cation binding to charged head groups and the screening effect in a simultaneous manner. We have formulated the observed phase separation in the framework of the Poisson-Boltzmann theory by taking into account the increase in the screening effect by cationic species. As one of the future experimental targets, one needs to evaluate the effect of direct binding of small cations by comparison of the effect of Ca and Mg ions as well as monovalent cations.

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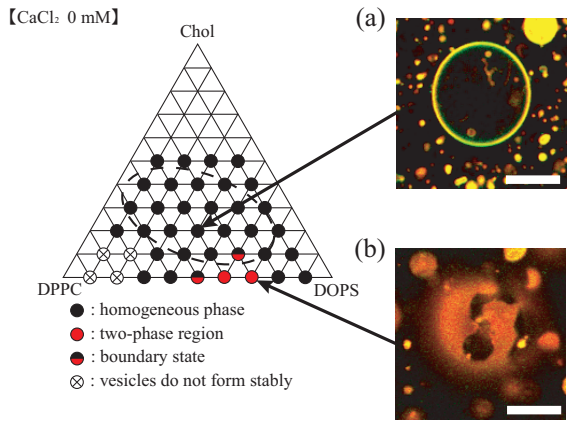

Fig 1: Ternary phase diagram of DPPC/DOPS/Chol at room temperature and corresponding giant vesicles observed by confocal microscopy at compositions of (a) DPPC/DOPS/Chol=4:4:2 (cross-sectional image) and (b) DPPC/DOPS/Chol=3:7:0 (plan view image). The dashed ellipsoid in the phase diagram indicates the approximate phase separation region in the case of a neutral lipid system that includes DOPC instead of DOPS [5]. The red regions in the confocal micrograph are the DOPS rich domains, the green regions are DPPC and Chol rich domains, and the homogeneous phase is indicated as yellow. Scale bars: 10 μm.

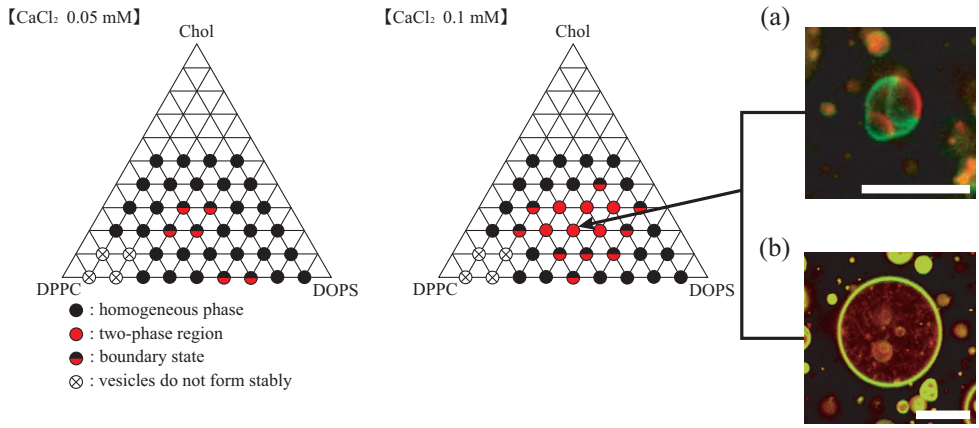

Fig 2: Phase diagrams of DPPC/DOPS/Chol after the addition of calcium chloride solution (left: 0.05 mM, right: 0.1 mM) at room temperature. The cross-sectional confocal micrographs in (a) and (b) have the same composition (DPPC/DOPS/Chol=4:4:2 at $CaCl_2$=0.1 mM). Scale bars: 10 μm.

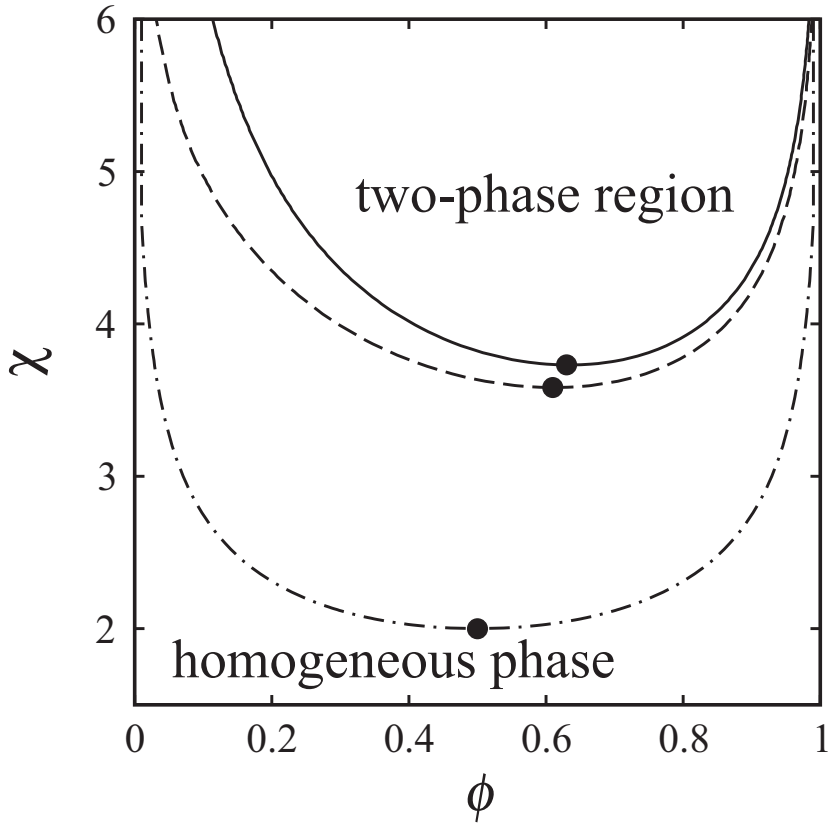

Fig 3: Phase diagram calculated as a function of the negatively charged lipid mole fraction ϕ and the interaction strength χ. The solid and dashed lines are the binodal lines for long and short Debye lengths, respectively. The dot-dashed line denotes the binodal line of a neutral membrane. The filled circles indicate the critical points.