Electrostatic cluster formation in lipid monolayers

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We study phase separation in mixed monolayers of neutral and highly negatively charged lipids, induced by the addition of divalent positively charged counterions. We find good agreement between experiments on mixtures of PIP₂ and SOPC and simulations of a simplified model in which only the essential electrostatic interactions are retained. Thus, our results support an interpretation of PIP₂ clustering as governed primarily by electrostatic interactions, in which divalent ions such as calcium mediate an effective attraction between like-charged lipids. Surprisingly, the mediated attractions are strong enough to give nearly complete phase separation, so that clusters can even form when the overall concentration of PIP₂ is low, as is the case in the cell membrane.

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One of the truisms of biology is that electrostatics play a relatively unimportant role in determining structure on scales above the Debye screening length, which is on the nanometer scale. Highly charged biomolecules such as DNA [¹] and actin [²] form an exception to this rule by aggregating into large bundles in the presence of multivalent ions. Another biomolecule that carries a very high negative charge density is the membrane lipid PIP₂. Despite the fact that its concentration in the membrane is extremely low (of order 1%), this lipid plays a critical role in many processes involving the cell membrane, including cell division [³], exchange of chemicals with the environment through endocytosis and exocytosis [⁴], and cell motility [⁵]. Evidence exists that PIP₂ forms clusters [⁶] at the sub-micron scale under roughly physiological conditions. It has been conjectured that this clustering is crucial to its effectiveness at such low overall concentration [⁷,⁸].

Various mechanisms for the clustering have been proposed, including PIP₂-protein interactions [⁹,¹⁰], exclusion from cholesterol-enriched ordered domains [¹¹,¹²], and hydrogen bonds [¹²,¹³]. However, recent experiments showed that PIP₂-clusters are induced simply by adding calcium or other divalent ions [⁶,¹⁴]. This raises the question of whether a purely electrostatic, counterion-mediated mechanism is consistent with experimental observations of calcium-induced PIP₂-clustering.

In this letter, we study ion-induced clustering of negatively charged lipids as a function of lipid charge and ion properties, both numerically and experimentally. We conduct simulations on a model designed to retain only the most critical features of the electrostatics and compare the results to experiments on Langmuir monolayers of a mixture of PIP₂ with neutral lipids with added divalent salts. We find semiquantitative agreement between the simulations and experiments, with similar trends for the dependence on lipid charge and ion size, suggesting that multivalent-ion-mediated attractions are indeed responsible for the observed clustering.

Counterion-mediated attractions are the collective result of a near-cancellation of repulsive and attractive interactions between like and unlike charges, respectively, in strongly correlated electrostatic systems. They are not captured in mean-field theory [¹⁵] and are typically quite weak [¹⁶]. Here, we show that counterion-mediated attractions are surprisingly strong so that phase separation is nearly complete even at reasonable values of the PIP₂ charge, implying that clustering can occur even at very low PIP₂ concentration.

Experiments—We look for phase separation using visual analysis of fluorescence micrographs of mixed lipid monolayers prepared in a Langmuir trough (Kibron, Inc.) and imaged on an inverted epifluorescence microscope. We use a molar fraction $\phi_{\text{PIP}}$ of L-α-phosphatidylinositol-4,5-bisphosphate (PIP₂, Avanti) in a monolayer otherwise consisting of 1-stearoyl-2-oleoyl phosphatidylcholine (SOPC, Avanti). SOPC is zwitterionic, and known to be net-neutral over a wide range of pH. Part of the PIP₂ (0.5 mol% of the total lipid content) is replaced by a fluorescently labeled analogue (Bodipy FL-PIP₂, Echelon, Inc.). A lipid monolayer is formed on a buffered sub-phase (10 mM HEPES, 100 µM EDTA, 5 mM DTT) by addition of the lipids dissolved in a 2:1 chloroform/methanol mixture to the air-water interface. After formation, the monolayer is visualized by epifluorescence to determine a baseline frequency of structural inhomogeneity. Divalent salts CaCl₂ or MgCl₂ were added at 1 mM to the sub-phase, followed by gentle mixing to avoid disrupting the monolayer. We image the monolayers about 50 minutes after cation addition to allow sufficient time for domain
coarsening. The existence of bright spots at a higher frequency than baseline serves to determine the existence of PIP2-rich clusters.

