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Effect of divalent counterions on asymmetrically charged lipid bilayers

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We study an asymmetrically charged lipid bilayer in which the inner layer is negatively charged, while the outer one is neutral. In particular, we focus on the interplay between the asymmetrical charge distribution and counterion valency in determining the preferred curvature state of the bilayer. We show that, at low ionic strength, an entropic effect associated with counterion release tends to expand the inner layer—surprisingly, the excess charge of the bilayer opposes this. In the absence of multivalent ions, the entropic effect is dominant and gives rise to a tendency towards negative mean curvature. The presence of divalent counterions, however, counterbalances the entropic effect—they tend to shrink the inner layer, leading to positive mean curvature.

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Cell membranes are lipid bilayers (studded with membrane proteins) that primarily isolate the cell's contents from their environment, while also serving as a binding site for numerous membrane binding ligands. Two faces of the bilayers often consist of strikingly different selections of their constituent lipids [1]. For example, the inner leaflets of the red blood cell (RBC) membranes are rich in anionic lipids, while the outer ones are essentially neutral. Thus, the inner leaflets strongly interact with basic ligands [2]. These ligands can influence the stability, shape, and preferred curvature of their target membranes by modifying the physical properties of the lipids of their binding layers. Despite significant effort [3–6], the precise mechanism for membrane stabilization or destabilization by ligands in living cells is largely unknown. A number of early RBC lysis experiments, however, suggest that the asymmetrical phospholipid distribution between the bilayers of the RBC membrane is crucial to the membrane stability againt inverted structures [3,4].

Despite the wealth of RBC experiments [3,4], a systematic theoretical approach to the membrane stability has so far been lacking. Analysis of experimental data on biological membranes is often hindered by various unknown factors. Consideration of pure lipid bilayers is useful as it allows more tractable analysis of key parameters that regulate the membrane stability. To shed some light into the problem, we consider asymmetrically charged lipid bilayers immersed in salty solution. This is invoked by the fact that negatively charged lipids, i.e., phosphotidylserene, mainly reside in the inner layer of the RBC membrane. In this study, the asymmetrical lipid distribution is taken into account through the asymmetrical charge distribution—only the inner layer is (negatively) charged as in the RBC membrane. We, however, largely ignore other complexities of the cell membrane and assume that the two layers of the membrane are otherwise identical. Even with this simplification, the resulting problem is still challenging and intriguing as will be evidenced later.

Using this model, we study the preferred curvature state of the bilayer. Throughout this paper, this is quantified in terms of the relaxed area difference between the inner and outer layers ΔA_0. In particular, we focus on the effect of multivalent counterions (i.e., cations) on ΔA_0. We find that ΔA_0 is highly sensitive to counterion valency. The presence of a tiny concentration ($\sim$0.1 mM) of divalent cations can even change the sign of ΔA_0 for a wide range of physical parameters, leading to a tendency towards positive mean curvature. We find that the preferred curvature as implied by ΔA_0 is determined by the balance of a few competing effects: the repulsion between charged headgroups, charge correlations, and an entropic effect arising from counterion release. At low ionic strength, the repulsion (when combined with counterion condensation) rather tends to shrink the inner layer, contrary to our intuition; in this case, the tendency towards negative mean curvature (corresponding to a tendency towards bending outward) is only triggered by the entropic effect arising from counterion release of the membrane. As the inner leaflet expands its area, the surface charge density decreases. As a result, some condensed counterions are free to return to the solution, since the attraction of counterions to the membrane is weakened. It is the gain in the entropy that derives the negative curvature. In the absence of multivalent ions, the entropic effect is dominant and favors negative curvature. The presence of even a small concentration of divalent counterions, however, ensures the positive-curvature state; they preferentially bind to and tend to shrink the inner layer.

The model we consider here is a thin flat membrane with charge density $-e\sigma_0$ in the xy plane, in the presence of a monovalent (1:1) salt such as NaCl, and in the presence or absence of Z valent (Z:1) salts, such as CaCl_2. The inner leaflets are capable of adsorbing counterions of the opposite charge, reducing the surface charge density of the membrane [9]. The magnitude of the reduced charge density can be estimated by equating the chemical potentials of the "free" and "condensed" counterions, i.e., those adsorbed onto the membrane surface. In the following descriptions, the subscripts $i=1$ and 2 refer to monovalent and multivalent counterions (of valency Z), respectively. If $e\sigma_i$ is the charge density of condensed counterions, then the effective surface charge density on the membrane is $-e\sigma^* = -e(\sigma_0 - \sigma_1 - Z\sigma_2)$ [10]. The chemical potential of free counterions is mainly associated with the configurational entropy of mixing: $\beta\mu_i^{free} \simeq \ln(n_i v_0)$, where $\beta = 1/k_B T$, k_B is the Boltzmann constant, T is the temperature, and n_i is the counterion concentration. Finally, $v_0 = 4\pi r_c^3/3$, where r_c the size of counterions. On the other hand, the chemical potential of con-

