

Stabilization and destabilization of cell membranes by multivalent ions

Bae-Yeun Ha

Department of Physics, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6
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We propose a mechanism for the stabilization and destabilization of cell membranes by multivalent ions with an emphasis on its implications for the division and fusion of cells. We find that multivalent cations preferentially adsorbed onto a membrane *dramatically* change the membrane stability. They not only reduce the surface charge density of the membrane but also induce a barrier to pore growth. While both of these effects lead to enhanced membrane stability against vesiculation and pore growth, the induced barrier arises from correlated fluctuations of the adsorbed cations and favors closure of a pore. Finally, the addition of a small amount of multivalent anions can reverse the membrane stabilization, providing an effective way to regulate membrane stability.

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Lipid bilayer membranes are resistant to rupture, primarily serving as a barrier to the leakage of the cell's contents, while also being dynamic structures that undergo various topological transitions. The capability of living cells to regulate the stability of their bounding membranes is crucial to their maintenance and reproduction [1]. Membrane stability against rupture changes most dramatically during cell division and fusion. The precise mechanism for achieving this complex task in living cells is complicated by various membrane-associated or -bounded proteins [1] and is not yet clear. Numerous studies, however, suggest that membrane stability is influenced by several factors such as the ionic strength, external fields, and thermal fluctuations [2–11]. Osmotic lysis of cell membranes is an efficient experimental means to study the membrane stability [2–4]. It has been known for a few decades that red blood cells can be converted into vesicles by osmotic lysis in a solution of low ionic strength lacking multivalent cations [1–4]. The presence of divalent cations, however, prevents this vesiculation [2–4]. In fact, a number of experiments [2–4] have unambiguously demonstrated that the stability of red cell membranes against vesiculation can be greatly enhanced by multivalent cations. Despite this, a consistent theoretical description of this phenomenon has so far been lacking.

The strong valency dependency of membrane stability [2–4] motivated this work. Not only can osmotic lysis lead to vesiculation, but it can also create large pores in the cell membranes that subsequently contract to a size that is controlled by the ionic strength. Pore closure can be stimulated by cations, and remarkably the rate of pore closure *strongly* depends on the valency of cations [3]; Ca^{2+} is roughly 60 times as potent on a molar basis as Na^+ . The potency of divalent cations, which essentially prevents vesiculation, was first demonstrated experimentally three decades ago [2], but it has yet to be examined theoretically. Here we propose a theoretical mechanism to explain this phenomenon. We find that multivalent counterions adsorbed onto a charged membrane *dramatically* enhance the membrane stability through two effects. First, they can significantly reduce the surface charge density of the membrane, enhancing the membrane stability. Second, they induce an attraction between charged head groups of the membrane and thus a barrier to pore

growth. The induced barrier originates from the correlated fluctuations of adsorbed counterions and favors closure of a pore, further stabilizing the membrane. Upon adding a small content of multivalent anions, the adsorbed cations are released into solution, thus reversing the membrane stabilization. Adsorption and desorption of multivalent cations provide an effective way to regulate the membrane stability.

The model we consider here is a thin flat membrane [12] in the xy plane (as shown in Fig. 1), 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 . Each leaflet of the membrane is composed of neutral and anionic lipids. In reality, the inner leaflet of red cell membranes is known to be more highly charged than the outer leaflet [3] as schematically shown in Fig. 1(a). We, however, ignore this asymmetrical charge distribution in the following discussions. As a result, each side of the membrane is assumed to be negatively charged with constant charge density $-e\sigma_0$ and attracts ions of the opposite charge. For simplicity, we consider the case of a single circular pore of radius R , al-

