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Complex Behavior of Phosphatidylcholine – Phosphatidic Acid Bilayers and Monolayers: Effect of Acyl Chain Unsaturation

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ABSTRACT

Phosphatidic acids (PAs) have many biological functions in biomembranes, e.g., they are involved in the proliferation, differentiation, and transformation of cells. Despite decades of research, the molecular understanding of how PAs effect the properties of biomembranes remains elusive. In this study, we explored the properties of lipid bilayers and monolayers composed of PAs and phosphatidylcholines (PCs) with various acyl chains. For this purpose, the Langmuir monolayer technique and atomistic molecular dynamics (MD) simulations were used to study the miscibility of PA and PC lipids and the molecular organization of mixed bilayers. The monolayer experiments demonstrated that the miscibility of membrane components strongly depends on the structure of the hydrocarbon chains, and thus, on the overall lipid shape. Interactions between PA and PC molecules vary from repulsive, for systems containing lipids with saturated and unsaturated acyl tails (strongly positive values of the excess free energy of mixing), to attractive, for systems in which all lipid tails are saturated (negative values of the excess free energy of mixing). The MD simulations provided atomistic insight into polar interactions (formation of hydrogen bonds and charge pairs) in PC-PA systems. H-bonding between PA monoanions and PCs in mixed bilayers is infrequent, and the lipid molecules interact mainly via the electrostatic interactions. However, the number of charge pairs significantly decreases with the number of unsaturated lipid chains in the PA-PC system. The PA dianions weakly interact with the zwitterionic lipids, but their headgroups are more hydrated as compared to the monoanionic form. The acyl chains in all PC-PA bilayers are more ordered comparing to single-component PC systems. In addition, depending on the combination of lipids, we observed a deeper location of the PA phosphate groups compared to the PC phosphate groups, which can alter the presentation of PAs for the peripheral membrane proteins, affecting their accessibility for binding.

INTRODUCTION

Phosphatidic acids (PAs) constitute the simplest subgroup of phospholipids and occur in cellular membranes in rather small amounts of about 1 mol% of all lipids. Nevertheless, their concentrations are elevated under specific physiological conditions. For instance, the level of PA in the bacterial membrane increases in response to surfactin, a lipopeptide type antibiotic.¹ In animal cells, PAs, similarly to other negatively charged phospholipids, are asymmetrically distributed in the cell membrane with a preference for the cytosolic leaflet.² PAs are central molecules in lipid metabolism, as they are used in the synthesis of all other types of phospholipids. Interestingly, they also initiate the metabolic pathway of triglyceride synthesis.³ The main functions of PAs are related to cellular signaling in a vast number of physiological and cellular processes. In animal cells, PA is involved in the proliferation, differentiation, and transformation of cells, as well as in the survival signaling and tumor progression.⁴ PA was also found to play numerous roles in neurons, where it participates in neurotransmission.⁵ In plants, PAs take part in the response to stress associated with water deficit, temperature, saline conditions, starvation, free radicals, and interactions with microbes.⁶ Growth and development of plants are also regulated by PA, e.g., germination and aging of seeds, leaf senescence, root hair patterning, root growth, pollen germination, and pollen tube growth.⁶ In most of these processes, PA was found to act as a second messenger in hormone signaling.^{5,6} Although the mitochondrial level of PA is only ~1-2 mol%, it is necessary for mitochondria fusion and fission. An inhibition of PA synthesis leads to mitochondria fragmentation.⁷ A comprehensive overview of the PA functions can be found in papers of Wang et al.⁴ and Testerink and Munnik.⁶

PAs, as signaling molecules, interact with numerous proteins. The interaction network, provided in ref. 8, shows fifty different proteins as PA binding partners including kinases, phosphatases, and nucleotide binding proteins.⁹ Analysis of the subcellular localization of these proteins showed that PA plays important roles in almost all cellular compartments. Functional

analysis of the PA binding partners indicates that PA is involved in the cytoskeleton organization, regulation of apoptosis, cell proliferation, migration, and morphogenesis, vesicular transport, and response to mechanical stimuli.⁹ Despite a large number of proteins interacting with PA, no common PA binding motif was found.¹⁰ Several short sequences of 3-4 residues, rich in arginine and lysine, were documented for specific proteins and hydrophobic residues were also shown to be involved in the PA binding.¹⁰ Interestingly, the direct interaction of the PA phosphate group with arginine and lysine causes the phosphate group to be more acidic, increasing the strength of the interaction.^{11,12} This mechanism called the electrostatic/hydrogen bonding switch was documented for several proteins.¹³ Another interesting feature of PA-protein binding is its dependence on the membrane curvature.¹⁴ Finally, PA was shown to increase the binding of low-molecular-weight ligands, such as neurotransmitters and anesthetics, to the lipid bilayer.¹⁵

PA has unique biophysical properties in lipid bilayers, resulting from its molecular shape and negative charge of the headgroup.¹⁶ PA molecules have an inverted conical shape with a relatively small headgroup in comparison with the hydrophobic part of the molecule, which promotes a negative spontaneous curvature. Interestingly, lyso-PA (LPA) (with only one acyl chain), has a larger cross-section of the headgroup than the cross-section of the hydrophobic part of the molecule, which favors a positive spontaneous curvature.¹⁷ For this reason, PA destabilizes the lamellar structure of dioleoyl-phosphatidylethanolamine (DOPE) by lowering the lamellar-to-hexagonal phase transition temperature, while LPA stabilizes the lamellar phase.¹⁸ The destabilizing effect of PA was found to be stronger in the acidic pH and in the presence of divalent cations.¹⁸ Also, the presence of NaCl in physiological concentration affects the curvature induced by PA.¹⁸ These properties of PA and LPA suggest the possible role of these lipids and enzymes that convert them, in the vesicle fission and fusion in cells. Moreover, the PA cellular concentration was shown to induce the formation of supersized lipid droplets.¹⁹

