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Calcium-induced lipid nanocluster structures: sculpturing of the plasma membrane

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Abstract

The plasma membrane of the cell is a complex, tightly-regulated, heterogeneous environment shaped by proteins, lipids, and small molecules. Ca^{2+} ions are important cellular messengers, spatially separated from anionic lipids. Upon cell injury, disease, or apoptotic events, anionic lipids are externalized to the outer leaflet of the plasma membrane and encounter Ca^{2+} , resulting in dramatic changes in the plasma membrane structure and initiation of signaling cascades. Despite high chemical and biological significance, the structures of lipid- Ca^{2+} nanoclusters are still not known. Previously, we demonstrated by solid-state NMR spectroscopy that upon binding to Ca^{2+} , individual phosphatidylserine lipids populate two distinct yet-to-be-characterized structural environments. Here, we concurrently employ extensive all-atom MD simulations with our accelerated membrane mimetic and detailed NMR measurements to identify lipid- Ca^{2+} nanocluster conformations. We find that major structural characteristics of these major structural characteristics of these nanoclusters, including inter-lipid pair distances and chemical shifts, agree with observable NMR parameters. Simulations reveal that lipid-ion nanoclusters are shaped by two characteristic, long-lived lipid structures induced by divalent Ca^{2+} . Using ab initio quantum

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Additional analysis figures (PDF)

mechanical calculations of chemical shifts on MD-captured lipid-ion complexes, we show that computationally observed conformations are validated by experimental NMR data. Both NMR measurements on diluted specifically-labeled lipids and MD simulations reveal that the basic structural unit that reshapes the membrane is a Ca^{2+} -coordinated phosphatidylserine tetramer. Our combined computational and experimental approach presented here can be applied to other complex systems where charged membrane-active molecular agents leave structural signatures on lipids.

Graphical Abstract

Introduction

The plasma membrane of the mammalian cell is a tightly regulated environment with complex chemical and biophysical characteristics. Though a small part of the total plasma membrane, anionic phospholipids play key regulatory roles in diverse cellular processes, such as apoptosis, vesicle fusion, blood coagulation, and signaling.^{1,2} Phosphatidylserine (PS), an anionic phospholipid with a central role in cell signaling and lipid synthesis, is present in most cellular organelles including the plasma membrane (PM), where it can account for as much as 25% of all lipids.^{1,3} PS lipids usually reside in the inner leaflet of the plasma membrane, a process that is regulated by lipid transfer proteins.^{4–6} Likewise, the cell strictly controls concentrations of ions in an asymmetric manner; for example, to modulate signaling, concentration of Ca^{2+} varies up to five orders of magnitude from 0.01–1 μM in the cytoplasm and ~3 mM outside the cell.^{7,8} As a part of regulation, Ca^{2+} and anionic lipids are spatially separated^{1,3} where the latter mostly reside in the inner leaflet of the PM, while the former is primarily at high concentrations outside of the cell.

One prominent example of a signaling pathway where ion-lipid chemistry is vital is blood clotting, where the presence of Ca^{2+} increases protein activity thousands of times.⁹ Recently, using experimental and computational approaches, we presented the “Anything But Choline” (ABC) hypothesis,⁹ which reveals how blood clotting proteins bind to membranes containing PS. We found that both PS-specific interactions, mediated by Ca^{2+} , as well as non-headgroup-specific interactions with lipids other than phosphatidylcholine (PC) can yield synergistic activation of these enzymes, as the functional state of the lipids appear to depend on the exposure of the phosphate group.⁹ The atomistic details underlying these effects remain incompletely described, and are significant for understanding the precise roles of each lipid and have potential to drive the design of small molecules that selectively modify the chemistry of protein-lipid interactions.

