Increased Binding of Calcium Ions at Positively Curved Phospholipid Membranes

*Aniket Magarkar[1, 2] Piotr Jurkiewicz[3], Christoph Allolio[1, 4] Martin Hof [3] & Pavel Jungwirth[1,5]**

[1] Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 16610 Prague 6, Czech Republic

[2] Faculty of Pharmacy, University of Helsinki, Viikinkaari 5E, Helsinki, 00014 Finland

[3] J. Heyrovský Institute of Physical Chemistry, Academy of Sciences of the Czech Republic, v.v.i., Dolejškova 3, CZ-182 23 Prague, Czech Republic

[4] Institut für Physikalische und Theoretische Chemie, Universität Regensburg, 93040 Regensburg, Germany

[5] Department of Physics, Tampere University of Technology, P.O. Box 692, FI-33101 Tampere, Finland

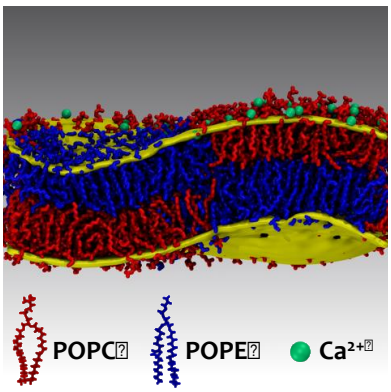
Corresponding Author

**pavel.jungwirth@uochb.cas.cz*

Abstract

Calcium ion is the ubiquitous messenger in cells and plays a key role in neuronal signaling and fusion of synaptic vesicles. These vesicles are typically ~20-50 nm in diameter and thus their interaction with calcium ions cannot be modelled faithfully with a conventional flat membrane bilayer setup. Within our newly developed molecular dynamics simulations setup, we characterize here interactions of the calcium ion with curved membrane interfaces with atomistic detail. The present molecular dynamics simulations together with time-dependent fluorescence shift experiments suggest that the mode and strength of interaction of calcium ion with a phospholipid bilayer depends on its curvature. Potential of mean force calculations demonstrate that the binding of calcium ion to the positively curved (convex) side of the bilayer is significantly stronger compared to that to a flat membrane.

TOC GRAPHICS



KEYWORDS: Membrane curvature, Ion-membrane interactions, Molecular dynamics simulation, Time-Dependent Fluorescence Shift

Many biological functions of a cellular membrane are closely related to its curvature. [1] In cells the curvature of the membrane is dynamically controlled by various means such as lipid composition, curvature-scaffolding proteins, or by adsorption of molecules at the membrane. [1,2] Mechanical properties of curved membranes have been studied extensively both in experiments [3-6] and molecular dynamics (MD) simulations. [7-11] However, the interactions of ions and biomolecules with these curved membrane interfaces remains mostly unexplored. Here we present results from a novel molecular dynamics setup that can faithfully model the curvature of stable highly curved vesicles with diameters of 20-50 nm by an asymmetric arrangement of the 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphotydilcholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoehanolamine (POPE) lipids. We investigate how calcium ions interact with curved membrane interfaces and unravel the differences in the interaction mode and strength as compared to the flat membrane bilayer. Calcium ions are known to play an important role in vesicle membrane fusion during the release of neurotransmitters at the neuronal synapses. [12] Thus, interactions of Ca^{2+} with curved membrane interfaces are particularly important in the Ca^{2+} mediated vesicular fusion as revealed by the in vitro experimental studies. [12,13] The major finding of the present study is that the modes of binding of Ca^{2+} to curved vs. flat bilayers are significantly different from each other and so is the energetics, which favors calcium binding to the former systems.

It is well known that a different number of lipid molecules in the two leaflets induces spontaneous curvature in a lipid bilayer.[14,15] Also, lipid molecules, based on their geometrical shape, exhibit varying preferences for their location in curved membrane. [13] For instance, POPE with its conical geometrical shape due to a smaller head-group volume as compared to that of lipid tails induces negative curvature in lipid

bilayers.[13] Taking these facts into account we induced curvature by assembling lipid bilayers composed of patches of POPC and POPE lipids, as depicted in Figure 1.