Another interesting feature of PA is the sensitivity of its phosphate group to the chemical environment. In particular, the presence of polyvalent cations²⁰⁻²² and hydrogen bonding groups²³ stabilizes the fully deprotonated form (dianion) of PA²⁻.^{24,25} It was shown that *pK* of LPA, with a hydroxyl group capable of forming hydrogen bonds with the phosphate group, carries more negative charge compared to chemically modified LPA with a hydroxyl group removed.²⁴ Similarly, the presence of phosphatidylethanolamine, containing a positively charged amine group, stabilizes the fully deprotonated form of PA²⁻, while the presence of negatively charged phosphatidylserine has the opposite effect.²⁴ Even more interesting is the fact that the *pK* of the PA phosphate group is sensitive to the bilayer curvature.²⁶ Since the protonation state of the phosphate group changes in the pH range close to physiological (pH 7.4), the PA signaling was shown to be pH sensitive.^{27,28} It was also shown that the interactions of PA with cations induce the fusion of PA-containing vesicles. This effect was observed for divalent cation concentrations below 1 mM,^{29,30} and for Na⁺ concentrations above physiological values.³¹ As a result of the PA-cation interaction, the formation of PA-rich domains was also observed.³² The shape and size of these domains were shown to be sensitive to the ratio of monovalent to divalent cations.³³ The cation-induced domain formation was also demonstrated in PA monolayers.^{34,35} The shape of these domains is sensitive to the concentration of mono- and divalent cations, namely Na⁺ induces round domains, while Ca²⁺ induces dendritic domains.

In this study, we focus on the molecular organization of mixed PA-phosphatidylcholine (PC) membranes. We are particularly interested in the effect of hydrocarbon chain saturation on the mutual miscibility and intermolecular interactions in these membranes. For this purpose, we performed atomistic molecular dynamics (MD) simulations of several systems containing PA and PC lipids with various acyl chains. The calculations were carried out for mono- and divalent anionic forms of PA to determine the effect of the protonation state on the molecular structure of the bilayer. The simulations provided also insight into the polar interactions between lipids

(formation of hydrogen bonds and charge pairs) in PC-PA systems. Langmuir monolayer and Brewster angle microscopy (BAM) experiments were then used to complement the MD simulation results. Our data show complex behavior of both mono- and bilayers, which originates from the type of hydrocarbon chains (unsaturated or saturated), and thus the effective shape of a lipid molecule. In addition, depending on the combination of lipids, we observed a deeper location of PA phosphate groups compared to those in PC, which also contributes to the monolayer properties.

Material and methods

Materials. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (sodium salt) (DPPA), and 1,2-dioleoyl-*sn*-glycero-3-phosphate (sodium salt) (DOPA) were synthetic products of high purity ($\geq 99\%$) purchased from Avanti Polar Lipids, Inc. Chloroform and methanol were received from Sigma-Aldrich (HPLC grade, $\geq 99.9\%$). KH_2PO_4 and $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ were obtained from Sigma-Aldrich. The ultrapure Milli-Q water, used in the experiments, had a surface tension of 72.6 mN/m (at 20°C) and a resistivity of 18 M Ω cm. A phosphate buffer (pH 7.4) was prepared by mixing appropriate volumes of 10 mM KH_2PO_4 and 10 mM Na_2HPO_4 solutions in Milli-Q water.

Langmuir Monolayer and Brewster Angle Microscopy (BAM) Experiments. The measurements were performed using a KSV 2000 Langmuir trough (KSV Instruments Ltd., Helsinki, Finland) equipped with an ultraBAM (Accurion GmbH, Goettingen, Germany) microscope, as previously described.³⁶ Lipids were dissolved in chloroform/methanol (4:1 v/v) to form stock solutions (DPPA is completely dissolved to a concentration of less than 0.2 mg/mL). The phosphate buffer (pH 7.4) was used as the subphase. All experiments were repeated at least twice to ensure consistent results. Surface pressure–area (π –A) isotherms were reproducible within an error of $\pm 0.02 \text{ nm}^2 \text{ molecule}^{-1}$.

MD Simulations. We performed atomistic MD simulations of 18 model bilayers composed of a mixture of phosphatidylcholine and phosphatidic acid (~10 mol%). Two protonation states of PA (monoanionic, PA^- , and dianionic, PA^{2-}) were used in calculations. We considered three combinations of lipid acyl chains: dioleoyl (DOPC, DOPA), palmitoyl-oleoyl (POPC, POPA), and dipalmitoyl (DPPC, DPPA). The chemical structures of the lipids are shown in **Figure 1**. Using these lipids, we constructed 9 lipid bilayers: DOPC-DOPA, DOPC-POPA, DOPC-DPPA, POPC-DOPA, POPC-POPA, POPC-DPPA, DPPC-DOPA, DPPC-POPA, and DPPC-DPPA. All bilayers consisted of 128 lipids (116 PCs and 12 PAs), Na^+ ions (as counterions that neutralize the system), and 6400 water molecules. Figure 1 presents a typical snapshot showing the organization of the POPC-POPA²⁻ membrane at the end of the simulation. The POPA molecules are uniformly dispersed in the mixed bilayer with the acyl chains are arranged parallel to the POPC tails. The POPA headgroups are located slightly deeper compared to the POPC molecules.

