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Charged Lipids Modulate the Phase Separation in Multicomponent Membranes

Journal:	<i>Langmuir</i>
Manuscript ID	la-2023-011996.R1
Manuscript Type:	Article
Date Submitted by the Author:	n/a
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24 **ABSTRACT**
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29 Phase separation in lipid membranes controls the organization of membrane components and thus, regulates
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32 membrane-mediated processes. Membrane phase behavior is influenced by its components' molecular properties
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35 and their relative concentrations. Charged lipid species are among the most essential components of lipid
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38 membranes, and their impact on membrane phase behavior is yet to be fully understood. Aiming to provide an
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41 insight into this impact, this paper investigates how the presence and amount of anionic and cationic lipids affect
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44 the phase behavior of multicomponent membranes. Membranes of a ternary composition DOPC:DPPC:Chol with
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47 two distinct molar ratios were used to test the hypothesis that inclusion of charged lipids with saturated tails,
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50 beyond a certain concentration, would impede the phase separation in an otherwise phase-separating membrane.
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53 Fluorescence microscopy examination of electroformed giant liposomes revealed that when more than half of
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56 DOPC in the examined mixtures was replaced with DOPA or DOTAP, phase separation in liposomes was
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1 somewhat suppressed and this effect increased with increasing the charged lipid content. This effect depended on
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4 the membrane surface charge density as the half-maximal effect was observed at around $0.0072 \text{ C} \cdot \text{\AA}^{-2}$ in all
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7 examined cases. The phase-separation suppressing effect of DOPA was neutralized when the oppositely-charged
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10 lipid DOTAP was included in the mixture. Likewise, presence of divalent cation Ca^{2+} in the solution neutralized
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13 the impact of negatively-charged DOPA. These results underline the detrimental influence of surface charge
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16 density on membrane phase behavior. More importantly, these findings suggest that the charged lipid content in
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19 membranes may be a regulator of their phase behavior, and open new opportunities for the design of synthetic
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22 lipid membranes.
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29 Introduction

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33 Biological membranes have evolved as semipermeable barriers that envelop cells and delimit
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36 compartments within the cytoplasm.¹ These membranes are heterogeneous and host a range of raft-like domains
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39 with various size and lifetimes.² In plasma membrane, lipid rafts are often referred to domains enriched in
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42 sphingolipids and cholesterol.³ Sphingolipids with their long and saturated acyl chains favor interactions with
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45 cholesterol, leading to the formation of a tightly-packed liquid ordered (L_o) phase in the membrane.⁴ In contrast,
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48 a loosely-packed and more fluid liquid disordered (L_d) phase is mainly composed of phospholipids with
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51 unsaturated acyl chains.⁴ The difference in lipid packing is believed to derive the lipid segregation and separation
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54 of phases in multicomponent membranes.⁵
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1 Phase separation affects the organization of membrane molecules and is thus, involved in a wide range of
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4 cellular functions such as vesicle trafficking,^{5, 6} cell adhesion,⁷ and metabolic processes.⁸ As a result, membrane
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7 phase separation has been the subject of much research using variety of model membranes.⁹⁻¹² These studies have
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10 revealed that factors such as lipid chain-length,¹³ sterol concentration,¹⁴ and concentration of membrane proteins¹⁵
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13 and membrane-bound polymers¹⁶ can influence the lipid phase separation in membranes. Moreover, few studies
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16 have demonstrated that negatively charged lipids can impact the membrane phase behavior.¹⁷⁻¹⁹ For instance,
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19 Dimova and her colleagues reported that in a ternary composition of DOPC:eSM:Chol, replacement of DOPC
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22 with the negatively charged DOPG led to changes in its phase diagram and shifted the miscibility transition
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25 temperature of this mixture to lower temperatures. This effect was attributed to electrostatic repulsion among the
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28 charged headgroups of PG lipids.¹⁷ Another study by Takagi's group demonstrated that phase separation in
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31 DOPC:DPPC:Chol membrane was suppressed by the negatively-charged unsaturated lipid DOPG while DPPG,
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34 with a similarly charged headgroup but saturated acyl chains, had an opposite effect on phase separation.¹⁹ In
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37 another report by Shimokawa et al., replacing DOPC in the ternary composition of DOPC:DPPC:Chol with DOPS
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40 halted the phase separation in this composition.²⁰ Interestingly, a study by Keller's group found that replacing
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43 DiphyPC in DiphyPC:DPPC:Chol ternary composition, with DiphyPG lipid had a minimal effect on phase
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46 separation in liposomes formed by gentle hydration.¹⁸ Herein, we aim to further explore the impact of membrane
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49 charged lipid content on its phase behavior by testing the hypothesis that presence of charged, anionic or cationic,
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52 lipids with unsaturated acyl chains in a phase-separating multicomponent membrane beyond a certain
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concentration, can impede the phase separation (Figure 1A). To this end, we focus on a ternary membrane system with well-established phase behavior DOPC:DPPC:Chol at two different molar ratios (Figure 1B), and include various amounts of charged lipids in these membranes, to partially replace DOPC. Phase separation in these membranes is studied in electroformed giant unilamellar vesicles (GUVs) using fluorescence microscopy.