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densed counterions arises from electrostatic interactions and the entropic penalty for condensation [11]:

$$\beta\mu_i^{cond} \simeq -2\pi Z_i \ell_B \sigma^* \kappa^{-1} + \ln(\sigma_i a_0) + \beta\mu_i^{corr}, \qquad (1)$$

where $Z_1 = 1$, $Z_2 = Z$, $a_0 = 4\pi r_c^2$, $\ell_B = e^2/\epsilon k_B T$ is the Bjerrum length, ϵ is the dieletric constant, and κ^{-1} is the Debye screening length given by $\kappa^2 = 4\pi\ell_B[2n_1 + Zn_2(Z+1)]$. The last term is the charge correlation contribution to the chemical potential of condensed counterions that has yet to be specified. An early study suggests that charge correlations among condensed counterions trigger extra condensation [12]. If $\mathcal{F}_{corr}$ is the charge correlation contribution to the free energy of ions on the membrane surface [cf. the last term in Eq. (3)], we find

$$\beta\mu_i^{corr} = \beta\frac{\partial\mathcal{F}_{corr}}{\partial\sigma_i} = -\frac{Z_i^2 \ell_B}{2\lambda}\ln\left[\frac{\lambda^{-1} + \sqrt{k_0^2 + \kappa^2}}{\lambda^{-1} + \kappa}\right], \qquad (2)$$

where $\lambda^{-1} = 2\pi\ell_B(\sigma_0 + \sigma_1 + Z^2\sigma_2)$. The equilibrium values of σ_i can then be obtained by requiring $\mu_i^{free} = \mu_i^{cond}$.

The total free energy of the membrane (per unit area) is then given as a sum of the entropy of mixing, the repulsive and the charge correlation contributions [8,13]:

$$\beta\mathcal{F}_{cond} = \sum_{j=1}^{2} \sigma_j(\ln\sigma_j a_0 - 1) + \pi\ell_B\sigma^{*2}\kappa^{-1} + \frac{1}{2}\int \frac{d\mathbf{k}_\perp}{(2\pi)^2}\{\ln[1 + \mathcal{M}(\mathbf{k}_\perp)] - \mathcal{M}(\mathbf{k}_\perp)\}, \qquad (3)$$

where $\mathbf{k}_\perp = (k_x, k_y)$ and $\mathcal{M}(\mathbf{k}_\perp) = 1/\lambda\sqrt{k_\perp^2 + \kappa^2}$. The last term arises from in-plane charge correlations and can be evaluated by integrating out the charge fluctuations at the Gaussian level [8,13].

The preferred curvature state can be quantified in terms of the difference between the (relaxed) areas of the outer and the inner layers denoted by $\Delta A_0 = A_0^{out} - A_0^{in}$ [7], where the superscripts $\alpha =$ "in" and "out" refer to the inner and outer layer, respectively. Membranes with $\Delta A_0 > 0 (< 0)$ have a tendency to bend inwards (outwards) or a tendency towards positive (negative) mean curvature. Note that the relaxed area of each layer (thus, ΔA_0) is determined by the balance of forces at the layer. Since we assume here that the two layers are identical, except for their charge properties, the sign of ΔA_0 will be essentially determined by the excess free energy or the free energy difference between the two layers, $\Delta\mathcal{F}$, which is a sum of $\mathcal{F}_{cond}$ and an entropic effect arising from counterion release [cf. Eq. (5) or Eq. (6)]; if $\Delta\Pi = -\partial[A^{in}\Delta\mathcal{F}]/\partial A^{in} > 0 (< 0)$, then $\Delta A_0 < 0$ (> 0).