The OPLS-AA force field^{37,38} was employed to parameterize all molecules using the recently derived lipid models.^{39,40} Molecular topologies of DPPC, POPC, and DOPC can be found in the Supporting Material of the reference 41. Parameters for the PA phosphate group in two different protonation states were derived in our previous studies,³⁶ and molecular topologies are included in the Supporting Information. The TIP3P model was used for water molecules.⁴²

Figure 1. (a) Chemical structures of lipids used in this study. (b) Snapshot of the POPC-POPA²⁻ lipid bilayer after 500-ns simulation; POPC is shown in yellow with polar atoms (N, P, O) in orange; POPA is shown in blue with phosphate groups in dark blue. (c) Snapshot of the DPPC-DPPA²⁻ lipid bilayer in gel phase after 500-ns simulation; DPPC is shown in gray, DPPA is shown in orange. Three periodic images are shown to clearly illustrate the ripple phase structure.

All simulations were carried out with GROMACS 4.6.7⁴³ and lasted 500 ns. The last 300 ns of the trajectories were used for analysis, unless stated otherwise. The calculations were performed at a constant temperature of 310 K and pressure of 1 atm. Systems with DPPC-based bilayers were additionally simulated at 323 K (above of the DPPC main phase transition temperature, $T_m = 314$ K). Temperature and pressure were controlled by the Nose-Hoover algorithm^{44,45} and by the Parrinello-Rahman algorithm,⁴⁶ with 0.4 ps and 1 ps time constants, respectively. A semi-isotropic algorithm was applied for pressure. The temperature of water and solute were controlled independently. The LINCS algorithm⁴⁷ was used to maintain the length of bonds and the time step was set to 2 fs. The 1 nm cutoff was used for nonbonding

interactions, and long-range electrostatic interactions were calculated using the Particle Mesh Ewald algorithm.⁴⁸ The list of neighbors was updated every 10 steps.

RESULTS

MD Simulations.

The surface area per lipid (APL) was calculated by dividing the xy area of the simulation box by the number of all lipids in the bilayer leaflet (Table 1). In all cases of bilayers in the liquid crystalline phase, the APL for mixed bilayers is smaller than that for the pure PC bilayer. APL is larger for the bilayers containing PA with a larger volume occupied by the acyl chains in the order DPPA < POPA < DOPA. In addition, the bilayers in the liquid crystalline phase containing PA^{2-} have a slightly larger APL compared to those with PA^- , but the differences are often within the error bars. This result agrees with previous studies of DPPA monolayers performed under three different conditions: pH 3, 6, and 10, in which DPPA is in neutral, monoanionic, and dianionic form, respectively.⁴⁹ In these studies, a larger charge of PA molecules correlates with larger surface areas at the same surface pressure. In the case of bilayers in the gel phase, there is no clear trend, as both the increase and the decrease in the area are observed. This, however, may be the artifact of not fully equilibrated systems, since lipid diffusion in the gel phase is much slower as compared to the liquid crystalline phase. Next, the area per PC (APC) and the area per PA (APA) were calculated using the Voronoi tessellation method, implemented in the APL@VORO software.⁵⁰ APC and APA values are collected in Table 1. As expected, APAs are smaller than APCs. Additionally, the APA is further modulated by the host lipid being the smallest in the DPPC bilayer and the largest in the DOPC bilayer. There is no statistically significant difference between APA for PA^- and PA^{2-} .

Table 1. Values of the surface area per lipid (APL), the surface area per PC (APC), and the surface area per PA (APA).

	DPPC at 37°C	DPPC at 50°C	POPC	DOPC
APL $\pm$ 0.004 [nm ²]				
DPPA ⁻	0.516	0.547	0.645	0.692
DPPA ²⁻	0.537	0.543	0.648	0.695
POPA ⁻	0.529	0.596	0.652	0.700
POPA ²⁻	0.508	0.610	0.656	0.700
DOPA ⁻	0.537	0.615	0.657	0.700
DOPA ²⁻	0.523	0.619	0.660	0.704
-	0.533	0.620	0.683	0.722
APC $\pm$ 0.004 [nm ²]				
DPPA ⁻	0.518	0.559	0.650	0.699
DPPA ²⁻	0.538	0.560	0.654	0.701
POPA ⁻	0.530	0.599	0.660	0.706
POPA ²⁻	0.509	0.620	0.662	0.705
DOPA ⁻	0.533	0.621	0.664	0.704
DOPA ²⁻	0.521	0.632	0.663	0.709
APA $\pm$ 0.007 [nm ²]				
DPPA ⁻	0.499	0.508	0.596	0.638
DPPA ²⁻	0.516	0.512	0.608	0.627
POPA ⁻	0.519	0.578	0.613	0.648
POPA ²⁻	0.527	0.579	0.609	0.636
DOPA ⁻	0.549	0.592	0.609	0.649
DOPA ²⁻	0.544	0.612	0.620	0.642

The solvent accessible surface area (SASA) is a good measure of exposure of a given group to solvent.⁵¹ For a given molecule, the SASA is the sum over all its atoms. In this work, SASA was computed using the GROMACS tool *gmx sasa*. The SASA values calculated for PAs are given in Table 2. As expected, SASA increases with the volume occupied by the acyl chains in the order DPPA<POPA<DOPA, however, the differences between SASA values are greater in the bilayers with a smaller surface area in the order DPPC>POPC>DOPC. The SASA is also correlated with the surface area of the whole bilayer, being the smallest for the DPPC bilayer in the gel phase, followed by the DPPC, POPC and DOPC bilayers. Finally, the SASA values are always larger for PA²⁻ than PA⁻.

Table 2. Values of solvent accessible surface area (SASA) per PA molecule.