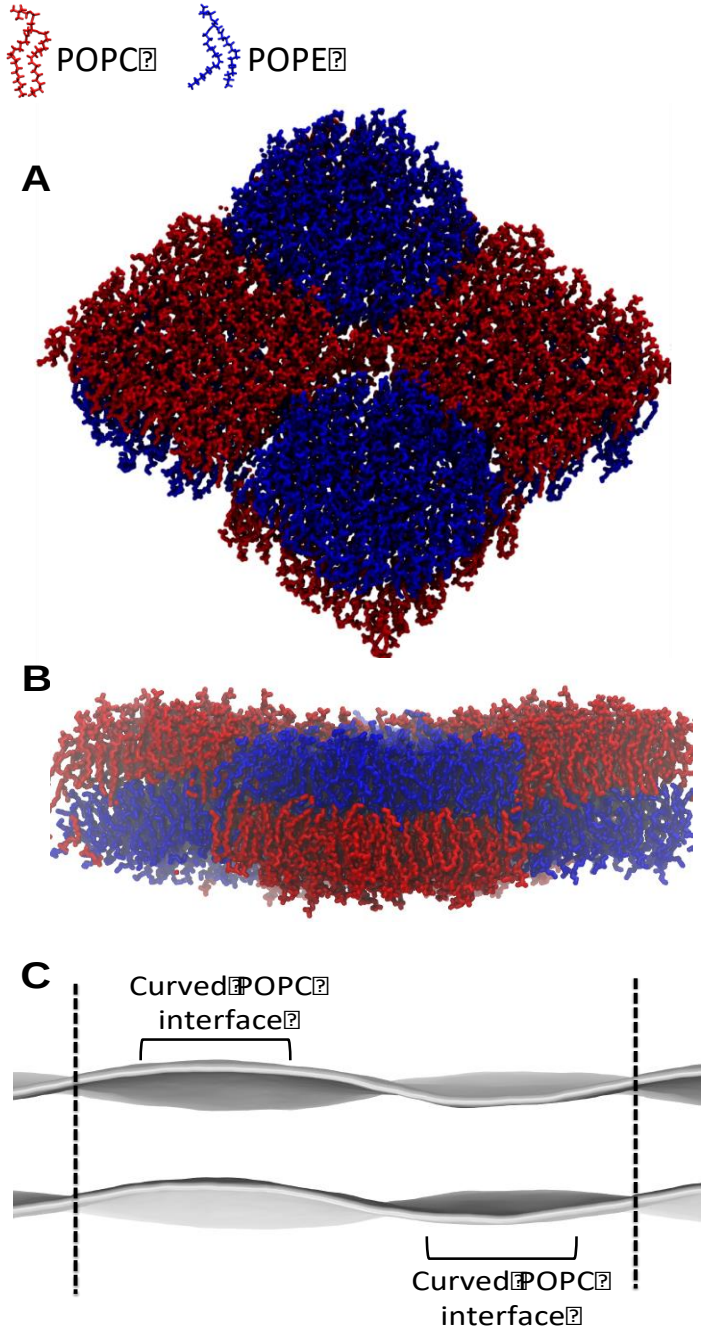


Figure 1: A newly designed simulation setup yielding a curved lipid bilayer. (A) Initial simulation setup with POPC (shown in red) and POPE (shown in blue) (B) Stable curved lipid interface obtained after ~70 ns. (C) Cross-section of a curved membrane surface generated by rendering of phosphate atoms. The setup generates four positively curved interfaces and 4 negatively curved ones.

