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Path Dependence of Three-Phase or Two-Phase Endpoints in Fluid Binary Lipid Mixtures

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Abstract

The phase behavior of anionic/zwitterionic mixtures can be controlled by tuning the charge state of the anionic lipid. In the case of dioleoylphosphatidic acid (DOPA)/dioleoylphosphatidylcholine (DOPC) mixtures, demixing occurs either when DOPA is protonated or when $\text{DOPA}^{2-}:\text{Ca}^{2+}$ complexes form. Herein it will be shown that the final endpoint, a three-phase or two-phase system, depends on the order in which the charge state is manipulated. The facile accessibility of different endpoints is a clear demonstration of the inherent flexibility of biological systems.

Introduction

Surfaces can acquire charge by two processes: (i) the ionization of surface groups, e.g. by dissociation of protons, or (ii) the adsorption of ions, such as calcium.¹ These processes depend on both the surface charge density (σ) and the surface potential (ψ_0).^{1–3} As such, they are sensitive to bulk solution properties, including pH, ion concentration, valency and type (Hoffmeister effect). Consequently there are a myriad of routes by which the charge on a surface can be modulated. The manipulation of surface charge density by changing solution conditions has been observed in a wide variety of systems, including (but not limited to): weak polyelectrolyte brushes,⁴ proteins,^{5–8} phospholipid bilayers,^{9,10} bacterial cell surfaces,¹¹ glass,¹² metal oxide surfaces,¹³ and polymers interacting with surfaces.¹⁴

Regulation of surface charge controls adsorption processes, and in the case of multi-component fluid surfaces such as membranes, it can also control the miscibility.^{3,15–22} In an ideal solution mixing is driven solely by entropy. If the intermolecular interactions in the pure liquids are different, the mixture is non-ideal, and both entropic and energetic factors contribute to miscibility.^{23,24} For example, in a binary mixture of molecules A and B, phase separation can occur if A-A or B-B interactions are significantly stronger than A-B interactions (i.e. oil and water). By tuning the charge on one of the components, the interactions strengths are altered; this in turn can switch the system from a mixed to demixed state, or vice versa.

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We have previously shown that this is the case in the binary mixture of the phospholipids DOPA (1,2-dioleoyl-*sn*-glycero-3-phosphate) and DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine).⁹ DOPC is zwitterionic, while DOPA has two acidic protons (pK_a values 2.5, 2.6 ~3 and 8). Over a pH range of 5 – 8 (and at room temperature), DOPA carries a charge when the bulk ion concentration is high, and electrostatic repulsion drives DOPA to mix uniformly with DOPC.^{9,10} However, when the bulk ion concentration is lowered at pH 5, DOPA protonates and phase separates from DOPC.⁹ The observed protonation is consistent with predictions from Poisson-Boltzmann theory,² and the phase separation is not surprising considering the melting temperatures of DOPA and DOPC are $-8\text{ }^{\circ}\text{C}$ and $-20\text{ }^{\circ}\text{C}$, respectively.²⁷ The higher melting temperature of DOPA implies that PA-PA interactions are stronger than PC-PC interactions, which could be due to hydrogen bonding among PA headgroups.²⁰ Thus it is expected that when DOPA is neutral (fully protonated) it will not mix ideally with DOPC. These results have also been seen in binary mixtures of egg PA:egg PC and DOPA:POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), further indicating that the observed phase behavior is headgroup mediated (Cambera LR, Lamberson ER, and Hovis JH, unpublished results). Kinetic analysis has shown that the phase separation proceeds through a nucleation process, fluorescence recovery after photobleaching (FRAP) and attenuated total reflection – Fourier transform infrared spectroscopy (ATR-FTIR) experiments have confirmed that the phase separation is fluid-fluid, and as far as we have observed, the demixing is irreversible.⁹