	SASA [nm^2] ± 0.01			
	DPPC at 37°C	DPPC at 50°C	POPC	DOPC
DPPA ⁻	0.56	0.62	0.786	0.88
DPPA ²⁻	0.92	0.80	1.01	1.12
POPA ⁻	0.63	0.75	0.80	0.91
POPA ²⁻	0.92	0.94	1.09	1.19
DOPA ⁻	0.72	0.80	0.82	0.92
DOPA ²⁻	0.91	1.08	1.11	1.17

Figure 2 shows the mass density profiles of PC and PA phosphorus atoms in the bilayers in the liquid crystalline state. In systems containing POPC and DOPC, the PA phosphorus atoms are located 0.10-0.15 nm deeper than the PC phosphorus atoms. In most cases, the PA⁻ phosphorus atoms are located slightly deeper than those in the PA²⁻ headgroup. Similar behavior was observed for the DPPC bilayers mixed with DOPA and POPA, but the presence of DPPA in the DPPC bilayer leads to the distortion of the bilayer observed as a bimodal distribution of phosphorus atoms (Figure 2a). The average positions of PA and PC phosphorus atoms in the DPPC-DPPA bilayer are almost the same with only one visible difference – their distributions are narrower for the PA phosphorus atoms. **Figure 3** shows the mass density profiles of PC and PA phosphorus atoms in the DPPC-DPPA bilayer in the gel state. The profiles are bimodal, possibly due to the ripple structure of the bilayer (Figure 1c). The mass density profiles show that the DPPA²⁻ phosphorus atoms are located closer to the water phase than the DPPC phosphorus atoms. In all other cases, the observed differences are not significant.

Figure 2. Mass density profiles of phosphorus atoms in the lipid bilayers being in the liquid crystalline phase: dark blue line – phosphorus atoms in PC with PA^- , light blue line – phosphorus atoms in PC with PA^{2-} , black line – phosphorus atoms in PA^- , and green line – phosphorus atoms in PA^{2-} . Mass density profiles of PC phosphorus atoms (blue) in the bilayers with PA^- and PA^{2-} are shown separately for the cases where overlap was not exact. The depth equals zero corresponds to the center of the lipid bilayer.

Figure 3. Mass density profiles of phosphorus atoms in the DPPC bilayer at a temperature below T_m . Dark blue lines – phosphorus atoms in PC with PA^- , light blue lines – phosphorus atoms in PC with PA^{2-} , black lines – phosphorus atoms in PA^- , green lines – phosphorus atoms in PA^{2-} . The depth equal to zero corresponds to the center of the lipid bilayer.

The molecular hydrogen order parameter (S_{CH}) is defined as:

$$S_{\text{CH}} = \left\langle \frac{3}{2} (\cos^2 \theta_i) - \frac{1}{2} \right\rangle, \quad (1)$$

where θ_i is the angle between the C-H bond of the i -th carbon atom and the bilayer normal. The angle brackets denote averaging over time and over appropriate C-H bonds in the bilayer. S_{CH} parameter profiles along the PA *sn*1 chains are depicted in **Figure 4**. It is clear that the DPPA is the most ordered, when incorporated into the DPPC bilayer (both in the liquid and gel states),

while POPA and DOPA became more ordered only in the DPPC bilayer in the gel state. The order parameter profiles for the PC *sn*1 chains are shown in **Figure 5**. In the liquid-crystalline phases, the presence of PA in the lipid bilayers leads to the increase in the chain order. In the case of DPPC bilayers, the strongest effect is observed for DPPA and the weakest for DOPA. In contrast, the increase in the chain order in the POPC and DOPC bilayers is almost the same for each type of PA. The observed ordering effect is the weakest in the DOPC bilayers. We did not observe any qualitative differences between the behavior of the *sn*1 and *sn*2 chains (data not shown).

Figure 4. Hydrogen order parameters (S_{CH}) of the PA *sn1* chain. (a) DPPA-DPPC at 50°C (black line), DPPA-DPPC at 37°C (gray line), DPPA-POPC (red line), DPPA-DOPC (green line). (b) POPA-DPPC (black line), POPA-POPC (red line), POPA-DOPC (green line), POPA-DPPC at 37°C (gray line). (c) DOPA-DPPC at 50°C (black line), DOPA-POPC (red line), DOPA-DOPC (green line), DOPA-DPPC at 37°C (gray line). Lipid bilayers with PA^- are shown as solid lines, while lipid bilayers with PA^{2-} are shown as dashed lines.

Figure 5. Hydrogen order parameters along the *sn1* chain of: (a) DPPC in temperature above T_m (at 50°C), (b) DPPC in temperature below T_m (at 37°C), (c) POPC at 37°C, and (d) DOPC at 37°C. Lipid bilayers with DPPA are shown as black lines, with POPA as red lines, with DOPA as green lines. Lipid bilayers without PA are depicted as gray lines. Lipid bilayers with PA^- are shown as solid lines, while bilayers with PA^{2-} as dashed lines.

Finally, we evaluated the polar interactions between PAs, PCs, and water (Table 3) using the geometric criteria derived in our previous studies.⁵² For PC-PA pairs, hydrogen bonds (H-bonds) can only be formed between the PA^- hydroxyl group and the oxygen atoms in the PC phosphate group. The charge pairs may result from electrostatic interactions between positively charged quaternary ammonium groups of PC and negatively charged oxygen atoms of PA^- and PA^{2-} . In the case of lipid-water pairs, the H-bonds can be formed between the oxygen atoms of the lipid phosphate and carbonyl groups and the OH group of water molecules or between the PA^- hydroxyl group and the oxygen atom of water molecules. The cartoon representation of the polar interaction network is shown in **Figure 6**. A detailed description of polar interactions

at the water-membrane interface can be found in the review article by Pasenkiewicz-Gierula et al.⁵³

Figure 6. Cartoon representation of all possible hydrogen bonds (red dashed lines) and charge pairs (blue dashed lines) in PC bilayers containing PA monoanions (a) and PA dianions (b).