We perform this procedure for a range of $\phi_{\text{PIP}}$-values and several pH values: 3, 4, 5, 6, 7, 4, 9. At these values of the pH, $q_{\text{PIP}}$ is roughly $-1.5$, $-2.7$, $-3.2$, $-4.2$, $-5.0$, respectively, based on acid dissociation constants from Ref. [17]. However, because the ionization state of PIP2 may be influenced by various geometric and chemical factors [17], we do not assume that these $q_{\text{PIP}}$ values are exact for our system.

**Simulations**—We retain only the competition between electrostatic interactions and excluded volume repulsions by adopting a model in which both lipids and small ions are represented as charged spheres (radius $\sigma$) with an excluded volume interaction given by the purely repulsive (truncated at its minimum and shifted) Lennard-Jones potential (the WCA potential [18]). Parametrized by an energy scale $\epsilon = k_B T \equiv 1$ (our unit of energy) and length scale $\sigma_{ij} = R_i + R_j$, this potential takes the following form as a function of center-to-center distance $r_{ij}$,

$$V_{\text{WCA},ij}(r_{ij}) = 4\epsilon \left[ \left( \frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left( \frac{\sigma_{ij}}{r_{ij}} \right)^{6} + \frac{1}{4} \right],$$

for $r_{ij} < 2^{1/6}\sigma_{ij}$, and $V(r_{ij}) = 0$ otherwise. Note that $\sigma_{ij}$ is the distance at which the potential equals $k_B T$. $N = 1600$ lipid particles are confined to the $z = 0$ plane, to mimic the effect of the hydrophobic interaction that keeps them at the air-water interface. We use $R_i = R_L = 3$ Å for the lipids and $R_i = R_{\text{CI}} = 2$ Å for the small cations that are allowed to explore the entire simulation box. In a study of the dependence of the clustering on cation size, we vary it in the range 0.5 Å $\leq R_{\text{CI}} \leq 2.5$ Å. The box is periodic in $x$- and $y$-directions (size $L_x = 320 \times L_y = 320$ Å) and has hard walls at $z = 0$ and $z = L_z = 200$ Å.

The typical distance between lipids in the monolayer at $z = 0$ is therefore 8 Å.

In addition, the charged spheres interact via the Coulomb interaction, $V_{C,ij} = q_i q_j / r_{ij}$, where we measure charges $q$ in units of the proton charge. In room temperature water the Bjerrum length $l_B \approx 7$ Å.

We run molecular dynamics simulations using LAMMPS [19], with a Nosé-Hoover thermostat [20] and PPPM for the long range Coulomb interactions [21].

The strong Coulomb attraction between the anionic lipids and the small cations allows them to bind at a distance of roughly $\sigma_{ij}$. The essence of ion-mediated attractions is that these bonds are strong and long-lived enough so that one or two counterions can draw together two lipids and be bound to both simultaneously [22]. Due to its coarse-grained nature, our model underestimates the binding energy of such bonds in two ways. Firstly, in real PIP2 the negative charges are localized mainly in phosphate groups that lie close to the surface of the molecule, so that the distance between the phosphate groups and the cations is much closer than the typical 5 Å allowed by our “spherical” lipids. To determine the effect of the inter-charge distance on the binding energy, we performed two test calculations with two “lipids” ($q_{\text{PIP}} = -4$) and four divalent ions. We found that the binding energy is 2.2 times larger for a structured lipid with two charges ($q = -2$) about 4 Å apart, than for the spherical lipids in our model. Secondly, Ca$^{2+}$ can be expected to lose some of its hydration shell when it binds to a phosphate group, so that the effect of water as a dielectric medium is partially eliminated. This further increases the net binding energies by an unknown amount. As an estimate, we assume the binding energies produced by our model interaction are a factor of 3 lower than those in the real system and compensate for this by using a dielectric constant lower by a factor of 3; i.e., we

FIG. 1: Snapshots of the experiment (a) and simulation (b) at $\phi_{\text{PIP}} = 25\%$. (a) The (inverted) epifluorescence micrograph, taken 50 minutes after mixing 1 mM CaCl$_2$ into the subphase (pH 7.4). PIP2-rich domains are shown as very dark green spots. (b) The simulation (PIP2-charge $q_{\text{PIP}} = -4$, divalent ion radius $R_{\text{CI}} = 2$ Å) after 3.5 ps of coarsening. Charged and neutral lipids are drawn green and light grey, respectively, and divalent ions that are close to the lipid monolayer are plotted in dark red. (c) Strength (shaded contours) and direction (streamlines) of the electric field around a string-like domain (geometry taken from the simulation), illustrating that further growth of the domain is likely to occur at the end.