Towards this goal, experimental investigations—utilizing a variety of analytical techniques and spanning more than five decades—have revealed increasing details about how Ca^{2+} cations interact with anionic lipids and lead to dramatic reshaping of the membrane surface.^{10–13} A number of early experimental studies¹⁴ concentrated on bulk properties of the membrane surface in the presence of ions, including microdomain formation.¹⁵ Novel applications of tether-pulling methodologies revealed that a high concentration of Ca^{2+} ions is needed for a global membrane reshaping and formation of the positive curvature.¹⁶ Membrane curvature can also be influenced by asymmetric concentration of Ca^{2+} interacting with anionic lipids¹⁷ and can be caused by local increase of the concentration to the point of formation of tubular protrusions.¹⁸ A combined experimental and computational investigation reported that the strength of Ca^{2+} ion binding to membrane also depends on the membrane curvature.¹⁹ Recent breakthroughs in fluorescence nanoscopy and single particle tracking allowed to trace single anionic lipids in live cells, demonstrating that lipids tend to dwell within PM regions as small as 20 nm;^{20,21} this spatial nanoscale is now becoming accessible to molecular dynamics (MD) simulations. NMR spectroscopy was used to demonstrate that Ca^{2+} can lead to changes in the conformations of PS lipid headgroups.^{22,23} We were previously able to show with two-dimensional solid state NMR spectroscopy (SSNMR) that in the presence of Ca^{2+} , PS headgroups exist in two long-lived chemical environments, most likely reflecting two distinct lipid headgroup conformations which are yet to be fully resolved. Importantly, the same lipid systems did not show characteristic signatures in the presence of Na^+ ions.²⁴

Computational investigations into lipid-ion interactions previously used a number of approaches including traditional all-atom^{13,25} and coarse-grained²⁶ molecular dynamics (MD), free energy perturbation,²⁷ and metadynamics²⁸ simulations. MD simulations in combination with fluorescence probes, Laurdan, were used to show varying effects of Ca^{2+} and Na^+ ions on all-anionic membranes.¹³ Small-angle neutron and X-ray scattering PS- Na^+ data were refined with MD simulations, which demonstrated caging of sodium ions.²⁹ Very recently, time-resolved fluorescent and vibrational zero-sum spectroscopies, complemented by MD simulations, demonstrated the capacity of PS lipid bilayers to bind calcium with concentration dependence.³⁰ While these studies provided useful characterizations of the PS lipid bilayers, the conformational geometries of PS lipid headgroups in the presence of Ca^{2+} remain incompletely characterized in part because current conventional all-atom MD simulations cannot fully sample the slow lateral diffusion and exchange^{21,31} of full-tail lipids during a typical 100 ns MD simulation. With explicit MD simulations, adequate lipid mixing requires multiple microseconds.²⁶

Here we combine extensive all-atom MD simulations using the highly mobile membrane mimetic^{32,33} (HMMM), which is capable of expedited sampling of the lateral and rotational dynamics of lipids, with quantum chemistry calculations and SSNMR measurements of specifically-labeled PS lipids to address the question of how the presence of Ca^{2+} reshapes PS lipids on the nanoscale level and activates the membrane surface for interactions with proteins. The key innovation of our approach addresses the challenge of sampling Ca^{2+} ionlipid interactions by computationally capturing diverse complexes that are then validated by experimental NMR measurements.

Materials and Methods

To address this complex problem, we employ a tightly connected combination of extensive conformational sampling with MD simulations, providing lipid-ion structures to high-level QM calculations and geometric and dynamic characterization, that in turn, are compared to experimental data from selectively-labeled SSNMR measurements performed on diluted samples. The latter approach is an innovation that allows for resolution of inter- and intralipid contributions to interatomic distances, which cannot be distinguished in samples with high lipid concentrations of selectively-labeled lipids (Figure 1C).