On average, PA⁻ forms a small number of H-bonds with PC, while the H-bonding of water to lipid molecules is significant. The number of H-bonds between PA and water depends on APA and APL. The number of H-bonds with water is the smallest in systems where PA is incorporated in the DPPC bilayer (the smallest APA) and the largest in systems where PA is incorporated in the DOPC bilayer (the largest APA). In addition, the number of hydrogen bonds between water and PA is higher for PA²⁻ than PA⁻. The number of H-bonds between water and PC molecules also depends on the surface area per lipid and increases in order of DPPC < POPC < DOPC.

Table 3. Summary of the polar interactions between lipids and water. Errors are less than 0.02.

	DPPC at 37°C	DPPC at 50°C	POPC	DOPC
H-bonds for PA ⁻ – PC /per PA $\pm$ 0.02				
DPPA ⁻	0.36	0.34	0.32	0.27
POPA ⁻	0.33	0.34	0.34	0.28
DOPA ⁻	0.36	0.33	0.33	0.31
H-bonds for PA – water /per PA $\pm$ 0.02				
DPPA ⁻	7.6	7.7	8.2	8.5
DPPA ²⁻	10.3	9.6	9.6	10.1
POPA ⁻	7.8	7.8	8.2	8.5
POPA ²⁻	10.3	9.4	10.2	10.3
DOPA ⁻	8.1	7.9	8.2	8.5
DOPA ²⁻	10.2	10.0	10.0	10.1
H-bonds for PC – water /per PC $\pm$ 0.02				
DPPA ⁻	8.2	7.2	7.5	8.0
DPPA ²⁻	8.2	7.2	7.4	8.0
POPA ⁻	7.4	7.3	8.1	8.3
POPA ²⁻	7.2	7.7	8.0	8.2
DOPA ⁻	7.5	7.7	8.1	8.3
DOPA ²⁻	7.3	7.7	8.1	8.3
Charge Pairs for PA – PC /per PA $\pm$ 0.02				
DPPA ⁻	2.66	2.60	1.84	1.71
DPPA ²⁻	0.47	0.57	0.21	0.19
POPA ⁻	2.76	2.46	1.81	1.68
POPA ²⁻	0.48	0.28	0.13	0.18
DOPA ⁻	2.64	2.28	1.80	1.56
DOPA ²⁻	0.57	0.31	0.21	0.15
Charge Pairs for PC – PC /per PC $\pm$ 0.02				
DPPA ⁻	3.00	2.84	3.39	3.18
DPPA ²⁻	2.93	2.82	3.41	3.19
POPA ⁻	2.77	2.50	3.32	3.13
POPA ²⁻	2.80	2.47	3.36	3.19
DOPA ⁻	2.65	2.36	3.32	3.13
DOPA ²⁻	2.93	2.39	3.36	3.16

We calculated the number of charge pairs formed between the positively charged quaternary ammonium groups in PCs and negatively charged oxygen atoms from PAs and PCs phosphate groups. The number of PA-PC charge pairs is greater in the bilayers with smaller APLs in the order DPPC-gel>DPPC>POPC>DOPC, and is higher for PA species with smaller APA. In all cases, PA⁻ forms much more charge pairs than PA²⁻, which results from the competition between charge pairs and H-bonds with water. PC-PC charge pairs in POPC- and DOPC-

containing bilayers are not affected by the type of PAs, but for the DPPC-containing bilayers in the liquid crystalline state, the number of charge pairs decreases with increasing size of the PA hydrophobic part in the order DPPA>POPA>DOPA.

Monolayer experiments. We studied three mixed systems: DPPC-DPPA, DOPC-DPPA, and DOPC-DOPA. The $\pi - A$ isotherms recorded during the monolayer compression are presented in **Figure 7**, while the calculated values of the compression modulus (C_s^{-1}) are shown in Figure S1 (the Supporting Information). The isotherms collected for pure DPPC and DOPA monolayers agree with those published previously.³⁶ The DPPA film is much more condensed compared to the DOPA and DPPC monolayers. This is because the DPPA molecules, in contrast to DOPA, have fully saturated acyl chains and smaller headgroups than the DPPC molecules, which allows dense packing of molecules in the monolayer. In addition, the PA groups are capable of forming intermolecular hydrogen bonds. The analysis of the shape of $\pi - A$ isotherm for the DPPA film and its $C_s^{-1} - \pi$ dependency (Figure S1A) reveal that the monolayer changes its physical state twice during the compression. The first phase transition is associated with a change from the liquid (L) to liquid condensed (LC) state, while the second one corresponds to the transition from the LC to solid (S) state.

The addition of DPPA molecules to the DPPC film causes a liquid expanded (LE)-LC phase transition, characteristic for DPPC monolayers, to occur at lower surface pressures, indicating that the monolayers become more condensed. In addition, for the DPPA mole fraction (X_{DPPA}) ≥ 0.3 , additional inflections resulting from the L-LC phase transition can be observed in the isotherms, as well as in the $C_s^{-1} - \pi$ plots. The surface pressure, at which monolayers undergo this transition, depends on the composition and decreases with increasing DPPA content ($\pi_{\text{L-LC}} = 20, 17, 14, 7.5, \text{ and } 4.7 \text{ mN/m}$ for $X_{\text{DPPA}} = 0.3, 0.5, 0.7, 0.9, \text{ and } 1.0$, respectively). Similarly, to the pure DPPA film, the mixed monolayers containing a higher DPPA content ($X_{\text{DPPA}} \geq 0.7$) show an LC-S phase transition, for which the surface pressure decreases with increasing DPPA

content ($\pi_{LC-S} = 33, 30$, and 29 mN/m for $X_{DPPA} = 0.7, 0.9$, and 1.0 , respectively). In the DOPC-DPPA system, the collapse surface pressure is the same as in the pure DOPC monolayer up to $X_{DPPA} \leq 0.3$, while in films with $X_{DPPA} \geq 0.5$, there are two collapse regions, which may suggest immiscibility between the film components. In the case of the DOPC-DOPA system, both the pure components and the mixtures form liquid monolayers for which the C_S^{-1} values practically do not vary with the composition.