Prior studies on impact of charged lipids on membrane phase behavior have heavily focused on anionic lipids, PG and PS, perhaps due to the prominent role of these lipids in biological membranes.¹⁷⁻²⁰ Here, we decided to explore both anionic and cationic lipids as cationic lipids have also found growing application in liposomal delivery systems and have even been explored for use in phase-separating membranes.²¹⁻²³ For anionic lipid, we focused on DOPA as PA is known for its key role in cell signaling²⁴ and lipid metabolism²⁵ and as an important precursor for biosynthesis of other lipids.²⁶ For cationic lipid, we selected DOTAP due to its wide application in fusogenic liposomes and delivery of nucleic acids.²⁷⁻³⁰ Both DOPA and DOTAP have acyl-chains similar to DOPC and were therefore, used to replace DOPC in the examined DOPC:DPPC:Chol membranes assuming that they would have similar phase partitioning behavior as DOPC.

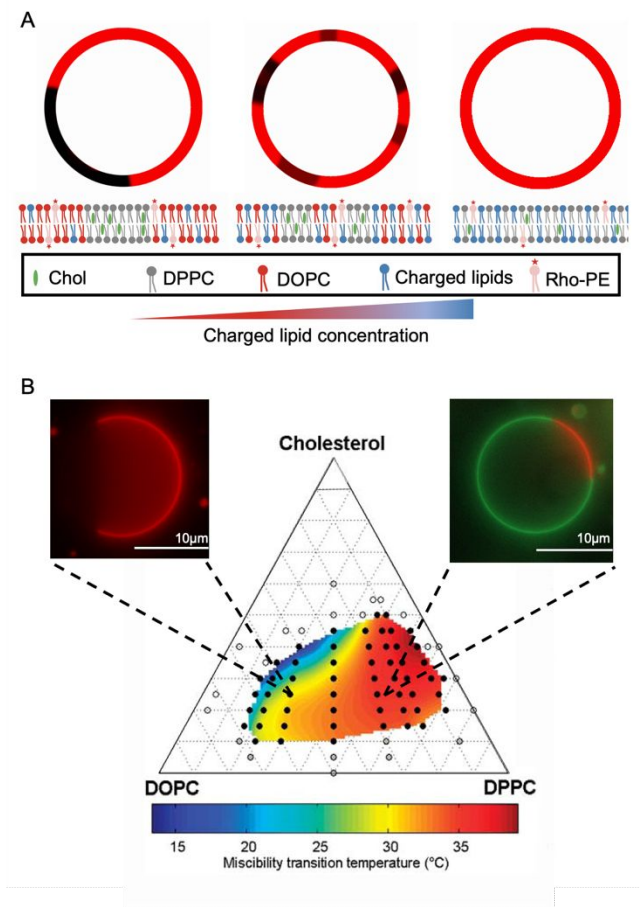


Figure 1. (A) Schematic illustration of the hypothesized concept that charged unsaturated lipids, beyond a certain concentration, would impede the phase separation in an otherwise, phase-separating multicomponent membrane. Top row illustrates liposomes of phase-separating composition with increasing content of unsaturated charged lipids (left to right), where red and black regions on liposomes represent L_o and L_d phases, respectively, and bottom row shows the lipid bilayers of corresponding compositions. (B) Phase diagram of DOPC:DPPC:Chol composition with a map of miscibility transition temperatures.¹⁴ The two compositions studied here are marked and epifluorescence images show representative GUVs composed of DOPC:DPPC:Chol at molar ratios of 2:1:1 (left) with 0.6% Rho-PE, and 1:2:1 (right) with 0.3% Rho-PE and 0.5% NAP.

Materials and Methods

Materials

Lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N- (lissamine rhodamine B sulfonyl) (Rho-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). We purchased the sucrose and glucose from VWR (West Chester, PA). Cholesterol and sodium chloride were from Sigma Aldrich (Saint Louis, MO) and chloroform was from Omnipure (Caldwell, Idaho). Naphtho[2,3-a] pyrene (NAP) and calcium chloride were purchased from Thermo Fisher Scientific (Waltham, MA). All lipid stock solutions were prepared in chloroform.

Indium tin oxide (ITO) coated glasses and microscope glass slides were from Thermo Fisher Scientific (Waltham, MA) and coverslips were from Corning Inc. (Corning, NY). ITO plates were cleaned using chloroform, ethanol, and DI water prior to use. Microscope slides and coverslips were cleaned with ethanol and DI water before usage.

Methods

Preparation of GUVs

GUVs were prepared by following a modified form of electroformation technique described in our pervious studies.^{31, 32} Briefly, we started with formation of small liposomes using dehydration-rehydration

method. A mixture of DOPC, DPPC, Chol with DOPA and DOTAP at described molar ratio was prepared in chloroform. Specified amounts of Rho-PE and NAP were also added for visualization and imaging. The lipid solution was next dried out in a pearl-shaped flask to create a thin lipid film using a rotary evaporator (Hei-Vap, Heidolph, Germany) for ~2 hours. A milky suspension was formed after rehydrating the lipid film with 60°C DI water. We then used a tip-sonicator (55-Watt Sonicator Q55, Qsonica, Newtown, CT) to produce small unilamellar liposomes. The resulting small liposomes were next deposited on two ITO-coated glasses and were left to dry overnight. A 235 mM sucrose solution was then injected into the electroformation chamber that was formed by sandwiching a PDMS frame (with integrated tubing) between the two ITO glasses, with their lipid-coated sides facing inside. Electroformation was performed at ~60°C (in oven) to exceed the highest melting temperature in the lipid mixture (41°C, for DPPC). A function waveform generator (4055, BK Precision, Yorba Linda, CA) produced an AC electric field of 50 Hz frequency and increased 2 Vpp at 100 mVpp/min for 3 hours. Then, the frequency was decreased to 1 Hz for 30 min to detach the formed GUVs. Afterwards, we removed the chamber from oven and let it cool down to temperatures of ~16°C at microscopy room. Vesicles were taken out from the chamber for further experimentation and imaging as detailed below. Solutions of 1, 5, 50 mM NaCl and 1 mM CaCl₂ were used to investigate the effect of salts on membrane phase separation behavior. Excess glucose was added to the system to maintain the osmotic pressure unchanged. The solution was well-mixed and kept for ~30 min before visualization.