Molecular Dynamics Simulations

HMMM Membrane System Preparation—To model membrane systems, we used the recently developed HMMM model.^{32–34} HMMM membranes consist of short-tail lipids flanking organic hydrophobic solvent.³² Due to the reduced length of the short-tail lipids, their lateral diffusion is one to two orders of magnitude faster than conventional full-tail lipids, which leads to an increased rate of sampling of the lipid-lipid and lipid-ion interactions. It was demonstrated that the free energy of amino acid insertion into the interface of the HMMM membranes³³ agrees with the experimental interfacial hydrophobicity scale³⁵ and the computational scale with full-tail membranes.³⁶ HMMM membranes used in this study were assembled using the HMMM Builder³⁷ module of the CHARMM-GUI³⁸ web portal. The HMMM membranes consist of 120 lipids with an initial density of approximately 60 Å² per lipid. The membrane composition is 70:30 molar ratio of HMMM-PS to HMMM-PC lipids. The membrane core in the HMMM membranes is replaced by 1,1-dichloroethane (DCLE) as a hydrophobic mimic. Ion concentrations were 150 mM of Ca²⁺ or 260 mM of Na⁺, in order to match the lipid-ion ratio used in our NMR experiments. The modeled membrane systems were hydrated with ~4160 water molecules and overall each system contained ~25,000 atoms (Figure 1). Ten independent replicas containing Ca²⁺ and one control containing Na⁺ were prepared. Each replica was simulated for 300 ns, which due to the expedited lateral motion of lipids in HMMM, is equivalent to sampling obtained from at least 2 μs when using a full-tail membrane representation.

MD Simulation Protocols—MD simulations were performed using NAMD 2.11.³⁹ Lipids and ions were modelled with CHARMM36⁴⁰ parameters and DCLE molecules with CGenFF⁴¹ force field. Water molecules were represented explicitly by the TIP3P model.⁴² Traditional force fields use ion parameters developed based on matching calculated and measured ion solvation free energies.^{43,44} We used standard ion parameters with no additional adjustments for carbonyl, phosphate, or carboxyl groups. Simulations were performed at constant temperature of 303 K and constant pressure of 1 atm maintained by Langevin dynamics,⁴⁵ with damping coefficient of 0.5 ps⁻¹ and Langevin Nose-Hoover method,⁴⁶ respectively. Non-bonded cutoff distance for short range interactions was 12 Å with switching at 10 Å and long-range electrostatics were included using the particle-mesh Ewald (PME) method^{47,48} with 1 Å grid spacing. SETTLE⁴⁹ was used to restrain hydrogen atom bond length. Integration step was set to 2 fs. The surface area was held constant at ~62 Å², which prevents the overall membrane curvature from changing due to the use of the short-tail lipid. While the use of constant area simulation in HMMM membranes does not

allow capturing of any Ca^{2+} -mediated changes in the total area⁵⁰ of the lipid bilayer, a local decrease of the area per lipid caused by formation of nanoclusters is still allowed in our setup. A mild restraint ($1 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$) along the membrane normal was applied to the carboxy carbon atoms of each lipid tail to minimize the small chance of short-tail lipids partitioning into the solution, while still reproducing the vertical mobility of the full-tail lipid ($\pm 3.5 \text{ \AA}$). The lipids are unrestrained in lateral motion.

These approaches were extensively tested and successfully used for a number of studies of membrane-associated phenomena (See reviews^{34,51}).

Analysis of MD Simulations—The MD trajectories were analyzed using VMD⁵² to extract dihedral angles of headgroup atoms and intra-lipid atomic distances for each PS molecule. Lipid-ion coordination was determined by locating ions that were within $2.5 \text{ \AA}$ of any oxygen atom of a PS lipid. These coordination events are categorized by the oxygen atoms involved: (1) a carboxyl coordination for either oxygen atom on the PS C_γ carboxyl group; (2) a phosphoryl coordination for the four oxygen atoms of the phosphate moiety, or (3) a tail coordination for the two carbonyl oxygens linking the acyl chain. Polydentate coordination events are also categorized, with the two most prevalent categories being phosphoryl-carboxyl and carboxyl-tail.