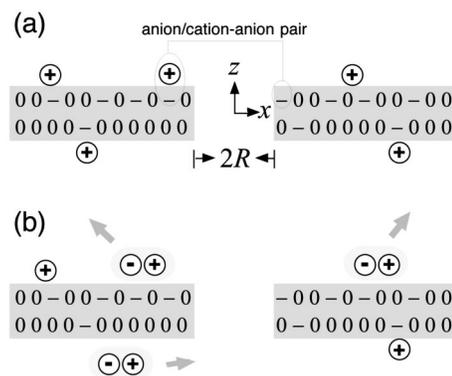


FIG. 1. (a) Schematic view of a charged membrane with a pore of a radius R . Charged and neutral lipids are denoted by $-$ and 0 , respectively, while the adsorbed multivalent cations are denoted by encircled $+$'s. A pair of an attracting lipid and a lipid and cation is also shown that stabilizes the membrane against pore growth. (b) Illustration of multivalent anions (encircled $-$'s) forming pairs with the adsorbed multivalent cations, then leaving into solution. Depletion of the adsorbed layer destabilizes the membrane.

readily formed in the membrane by, for example, osmotic stress. The stability of the membrane against rupture can be quantified in terms of a line tension, i.e., the energetic penalty for creating a pore per unit length. The electrostatic repulsion between charges on the membrane favors pore formation [6], but the hydrophobic effect opposes this. If mobile ions are treated as screening objects that simply reduce the electrostatic repulsion via Debye screening, then the electrostatic contribution to the line tension γ_{DH} can be estimated using Debye-Hückel (DH) theory [6]: $\gamma_{DH} \sim -e^2\sigma_0^2\kappa^{-1}\epsilon^{-1}R$ if $R < \kappa^{-1}$ and $\gamma_{DH} \sim -e^2\sigma_0^2\kappa^{-2}\epsilon^{-1}$ if $R > \kappa^{-1}$, where κ^{-1} is the screening length and ϵ is the dielectric constant of the solvent. Obviously, the electrostatic repulsion favors creation and expansion of a pore, i.e., $\gamma_{DH} < 0$.

Charged membranes are, however, capable of adsorbing counterions of the opposite charge [see Fig. 1(a)], reducing the surface charge density of the membrane [13]. 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 the monovalent and multivalent counterions, respectively. If $e\sigma_i$ is the charge density of condensed counterions, then the effective surface charge density on the membrane is $-e\sigma^* = -e(\sigma_0 - \sigma_1 - Z_+\sigma_2)$. The chemical potential of free counterions is mainly associated with the configurational entropy of mixing: $\mu_i^{free} \sim k_B T \ln(n_i a_i^3)$, where n_i and a_i are the concentration and size of counterions, respectively. On the other hand, the chemical potential of the condensed counterions arises from electrostatic interactions and the entropic penalty for condensation; $\mu_i^{cond} \sim -2\pi Z_i \ell_B \sigma^* \sqrt{S} + \ln(\sigma_i a_i^2)$ if $\kappa^{-1} > \sqrt{S}$ and $\mu_i^{cond} \sim -2\pi Z_i \ell_B \sigma^* \kappa^{-1} + \ln(\sigma_i a_i^2)$ otherwise, where S is the area of the membrane, $Z_1 = 1$, and $Z_2 = Z_+$. The equilibrium values of σ_i can then be obtained by requiring $\mu_i^{free} = \mu_i^{cond}$.

The condensed counterions do not simply renormalize the membrane charge density but also give rise to charge fluctuations in the plane of the membrane surface that tend to be correlated with each other [14,15]. A typical attracting pair of a lipid and a cation is illustrated in Fig. 1. Creation of a pore makes charges at the edge less efficiently correlated and is discouraged by the charge-correlation effects. In the following derivations, we take the continuum limit and suppress the finite thickness of the membrane. Consequently the charge distribution on the membrane is described by the local surface charge density [16], $e\hat{\sigma}(\mathbf{r}_\perp) = -e\sigma_0 + em_1 + em_2 Z_+$, where e is the electronic charge, and $m_1, m_2 = 0, 1, 2, 3$, etc., are, respectively the number of condensed monovalent and multivalent counterions per unit area at $\mathbf{r}_\perp \equiv (x, y)$. Then the interaction Hamiltonian is simply