Microscopy imaging and analysis

GUVs imaging and analysis were performed as described before.^{11, 32} In brief, an imaging chamber was created by sandwiching a frame of double-sided tape between a clean microscope glass slide and a coverslip. 12 μ l of GUV solution was sealed in the chamber for imaging. An inverted Zeiss microscopy, Zeiss LSM 800 confocal laser scanning microscope (Zeiss, Germany) equipped with epi-fluorescence, was used to monitor and image the membrane phase-behavior in GUVs. Confocal images were collected using a 63x oil objective with NA of 1.40 using 488 nm and 561 nm wavelength lasers. For the confocal 3D reconstruction, approximately 20 confocal slices were collected and then reconstructed using ZEN software (ZEN 3.1 lite, Zeiss, Germany).

To calculate the percentage of L_o/L_d phase-separated vesicles, we collected the data from at least 3 independent experiments. In each experiment, more than 50 vesicles were imaged, and the number of phase-separated vesicles was counted manually and divided by the number of total vesicles in captured frames. It should be noted that inclusion of DOTAP in GUV formulation led to some level of aggregation, making the image analysis more challenging for these GUVs.

For domain area fraction calculations, we assumed that the GUVs were spherical shape and their domains were in the form of spherical caps. Confocal images of GUVs were analyzed to find the diameter and area of domains as detailed in our previous studies.^{11, 32} The analysis was performed on at least 10 GUVs from 3 independent experiments for all compositions except 25 mol% DOTAP, 50 mol% DPPC, 25 mol% Chol with 0.3 mol% Rho-PE and 0.5 mol% NAP.

Zeta potential measurements

Zeta potential was measured using Zetasizer (Malvern Instruments, Malvern, UK) at 25°C. The vesicles were 10 times diluted with 235 mM sucrose (for GUVs) to produce enough solution for accurate measurement. For each measurement, at least 3 replicates were used to calculate the average.

Results and Discussion

Focusing on DOPC:DPPC:Chol composition, we selected to work on two different molar ratios of this composition that undergo phase separation to form L_o and L_d phases at temperature range studied here (15-17.5°C), but exhibit distinct phase behavior. Specifically, we selected a membrane composition of 50 mol% DOPC, 25 mol% DPPC and 25 mol% cholesterol (referred to as 2:1:1 composition) as this mixture has a miscibility transition temperature of ~30°C and a membrane composition of 25 mol% DOPC, 50 mol % DPPC, and 25 mol% cholesterol (referred to as 1:2:1 composition) with a miscibility transition temperature that is ~5°C higher than the other mixture (Figure 1B).¹⁴ In 2:1:1, L_d phase is dominant and inclusion of trace amounts (0.6 mol%) of Rho-PE (red fluorescence) that is known to partition preferentially into L_d phase,³³ enabled visualization of this dominant phase while L_o phase on these liposomes appeared dark. In contrast, in 1:2:1, the L_d phase is the minority phase and thus, we also included 0.5 mol% NAP (green fluorescence) which preferentially partitions into L_o phase³³ in the liposome formulation to enable visualization of the dominant L_o phase while the L_d phase

1 appeared red (due to the presence of 0.3% Rho-PE). We prepared GUVs of these two compositions with various
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3 amounts of DOPA or DOTAP using a modified version of electroformation^{31, 32} and examined the phase behavior
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6 of resulting GUVs using epifluorescence and confocal microscopy.
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11 To test the hypothesis that unsaturated charged lipids can suppress phase separation in multicomponent
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13 membranes in a concentration-dependent manner, we started with examining the 2:1:1 mixture with different
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15 contents of DOPA or DOTAP. To keep the membrane's content of unsaturated lipids unchanged, the
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17 concentration of DOPC was lowered to accommodate the incorporation of DOPA or DOTAP. We first assessed
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19 the overall impact of charged lipids on phase separation in GUVs through the fraction of GUVs that exhibited
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21 phase separation, focusing on GUVs with coexisting L_o - L_d phases (round domains with smooth borders). For
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23 2:1:1 mixture, replacement of DOPC with the unsaturated charged lipids up to ~ 25 mol% in the membrane did
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25 not have a significant impact on phase separation on GUVs, as nearly all examined GUVs presented one round
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27 dark domain (L_o domain) with smooth borders at equilibrium. Upon increasing the charged lipid content beyond
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29 25 mol%, the population of GUVs displaying L_d - L_o coexisting phases dropped drastically as illustrated in Figures
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31 2A-B. Additionally, on GUVs that exhibited phase separation, the large dark L_o domain appeared to be replaced
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33 by several smaller domains and this trend continued with increasing the charged lipid content till the charge
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35 content in the membrane reached about 50 mol%, where nearly no GUV had detectable L_o domains and all
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37 examined GUVs appeared homogenous. For instance, with increasing DOPA from 25 mol% to 50 mol%, fraction
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39 of phase-separating liposomes reduced from above 98% to less than 2% (Figure 2B).
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This trend was similar for DOPA and DOTAP, suggesting that beyond a certain charge content, either negative or positive, they can suppress the phase separation in this mixture. A closer examination of this data revealed that DOPA had a stronger suppressing effect on phase separation in these GUVs compared to DOTAP, as the half-maximal effect of DOPA was reached at 34 mol% DOPA content, while this effect was reached at 36 mol% DOTAP content (Figure 2B). This difference may be due to a lower concentration of DOTAP in GUVs compared to DOPA. Given the charged nature of these lipids, their distribution in membrane may be influenced by the electric field applied in electroformation.³⁴ Such effect may vary for anionic DOPA and cationic DOTAP and may influence their incorporation into membranes differently. It may be harder to incorporate DOTAP into electroformed GUVs, leading to a lower concentration in the membrane compared to DOPA.