Another way to tune the surface charge density and mixing behavior of membranes is through the adsorption of multivalent ions such as calcium.^{3,10,28–39} We have previously demonstrated that in mixtures of DOPA/DOPC at pH 7, calcium binds to DOPA and forms a 1:1 $\text{DOPA}^{2-}:\text{Ca}^{2+}$ complex which demixes from DOPC. (It is likely calcium also forms a 2:1 $\text{DOPA}^{1-}:\text{Ca}^{2+}$ complex; however, this species remains miscible with DOPC.)¹⁰ FRAP experiments have confirmed that this phase separation is also fluid-fluid, unlike the gel-fluid or cocholeate-fluid phase separation typically reported for PA/PC systems.^{3,28–32,34–38} Complex formation is sensitive to the bulk electrostatic environment and can be enhanced (leading to more extensive phase separation) by increasing the pH, increasing the calcium concentration, or decreasing the monovalent ion concentration. Individually these variations are straightforward to explain: At higher pH, the surface charge density is greater, and more DOPA^{2-} ions are available for complex formation. The same is true when calcium concentrations rise. The observation that phase separation is more extensive at lower monovalent concentrations seems contrary to what is predicted by Poisson-Boltzmann theory, since decreasing the monovalent concentration decreases the overall ionic strength of the bulk solution and should lower the surface charge density. However, Poisson-Boltzmann theory does not take into account the specific adsorption of ions. As the monovalent ion concentration decreases in the presence of calcium, the binding constant of calcium increases and more immiscible $\text{DOPA}^{2-}:\text{Ca}^{2+}$ complexes form. Phenomenologically, a linear relationship is observed between the area coverage (extent of phase separation) and the $\text{K}^+:\text{Ca}^{2+}$ ratio.

To this point we have demonstrated two ways to create an immiscible DOPA species (protonated (DOPA^0) or $\text{DOPA}^{2-}:\text{Ca}^{2+}$) that switches the binary mixture of DOPA/DOPC

from a uniform one-phase system to a two-phase system. In all cases, it has not been possible to reverse the phase separation and force the components to remix. Irreversibility implies memory and history-dependence; therefore we investigated the behavior of the bilayer when both DOPA⁰-rich and DOPA²⁻:Ca²⁺-rich phases were created. Herein it will be shown that a binary lipid mixture can have two endpoints, two-phase or three-phase coexistence, depending on the order in which the solution conditions are manipulated.

Experimental Methods

Materials

Chloroform stock solutions of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA), and 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphocholine (NBD PC) were purchased from Avanti Polar Lipids, Inc. and used without further purification. CoverWell™ perfusion chamber gaskets were purchased from Invitrogen-Molecular Probes, Inc. Glass coverslips, 22 × 30 #1.5 were purchased from Fisher Scientific, and ICN 7X detergent was purchased from MP Biomedicals, Inc. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and MES (2-(*N*-morpholino)ethanesulfonic acid hydrate) were purchased from Sigma Chemical Co. Potassium chloride (KCl), potassium hydroxide (KOH), and calcium chloride dihydrate (CaCl₂) were purchased from Mallinckrodt Chemicals. Alexa Fluor 647 carboxylic acid, succinimidyl ester was purchased from Invitrogen. The Superdex 200 gel-filtration column was purchased from GE Healthcare Life Sciences, and the Microcon YM-100 centrifugal filter units were obtained from Millipore. All buffers were prepared using 18 MΩ-cm water.

Vesicle and supported lipid bilayer preparation

Large unilamellar vesicles (LUVs) were prepared by the extrusion method. Briefly, the chloroform solvated lipids were mixed to give a 30:69:1 mol ratio DOPA:DOPC:NBD PC, dried under nitrogen, and held under vacuum for 1 hour. The dried lipids were rehydrated in a 50 mM MES, 250 mM KCl buffer, pH 5 (pH adjusted with concentrated KOH), and the lipid suspension was extruded 21 times through a polycarbonate membrane with 50 nm pores. Following extrusion, the LUV solution was centrifuged for 5 min at 14,000 rpm (Eppendorf Minispin Plus). The extruded vesicles were stored at 20 °C, shielded from light, and used within 12 hr. Supported lipid bilayers were formed by vesicle fusion inside a 60 μL perfusion chamber on a glass slide that had been washed in hot, dilute ICN 7X detergent, rinsed extensively in 18 MΩ-cm water, and baked at 450 °C for 4 hrs (slides were used within a day of preparation). After 5 min, excess vesicles were removed by flushing the perfusion chamber with 3 mL of the same buffer used in vesicle preparation. The bulk solution was then exchanged with 3 mL of a buffer composed of 50 mM HEPES and 250 mM KCl, pH 7.4 or 8.0. Bulk monovalent and divalent ion concentrations were changed by rinsing the perfusion chamber with 1 mL of an exchange buffer composed of 50 mM HEPES, 0–250 mM KCl and 0–5 mM CaCl₂, pH 7.4 or 8.0. α-Synuclein binding was observed using 0.26 μM protein in a buffer of appropriate KCl concentration and pH. Complications resulted from the interaction of α-synuclein with bulk calcium; therefore the