$$\frac{\mathcal{H}}{k_B T} = \frac{\ell_B}{2} \int \int d\mathbf{r}_\perp d\mathbf{r}'_\perp \hat{\sigma}(\mathbf{r}_\perp) \hat{\sigma}(\mathbf{r}'_\perp) \frac{e^{-\kappa|\mathbf{r}_\perp - \mathbf{r}'_\perp|}}{|\mathbf{r}_\perp - \mathbf{r}'_\perp|}, \quad (1)$$

where the integrations are over the membrane surface, $\ell_B = e^2/\epsilon k_B T$ is the Bjerrum length, $\kappa^2 = 8\pi\ell_B I$, and I is the ionic strength of the solution. The electrostatic pore free en-

ergy is given as a sum over all realizations of $\hat{\sigma}(\mathbf{r}_\perp)$. Computation of this quantity is highly involved, partly because membrane charges at different sites are *not* correlated pair by pair; the interaction between two charged lipids is influenced by the presence of a third charged lipid and thus all fluctuating charges on the membrane should be explicitly taken into account [14]. This is complicated by yet another factor: the specific geometry of the membrane with a pore. The electrostatic effects at the mean-field level, suppressing both adsorption and charge fluctuations, has only recently been addressed [6]. We therefore incorporate the in-plane charge fluctuations at the Gaussian level as in previous cases [14,15]. In other words, we keep terms to the second order in the charge fluctuations in the computation of the free energy. The resulting charge fluctuation contribution to the pore free energy, i.e., the change in the charge fluctuation free energy by creating a pore, is formally given by

$$\frac{\Delta\mathcal{F}_{pore}}{k_B T} = \frac{1}{2} \ln \det[1 + (Q - \mathcal{Q})\mathcal{Q}^{-1}]. \quad (2)$$

Here, the matrix Q is defined by the matrix elements

$$Q_{\mathbf{x}_\perp \mathbf{x}'_\perp} = 1 + \ell_B \sigma_{cc} \zeta_{\mathbf{x}_\perp \mathbf{x}'_\perp} \frac{e^{-\kappa|\mathbf{x}_\perp - \mathbf{x}'_\perp|}}{|\mathbf{x}_\perp - \mathbf{x}'_\perp|}, \quad (3)$$

where $\sigma_{cc} \equiv \sigma_1 + Z_+^2 \sigma_2$ and $\mathcal{Q} \equiv \lim_{R \rightarrow 0} Q$. Finally, $\zeta_{\mathbf{x}_\perp \mathbf{x}'_\perp} = 1$ if \mathbf{x}_\perp and \mathbf{x}'_\perp are on the membrane and 0 otherwise. In the case of $S > \kappa^{-1}$, as is the case for red cell experiments [2,3], \mathcal{F}_{pore} in Eq. (2) can be calculated without making further approximations. This follows from the fact that $\delta \equiv (Q - \mathcal{Q})\mathcal{Q}^{-1} \sim S^{-1}$ and \mathcal{F}_{pore} can then be expanded in powers of δ . In the case $S > \kappa^{-1}$, we can take the limit $S \rightarrow \infty$ without introducing any appreciable error. In this limit, only the leading term survives in the expansion.

To calculate the free energy in Eq. (2), it proves useful to Fourier transform it from \mathbf{x}_\perp to \mathbf{k}_\perp . We find

$$\begin{aligned} \frac{\Delta\mathcal{F}_{pore}}{k_B T} &\simeq \frac{1}{2\lambda_{cc}} \left[\int \int_{R=0} - \int \int_{R>0} \right] r_\perp dr_\perp r'_\perp dr'_\perp \\ &\times \int_0^{2\pi} d\theta \int_0^\infty \frac{k_\perp dk_\perp}{1 + \lambda_{cc} \sqrt{k_\perp^2 + \kappa^2}} \\ &\times \frac{e^{-\kappa \sqrt{r_\perp^2 + r'_\perp^2 - 2r_\perp r'_\perp \cos \theta}}}{\sqrt{r_\perp^2 + r'_\perp^2 - 2r_\perp r'_\perp \cos \theta}} \\ &\times J_0(k_\perp \sqrt{r_\perp^2 + r'_\perp^2 - 2r_\perp r'_\perp \cos \theta}), \quad (4) \end{aligned}$$