- Periodic in $x$- and $y$-directions (size $L_x = 320 \times L_y = 320$ Å) and has hard walls at $z = 0$ and $z = L_z = 200$ Å.

- The observed bright spots occur at a higher frequency than baseline.

- The simulation shows that PIP2-rich clusters exist.

- Electrostatic interactions and excluded volume repulsions are modeled using a Lennard-Jones potential.

- The model uses charged spheres with a particular radius and an attractive term that is truncated at its minimum and shifted.

- The simulation is performed using LAMMPS with a Nosé-Hoover thermostat and PPPM for the long range Coulomb interactions.

- The binding energy of ion-mediated attractions is estimated to be a factor of 3 lower than in real systems due to the effect of water as a dielectric medium.
use a Bjerrum length of 21 Å.

Results—At high PIP2-charge, for example at pH 7.4 in the experiment, where \(q_{\text{PIP}} \approx -4.2\), or at \(q_{\text{PIP}} = -4\) in the simulation, cluster formation is readily observed (Fig. 1). The experimental image (Fig. 1a) shows the larger-scale picture of bright fluorescent spots marking the regions of large PIP2-concentration, while the simulation image (Fig. 1b) shows still growing clusters at a length scale that is 1000 times smaller, after 3.5 ps of simulation time. As expected, the positions of the condensed calcium ions (red discs in Fig. 1b) clearly indicate their role in binding the charged lipids (green discs) together.

The morphology observed in the early stages of coarsening in the simulations illustrates some particular features of ion-mediated attractions that set them apart from simple attractive potentials. As can be seen in Fig. 1b, the PIP2-rich clusters are often irregularly shaped, and even string-like. This occurs because the attraction is the net result of strong attractions (PIP2-Ca\(^{2+}\)) and strong repulsions (PIP2-PIP2 and Ca\(^{2+}\)-Ca\(^{2+}\)) that can both be several tens of \(k_B T\). Hence, a rearrangement of the lipids in a cluster typically involves energy barriers that are much higher than the net attraction energies, so that evolution towards more compact shapes is severely hindered kinetically. In the earliest stages of coarsening, most domains are string-like, because for very small clusters such linear arrangements have the lowest Coulomb energy. As the domains grow, compact shapes become energetically favorable but are difficult to reach for two reasons. First, once there is a string-like cluster, the electric field in its neighborhood is focused towards the end of the string (see Fig. 1b), which makes it more likely for the next lipid to bind at the end, thus extending the string. Second, the energy barrier for the string to fold onto itself is quite high. Therefore, string-like domains can persist even in the later stages of coarsening, as seen for example in the bottom left of Fig. 1b. Such irregular domains have been seen experimentally [8, 9].

To determine the conditions under which cluster formation occurs, a grid of parameter values (\(\phi_{\text{PIP}}, q_{\text{PIP}}\)) was explored. Experimentally, we identify which images have more bright spots than the baseline level (which we found to be at most two spots per frame). The resulting phase diagram is shown in Fig. 2a, where the region of cluster formation is shaded. In the simulations, we follow the coarsening dynamics by keeping track of the static structure factor of the charged lipids,

\[
S(k) = \frac{1}{N} \sum_{i,j} \exp[i k \cdot (r_i - r_j)],
\]