Statistical analysis of the data was performed using the R programming language,⁵³ relying heavily on the dplyr,⁵⁴ reshape2,⁵⁵ and ggplot2⁵⁶ libraries, via the RStudio⁵⁷ interface. PS lipids were grouped into similar structural classes by utilizing unsupervised k-means clustering, with the $\text{O-C}_\alpha\text{-C}_\beta\text{-N}$ dihedral angle as the distance metric. A value of $k = 3$ was chosen based on visual inspection of the histogram of $\text{O-C}_\alpha\text{-C}_\beta\text{-N}$ dihedral values over the entire length of the trajectory (Figure 4B). The sequence of class assignments for a lipid was used to estimate the amount of time lipids spend in each conformation, and the frequency each lipid transitioned to another conformation. A tabulation of all of the observed conformational transitions yielded a transition matrix with a lag time of 100 picoseconds, and was used to build a Markov State Model to quantify the probabilities of a lipid in a particular conformational class to either stay in that class or transition to another.

Quantum Mechanical Simulations—Using VMD, the atomic coordinates for each PS lipid and nearby Ca^{2+} ions located within $2.5 \text{ \AA}$ were extracted to use as the system for a quantum mechanics calculation. Coordinates were taken from the final frame of each trajectory, for a total of 840 lipid-ion systems. Care was taken in extracting coordinates such that they were well-centered about the origin, and molecules were not bifurcated over the MD periodic boundary. Gaussian09⁵⁸ was used to predict the NMR shielding tensors for each system using the GIAO method^{59,60} via the DFT method using the B3LYP functional.^{61,62} Calculations were performed using the 6-31+G(d,p) basis set and included chloroform using the SMD Model.⁶³ These simulation parameters match computational studies collected in the CHESHIRE database, where conversion factors to convert computed isotropic shielding into experimentally observable chemical shift values are reported.⁶⁴

Materials—Isotopically labeled phospholipids were prepared as previously described.²⁴ The choline head-group of POPC (Avanti Polar Lipids, Alabaster, AL) was replaced with

either ^{15}N -L-serine or $1\text{-}^{13}\text{C}$ -L-serine (Cambridge Isotope Laboratories, Andover, MA) using phospholipase D (PLD) from *Streptomyces*.⁶⁵ A mixture of POPC, serine, PLD, and CaSO_4 in 50 mM sodium acetate buffer, pH 5.8, was incubated overnight at 37 °C. POPS product was extracted into hexane and purified with CM52 ion-exchange resin (GE healthcare, Malborough, MA) in chloroform/methanol. Product identity and labeling were confirmed by thin layer chromatography and solution NMR. Phospholipids were combined with a molar ratio of 10:60:30 $1\text{-}^{13}\text{C}$ POPS: ^{15}N POPS:POPC and incorporated into Nanodiscs in 20 mM Tris-HCl pH 7.4 and 100 mM NaCl with a ratio of 70:1 phospholipid:MSP1D1 (membrane scaffold protein 1D1). Nanodiscs were purified by FPLC on a Superdex 200 column (GE Healthcare, Chicago, IL). Calcium was added for a final ratio of 1:1 POPS: Ca^{2+} , and the lyoprotectant trehalose was added at a ratio of 1:1 trehalose:phospholipid. (Relative concentrations of POPS and POPC were confirmed by ^{31}P solution NMR, phospholipid concentrations were determined by phosphate analysis, and calcium concentration was determined by Fura-2 assay.⁶⁶ The sample was then frozen and lyophilized, the powder was packed into a 1.6 mm SSNMR rotor (Revolution NMR, Fort Collins, CO), and water was added for a total hydration of 38% (determined by comparing the water signal at ~5 ppm in the ^1H 1D SSNMR spectrum to the total signal). The rotor was centrifuged in a custom-designed rotor-packing device⁶⁷ to pellet the sample material into the center of the rotor, with geometry defined by Kel-F spacers to be in the center 80% region of the coil volume, sealed with rubber disks.