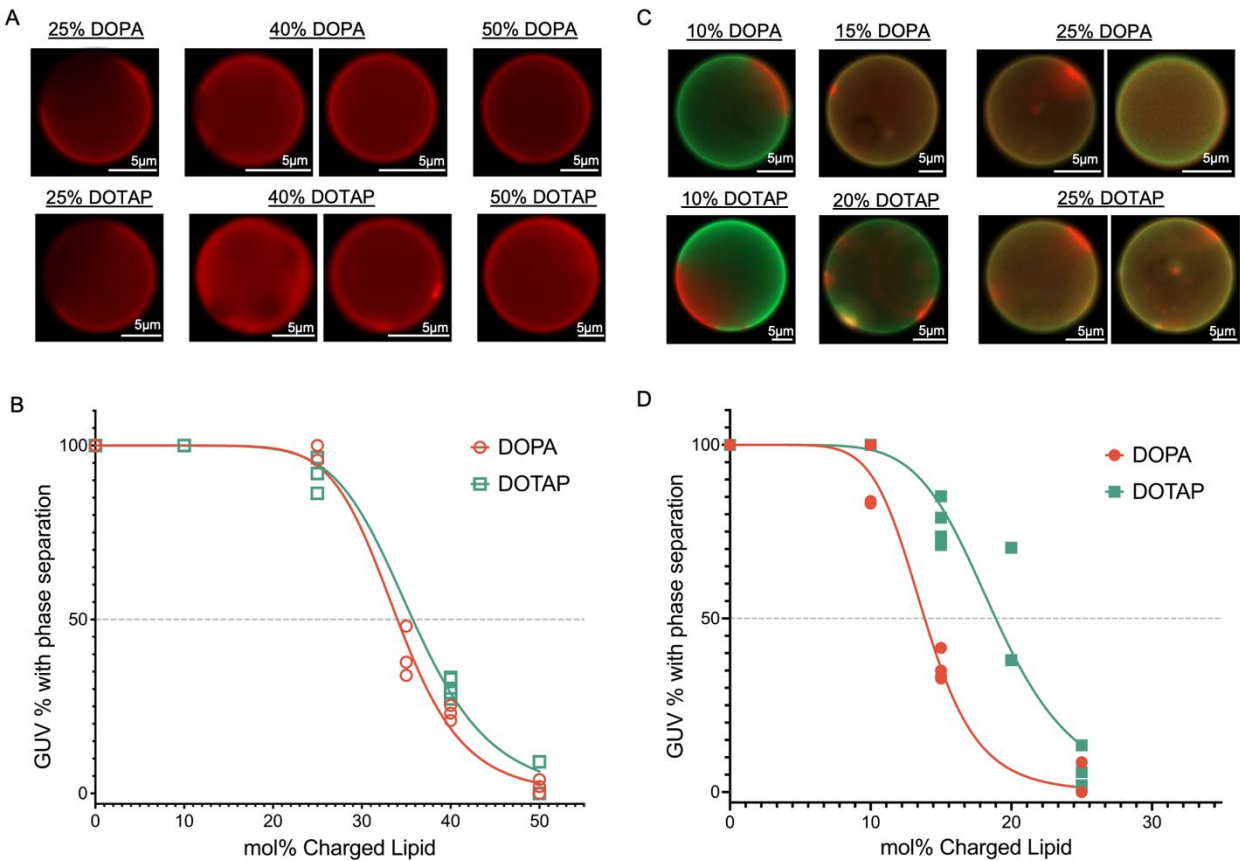


Figure 2. The effect of charged lipids DOPA and DOTAP on phase separation in GUVs with DOPC:DPPC:Chol composition at molar ratio of 2:1:1 (A, B) and 1:2:1 (C, D). Epifluorescence images of representative GUVs with different amounts of DOPA (top row) and DOTAP (bottom row) showing the predominant membrane behavior observed at $\sim 16.5^{\circ}\text{C}$ in (A) 2:1:1 labeled with 0.6 mol% Rho-PE, and (C) 1:2:1 labeled with 0.3% Rho-PE and 0.5% NAP. Graphs showing the percentage of GUVs exhibiting L_o - L_d phase coexistence as a function of charged lipid content in the membrane with (B) 2:1:1 and (D) 1:2:1 mixture. Data points represent the average area fractions from independent experiments. Dashed lines mark the half-maximal effect.