where \(N\) is the number of PIP2-particles. As a function of \(k \equiv |k|\), a maximum in this function at \(k = k_{\text{peak}}\) indicates that the PIP2-particles are developing structure at a length scale \(2\pi/k_{\text{peak}}\). For the more pronounced cases of cluster formation (deep in the phase-separated regime), we followed this peak as a function of time and verified that it scales with time as \(k_{\text{peak}} \sim t^{-1/3}\), consistent with the general theory of coarsening of a binary fluid mixture [23]. Thus, even though the counterion-mediated origin of phase separation yields irregularly shaped clusters instead of circular ones, this does not seem to affect the kinetics of coarsening. In the phase diagram in Fig. 2a, all parameter values (\(\phi_{\text{PIP}}, q_{\text{PIP}}\)) for which an appreciable peak appears that approaches \(k_{\text{peak}} = 0\) in \(S(k)\) for long times were marked as cluster-forming (within the coexistence region). Both in the experiment and simulation, we found that divalent cations cause phase separation provided the lipid charge is high enough (pH 4.5 or higher in experiment, \(q_{\text{PIP}} \leq -2\) in simulation). Monovalent cations were never seen to induce clusters.

Larger divalent ions than Ca\(^{2+}\) should mediate weaker attractions, because larger binding distances imply lower Coulomb energies. This effect should manifest itself in a higher charge on the PIP2 needed to obtain cluster formation with larger ions. We verified this in experiments.
using Mg$^{2+}$, which is known to have a larger hydrated radius than Ca$^{2+}$, even though precise values appear to be lacking [25]. We tentatively indicate this size difference in Fig. 3a. In agreement with this observation and the theoretical expectation, the ability of divalent cations to drive cluster formation in our simulations also decreases with increasing ion size (Fig. 3b). A subtle effect that could influence the effective size of the ions is the level of dehydration that occurs: the more of the hydration shell is removed, the closer and hence stronger the binding. We have therefore refrained from attempting to assign numerical values to the radii of the cations in Fig. 3a.

Discussion—The phase diagram of our model compares surprisingly well with our experiments without any parameter optimization. The only free parameter is the dielectric correction factor we apply to compensate for the underestimation of the mediated binding energy, which we set to 1 [9] and experiment at all parameter values studied, surface pressures were of order 10 to 30 mN/m and dropped by several mN/m upon addition of Ca$^{2+}$ (data not shown).

It should be noted that, while hydrogen bonds between the PIP$_2$-molecules exist and may play a role when the charges are small [12], our work strongly suggests that they do not play a dominant role in multivalent ion-induced clustering — if they did, having a higher PIP$_2$-charge would make it harder to form clusters, rather than easier, as we report in Fig. 2.

Since the interactions in our model have been stripped down to the bare minimum of electrostatics and steric repulsion, the only attractive interaction in the simulations is the Coulomb attraction between the anionic lipids and the divalent cations. Therefore, the observed phase separation must be due to cation-mediated attractions. The fact that these attractions are the result of near-cancellation of even larger attractions and repulsions leads to several special features. We have already mentioned the high energy barriers for lipid rearrangements that lead to long lived irregular or string-like domain shapes in our simulations. Such domains are observed experimentally [14] at pH values close the physiological value (where $q_{\text{tip}}$ is high). It is therefore possible that stringlike clusters appear in biological contexts.

Another striking aspect of ion-mediated attractions is the strong dependence of the effective attraction strength on the lipid charge. To illustrate this point, we numerically calculated the binding energy per lipid for a cluster of 30 lipids [26], and found that it increases from 3$k_B T$ at $q_{\text{tip}} = -2$ to 6$k_B T$ at $q_{\text{tip}} = -3$ and 11$k_B T$ at $q_{\text{tip}} = -5$. At $q_{\text{tip}} = -2$ phase separation first appears, and at $q_{\text{tip}} = -3$ it is already strong enough to lead to nearly complete phase separation (see Fig. 2). This has the potentially important biological consequence that clusters can form even at the extremely low concentrations of PIP$_2$ (~1%) found in the cell membrane.

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[25] The reported hydrated radii vary from source to source, mainly due to different methods to determine it, but the literature consistently reports Mg$^{2+}$ to be larger (3 to 7 Å) than Ca$^{2+}$ (2.6 to 6.3 Å) [24].
[26] The reference state for this binding energy is the state in which they form 15 lipid dimers, neutralized with Ca$^{2+}$, so that we can consider charge neutral clusters and monopole terms will not dominate the result.