Solid-State NMR Spectroscopy and Data Analysis—MAS solid-state NMR experiments were performed at 14.1 T (600 MHz) on a Varian Infinity-Plus (Varian NMR Inc., Fort Collins, CO) spectrometer with a 1.6-mm T3 HXY MAS probe in ^1H - ^{31}P - ^{13}C mode. A Varian MAS controller was used to control spinning at $15,000 \pm 5$ Hz. ^{13}C -dephased, ^{31}P -observed rotational-echo double-resonance (REDOR) experiments were performed with variable-temperature gas set to -20 °C. NMR experiments used TPPM⁶⁸ and SPINAL-64⁶⁹ ^1H decoupling during evolution and acquisition periods, respectively, at a nutation frequency of ~80 kHz. Tangent ramped cross-polarization^{70,71} and active rotor-synchronization of REDOR periods⁷² were also employed. Spectra were referenced externally to adamantane, using the downfield peak of 40.48 ppm.⁷³ REDOR data were processed and integrated in Spinsight (Varian) using Lorentzian-to-Gaussian apodization and zero filling prior to Fourier transformation. REDOR trajectories were analyzed using the Bessel function expansion of Mueller⁷⁴ and the S/S' normalization,⁷⁵ which was previously shown to be most accurate for frequency-selective REDOR fitting. We used this normalization in a recent study of phosphorylated amino acid crystals⁷⁶ and present the details of the approach there. Fit parameters included a characteristic ^{13}C - ^{31}P internuclear distance d and a scaling factor λ that reflects the dilution factor and the number of ^{31}P nuclei within the range of influence of a given ^{13}C nucleus.

Results and Discussion

Capturing lipid-ion oligomers

To investigate what types of oligomers can be formed by PS lipids and ions, we performed equilibrium MD simulations of ten independent replicas of the HMMM membrane systems

with Ca^{2+} , and one replica of the system with Na^+ to serve as a control. HMMM-based simulations cover total simulated time of 3.3 μs , which would correspond to at least 30 μs if sampling were to be performed with conventional full-tail lipids, as lateral diffusion of the HMMM short-tail lipids is one to two orders of magnitude faster.⁷⁷ We observe that up to four distinct PS lipids can be captured by a single Ca^{2+} , such that the ion is within 2.5 Å from an oxygen atom on each lipid. The number of Ca^{2+} ions over time that have captured one, two, three, or four PS lipids is seen in Figure 2. Though to our knowledge no modern computational methodology can sufficiently sample Ca^{2+} unbinding events, HMMM sampling combined with NMR verification presents an opportunity to capture structures of lipid-ion complexes. Lipids are also observed to be simultaneously coordinating with multiple ions, creating networks of interactions, however we will restrict analysis of oligomers to a single ion and only the directly adjacent lipids.

PS monomers (an ion with only one captured lipid) form rapidly, within the first 10 ns of the simulations. The lipid monomer population starts to decay as more lipids become captured by ions to form higher oligomers. Though the monomer population is fast to decay the number of unbound ions decayed even faster (Figure S1). The population of lipid dimers (an ion coordinating two lipids) reaches a steady count within 25 ns and serves as an intermediate as the monomers are depleted in favor of trimers and tetramers. In contrast, presence of Na^+ leads to large fluctuations in the number of lipid oligomers, indicating fleeting nature of the interactions (Figure S2). It is also interesting that on average a PS lipid binds similar number of ions, Ca^{2+} or Na^+ (Figure S10).

Small number of key lipid conformations are observed

Previous work²⁴ determined that two distinct chemical environments exist for PS in the presence of Ca^{2+} , however only when a sufficient amount of PS is present, indicating that there must be calcium-mediated inter-PS interactions. Now we measured unique P- C_γ distances of these two environments, named PS1 and PS2. When compared to MD simulations, a correlation was shown between the P- C_γ distance and the O- C_α - C_β -N dihedral for PS molecules in the presence of Ca^{2+} . Extending upon this work, we characterised the same lipid structural measures in order to maintain the same mapping from SSNMR to MD.