Next, we performed these experiments on GUVs with 1:2:1 mixture, in which L_d phase is the minority phase. While the overall trend of reduced fraction of phase-separated GUVs with increasing the charged lipid content in the membrane was similar to 2:1:1, charged lipids exerted a stronger effect on membrane phase behavior in this composition. In other words, the phase-separation suppressing effect of the examined charged lipids were detected at substantially lower concentrations of charged lipids. For instance, presence of DOPA beyond 10 mol% in this membrane composition led to a drop in the fraction of phase-separated GUVs and this fraction approached zero when about 25 mol% of membrane's DOPC was replaced with DOPA (Figure 2C-D). The phase-suppressing effect of DOTAP was also more pronounced in this composition, compared to composition 2:1:1. Notably, similar to 2:1:1, DOPA in this composition showed a stronger influence on membrane phase

behavior compared to DOTAP. The half-maximal effect of DOPA was reached at 14 mol% of DOPA content, while this effect was reached at 20 mol% for DOTAP in the membrane (Figure 2D), again possibly due to a lower concentration of DOTAP in electroformed GUVs compared to DOPA.

The stronger influence of DOPA and DOTAP on phase behavior in composition 1:2:1 compared to 2:1:1 was attributed to the fact that these unsaturated lipids make up a higher portion of lipids in L_d phase in 1:2:1, leading to a higher charge concentration in this phase and thus, stronger electrostatic repulsion among the charged lipids. In fact, when the fraction of phase-separated GUVs is plotted as a function of charge density in L_d phase, the differences between 2:1:1 and 1:2:1 mixture shrink drastically, as illustrated in Figure 3, and the half-maximal effect for all the examined membrane compositions is reached within $0.0062 - 0.0082 \text{ C} \cdot \text{\AA}^{-2}$. This result indicates that the membrane charge-density is a detrimental player in membrane's phase behavior.

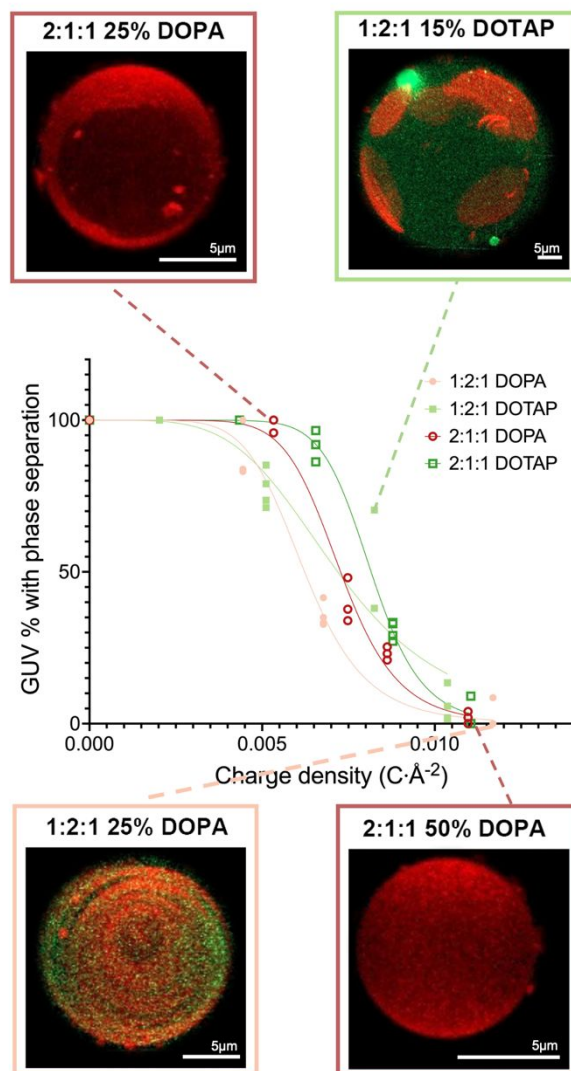


Figure 3. Charge density-dependent effect of DOPA and DOTAP on phase separation in GUVs of DOPC:DPPC:Chol with 2:1:1 and 1:2:1 molar ratios. Graph showing the percentage of phase-separated GUVs as a function of membrane charge density in L_d phase. Confocal 3D reconstructed images show representative GUVs from different examined GUV formulations.

Focusing on phase-separated liposomes in the above studies on 2:1:1 mixture, we noted that inclusion of DOPA and DOTAP had an effect on the area fraction of L_o domains on phase-separated GUVs (Figure 4A). With increasing the amount of charged lipids in the membrane, the average area fraction of L_o domains increased from

~0.25 for a 2:1:1 membrane without charged lipids to ~0.38 for a membrane with 40 mol% DOPA, and to ~0.44 for a membrane with 40 mol% DOTAP. This increase in L_o domain area fraction on GUVs, i.e. reduction in L_d area fraction, may be attributed to the smaller cross-sectional molecular area of DOPA ($65\text{\AA}^2$)³⁵ and DOTAP ($70\text{\AA}^2$),^{36, 37} compared to DOPC ($72.5\text{\AA}^2$).³⁸ These unsaturated lipids make up a high lipid fraction in L_d phase, and their presence can thus reduce the area fraction of this phase, leading to an apparent increase in L_o area fraction on GUVs. In addition, presence of charged lipids can result in reorientation of PC dipoles and hence, a tighter packing in the bilayer³⁹ further reducing the area fraction of L_d phase, and thus contributing to the apparent growth of L_o phase area fraction. Additionally, inclusion of larger amount of charged lipids may lead to a shift in lipid distribution among L_d and L_o phases, affecting the domain area fraction. For instance, with increasing the charge density, the fraction of unsaturated charged lipids may increase in L_o phase to keep the charged lipids further apart during phase separation.