Figure 7. Surface pressure – area ($\pi - A$) isotherms for mixed monolayers of DPPC-DPPA (A), DOPC-DPPA (B), and DOPC-DOPA (C) measured at 25°C. The phosphate buffer (a mixture of 10 mM KH₂PO₄ and 10 mM Na₂HPO₄ solutions, pH 7.4) was used as the subphase.

It has been shown that the properties of lipid monolayers at surface pressures in the range of 30-35 mN/m correspond to the properties of bilayers with the same lipid composition.⁵⁴ Therefore, to shed more light on the miscibility of monolayer components, the A values at the surface pressure of 30 mN/m were determined from isotherms and compared to those calculated assuming the ideal miscibility of the molecules (A^{id}) using the following equation:

$$A^{\text{id}} = X_1 A_1 + X_2 A_2, \quad (2)$$

where X_i and A_i are a mole fraction in the mixed film and a mean area per molecule of the i th component in its pure film at given π , respectively.⁵⁵ The results are shown in **Figure 8** A-C. In addition, the values of the excess free energy of mixing (ΔG^{Exc}) were calculated and plotted as a function of the monolayer composition (Figure 8D) to compare the magnitude of interactions between components in the investigated systems.

Figure 8. Dependence of the mean area per lipid molecule (A) on the composition for the DPPC-DPPA (A), DOPC-DPPA (B), and DOPC-DOPA (C) mixed monolayers at a surface pressure of 30 mN/m and at 25 °C. (D) Values of excess free energy of mixing (ΔG^{Exc}) versus the composition of the DPPC-DOPA monolayers at $\pi = 30$ mN/m. The dotted lines correspond to the ideal mixing.

Figure 8 clearly shows that the A values determined from the isotherms do not coincide with those calculated assuming ideal mixing (dashed lines). This suggests that interactions between lipid molecules in the mixed monolayers differ from those in the corresponding one-component films. In the case of the DPPC-DPPA mixture, deviations from the ideality are negative, while in the DOPC-DPPA and DOPC-DOPA films, they are positive. The negative deviations, observed in the DPPC-DPPA system, indicate that the interactions between the components are more attractive than those in the pure DPPC and DPPA monolayers (Figure 8D). The BAM images for selected compositions of the DPPC-DPPA films, presenting changes in the monolayer morphology during the film compression, are shown in Figure S2 (and Figure S3, the Supporting Information). These images show that the film components are miscible in the whole range of monolayer compositions. On the contrary, the mixture of DOPC with DPPA shows large positive deviations from the ideality and the ΔG^{Exc} values are highly positive indicating that the mixing of DOPC and DPPA molecules is thermodynamically unfavorable. This confirms the conclusion that the DOPC and DPPA molecules are immiscible. The phase separation, resulting from the immiscibility of the monolayer components, is clearly visible in the BAM images (**Figure 9**), where, regardless of the monolayer composition, bright-condensed DPPA domains dispersed in the fluid DOPC matrix can be seen.

For the DOPC-DOPA mixtures, the values of the mean molecular area determined from the isotherms are higher than those predicted for ideal mixing. However, the deviations are significantly weaker than those observed for the DOPC-DPPA films. The ΔG^{Exc} values are only slightly positive and do not exceed 84 J/mol. In addition, the BAM images (Figure S4, the Supporting Information) recorded for this system show that the monolayers are completely

homogenous regardless of the film compositions. This indicates that the mixing between DOPC and DPPA is close to ideal.

Figure 9. BAM images of the DOPC-DPPA monolayers taken at different stages of compression at 25°C.

DISCUSSION

This research aimed to study the effect of saturation of lipid acyl chains on the molecular organization of mixed lipid membranes containing phosphatidylcholine and phosphatidic acid. By changing the acyl chains from saturated (palmitoyl) to unsaturated (oleoyl), we modified the ratio of the cross-sectional area of nonpolar acyl chains (the hydrophobic part) to the cross-sectional area of the polar headgroup (the hydrophilic part), which is defined as a packing parameter, P .⁵⁶ The PC headgroup is relatively large, so for DPPC and POPC the packing parameter is smaller or close to one, indicating a slightly conical or nearly cylindrical shape of these molecules (see **Figure 10**). For DOPC, P is larger than one which indicates the inverted conical shape of this molecule. The PA headgroup is small, thus for all acyl chain combinations the packing parameter is greater than one, indicating the inverted conical shape of these lipids. In addition, PAs have two dissociation constants (pK_s) whose values depend on various parameters (e.g. acyl chains and membrane composition). The apparent pK_s for the pure DPPA monolayer were found to be ~ 8.0 and ~ 9.4 , while these values for the pure DOPA monolayer are 3.5 and 8.0.⁵⁷ The incorporation of PA into the PC membrane reduces the pK values. The apparent dissociation constants for 10 mol % of DOPA in the DOPC bilayer were determined to be 3.2 ± 0.3 and 7.92 ± 0.03 at 20°C .²³ Therefore, it can be assumed that at physiological pH, PA molecules, embedded into zwitterionic membranes, are mainly in the monoanionic form. Nevertheless, we simulated the mixed bilayers containing one of two forms of PA (monoanions and dianions).

Figure 10. Schematic representation of lipid molecules showing their shape and relationship to the packing parameter. The cartoon representation of monolayers depicts the difference in the location of the PA headgroup in comparison to PC.