A lipid bilayer prepared in this way forms a spontaneous curvature within ~10 ns of MD under semi-isotropic pressure coupling conditions. For 10-70 ns the curvature of the system fluctuates but after ~70 ns a stable curvature is obtained, see figure S1 in SI. In order to maintain this curvature and avoid mixing of POPC and POPE lipids, position restraints were applied to the central glycerol carbon atom of each of the POPE molecules, constraining their lateral motion. No other position restraints were applied to the system. Importantly, all the investigated properties in the present study concern the positively curved interface formed by unconstrained POPC lipids. Further technical details are presented in Methods section.

It has been recently reported that [14] for liposome diameters larger than 100 nm the local membrane surface can be considered as being flat for the sizes of up to ~10-15 nm^2. However, for diameters smaller than 100 nm, the vesicle curvature needs to be taken into account. Also the authors of this study calculated the number ratio of lipids in the outer leaflet to the inner monolayer for vesicles with diameters of 30-80 nm. Reproducing these ratios in our models we have built two curved lipid bilayer systems, which represent surfaces of vesicles with diameters of 20 nm and 50 nm (Table S1 in SI).

MD simulations were performed for two curved membrane bilayers representing surface patches of vesicles with diameters of the 20 and 50 nm and, for comparison, for a flat POPC membrane bilayer (pertinent also to vesicles with diameters larger than 100 nm). All bilayers were immersed in a physiological 150 mM solution of NaCl, with added 450 mM of $CaCl_2$. The relatively high nominal calcium concentration ensures sufficient exchange of the strongly membrane-bound and free calcium within the duration of the simulation. At lower concentrations of calcium ions,

all of the calcium ions bind to membrane bilayer, thus leaving no free calcium in the bulk solution. In contrast, for the present system size at 450 mM $CaCl_2$ concentration along with 150 mM NaCl, ~15% of calcium ions were always in bulk with an average exchange time between the solution and bilayer surface of 60 ns.