The excess interfacial tension at the inner layer due to the combined entropy and electrostatic effects reads

$$\Delta\Pi = -\mathcal{F}_{cond} + \sigma_0\frac{\partial\mathcal{F}_{cond}}{\partial\sigma_0} - A^{in}\sum_{i=1}^{2}\frac{\partial\sigma_i}{\partial A^{in}}\frac{\partial\mathcal{F}_{cond}}{\partial\sigma_i} + k_B T\sum_{i=1}^{2}\left[\left(\sigma_i + A^{in}\frac{\partial\sigma_i}{\partial A^{in}}\right)\ln n_i v_0\right]. \qquad (4)$$

The first two terms in Eq. (4) are contributions of condensed counterions to $\Delta\Pi$ estimated with $\Sigma_i \equiv A^{in}\sigma_i$ $(i = 1,2)$ fixed, while the third term arises from changes in Σ_i for a given A^{in}. The last term is a gain in the entropy due to counterion release. This can be readily derived by noting that the change in the entropy of counterions with A^{in} with reference to that of condensed counterions [corresponding to the first term in Eq. (3)] is simply $\Sigma_i(\partial\Sigma_i/\partial A^{in})\ln n_i v_0$.

We first study the asymptotic behavior of the excess interfacial tension in the limit of $\kappa\lambda \ll 1$. The contribution of the excess charge to the interfacial tension can be written as $\beta\Pi_{rep} \simeq -\pi\ell_B\sigma^{*2}\kappa^{-1} - 2\pi\ell_B\kappa^{-1}\sigma^* A^{in}(\partial\sigma^*/\partial A^{in})$. In the limit of small $\kappa\lambda$, σ^* is roughly independent of σ_0 and thus A^{in}. This results in $\beta\Pi_{rep} \simeq -\pi\ell_B\sigma^{*2}\kappa^{-1}$, which is negative. In other words, the repulsive contribution leads to positive curvature, contrary to our intuition. The key to understand this lies in a consistent treatment of Π_{rep} and counterion condensation; as A^{in} increases, the repulsive free energy also increases (recall σ^* is roughly constant), resulting in a negative tension. If we used the linearlized Poisson-Boltzmann or Debye-Hückel (DH) approach that suppresses counterion condensation, we would get $\beta\Pi_{DH} = \pi\ell_B\sigma_0^2\kappa^{-1}$, which is positive [14]. Counterion condensation, however, invalidates this approach at low ionic strength, as long as $\lambda < \kappa^{-1}$, as is the case for RBC experiments [3,4]—in this case, DH result *mistakenly* implies that the membrane charge tends to expand the charged layer, i.e., the inner layer.

In contrast, the entropic contribution Π_{ent} counterbalances Π_{rep}. This follows from the fact that $\Pi_{ent} \geqslant \sigma_1 + \sigma_2 = -[\partial(-TS)/\partial A^{in}]_{\Sigma_i} \geqslant 0$. Note that the third term is a tension one would get if Σ_j was held fixed as A^{in} increases. Due to counterion release, however, the entropic free energy is further reduced from this value, leading to the first inequality. More precisely, the entropic tension can be written as

$$\beta\Pi_{ent} = \sum_{i=1}^{2}\left[\sigma_i + \left(A^{in}\left|\frac{\partial\sigma_i}{\partial A^{in}}\right| - \sigma_i\right)\ln\left(\frac{\sigma_i a_0}{n_i v_0}\right)\right]. \qquad (5)$$

For monovalent cases at a low salt limit, this expression assumes a much simpler form:

$$\beta\Pi_{ent} \simeq \sigma_1 + \sigma^*\ln\left(\frac{\sigma_1 a_0}{n_1 v_0}\right). \qquad (6)$$

The second term, arising from counterion release, dominates the first term at a low salt limit and is the main cause for negative mean curvature. On the other hand, the charge correlation tension Π_{corr} is always more negative than $-(\partial\mathcal{F}_{corr}/\partial A^{in})_{\Sigma_i}$ $(\leqslant 0)$, which is a tension one would get if Σ_i were assumed to be constant. Note that charges corre-