Our experiments showed a significant effect of the lipid chain saturation on the mutual miscibility of PC and PA. The explanation of the complex miscibility of PC/PA mixtures most likely lies in the shape of lipid molecules and intermolecular interactions between lipid headgroups. The comparison of single component monolayers showed that the PA monolayers are more densely packed, and thus more condensed (and ordered) than the PC films. This originates from not only the smaller cross-sectional area of the hydrophilic PA headgroup but also from the PA's ability to form intermolecular hydrogen bonds. This affects the gel to liquid-crystalline phase transition temperatures of fully hydrated PC and PA bilayers, which are always higher for PAs than those for PCs (e.g., 65°C for DPPA versus 41°C for DPPC, -4°C for DOPA versus -17°C for DOPC).⁵⁸ The smaller size of the PA headgroups, compared to PC, causes the DPPA acyl chains to be oriented perpendicular to the air/water interface (in the single component monolayers at higher surface pressures), while the DPPC acyl chains are tilted under similar conditions. Thus, the addition of DPPA to the DPPC film results in the ordering of the

DPPC chains and shrinking the mean molecular area, as was observed in our monolayer experiments.

We observed an excellent miscibility (reflected in the negative values of ΔG^{Exc} , Figure 8) in systems where both lipids have fully saturated chains (the DPPC-DPPA systems). This result is supported by studies of DPPC-DPPA bilayers using differential scanning calorimetry (DSC),⁵⁹ which showed that the lipid miscibility in the DPPC-DPPA bilayers strongly depends on the pH of the surrounding environment, and thus on the degree of ionization of the PA headgroups. The authors suggested that at pH 7 (DPPA is negatively charged) electrostatic repulsion between the DPPA molecules and H-bonds between the DPPA and DPPC headgroups favor mixing of these two components.⁵⁹ Our MD simulations provide further data supporting strong interactions in the DPPA-DPPC mixtures. However, they indicate a large number of direct electrostatic interactions (charge pairs between DPPC and DPPA monoanion) and rather small number of H-bonds (Table 3). Importantly, the DPPA headgroups are located at the same depth as the headgroups of the hosting DPPC and the number of DPPC-DPPA charge pairs is comparable to that between the DPPC molecules, which indicates good mixing in the DPPC-DPPA⁻ pair. In addition, we observed a substantial increase in the order parameter of the lipid acyl chains in the PC-PA bilayers (Figure 5a). As a results, the DPPC-DPPA membrane is more tightly packed (much lower APL for the mixed bilayer in the liquid phase compared to the pure DPPC membrane, Table 1), thus the van der Waals interactions between the lipid acyl chains are stronger. Only in the case of the DPPC-DPPA⁻ in the gel state, we observed a slight decrease in the $-S_{\text{CH}}$ values of DPPC, but this may be a result of a slow equilibration of this bilayer.

Contrary to the DPPC-DPPA films, the ΔG^{Exc} values for the monolayers where both lipids contain unsaturated acyl chains (the DOPC-DOPA systems) were slightly positive indicating that the mixing of the components is almost ideal or slightly unfavorable. This is consistent with microscopic observations showing that DOPC and DOPA films are uniformly mixed in

the pH range 5–8 at room temperature and high ionic strength.³² In accordance with the experiments, the MD simulations indicate weak direct intermolecular interactions in the DOPC-DOPA[−] systems. The number of DOPC-DOPA[−] charge pairs is twice lower than that between DOPC molecules. This may be due to the deeper location of DOPA[−] in the mixed bilayer. In addition, both the DOPC and DOPA molecules have the shape of an inverted truncated cone and the $-S_{CH}$ values of the acyl chains in the mixed bilayer increased only slightly. Therefore, the van der Waals interactions between the lipid acyl chains are comparable to that in the pure DOPC membrane.

In the case of mixtures containing lipids with saturated and unsaturated acyl chains, the situation is more complicated. For the DOPC-DPPA system, the values of the excess free energy of mixing were very high suggesting complete immiscibility between the monolayer components, as confirmed by BAM microscopy (Figure 9). The MD simulations showed a tiny increase in the acyl chain order of the DOPC molecules after the addition of DPPA[−] to the membrane (Figure 5d). Additionally, DOPC has the shape of an inverted cone and its bent acyl chains may introduce disordering in the saturated DPPA chains, as the $-S_{CH}$ values of the DPPA[−] chains in the DOPC membrane are the lowest (Figure 4a). Therefore, it was expected that the van der Waals interactions between the lipid hydrocarbon chains are weaker. The DPPA phosphate groups tend to locate deeper in the mixed bilayer than DOPC phosphate groups, which reduces the effect of DPPA[−] on the host bilayer properties. Furthermore, DPPA[−] forms much less charge pairs with the surrounding DOPC molecules compared to polar interactions between the DOPC molecules. All those can contribute to the greater positive deviations from ideality in mixing between DOPC and DPPA (Figure 8) and lead to phase separation.

In contrast to the DOPC-DPPA system, our previously published results demonstrated that the components of the DPPC-DOPA monolayers are miscible without phase separation symptoms and the ΔG^{Exc} values changed non-monotonically from negative values for low

DOPA concentrations to positive values for higher DOPA concentrations.³⁶ As with the DPPC-DPPA mixture, the addition of small amounts of DOPA (up to 30 mol%) to the DPPC monolayer leads to monolayer condensation. Since the DPPC and DOPA molecules have a conical and inverted conical shape, respectively, the DOPA and DPPC molecules are capable of efficient packing up to a 1:4 molar ratio, as was shown previously,³⁶ reducing the mean molecular area compared to that predicted from the assumption of ideal mixing of components. However, the cross-sectional area of the hydrophobic part of the DOPA molecule is higher than that for DPPA. Thus, DPPC/DPPA monolayers are more densely packed than DPPC/DOPA films over the whole concentration range.