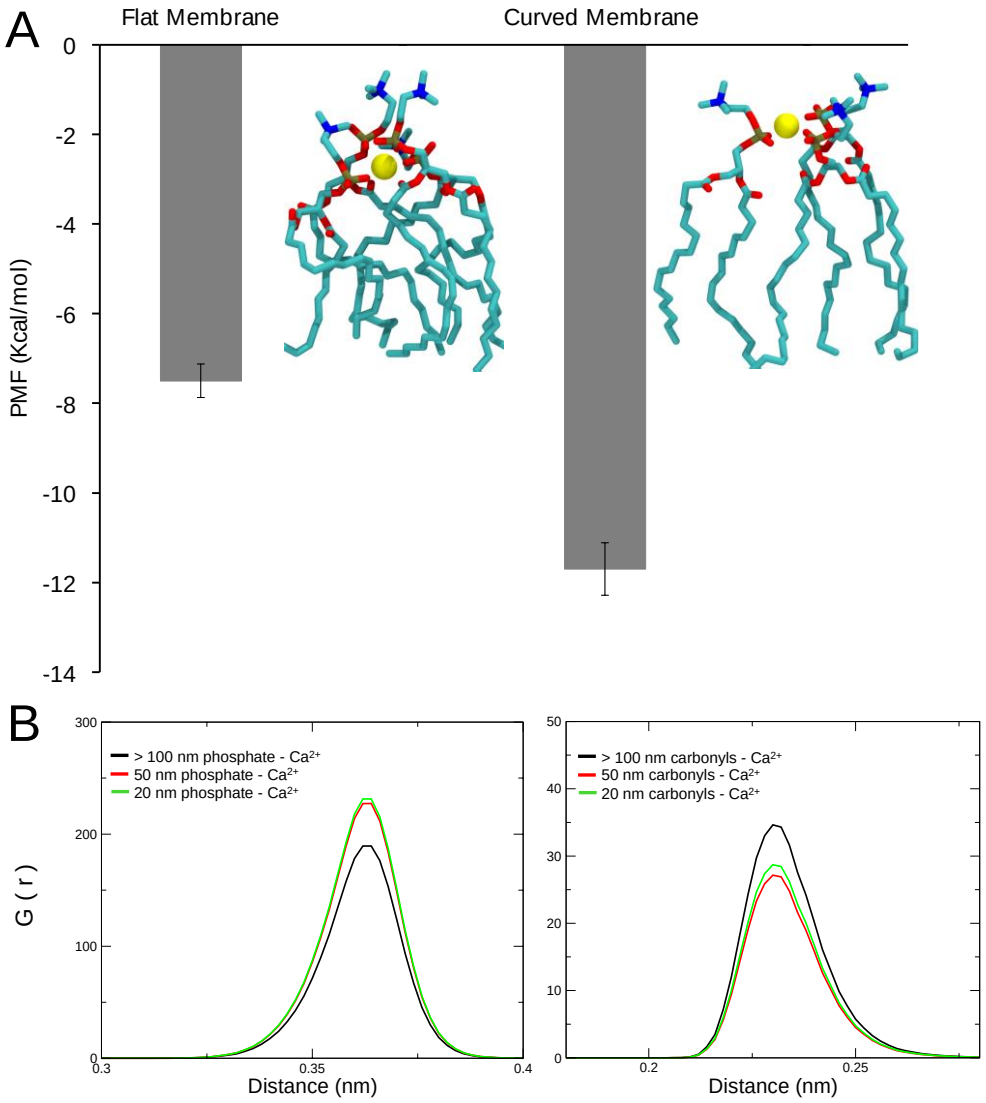


Figure 2: (A) Binding of Ca^{2+} (shown in yellow) to flat and curved POPC bilayers and respective interaction free energies (PMF) depicted as gray bars. (B) Radial distribution function (RDF) for the Ca^{2+} with phosphate group (left) and carbonyl oxygens (right).

Unlike sodium (which is at best very weakly attracted to the membrane) or chloride (which shows no attraction to the membrane), calcium exhibits an appreciable

binding to the lipid bilayer. Free energy calculations (see Methods for details) revealed that binding free energies of Ca^{2+} at flat vs. curved bilayers are significantly different from each other. Namely, in the case of Ca^{2+} at the planar interface the binding energy is ~7.6 kcal/mol whereas for the case of Ca^{2+} at the curved interface it increases to ~11.7 kcal/mol as shown in Figure 2. The result presented here is directly pertinent to the vesicle diameter of 50 nm, nevertheless, we found that calcium binding to the more curved bilayer (diameter of 20 nm) is of a comparable strength. We note here that partial inclusion of electronic polarization in a mean field way via rescaling of the ionic charges reduces slightly the calcium binding to the bilayer in agreement with experimental observations. [15] A more complete inclusion of electronic polarization effects (which would be rather difficult at this point) is thus likely to further reduce the absolute strengths of calcium binding to the bilayers, but will not change the principal result presented here, i.e., the increase of binding upon curving the membrane.

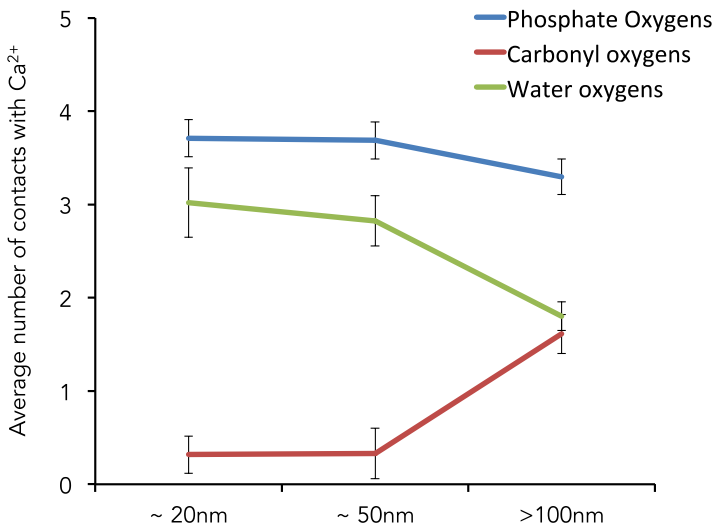


Figure 3: Average number of contacts of Ca^{2+} with POPC phosphate oxygens, carbonyl oxygens, and water oxygen atoms.