Examining the phase-separated GUVs of 1:2:1 composition revealed a similar effect for DOPA and DOTAP on L_o domain area fraction (Figure 4B). In the absence of these charged lipids, the average area fraction of L_o phase on GUVs with this ternary composition was 0.69, which is in agreement with the theoretically estimated value of 0.70 based on phase-diagrams, as detailed in our previous study.¹¹ Partial replacement of DOPC with DOPA or DOTAP led to an increase in L_o domain area fraction where, for instance, it reached to ~0.85 for 15 mol% DOPA and ~0.77 for 15 mol% DOTAP. It should be noted that area fraction measurements proved to be challenging for this composition as the green fluorescence signal was weak and the area fraction

analysis was performed based on the red fluorescence signal, which labeled the minority phase in this composition, resulting in higher levels of variations.

To further assess the charged lipid-containing liposomes, we next performed zeta potential measurements on these liposomes. These measurements are commonly used for assessment of surface charge of particles.⁴⁰ As shown in Figure 4C-D, in both compositions increasing amount of DOTAP led to a shift of zeta potential towards positive values, as anticipated, while increasing amounts of anionic DOPA resulted in negative zeta potential values. Interestingly, in all cases, the value of zeta potential appeared to drop around the point where the charged lipid content in the membrane had shown a suppressing effect on phase-separation and at least half of liposomes would be homogenous, according to the results shown in Figure 2. For instance, in the case of DOTAP-containing liposomes, zeta values appeared to drop around 40 mol% DOTAP for 2:1:1 mixture (Figure 4C), and around 20 mol% for 1:2:1 mixture (Figure 4D). This was in contrast with the trend of zeta potential changes in homogenous liposomes with DOTAP (see Figure S1) in which zeta values increased continuously with an increase in DOTAP content in membrane. Zeta potential values of DOPA-containing liposomes had a similar apparent drop in value at around 40 mol% DOPA for 2:1:1 mixture, and around 15 mol% DOPA for 1:2:1 mixture. We attribute this drop of zeta potential values to the change in surface charge distribution on liposomes as a result of phase-separation. Zeta potential is known to be affected by the charge symmetry on particles and has been shown to be higher for particles with asymmetrical surface charge compared to their symmetrical counterparts.⁴¹ The apparently higher zeta potential values have been attributed to the orientation of asymmetrically charged particles

in the electric field applied during zeta potential measurements, where the particle's side with higher surface charge density is oriented toward the oppositely charged electrode, leading to particle's faster movement in the electrical field and thus, higher zeta potential readout.^{41, 42} Accordingly, zeta potential values in phase-separated liposomes that contain charged lipids are expected to be larger compared to their homogenous counterparts with similar charged lipid content. As a result, when majority of liposomes are phase-separated, the zeta values may appear larger and once the population majority shifts to homogenous liposomes, the zeta values drop and then again start to increase with increasing the charge content in liposomes.