where $\lambda_{cc} \equiv 1/2\pi\ell_B\sigma_{cc}$ controls the strength of charge fluctuations (thus the charge fluctuation term $\Delta\mathcal{F}_{pore}$) and $J_0(x)$ is the zeroth-order Bessel function of the first kind. As already indicated earlier, the charge-fluctuation contribution $\Delta\mathcal{F}_{pore}$ in Eq. (4) prefers closure of the pore. To understand this more systematically, note that the membrane and the oppositely charged counterions on the membrane surface can be considered as forming a two-dimensional ionic fluid,

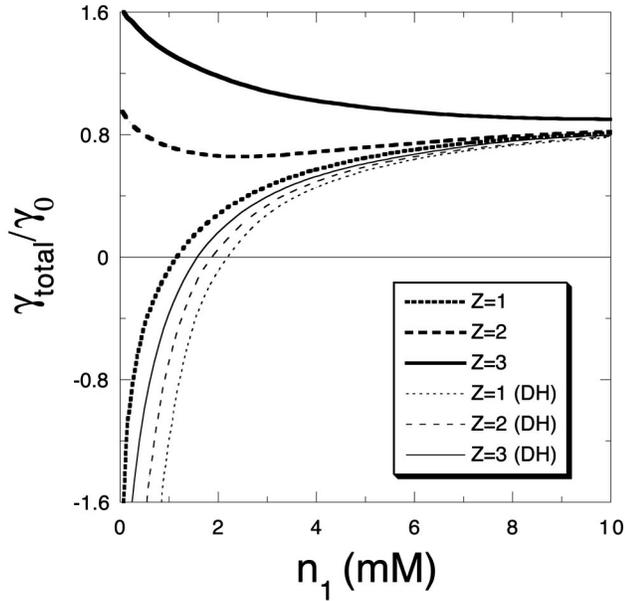


FIG. 2. Total line tension, in units of the hydrophobic contribution $\gamma_0 = 10^{-11}$ J/m, as a function of the monovalent salt concentration n_1 . We have chosen $T = 300$ and $\sigma_0 = 0.2$ nm $^{-2}$. In the absence of multivalent cations ($Z = 1$), there exists a finite range of the monovalent salt concentration where the membrane is unstable ($\gamma_{total} < 0$) to pore formation. The presence of as small a concentration as 0.1 mM of multivalent cations ($Z = 2, 3$) stabilizes the membrane against pore growth for the whole range of monovalent salt concentration. The distinction between the monovalent and multivalent cases is, however, minor in the DH approach, and 0.1 mM of multivalent cations only *slightly* enhances the membrane stability.

where ions are easily diffusive in the plane of the surface. This results in in-plane charge fluctuations that tend to be correlated, leading to an effective attraction between the head groups. This attraction between two head groups near the edge of the pore weakens as the pore expands. Thus the charge fluctuation contribution prefers to close the pore, as indicated by the sign of $\Delta\mathcal{F}_{pore}$. In the case of $\kappa R \gg 1$, the charge-fluctuation line tension, i.e., $\Delta\gamma = \Delta\mathcal{F}_{pore}/2\pi R$, shows two distinct scaling behaviors: $\Delta\gamma \sim \lambda_{cc}^{-1} \ln(1/\lambda_{cc}\kappa)$ for small $\lambda_{cc}\kappa$ and $\Delta\gamma \sim \lambda_{cc}^{-2}\kappa^{-1}$ for large $\lambda_{cc}\kappa$. If we calculated $\Delta\gamma$ using a form of a two-body interaction calculated for charges on the membrane, we would obtain $\Delta\gamma$, which shows a simple scaling behavior for all $\kappa > 0$: $\Delta\gamma \sim \lambda_{cc}^{-2}\kappa^{-1}$. In fact, the existence of multiple-scaling regimes is characteristic of the charge-fluctuation interactions that are nonpairwise additive [17].