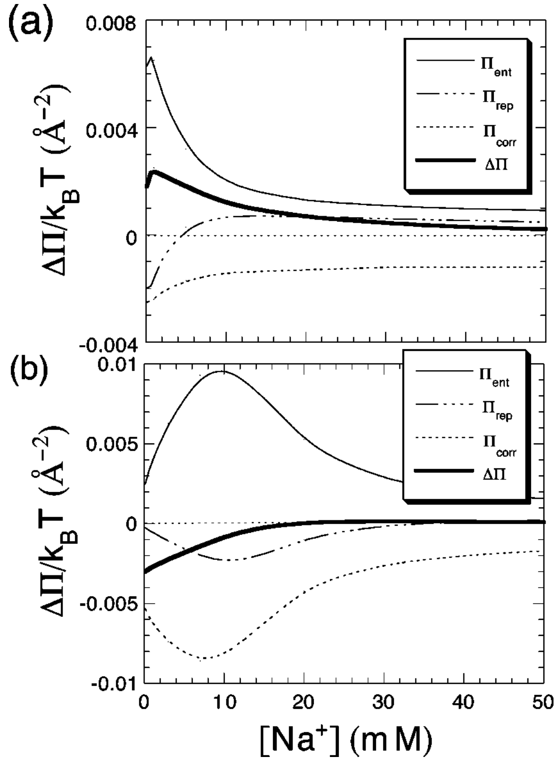

FIG. 1. The excess interfacial tension as a function of the sodium concentration in millimole, $[\mathrm{Na}^+]$. (a) In the absence of divalent ions $(Z=1)$, the entropic effect is dominant for the whole range of $[\mathrm{Na}^+]$, leading to $\Delta A_0<0$. (b) In the presence of 0.1 mM of divalent cations, however, the tension is negative for a much wider range of $[\mathrm{Na}^+]$, resulting in $\Delta A_0>0$.

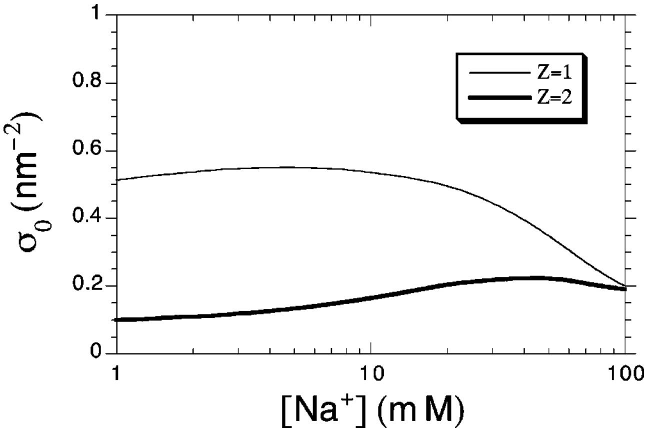

FIG. 2. Preferred curvature state of an asymmetrically charged (but otherwise identical) bilayer, consisting of a neutral leafleat on the outside and a charged one on the inside. In the upper (lower) side of each curve, $\Delta A_0>0$ $(\Delta A_0<0)$. In the presence of monovalent ions only $(Z=1)$, ΔA_0 is negative unless σ_0 is sufficiently large; in the presence of 0.1 mM of divalent cations, it is positive for a much wider regime.

late more efficiently at higher charge densities (corresponding to smaller A^{in}), and thus Π_{corr} prefers to shrink the inner layer, leading to a tendency towards $\Delta A_0>0$.

To study the preferred curvature state (ΔA_0 in this paper) for a wider parameter range, we have solved Eqs. (1) and (4) simultaneously. In Fig. 1, we plot the excess interfacial tension as a function of the concentration of monovalent cations, namely, Na^+–$[\mathrm{Na}^+]$ is the sodium concentration in millimole. We have chosen $\sigma_0=0.2\ \mathrm{nm}^{-2}$ (a typical value for the RBC membranes), $T=300$ K, $\epsilon=80$, and $r_c=2$ Å. In the absence of multivalent ions, the entropic tension is dominant and the resulting total tension is *always* positive, enlarging the headgroup area of lipids in the inner layer. This results in negative ΔA_0, a tendency towards negative curvature. In the presence of as small a concentration as 0.1 mM of divalent cations, however, the excess tension is negative for a wide range of $[\mathrm{Na}^+]$, up to 20 mM. In other words, the optimal headgroup area is reduced in this case, leading to positive ΔA_0. Note that Π_{corr} and Π_{rep} vary nonmonotonically with $[\mathrm{Na}^+]$ and take the minimum values at $[\mathrm{Na}^+]\simeq 8$ mM, in contrast to the monovalent case for which their minima fall around $[\mathrm{Na}^+]\sim 0$. The difference between the two cases can be attributed to the fact that, in the presence of divalent ions, counterion release is most pronounced around $[\mathrm{Na}^+]=8$ mM. Note that this value should depend on n_2 and will shift to a smaller value if n_2 decreases. Thus, both the charge fluctuation and the excess charge contribution to the third term in Eq. (4) are also most pronounced around this value, leading to the aforementioned behavior.