To determine how observed lipid-ion interactions shape lipid geometries we took a lipidcentric approach and projected all PS lipid data (~2.8 million total poses; every lipid every 100 picoseconds) into a 2-D Ramachandran-style plot using the O- C_α - C_β -N dihedral angle and P- C_γ distance as the coordinates. Data in the presence of Ca^{2+} is shown in Figure 4. While PS lipid headgroups sample a wide range of structural configurations, it is evident that a few preferred regions exist. To select an effective reaction coordinate, we applied the k-means unsupervised clustering algorithm to classify lipid geometries into characteristic categories. The MD-sampled O- C_α - C_β -N dihedral angle ensemble was clustered into three categories of conformations: gauche-negative (g^- , mean value $\sim 300^\circ$), trans (t, $\sim 180^\circ$), and gauche-positive (g^+ , $\sim 60^\circ$) (Figure 4B). The g^- is the most frequently observed confirmation. The t category, which is the most infrequently visited by the PS lipids, appears to have the unique structural designation that the P-N distance is at a maximum (Figure S3).

The O-C α -C β -N dihedral angle-based categories were applied to other potential reaction coordinates, e.g. P-C γ , P-N distances, and the P-O-C α -C β dihedral angle (Figure S3). It is important to note that presence of Na⁺ leads to similar sampling of the (O-C α -C β -N) – (P-C γ) space by PS (Figure S4), indicating that the conformational space that lipids explore is not ion dependent.

Dynamics of the conformational exchanges are characterized with a three-state Markov chain models of MD data that show that PS lipids in the presence of Ca²⁺ retain longer in the compact g⁺ state (Figure 4C). The fleeting character of the PS-Na⁺ interactions is evident by lower retention probability of the intermediate t state confirming our previous analyses.

Chemical environment of the characteristic lipid conformations

To characterize the chemical environment experienced by carbon atoms of the lipid head-group, coordinates were extracted from the final frame of each trajectory and NMR shielding tensors were computed for each lipid and surrounding ions (total of 840 lipid-ion complexes). Isotropic shielding values were converted to chemical shifts and compared against our previous experimental SSNMR measurements.²⁴ The distributions of computed shifts, seen in Figure 4D, are in a good agreement with experiment. While calculated distributions are relatively wide, the mean computed chemical shift are within 1 ppm of the shifts measured for C β and C γ , and within 4 ppm of C α atoms (Figure 4D). Though current calculations lack the precision to differentiate directly between the PS1 and PS2 environments (in the nomenclature of our previous study), which we previously measured to be within less than 1.6 ppm,²⁴ the agreement between computed and measured chemical shifts is a testament to the accuracy of the force field we used for the MD simulations and of the quantum mechanical calculations performed on the MD-based lipid-ion complexes.

Long-lived PS clusters are mediated by Ca²⁺

MD simulations reveal that ion-PS lipid monomer interactions occur via diverse lipid moieties (Figure 3). The classification of these interactions is named according to which of the oxygen atoms on the lipid are in coordination with the ion. The most populated categories are ions interacting with carboxyl and phosphate groups of PS. The dominance of carboxyl coordination is expected as that portion of the lipid is the most accessible moiety to the environment. We observed a dramatic difference in lifetime of interactions between PS-Ca²⁺ and PS-Na⁺, for example, when Na⁺ binds to carboxyl oxygen atoms of PS the interactions are short-lived (less than 50 ns), whereas Ca²⁺ ions remain bound much longer, often for the remainder of the simulation. MD simulations also show that pairs of PS lipids that are coordinated by an ion exhibit the similar lifetime distributions as the case of lipid monomers (Figure S5). In the case of Ca²⁺ ions, the four most prominent types of interactions all involve the carboxyl and phosphate groups. The most prevalent long-lived configurations were those where an ion coordinates the carboxyl group of one lipid and the phosphoryl group of the other lipid—suggesting a head-to-tail assembly of two lipid monomers. In the presence of Na⁺ ions, the category of carboxyl-phosphoryl/carboxyl (one lipid coordinated via the carboxyl group and the other lipid coordinated by both the carboxyl and phosphoryl group) is nearly absent, due to high mobility of monovalent Na⁺. Regardless of moieties captured by the ion, the net effect is that Ca²⁺ is able to form long-term

interactions with lipids in a way that Na^+ does not. Lifetimes of higher oligomers coordinated by Ca^{2+} are longer than those in the presence of Na^+ , in many cases reaching the length of an individual simulation at 300 ns (Figure S6). The high-frequency of g^- headgroup confirmation (Figure 4B) agrees well with the fact that the carboxyl group participates in most observed PS-ion interactions (Figure 3).