The charge fluctuation ($\Delta\gamma > 0$) and hydrophobic contributions ($\gamma_0 > 0$) favor closure of the pore, while the DH ($\gamma_{DH} < 0$) or renormalized DH term ($\gamma_{DH}^* < 0$) tends to expand the pore. To study the membrane stability, we have solved for σ^* and $\gamma_{total} \equiv \gamma_0 + \gamma^*$ (or γ_{DH}^*) + $\Delta\gamma$ simultaneously. We have chosen $a_1 = a_2 = 2$ Å, $T = 300$, and $\sigma_0 = 0.2$ nm $^{-2}$, which is in the physiological range. To suppress the R dependency, we have assumed that $R > \kappa^{-1}$. Figure 2 shows γ_{total} in units of $\gamma_0 = 10^{-11}$ J/m [5] as a func-

tion of the monovalent counterion concentration n_1 . In the absence of multivalent counterions ($Z = 1$), γ_{total} is negative when n_1 is in the range $0 \leq n_1 \leq 1$ mM. This implies that the membrane is unstable to pore growth as long as n_1 is in this range. The presence of 0.1 mM of multivalent counterions ($Z = 2$ and 3), however, dramatically enhances the membrane stability. In this case, γ_{total} is positive for the whole range of n_1 and is in the range $0.6\gamma_0 \leq \gamma_{total} \leq 0.7\gamma_0$. In order to enhance the membrane stability up to this level by monovalent counterions, about 5 mM concentration would be needed. This is approximately 50 times higher than that of the divalent counterion concentration. This estimate is *remarkably* consistent with the experimental finding that Ca^{2+} is roughly 60 times more effective on a molar basis than Na^+ in stimulating pore closure [3].

Our results are striking; the presence of multivalent counterions is more crucial to the membrane stability than that of the monovalent salt, though the ionic strength is mainly determined by the latter. The plausible reason for the potency of multivalent counterions as implied by our results in Fig. 2 (which essentially changes the sign of γ_{total} when Z changes from 1 to 2) is as follows: Recall that it is the electrostatic repulsion between charged lipids that destabilizes the membrane. Since the membrane preferentially binds multivalent counterions over monovalent ones, multivalent counterions are more efficient, on a molar basis, than monovalent ones in reducing the lipid-lipid repulsion, counteracting the membrane destabilization. Moreover, multivalent counterions induce an attraction between lipids more efficiently than monovalent ones. When combined, these two effects lead to significantly enhanced membrane stability against pore growth and vesiculation; at low ionic strength, the charge fluctuation contribution dominates the repulsive term in the presence of a small concentration of multivalent cations. For sufficiently screened cases, the sum of the charge fluctuation term and the hydrophobic contribution outweighs the repulsive term γ_{DH}^* . Thus the membrane is stabilized by the presence of multivalent cations for the whole range of the concentration of monovalent ions. Also note that trivalent counterions are even more efficient in enhancing the membrane stability than the divalent counterions.

In the absence of multivalent cations, however, a relatively much higher concentration of monovalent salts is required to stabilize the membrane. The main effect of monovalent ions is simply to screen the electrostatic interactions via Debye screening, reducing the lipid-lipid repulsion; the affinity of monovalent cations for the membrane is relatively weak, and they do not lead to strong charge fluctuations, since a monovalent cation on the membrane surface tends to simply neutralize a neighboring lipid headgroup. Thus the binding of monovalent cations to the membrane has a relatively minor effect on the membrane stability. The resulting system is well in the mean-field regime as indicated by the results in Fig. 2, and small changes in n_1 would not lead to any significant effect on the membrane stability; as shown in the figure, the dramatic distinction between the monovalent and multivalent cases is missing in the DH theory. The enhanced membrane stability by multivalent counterions seen in the experiments [2,3] can be explained