The curvature-induced variations in ion binding energetics are connected with structural changes. In the case of a flat POPC membrane, we find that single Ca^{2+} ion

binds in average 2 oxygen atoms of water, 2 oxygen atoms of POPC carbonyls, and 3-4 oxygen atoms of POPC phosphates, as shown in Figure 3 and Figure S3. These findings are consistent with previous simulation studies. [16-18,] In contrast, at curved bilayers of diameters of 20 and 50 nm, Ca^{2+} preferentially locates at the phosphate group and interacts with 4 oxygens of phosphate groups rather than with those of carbonyl groups of the PC lipids. Also the number of water molecules interacting with Ca^{2+} increases to ~3 upon inducing membrane curvature.

3. Experimental validation by fluorescence spectroscopy

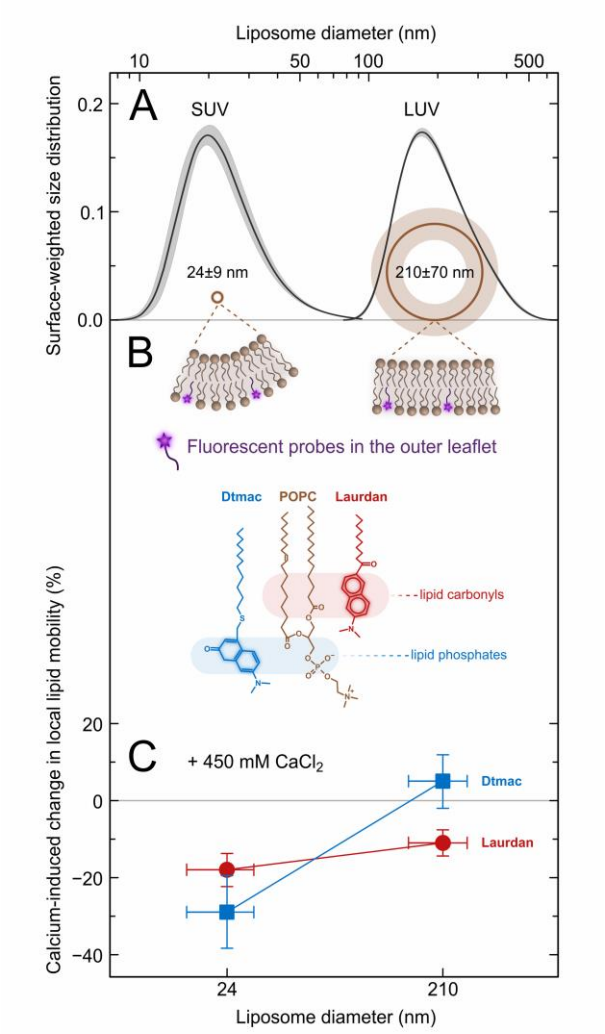


Figure 4: Calcium-induced changes in the properties of flat and curved lipid bilayer. Large and small POPC vesicles of well-defined sizes characterized using dynamic light scattering were investigated (A). Fluorescent probes Laurdan and

Dtmac, located at the lipid carbonyls and phosphates, respectively, were embedded in the outer leaflet of the vesicles (B). Relative changes in the local mobility of lipids obtained from time-dependent fluorescence shift experiments were calculated as a reciprocal of the mean integrated relaxation time measured at 293 K (C); see method section for details.