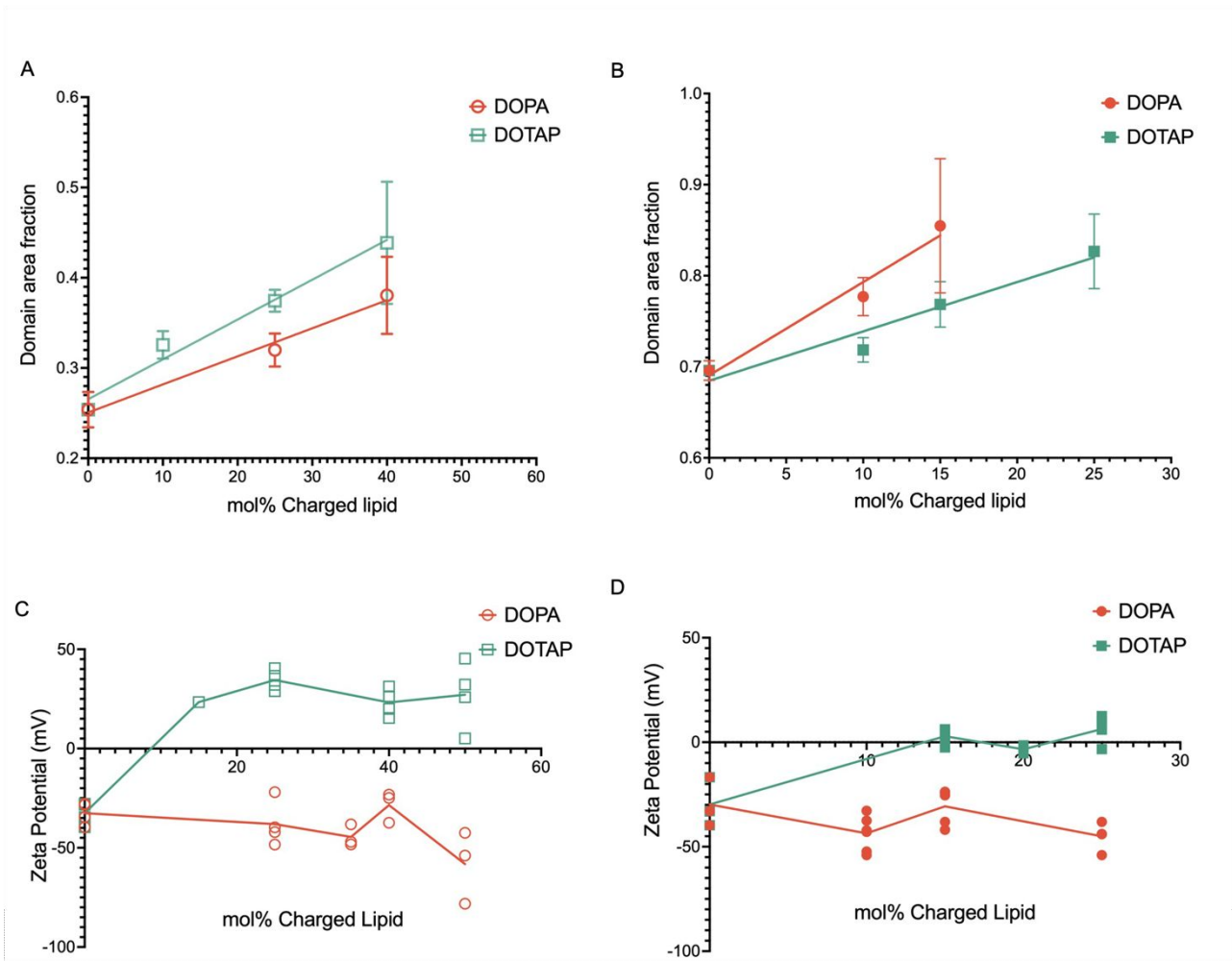


Figure 4. The impact of DOPA and DOTAP on domain area fraction in phase-separated GUVs and zeta potential of GUVs. A and B graphs show the changes in area fraction of L_o domains on phase-separated GUVs with (A) 2:1:1, and (B) 1:2:1 composition as a function of membrane's charged lipid content. Data points represent the mean of at least 3 independent experiments and error bars represent the standard deviation. C and D depict the zeta potential values of liposomes with (C) 2:1:1, and (D) 1:2:1 composition as a function of membrane content of DOPA or DOTAP. Data points represent the average measured values from independent experiments.

Next, we investigated if the above-mentioned phase-separation suppression effect of DOPA and DOTAP in GUVs could be countered by presence of the oppositely-charged lipid in the membrane. For this experiment, we used 2:1:1 mixture with a 35 mol% DOPA content and replaced the remaining DOPC (15 mol%) with DOTAP. We then compared the properties of the DOPA-containing GUVs with and without the 15% DOTAP. When DOPC in GUVs was only partially replaced with 35% DOPA, ~38% of liposomes underwent phase separation and when the remaining DOPC in this composition was replaced with DOTAP, this fraction went up to ~92%, as shown in Figure 5A. Addition of DOTAP presumably reduced the repulsion among DOPA headgroups and made the phase behavior closer to that expected for 2:1:1 composition with ~20% DOPA.

In these experiments, the area fraction of L_o domains on phase-separated liposomes had a slight but insignificant increase (Figure 5B) upon inclusion of DOTAP. Moreover, while inclusion of DOTAP in DOPA-

containing liposomes slightly reduced their negative zeta potential, this reduction was statistically insignificant, as depicted in Figure 5C.

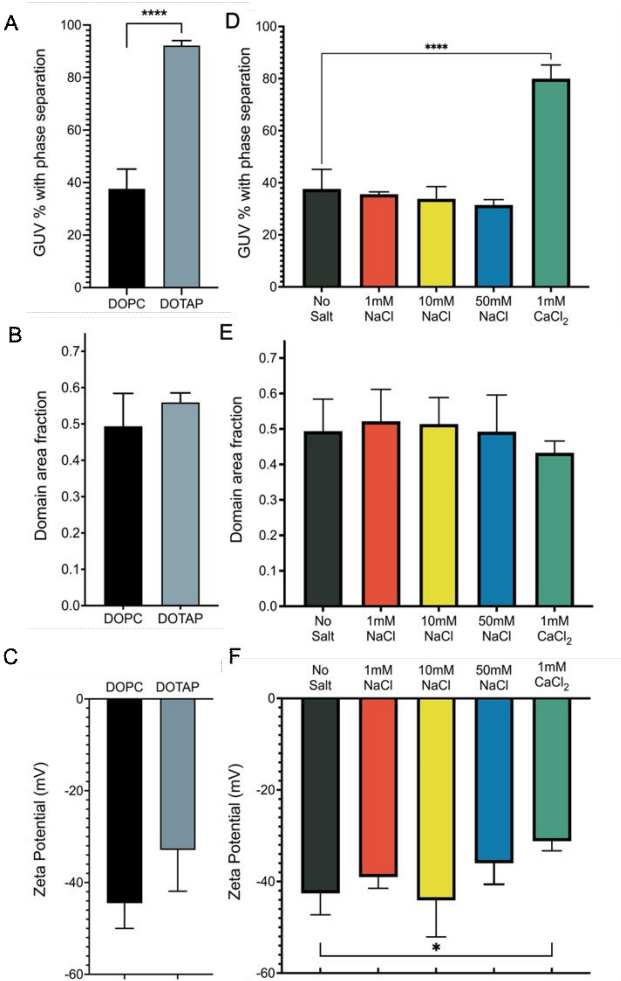


Figure 5. Phase separation, domain area fraction, and zeta potential in GUVs of 2:1:1 mixture with 35 mol% DOPA content, with and without 15 mol% DOTAP (A-C), and at different salt concentrations (D-F). Comparison of DOPA-containing GUVs with and without 15 mol% DOTAP for (A) phase-separated fraction of liposomes, (B) L_o domain area fraction on phase-separated liposomes, and (C) zeta potential. Comparison of DOPA-containing GUVs at different salt concentrations for (D) phase-separated fraction of liposomes, (E) L_o domain area fraction on phase-separated liposomes, and (F) zeta potential. In all these graphs, bars represent the mean

and error bars represent the standard deviation with $n \geq 3$. Data in (A-C) were statistically analyzed using an unpaired, 2-tailed student's t-test, data in (D-F) were statistically analyzed using one-way ANOVA and *****: p values < 0.0001 ; *: p value < 0.05 .