bulk calcium concentration was always dropped to 0 mM before the buffer containing α -synuclein was flushed into the perfusion chamber.

Calcium Contamination Analysis

Calcium contamination was identified in the chloroform stock solution of DOPA and in the KCl used to prepare buffers. Flame Atomic Absorption analysis of the DOPA stock solution, performed by Galbraith Laboratories, Inc. indicated that a 25 mg/mL sample contained <2.0 mg/L calcium, corresponding to a DOPA:calcium ratio of at least 700:1. The calcium contamination in the KCl was listed by Mallinckrodt Chemicals as <0.001%. Due to the excessive rinsing to change the buffer conditions, it is difficult to say exactly how much calcium was present at any given instant. If we assume that calcium is not strongly associated with the lipid bilayer when the bulk monovalent concentration is 250 mM KCl, then the calcium introduced by the first 6 mL of buffer rinsing can be ignored. A 1 mL solution of 25 mM KCl (the rinsing volume used to exchange the solution) contains less than $\sim 1.5 \times 10^{14}$ calcium ions, or ~ 6 calcium ions per DOPA molecule, while a 1 mL solution of 0.25 mM CaCl_2 contains $\sim 1.5 \times 10^{17}$ calcium ions, or ~ 6000 calcium ions per DOPA molecule. A 60 μL solution of 25 mM KCl (the total volume of the perfusion chamber) contains less than $\sim 9.0 \times 10^{12}$ calcium ions, or ~ 0.34 calcium ions per DOPA molecule, while a 60 μL solution of 0.25 mM CaCl_2 contains $\sim 9.0 \times 10^{15}$ calcium ions, or ~ 340 calcium ions per DOPA molecule. It was therefore concluded that the calcium contamination was insignificant because the system required three orders of magnitude more calcium to produce the large scale phase separation reported in this paper.

Protein preparation

The protein was expressed in *Escherichia coli* and purified as described previously.⁴⁰ It was preferentially labeled at the N-terminus: monomeric α -synuclein (0.56 mM in 225 mL PBS, pH 7.0) was mixed with Alexa Fluor 647 carboxylic acid, succinimidyl ester (0.25 mg in 25 mL dimethyl sulfoxide (DMSO)). The mixture was placed on a Microplate shaker at 800 rpm for 2 hr. The protein solution was then loaded onto a Superdex 200 gel-filtration column and eluted in 20 mM HEPES, pH 7.4. Protein concentration and degree of labeling were determined using ultraviolet-visible absorbance measurements (the degree of labeling of the protein was typically 20%, mol/mol). The protein was then aliquoted and stored at -20°C . Before each experiment an aliquot was thawed and centrifuged through a Microcon YM-100 centrifugal filter unit with a molecular mass cutoff of 100 kDa to remove any aggregates. For binding experiments, the concentrated protein eluted in HEPES, pH 7.4 was diluted 100-fold in HEPES buffer at pH 7.4 or 8 and used immediately.

Imaging of the supported lipid bilayer and protein

A Nikon TE2000U fluorescence microscope equipped with a Cascade 512B CCD camera (Roper Scientific) was used to image the bilayers. An X-Cite 120 arc lamp (EXFO) was used as a light source. The NBD and Alexa fluorophores were imaged using appropriate filter cube sets (Chroma Technology Corp.) and a 40 $\times$, 1.30 NA oil-immersion objective.