Summarizing, the MD simulations of lipid bilayers in the liquid crystalline state reveal an increase in the order of acyl chains in all simulated PC-PA bilayers compared to single-component PC bilayers. This effect is similar, but much weaker, to that exerted by cholesterol on the lipid acyl chains.^{60,61} One of the explanations of the cholesterol ordering effect, which is also relevant to the PA ordering effect, is the so-called umbrella model.^{62,63} In this model, increasing the order of hydrocarbon chains prevents their unfavorable interaction with water, because it reduces the cross-section area of hydrocarbon chains. Nevertheless, the imbalance of the hydrophobic to hydrophilic cross-sections of molecules is not the only factor here because the strength of the ordering properties of PA depends on both PC and PA acyl chains type (saturated or unsaturated). In the case of the DPPC-DPPA bilayer, we observed a strong ordering effect, while in other bilayers the increase in the order was moderate or insignificant. This is in contradiction with the imbalance between the cross-sections of PA headgroup and hydrocarbon chains. This imbalance is the smallest for the DPPA molecule compared to POPA and DOPA; thus, more factors should be considered. Our simulations showed the second molecular mechanism that prevents the PA hydrocarbon chains from being exposed to water. We observed that the PA molecules are located deeper in the bilayer than the PC molecules in

all systems except for the DPPC-DPPA bilayer. As a result, a part of the PA chains is inserted into the loosely packed center of the bilayer. These two mechanisms seem to act in opposite directions, and the balance between them is related to the presence of unsaturation, and therefore to the flexibility of the acyl chains.

Another result of our simulations is that the number of charge pairs between PA^- and PC decreases as the number of unsaturated acyl chains increases (Table 3, Figure S5). This demonstrates that the shape of lipid molecules affects intermolecular interactions in the bilayer. In addition, the changes in the electrostatic interactions inversely correlate with APL, and thus with the distance between the lipid headgroups. This result is consistent with our previous studies of polar interactions in PC bilayer where we showed the inverse correlation between the area occupied by individual lipid and number of polar interactions formed.⁶⁴ Lateral phase separation and domain formation are more likely when PA-PC interactions are much weaker than PC-PC and lipid-water interactions. In all PA-DPPC systems, the number of charge pairs between PA^- and PC is comparable to that between DPPC molecules. On the contrary, in PA-DOPC systems, the PA^- -PC interactions are less frequent than that between DOPC molecules.

MD simulations allow us to study the effect of the PA dianionic form on PC (zwitterionic) membranes. The acyl chains of DPPA^{2-} are more ordered than DPPA^- in all PC membranes in the liquid-crystalline state, and the difference is most significant in DPPC. There are no major differences between the ordering of monoanions and dianions of POPA and DOPA embedded in various PC membranes. In addition, PA^{2-} has a smaller ordering effect on the surrounding PC lipids in the DPPC membrane in the liquid-crystalline state than PA^- , while the ordering of the POPC and DOPC bilayers containing PA^- or PA^{2-} is similar. The PA dianions polar interactions with the PC lipids are very limited, while the number of H-bonds with water molecules is high. This indicates a possible phase separation in the PA-PC systems at higher

pH values where PA^{2-} dominates. Indeed, the DSC experiments suggest the immiscibility of the PA-PC bilayers at pH 12.⁵⁹

Conclusions

We performed MD simulations of lipid bilayers and Langmuir monolayer experiments of systems composed of PCs and PAs. The experimental results showed that the mixing of monolayer components strongly depends on the shape of lipid molecules, and thus on the packing parameter, which is determined by the ratio of the cross-section area of the acyl chain to the headgroup regions. The cross-section area of the acyl chain depends on the presence of the unsaturated bonds since the unsaturated chains have larger cross-section area. The miscibility of PA and PC can vary from entirely miscible systems (in which all lipid tails are saturated (negative values of the excess free energy of mixing)) to completely immiscible systems (containing lipids with saturated and unsaturated acyl tails (strongly positive values of ΔG^{Exc})). The MD simulations showed that mixing depends on changes in the acyl chains order, thus on the strength of the van der Waals interaction and on the strength of polar interactions formed between lipids. In the fully miscible DPPC-DPPA mixture, DPPA induces an increase of the order parameter of DPPC and forms an equal number of electrostatic interaction at the water-membrane interface with neighboring lipids as DPPC. On the contrary, in the immiscible system (DOPC-DPPA), the number of polar interactions formed by the DPPA molecules is much lower than that formed by DOPC and the order of DOPC chain is hardly affected. Moreover, we observed changes in the locations of PA phosphate groups with respect to the bilayer center. These changes were dependent on the type of acyl chains.

Changes in the location of the PA headgroups and associated changes of SASA are of high biological relevance. PA is the receptor for many peripheral membrane proteins. The deeper location of the PA headgroup changes its presentation to the peripheral proteins, rendering PA accessibility for binding. Besides, the deeper location may affect the pKs of the phosphate group

shifting the equilibrium towards -1 charge, again rendering PA accessibility for proteins. Interestingly, cells can use this mechanism to control the cellular signaling by altering the lipid composition of subcellular compartments and changing the PA surface presentation, which, in turn, leads to different PA recognition by peripheral proteins. Differences in the PA headgroup location caused by the varying lipid composition might explain the lack of a unique recognition motif in the PA-recognizing proteins.

Supporting Information:

Additional figures showing compression modulus as a function of surface pressure, and correlation between number of unsaturated tails and number of PA-PC charge pairs. BAM images for DPPC-DPPA and DOPC-DOPA monolayers.

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