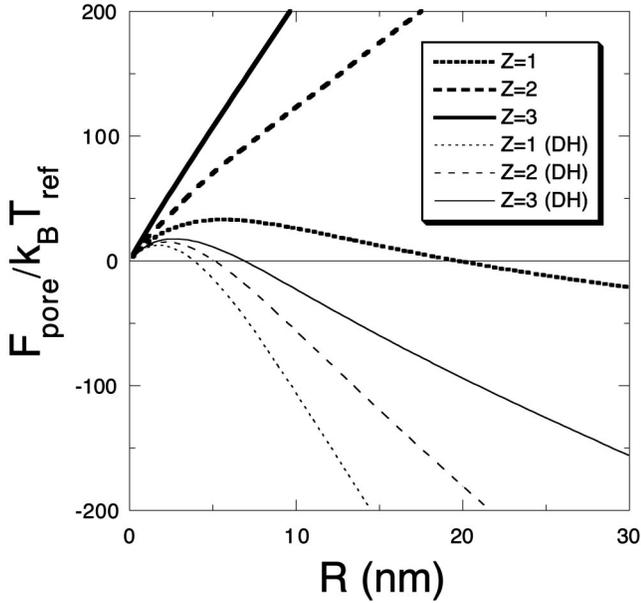


FIG. 3. Pore free energy as a function of the pore size R . We have chosen the same parameters as were used to generate Fig. 2. The pore free energy is estimated in units of $k_B T$. For the monovalent salt case ($Z=1$), the pore free energy has a finite barrier. In the presence of 0.1 mM of multivalent counterions ($Z=2,3$), the pore free energy grows *indefinitely* with R . In the DH approach, the barrier height is roughly *insensitive* to the counterion valency Z .

only when both the preferential adsorption of multivalent counterions and the effects of charge correlations are properly taken into account.

We have also found [18] that, in the presence of as small a concentration as 0.1 mM of multivalent cations, the membrane is stabilized against pore formation for the whole physical range of σ_0 , i.e., $0 < \sigma_0 < \sigma_0^{max}$, where $\sigma_0^{max} \sim 1/\text{nm}^2$ corresponds to a fully charged case; for weakly charged cases, the hydrophobic attraction mainly stabilizes the membrane, while the charge-fluctuation contribution is the main stabilizing effect for highly charged cases. In the absence of multivalent cations, however, there is always a finite range of n_1 over which the membrane is unstable to pore growth, as long as σ_0 is not too small.

Whether a pore grows or closes also depends on the height of the barrier as a function of the pore size R . In Fig. 3, we have plotted the pore free energy as a function of R , in units of $k_B T_{ref}$. We have chosen $T_{ref}=300$ K, $n_1=0.1$ mM, and $\sigma_0=0.2$ nm^{-2} . The barrier height is finite in the presence of monovalent ions only ($Z=1$). In contrast, the pore free energy in the presence of 0.1 mM of multivalent counterions ($Z=2,3$) grows *indefinitely* with R . This implies that the formation of a large pore is energetically greatly *disfavored* in the presence of multivalent counterions. The results are indeed consistent with the experimental observation that the presence of 0.1 mM of MgSO_4 stabilized the red blood cell ghosts against vesiculation [2]; a pore originally created by osmotic stress will grow into a large one or shut down, depending on the buffer quality and the strength of the restoring force provided by the spectrin net-

work [3]. Note that the osmotic stress will eventually be removed. When $Z=1$, the pore can grow into a large one once the barrier is overcome by osmotic stress. This will lead to vesiculation if the restoring force is outweighed by the repulsion between the charged groups on the red cell membranes. In contrast, there is a subsequent barrier to pore growth in the case $Z=2,3$. This prevents the ghosts from breaking into vesicles. In contrast, the DH approach mistakenly implies that the barrier height is roughly *insensitive* to the valency of counterions. Thus our results in Fig. 3 further support the importance of the counterion valency and charge correlations to the membrane stability, consistent with experiments [2,3].