Finally, from the lipid-centric view, lifetimes of the individual PS lipids reveal that there are more long-lived lipid geometries in the presence of Ca^{2+} ions than in the presence of Na^+ (Figure S7). Additionally, the portion of the extended g^- long-lived PS conformations is higher in the presence of Ca^{2+} , hence phosphate groups are exposed for longer time, enabling lipid-protein interactions.

Lipid nanodomains characterized by SSNMR and MD reveal specific interatomic distances

To measure characteristic lipid-lipid distances in Ca^{2+} -induced lipid nanoclusters, we performed ^{13}C -dephased, ^{31}P -observed frequency-selective REDOR⁷⁷ experiments on a Nanodisc sample composed of 10% specifically-labeled ($1\text{-}^{13}\text{C}_\gamma$) POPS with 60% natural abundance POPS and 30% natural abundance POPC, with 1:1 POPS: Ca^{2+} . The extent of dephasing (λ) in a ^{13}C -dephased ^{31}P -observed REDOR experiment informs on the number of close ^{13}C - ^{31}P contacts, while the fitted dipolar coupling informs on the characteristic ^{13}C - ^{31}P distance (Figure 5A). We achieved a very good agreement between data and fit with single distances of 4.8 Å and 4.9 Å for the ^{31}P atoms of PS1 and PS2, respectively, and scaling factors ($\lambda = 0.581\text{--}0.585$) very consistent with four $\text{C}_\gamma\text{-P}$ couplings per phosphorus atom (Figure 5B). Numerical simulations (see Figure S8) suggested that a rather tight distribution of couplings (corresponding to distances within 4.1 Å–5.1 Å) was necessary to accurately reproduce the data.

The pair-distance distribution function $g(r)$ of MD-observed inter-lipid P-C_γ distances (Figure 5C) shows a peak at ~5 Å for both Ca^{2+} and Na^+ , consistent with the internuclear distance determined by REDOR. The longer distances (~9 Å) appear to correspond the second shell of lipids. Importantly, the sharp maximum ~6 Å in the presence of Ca^{2+} corresponds to a particular $\text{COO}^- \text{-Ca}^{2+} \text{-PO}^{4+}$ coordination geometry not observed in the presence of Na^+ . Integration of $g(r)$ reveals that there are about 4 PS neighbors present within radius of ~5 Å and about 8 PS lipids present in nanoclusters with a radius of ~9 Å. This is an excellent agreement between our MD simulations and SSNMR measurements and it informs us of the arrangement of lipids in the presence of Ca^{2+} . Moreover, it indicates there is a characteristic inter-lipid spacing created by the ions. However, the varied timescales of lipid-ion interactions make this ordering detectable by NMR only when Ca^{2+} is present and not in the presence of Na^+ . Finally, as we observe only a few Ca^{2+} ions that solely coordinate four PS lipids at once, the experiment and computations suggests that these nanoclusters are formed from networks of PS lipids, coordinated by multiple Ca^{2+} ions.