Results and Discussion

The experiments discussed below were performed at room temperature using supported bilayers composed of 30:69:1 mol ratio DOPA:DOPC:NBD PC (1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphocholine, tail-labeled). The bilayers were formed on glass coverslips by vesicle fusion in a 250 mM KCl, 50 mM MES buffer, pH 5. After excess vesicles were rinsed away, the bulk solution was exchanged for either a pH 7.4 or 8 buffer containing 250 mM KCl and 50 mM HEPES. The bilayers were imaged using epifluorescence microscopy, and the mobility of each phase was verified by FRAP. All regions recovered on timescales consistent with fluid phase bilayers. The images shown below have been contrasted for clarity; the intensity difference between the DOPC-rich (light) phase and the DOPA-rich (dark) phase ranged from 13–20%. The phase separations reported below are highly reproducible, with no deviance from the reported behavior observed in > 10 samples each.

As mentioned previously, two ways to cause a fluid binary mixture of DOPA/DOPC to phase separate have been demonstrated; each of these methods results in a two phase endpoint. However, the chemical identity of the DOPA-rich phase is different depending on the end conditions of the bulk solution, indicating that it may be possible to cause the DOPA/DOPC system to reach a three-phase endpoint. Figure 1 is a caricature qualitatively illustrating a method by which this three-phase endpoint could be reached. (This method is based on the experimental data reported herein, but for the sake of clarity the method will be presented first.) The starting point is a bulk solution with a high monovalent ion concentration to ensure that DOPA and DOPC mix uniformly (Figure 1A, D). In this electrostatically screened environment, the pK_{a1} of DOPA is ~ 3 and the pK_{a2} is ~ 8.17 . At any pH greater than ~ 3 , most of the DOPA molecules carry a charge, and electrostatic repulsion makes it energetically unfavorable for DOPA to cluster and separate from DOPC.

The first step is to create a DOPA⁰-rich phase by lowering the bulk monovalent ion concentration in the absence of calcium, Figure 1(B, E). Poisson-Boltzmann theory predicts that the charge of an ionizable surface group, such as a lipid headgroup, is sensitive to the bulk ionic strength. If the bulk ionic strength is lowered, the screening distance decreases and the surface potential increases. DOPA protonates to reduce electrostatic repulsion and DOPA⁰ demixes from DOPC. We have previously observed this behavior at pH 5 and herein will show that it also occurs at pH values up to at least 8. This observation is noteworthy because it demonstrates the relative nature of pK_a values: even though the bulk solution is several pH units above the nominal pK_{a1} of DOPA, lowering the bulk ionic strength still forces DOPA to protonate, resulting in phase separation. A decrease in the area coverage of the DOPA⁰-rich phase is observed as we go from pH 5 to pH 8 (unpublished data), which is consistent with the idea that although DOPA does protonate at pH values far above the pK_{a1} , it becomes increasingly difficult.

The final step to reaching a three-phase endpoint is to introduce calcium to the bulk solution. The interaction of calcium with the lipids on the surface is governed by the surface potentials it encounters. One scenario is illustrated in Figure 1(B,C). If the DOPA in the DOPA⁰-rich phase remains protonated after calcium is introduced, it should have a low

surface potential. However, what DOPA is left in the DOPC-rich phase still carries a charge, giving the DOPC-rich phase an appreciable surface charge density (σ_{PC}) and surface potential (ψ_{PC}). The binding constant of calcium can be written as a function of surface potential (ψ_o):

$$K_{Me} = K_{Meo} \exp(2e\psi_o/kT) \quad (1)$$

where K_{Meo} is the intrinsic binding constant.³ If the surface potential of the DOPC-rich region is greater than that of the DOPA⁰-rich region, then it is expected that calcium will bind more strongly to the DOPA in the former. As mentioned previously, calcium will form an immiscible DOPA²⁻:Ca²⁺ complex, which gives rise to a new phase separation. The end result is a system with three phases: DOPC-rich, DOPA⁰-rich, and DOPA²⁻:Ca²⁺-rich. (Figure 1C).