Lastly, we examined the impact of salts on the phase-separation suppressing effect of unsaturated charged lipids. Prior studies have shown that the presence of salts in the solution affect the miscibility transition temperature in multicomponent membranes containing charged lipids.^{17, 19} Here, we focused on liposomes with 2:1:1 composition with 35% DOPA content and exposed them to sodium and calcium ions, as model monovalent and divalent cations that have been examined for their influence on membrane properties including phase behavior.⁴³⁻⁴⁶ Upon electroformation, we diluted the DOPA-containing GUVs in an isotonic glucose solution containing either NaCl or CaCl₂. Notably, addition of 1-50 mM NaCl had no significant impact on the phase-separating fraction of GUVs, while presence of only 1 mM CaCl₂ led to a drastic increase in this fraction, as summarized in Figure 5D. The observed stronger effect of divalent cation Ca²⁺ on phase-behavior of the membrane containing negatively-charged lipid, compared to monovalent cation Na⁺ is in great agreement with previous literature reports.¹⁷ Interestingly, when compared to GUV sample with no salt, presence of 1-50 mM NaCl had slight impact on the area fraction of L_o domains, and presence of 1 mM CaCl₂ slightly reduced this area

fraction but the change was not statistically significant (Figure 5E). In these experiments, the zeta potential measurements on GUVs, showed no significant difference between the samples with no salt and those with 1-50 mM NaCl, but there was a statistically significant difference between the liposome samples without salt and that with 1 mM CaCl_2 . Together, the above studies demonstrated that the phase-separation suppressing effect of unsaturated charged lipids DOPA and DOTAP can be effectively countered by reducing the repulsive interactions among charged headgroups in the membrane.

It should be noted that experiments here were conducted at constant pressure and temperature and in unbuffered solutions, and thus the above findings should be carefully considered for a different set of conditions as temperature,¹⁴ pressure,^{47, 48} and pH^{10} are all known as critical parameters in membranes' phase behavior.

Conclusion

In summary, this study investigates the influence of positively- and negatively-charged lipids with unsaturated acyl-chains, DOTAP and DOPA, on phase behavior of multicomponent membranes by testing the hypothesis that beyond a certain concentration, these lipids can suppress the phase-separation in an otherwise phase-separating membrane. Using GUVs of a well-studied composition, DOPC:DPPC:Chol, at two distinct molar ratios, we demonstrate that replacing the zwitterionic DOPC with anionic DOPA or cationic DOTAP can lead to suppression of the phase-separation in GUVs. The suppressive effect of these lipids, presumably due to

the electrostatic repulsion among similar charges, differed among two examined lipid mixtures as it depended on the membrane surface charge density. Moreover, DOPA's suppressive effect in GUVs was countered by inclusion of DOTAP in the GUVs. Examination of the influence of salts revealed that while monovalent cation Na^+ did not have an impact on DOPA's suppressive effect at up to 50 mM concentrations, low concentrations of divalent cation Ca^{2+} effectively neutralized this suppressive effect. Closer examination of the phase-separated GUVs containing the charged lipids revealed that replacement of DOPC with either DOPA or DOTAP in GUVs resulted in an increase in L_o domain area fraction (i.e. decrease in L_d area fraction) in GUVs, presumably due to the smaller cross-sectional area of these charged lipids compared to DOPC and possible changes in lipid distribution among the two phases. Zeta potential measurements on charged lipid-containing GUVs showed that presence of these lipids had an impact on the overall surface charge of liposomes. Zeta potential values also appeared to be impacted by the charge distribution on GUVs that varied as a result of phase separation. Findings of this study suggest that the charged-lipid content in membranes (which may fluctuate as a result of, for instance, enzymatic activity) can serve as a modulator for phase separation at low ionic strengths. It should be noted that under physiological conditions (i.e. high ionic strengths) this effect may be shielded. More importantly, it underlines the significance of membrane charge density for its phase behavior. This concept can thus, be applied to other charged components in membranes and provides opportunities for the design of new and dynamic membrane-based materials.

1 ASSOCIATED CONTENT

5 **Supporting Information**

9 Zeta potential of homogenous liposomes. This material is available free of charge on the ACS publications
12 website.

17 AUTHORINFORMATION

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29 **Author Contribution**

33 Y.Wang designed and conducted experiments, analyzed data, and wrote the manuscript. S.Majd supervised all
36 aspects of work, and wrote the manuscript.

40 **Funding Sources**

44 This study was supported by US National Science Foundation (NSF) through grants DMS-1953535 (S.M.) and
47 DMR-1753328 (S.M).

51 ABBREVIATIONS

DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPG, dipalmitoylphosphatidylglycerol; DOPG, 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol); DOPS, 1,2-dioleoyl-sn-glycero-3-phospho-L-serine; diPhyPC, 1,2-diphytanoyl-sn-glycero-3-phosphocholine; DiPhyPG, 1,2-diphytanoyl-sn-glycero-3-phosphoglycerol; Chol, cholesterol; DOPA, dioleoylphosphatidic acid; DOTAP (dioleoyl-3-trimethylammonium propane).

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