The fact that multivalent cations can be preferentially adsorbed onto a charged surface implies that the layer of the adsorbed cations can be depleted by multivalent anions. Imagine an anion of valency Z_- making a pair with a cation in the condensed layer and leaving into the solution, as illustrated in Fig. 1(b). Whether this is feasible can be tested by calculating the change in the chemical potential: $\Delta\mu \sim -Z_+ Z_- \ell_B / (a_{2+} + a_{2-}) + Z_+ \ell_B \sigma^* \kappa^{-1} + \ln(n_{2-} a_{2-}^3 / n_{2+} a_{2+}^3)$, where the subscript 2 refers to multivalent ions and the subscripts + and - refer to the cations and anions, respectively. When the valency of anions is sufficiently high, this change can be negative. This implies that a certain fraction of the cations in the layer make pairs with multivalent anions and will return to the solution to maintain “chemical equilibrium,” reducing the membrane stability. Cells contain multivalent cations (e.g., Ca^{2+} and Mg^{2+}) as well as multivalent anions (e.g., PO_4^{3-} and anionic proteins) and thus could regulate their stability in this way. This may provide a new insight into the biological phenomena of “breakdown” and “reassembly” of a nuclear envelope during cell division. During cell division, a nuclear envelope disintegrates into vesicles, which eventually reassemble into daughter cells [1]. This is suggestive of the cyclic change in the membrane stability: stable \rightarrow unstable \rightarrow stable. The stabilization and destabilization of membranes by multivalent ions could be relevant to this flow of membrane stability in a dividing cell. This is, however, complicated by various membrane-bounded proteins [1] and further consideration is certainly warranted.

To summarize, we have presented a working mechanism for the stabilization and destabilization of membranes by multivalent ions. Multivalent cations preferentially adsorbed onto a charged membrane not only reduce the surface charge density of the membrane but also induce a barrier to pore growth that favors the closure of a pore. The main advantage of the membrane stabilization by multivalent cations lies in that this can be easily reversed; the addition of a small concentration of multivalent anions can reverse the membrane stabilization. Our results are also consistent with the experimental findings that membrane adhesion by multivalent cations does not necessarily lead to membrane fusion and that membrane fusion (in the absence of fusion peptides) should be followed by lateral phase separation of the lipid molecules into two distinct phases [19]: anionic lipid-poor phases and anionic lipid-rich phases “coated” with multivalent cations. It is the *uncoated* phases that undergo a topological change

and eventually fuse into each other, consistent with our picture of multivalent cations as efficient agents for stabilizing membranes against rupture.

Naturally occurring membrane systems contain a large concentration of monovalent ions (~ 100 mM corresponding to $\kappa^{-1} \sim 10$ Å) as well as a small concentration of multivalent ions. Thus the electrostatic interactions are more effectively screened in these cases than in typical red cell experiments [2–4]. Our simple approach to counterion condensation is not well suited to sufficiently screened cases, since it ignores the effects of charge fluctuations and the discrete nature of ions on the affinity of counterions for an oppositely charged surface—note that these effects become important for screened cases. More careful consideration [18] of these additional factors shows that the repulsion between lipids is minor but the charge-fluctuation effect remains important as long as $\lambda_{cc} < \kappa^{-1}$, as is often the case. In typical biological settings, a relatively small osmotic balance is maintained across membranes, since both the inside and outside of the membranes carry comparable concentrations of ions. There can be, however, other effects that destabilize the membrane. One such example is the transmembrane po-

tential maintained across the membranes and membrane proteins [10,11]. It should be noted here that multivalent counterions influence the transmembrane potential and the affinity of these proteins for the membranes much more efficiently, on a molar basis, than monovalent ones, since they preferentially adsorb onto the membrane. Thus the regulation of membrane stability in real cases can still be accomplished by counterion valency as in the red cell experiments even though the destabilizing effect is different there. So the adsorption and desorption of multivalent counterions can lead to dramatic effects on membrane stability. Further experimental studies that involve changes in the concentration of multivalent ions and/or the transmembrane potential are necessary to further unravel the intriguing roles of multivalent ions in membrane stability. We believe that our charge fluctuation theory presented here will stimulate further investigation along this line.

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