In order to reach a three-phase endpoint, the DOPA in the DOPA⁰-rich phase must remain largely protonated after calcium is introduced. This behavior is more likely to occur at lower bulk pH values. At higher bulk pH values, it is possible that the addition of calcium will mitigate the driving force for protonation enough that the DOPA in the DOPA⁰-rich phase will deprotonate and regain some charge. The DOPA in the DOPA⁰-rich phase can acquire charge without remixing with the DOPC-rich phase due to the irreversible nature of this phase separation.^{9,10} If the surface charge density, and therefore surface potential, in the DOPA⁰-rich phase is greater than or equal to that in the DOPC-rich phase, then calcium should be able to bind to the DOPA to a similar extent in both the DOPA⁰-rich and DOPC-rich phases (Figure 1E). Thus the addition of calcium would simultaneously (i) convert the DOPA⁰-rich phase to a DOPA²⁻:Ca²⁺-rich phase, and (ii) interact with the DOPA in the DOPC-rich region to create DOPA²⁻:Ca²⁺ complexes, which would also phase separate. The end result is an extensive separation into two phases (Figure 1F).

We have experimentally observed both of the scenarios described in Figure 1. At 25 mM KCl (where these experiments were carried out), the transition from a two-phase endpoint to a three-phase endpoint is ~pH 7.6–7.8. We therefore show an example of a three-phase endpoint at pH 7.4 (Figure 2) and a two-phase endpoint at pH 8 (Figure 3). At pH 7.4, dropping the monovalent ion concentration from 250 mM to 25 mM KCl caused a uniformly mixed bilayer to phase separate, forming a DOPC-rich phase and a DOPA⁰-rich phase (Figure 2A). When the divalent ion concentration was subsequently increased from 0 to 1 mM CaCl₂ (keeping the KCl concentration at 25 mM, pH 7.4), a new phase appeared (Figure 2B). Calcium increases the charge of DOPA in the DOPC-rich phase, and interacts with it to form an immiscible DOPA²⁻:Ca²⁺ species. We therefore assigned the newly created phase to be rich in DOPA²⁻:Ca²⁺.

To verify that the two DOPA-rich phases are chemically distinct, we examined the binding of α -synuclein to the different regions of the bilayer. (The protein was labeled on the N-terminus with Alexa 647.) α -Synuclein binding is environment sensitive, and thus can be used as a surface chemistry sensor.⁹ When 0.26 μ M α -synuclein was added to the system it preferentially bound to the region which was (at least initially) DOPA⁰-rich (Figure 2C). Line scans of the regions indicated in Figure 2A–C are shown in Figure 2D. The regions

where there was initial phase separation in Figure 2A (red dashed line) correspond to the most intense protein peaks (blue solid line), while the regions where a new phase was created in Figure 2B (black solid line) correspond to smaller protein peaks. The final identity of the initially DOPA⁰-rich phase is unknown. α -Synuclein preferentially binds to anionic lipids over zwitterionic lipids,⁴¹ so it is possible that the DOPA⁰-rich phase has acquired some charge. However, protein binding can also alter the charge state of a tunable surface, and consequently does not offer an unambiguous way to assess the *initial* charge state of the DOPA⁰-rich region. Nevertheless, the fact that α -synuclein binds ~twice as much to the DOPAO-rich phase as the DOPA²⁻:Ca²⁺-rich phase strongly suggests that the two regions are chemically distinct.

The path dependence of this endpoint becomes apparent when compared to what was observed when a uniformly mixed bilayer (250 mM KCl, pH 7.4) was exposed to a bulk solution containing both a low KCl concentration (25 mM) and a high CaCl₂ concentration (1 mM), pH 7.4. The bilayer phase separated into a DOPC-rich phase and a DOPA²⁻:Ca²⁺-rich phase (Figure 2E,G), and α -synuclein showed two levels of binding (Figure 2F,G). Simultaneously varying the ion concentrations resulted in two phases at pH 7.4, while varying the ion concentrations sequentially resulted in three: a DOPC-rich phase, a DOPA⁰-rich phase, and a DOPA²⁻:Ca²⁺-rich phase