To further demonstrate the efficiency of multivalent counterions, we plot in Fig. 2 regimes where $\Delta A_0>0$ and $\Delta A_0<0$. For simplicity, we have assumed that two leafleats of the membrane are indentical except that only the inner layer is charged with the charge density $-e\sigma_0$. In the upper (lower) side of each curve, the membrane has positive (negative) ΔA_0.We have chosen $T=300$ K, $\epsilon=80$, and $r_c=2$ Å. In the absence of multivalent ions, the bilayer has negative ΔA_0 for a wide range of σ_0 and $[\mathrm{Na}^+]$, as described by the thin curve. The presence of 0.1 mM of divalent cations (corresponding to the bold line) has a dramatic effect on ΔA_0. In this case, the membrane has positive ΔA_0 for a much wider parameter space.

Plausible explanations for the efficiency of multivalent counterions are as follows. In the absence of multivalent ions, the preferred curvature state is dominated by the entropic gain of released counterions, unless the surface charge density is sufficiently high. The entropic effect is more pronounced at a low salt limit [cf. Eq. (6)]. This follows from the fact that $\ln(\sigma_1 a_0/n_1 v_0)$ depends more sensitively on $[\mathrm{Na}^+]$ than σ_1 and decreases with increasing $[\mathrm{Na}^+]$. For sufficiently highly charged cases, however, the charge fluctuation term outweighs the entropic effect, making ΔA_0 positive. In the presence of multivalent counterions, however, the charge fluctuation effect is more pronounced than in the monovalent case, since this efect is more sensitive to counterion valency *Z* than other effects. This effect together with the excess charge contribution results in $\Delta A_0>0$. As $[\mathrm{Na}^+]$ increases, however, monovalent counterions start to replace divalent counterions in the condensed layer. The distinction between the monovalent and divalent case becomes minor as implied by the boundaries in Fig. 2. Even though each term individually does not show strong *Z* dependence, the balance between them is highly sensitive to *Z*, leading to valency-sensitive ΔA_0. The dramatic distinction between the monovalent and multivalent cases is, however, completely missing in the DH approach or the linearized Poisson-Boltzmann approach.This clearly indicates the efficiency of multivalent counterions is captured *only when* preferential adsorption and a balance of a few competing effects are properly taken into account.

Our findings in Figs. 1 and 2 appear to be remarkably consistent with the RBC experiments that demonstrate valency sensitivity of membrane stability. It has been known for several decades that the RBC membrane stability is highly sensitive to Z. In the absence of multivalent ions, osmotic lysis of the RBC membranes leads to mainly inside-out vesicles (implying $\Delta A_0 < 0$, if $\Delta A_0 < 0$ is assumed to induce the membrane instability), while, in the presence of divalent cations, vesiculation can be prevented; it leads to leaky ghosts instead. Vesiculation often requires mechanical disruption. When sheared, the leaky ghosts disintegrate into vesicles; in the presence of even a small concentration (~ 0.1 mM) of divalent counterions, this procedure leads to mainly right-side-out vesicles (implying $\Delta A_0 > 0$). In the absence of multivalent ions, most of the resulting vesicles are inside out. Our results given in Figs. 1 and 2 provide a quantitative basis of these experiments.

We have presented a theory to account for the valency sensitivity of the preferred curvature state of asymmetrically charged (pure lipid) bilayers. A couples of issues ignored in our approach can, however, further complicate the problem. First of all, counterions also change the headgroup size of negatively charged lipids and thus the so called packing ratio and the spontaneous curvature C_0. It should be emphasized that the preferred curvature is determined not only by ΔA_0 but also by C_0. Even though our results appear to be consistent with the RBC experiments, our neglect of the complexity of RBC makes it difficult to draw a definite picture of the RBC stability. Even when $\Delta A_0 (< 0)$ is assumed to induce the membrane instability, there are many other potential sources. For example, the spectrin network, attached to the inner layer of the membrane, could impose a lateral tension on the inner layer. Even though the spectrin network is detached during vesiculation, it may lead to a nontrivial effect on ΔA_0; the detachment of the spectrin network can change the lateral tension on the inner layer, possibly in a time dependent manner. Also asymmetrical insertion of molecules in one of the layers can change ΔA_0. Additionally, analysis of membrane undulations and lipid symmetrization, coupled to vesiculation, is required. Once a pore forms in a previously closed membrane by osmotic stresses, ΔA_0 tends to zero, due to the exchange of lipids between the two layers of the membrane. In this case, the driving force for the inversion into inside-out structures will be diminished. On the other hand, lipids with a smaller headgroup size (e.g., anionic lipids in the divalent case) tend to reside in the inner layer, since they fit in the inner layer better, unless the radius of curvature is too large. Further consideration is certainly warranted.

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