Conclusion

We investigated the atomistic details of how membranes containing anionic PS lipids are shaped into their functional state by Ca^{2+} ions. The lipid phosphate groups and the captured

ions are made readily accessible to interact with proteins. Using extensive MD simulations with a lipid-accelerated membrane representation we demonstrated that PS lipid nanoclusters are formed by two distinct PS headgroup conformations that are induced by Ca^{2+} ions, which bind to the lipids in a specific manner. Importantly, detailed characterization of MD-derived lipid nanoclusters as well as geometries of individual PS lipids agree with SSNMR measurements on two different spatial scales: 1) the chemical shifts show accuracy of capturing the chemical environment around a single lipid in either of the two long-lived conformations and 2) Ca^{2+} -coordinated PS lipid tetramer is a basic structural unit that is involved in membrane sculpturing. By studying the effects of ions on membranes containing anionic lipids, we have characterized a baseline behavior and structures of the lipids. These results will be important in interpreting the role of lipids in future protein–membrane studies and they demonstrate that MD HMMM simulations combined and validated by SSNMR methodologies are effective at capturing high-resolution signature lipid structures that are stabilized by membrane-active agents in complex membrane environments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HMMM	highly-mobile membrane mimetic
DCLE	1,1-dichloroethane
PS	Phosphatidylserine
PC	phosphatidylcholine
SSNMR	solid state NMR spectroscopy
REDOR	rotational-echo double-resonance

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Figure 1:

Lipid-ion systems overview. (A) HMMM short-tail PC (grey)/PS (yellow) lipids, DCLE solvent (green), and Ca²⁺ ions (pink) are shown. (B) Lipid headgroup structures: carbon (cyan), oxygen (red), nitrogen (blue), phosphorus (tan), and hydrogen (white). Naming convention for carbon atoms is shown. (C) Schematics of MD/NMR methodology developed and used here. Check marks symbolize comparison of computational and experimental data: diluted refers to concentration of specifically-labeled nuclei, CS – chemical shifts, and SpinEvolution – spin physics calculations of NMR observables (please see SI).

Figure 2:

A single Ca^{2+} ion is capable of coordinating with multiple PS lipids simultaneously. The number of ions observed to be coordinating one, two, three, or four distinct PS lipids at a time is shown in green, red, purple, and blue, respectively. An ion is considered to be coordinating a lipid when the ion is within $2.5 \text{ \AA}$ of an oxygen atom on the lipid's headgroup. The mean count over ten simulations is shown, with one standard deviation shaded.

Figure 3: Coordination lifetimes of ion-PS lipid interactions, color-coded by the type interacting moieties. The lifetime indicates how long an ion captured individual lipid. Characteristic conformations are framed in the same color scheme (insets).

Figure 4:

PS lipid conformations observed in the MD simulations with Ca^{2+} . (A) Distribution of the $O-C_{\alpha}-C_{\beta}-N$ dihedral angle and the $P-C_{\gamma}$ distances in all MD trajectories. (B) Distribution of the $O-C_{\alpha}-C_{\beta}-N$ dihedral angle colored-coded by detected structural category. Characteristic PS lipid structures are shown framed in the same color scheme and average values of observed dihedral angle are given; O, C_{α} , C_{β} , N atoms are marked by yellow circles for clarity (inset). (C) Three-state Markov models of observed lipid conformations based on MD data. (D) Chemical shifts of carbon atoms of the PS lipid headgroup: calculated on MD-based structures (distributions shown in black) and SSNMR measured.²⁴ Experimental chemical shifts of the two unique chemical environments (termed PS1 and PS2, ref.²⁴) shown as vertical lines (blue and pink)

Figure 5:

SSNMR and MD data both indicate tetrameric headgroup arrangement. (A) ^{13}C -dephased, ^{31}P -observed REDOR curves of diluted specifically-labeled (1- ^{13}C) PS (circles), fit to the Bessel function expression (solid curves) yielding characteristic P-C γ distances (d) of 4:8 and 4:9 Å; n is the number of C γ close enough to dephase ^{31}P and PS1 and PS2 are the two SSNMR-resolved states of PS. Dashed lines reflect expected long-time REDOR signal intensities for 1, 2, 3, and 4 close C γ atom (B) SSNMR-inferred POPS clustering, with approximately four neighboring C γ nuclei about 5 Å from each ^{31}P nucleus. (C) MD based distribution of P-C γ interlipid pair distances (solid) and number of PS neighbors (dashed). Characteristic distances are highlighted by the magenta circle.