Figure 3 shows the response of a bilayer to changes in monovalent and divalent ion concentrations at pH 8. Dropping the monovalent ion concentration from 250 mM to 25 mM KCl again caused the uniformly mixed bilayer to phase separate into a DOPC-rich phase and a DOPA⁰-rich phase (Figure 3A, line scan shown in 3D). Subsequently increasing the divalent ion concentration from 0 mM to 0.25 mM CaCl₂ resulted in a new phase (Figure 3B, D), which we assigned to be rich in DOPA²⁻:Ca²⁺. Note that it took less calcium to see a similar area coverage of this new phase at pH 8 than at pH 7.4, which is consistent with previous observations.¹⁰ When 0.26 μ M α -synuclein was introduced to the bilayer, it bound preferentially to the DOPA-rich phases, but showed no preference for one DOPA-rich phase over the other (Figure 3C, D). This observation indicates that the DOPA in the initially DOPA⁰-rich phase is chemically indistinct from that in the DOPA²⁻:Ca²⁺ phase. We therefore conclude that at pH 8, calcium displaces protons in what was originally the DOPA⁰-rich phase, resulting in only two phases: one rich in DOPC and the other rich in DOPA²⁻:Ca²⁺. At this point, the organization of the bilayer is the same as when the monovalent and divalent ion concentrations are changed simultaneously from 250 mM KCl and 0 mM CaCl₂, to 25 mM KCl and 0.25 mM CaCl₂ (Figure 3E–G). Thus at pH 8, the bilayer reached the same endpoint (organization into two distinct phases) following two different paths: sequential or simultaneous variation of the monovalent and divalent ion concentrations.

Forming a DOPA⁰-rich phase first was essential to reaching a 3-phase endpoint. If the calcium concentration was increased first and then the potassium concentration was decreased (at either pH 7.4 or 8), the system reached the same two-phase endpoint that resulted from changing monovalent and divalent ion concentrations simultaneously. The effect of adding large amounts of calcium at pH 7.4 or small amounts of calcium at pH 8 was also investigated. If competitive binding governs whether or not calcium can displace

protons from the DOPA⁰-rich phase, then adding enough calcium at pH 7.4 would hypothetically shift the equilibrium and allow calcium to displace protons. Conversely, adding very little calcium at pH 8 would allow the DOPA⁰-rich phase to stay protonated. This was not the case. At pH 7.4, calcium concentrations up to 500 mM did not result in a two-phase endpoint, while at pH 8 calcium concentrations as low as 0.05 mM (the lowest amount needed to see a DOPA²⁻:Ca²⁺ phase at 25 mM KCl) did not result in a three-phase endpoint. The parameter that determined the endpoint was pH, presumably by modulating proton binding to DOPA. As mentioned earlier, at 25 mM KCl (where these experiments were carried out), the transition from a two phase endpoint to a three phase endpoint was ~pH 7.6–7.8. It is interesting to note that such small pH variations are observed in physiological systems; for example, extracellular pH is ~7.4 while cytosolic pH is ~7.0.

The initial mixture of DOPA/DOPC, which is uniformly mixed at a high monovalent concentration in the absence of divalent ions, appears to be metastable. Given enough time (hours, versus the seconds timescale of our experiments), the mixture phase separates despite the high monovalent ion concentration (data not shown). If heated, the mixture also phase separates. In both cases the phase separation proceeds through nucleation and growth. These observations are consistent with the DOPA/DOPC mixture being kinetically trapped in a local energetic minima, a metastable state.^{23,24} At the pH values used in these experiments, DOPA is charged in an electrostatically screened environment and charge-charge repulsion drives the mixing of DOPA with DOPC. If several DOPA molecules in close proximity protonate, they form a nucleation point for phase separation. Long times and high temperatures increase the probability of forming these nucleation points.

At pH 8, introduction of calcium spontaneously converts DOPA⁰ and DOPA^{x-} to the immiscible DOPA²⁻:DOPCa²⁺ complex, regardless of what phase the DOPA is found in. This implies that the DOPA²⁻:Ca²⁺ complex is more energetically stable than DOPA⁰ or DOPA^{x-}. At pH 7.4, however, introduction of calcium converts DOPA^{x-} but not DOPA⁰ to DOPA²⁻:Ca²⁺. Because we do not see any other changes on the timescale of our experiments, we cannot say whether DOPA⁰ is as energetically stable as DOPA²⁻:Ca²⁺ or whether it is also kinetically trapped in a metastable state.

The DOPA-rich domains observed in this work, as well as in our previous work with DOPA, are highly branched. Two possible explanations are: (i) Charged DOPA is line active; charge-charge repulsion promotes branching. (ii) Pinning defects in the underlying solid support promote branching. In other lipid mixtures we observe circular domains,^{23,24} suggesting that the solid support is not entirely responsible. It is likely that both factors are at work.

The control over phase behavior demonstrated in this paper is a result of the metastability and hysteresis of the uniform mixture. It is a delicate balance between utilizing and maintaining a metastable state. In general, cells exist in a metastable state and use equilibration to drive necessary cellular processes. This equilibration must be carefully controlled, however, and the cell must have mechanisms to restore metastability; otherwise it loses some of its versatility. For example, oxidative stress in neurons leads to elevated calcium levels,^{25,26} which in turn can sequester PA. While the cell can probably

compensate to some extent (e.g. controlling membrane lipid composition), excessive equilibration will ultimately be debilitating.

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Figure 1.

Caricature outlining two scenarios to create a three-phase or two-phase endpoint in a fluid binary lipid mixture. Low pH (A–C): (A) At high KCl and low CaCl_2 DOPA carries a charge and is miscible with DOPC at pH values above the $\text{pK}_{\text{a}1}$. (B) KCl concentration is lowered, and DOPA protonates and demixes. (C) CaCl_2 concentration is raised, $\text{DOPA}^{2-}:\text{Ca}^{2+}$ complexes form in the DOPC-rich region and demix. High pH (D–F): (D) DOPA is miscible with DOPC. (E) KCl concentration is lowered, and DOPA protonates and demixes. (F) CaCl_2 concentration is raised, $\text{DOPA}^{2-}:\text{Ca}^{2+}$ complexes form in the DOPC-rich region and demix, and Ca^{2+} binds to the DOPA⁰-rich region creating $\text{DOPA}^{2-}:\text{Ca}^{2+}$ complexes.

Figure 2.

Epi-fluorescence images of two 30:69:1 mol ratio DOPA:DOPC:NBD PC bilayers at pH 7.4. Images A–B, E show the NBD PC (lipid) fluorescence, while images C, F show the Alexa 674 (protein) fluorescence. Under the initial bulk solution conditions (250 mM KCl, 50 mM HEPES, pH 7.4) the bilayers were uniformly fluorescent. Sequential: (A) Bulk KCl concentration was reduced to 25 mM. (B) Bulk CaCl₂ concentration was increased to 1 mM. (C) 0.26 μ M α -synuclein was added. (D) Line scans taken at indicated locations in A–C. Simultaneous: (E) Bulk KCl concentration was reduced to 25 mM and bulk CaCl₂ concentration was increased to 1 mM. (F) 0.26 μ M α -synuclein was added. (G) Line scans taken at indicated location in E–F. All images are 120 $\times$ 120 μ m.

Figure 3.

Epi-fluorescence images of two 30:69:1 mol ratio DOPA:DOPC:NBD PC bilayers at pH 8. Images A–B, E show the NBD PC (lipid) fluorescence, while images C, F show the Alexa 674 (protein) fluorescence. Under the initial bulk solution conditions (250 mM KCl, 50 mM HEPES, pH 8) the bilayers were uniformly fluorescent. Sequential: (A) Bulk KCl concentration was reduced to 25 mM. (B) Bulk CaCl₂ concentration was increased to 0.25 mM. (C) 0.26 μ M α -synuclein was added. (D) Line scans taken at indicated locations in A–C. Simultaneous: (E) Bulk KCl concentration was reduced to 25 mM and bulk CaCl₂ concentration was increased to 0.25 mM. (F) 0.26 μ M α -synuclein was added. (G) Line scans taken at indicated location in E–F. All images are 120 $\times$ 120 μ m.