The simulation results are complemented by those from Time-Dependent Fluorescence Shift (TDFS) measurements. TDFS employs fluorescent polarity probes to monitor dipolar relaxation in lipid bilayers carrying information about local hydration and mobility of lipids. [19] The method, used previously to study membrane curvature [20], can be successfully paired with MD simulations [21]. Recently, we combined TDFS and MD to show coexistence of multiple calcium binding sites in flat PC and PC/PS bilayers. [15] Here, the two fluorescent probes located at the phosphate (Dtmac) and carbonyl (Laurdan) groups of the lipids were used to study calcium binding to highly curved membranes. LUVs and SUVs were formed by extrusion and sonication, respectively. Their size distributions were measured using dynamic light scattering (DLS) (Figure 4A), yielding mean diameters of 24 (SUVs) and 210 nm (LUVs). These vesicles were labeled with the fluorescent probes added from a methanol solution. In this way only the outer leaflet of the liposomes was labeled. The labeling procedure and the probe location in POPC bilayer are depicted schematically in Figure 4B. A more detailed explanation of the technique, sample preparation, and additional results are presented in the Methods section and in the Supplementary Information.

The relative changes in the phosphate and carbonyl mobilities, recorded upon the interaction of the membranes with a 450 mM $CaCl_2$ solution are presented in Figure 4C. While calcium ions hinder the mobility of lipid carbonyls both in large and small vesicles, the mobility of the phosphates is altered significantly only in the SUVs. The domination of the phosphate effect over the one of the carbonyls in the SUVs is in very

good agreement with the MD results, where the interaction of Ca^{2+} ions with phosphates becomes stronger with increasing membrane curvature. The experiments also show that the hindrance of mobility is more pronounced in SUVs than in LUVs both for the carbonyls and the phosphates. This is because larger membrane curvature creates more space for the lipid headgroups; hence the stiffening effect of calcium becomes more pronounced.

In summary, a novel molecular dynamics simulation setup was developed to faithfully model curved lipid interfaces of vesicles with diameters of 20 and 50 nm. This allowed us to investigate interactions of calcium ions at these curved interfaces. We showed that the mode of Ca^{2+} binding depends on the shape of the membrane bilayer interface. While at a planar bilayer calcium ions interact with phosphate oxygens of the lipid headgroups and the carbonyl oxygens of the tails, in the curved system calcium collocates primarily with phosphate oxygens. The difference in the mode of the binding of the calcium ions also translates into the difference in the binding free energy, which is about 4 kcal/mol larger for the curved bilayer as compared to the planar one.

Recently, the first *in-vitro* evidence that calcium ion induces positive curvature at the interface of giant unilamellar vesicles was provided [22]. The authors attribute this positive curvature induction to binding of calcium ions to the lipid headgroups and subsequent repulsion between neighboring calcium ions. Alternatively, in our study we find that calcium simply prefers to bind to positively curved bilayers since its mode of binding is thermodynamically more favorable compared to a flat membrane. To further support the present explanation and exclude the effect of calcium-calcium repulsion we performed a simulation of a single calcium ion at a curved membrane bilayer, finding

that it binds in the same way as in the 450 mM calcium chloride solution, see Figure S4.

The fact that calcium ions prefer to bind to positively curved membrane regions is of a particular importance for the process of vesicle fusion with cellular membranes or other vesicles. The first step in the calcium-mediated membrane fusion is crosslinking of the fusing membranes. This cross-linking is enabled by the ability of calcium ions to bind at the same time to two membranes which are in a close vicinity[1], initiating thus the membrane fusion process. The existing picture based on calcium binding to a flat membrane interface points to calcium localization between the carbonyl oxygens and phosphate head group of the lipid [16-18]. However, within this picture it would be hardly possible for the calcium ions to cross-link the two bilayers, because it is embedded rather deep in the membrane. In contrast, at the curved interface where calcium ions are localized primarily at the phosphate region, i.e., closer to the contact between the two bilayers, crosslinking becomes more feasible leading to a more efficient membrane fusion. Moreover, the present free energy calculations indicate that calcium ions should spontaneously accumulate at the regions of high positive curvature. The preferential location of calcium ions at the phosphate group in the highly curved membrane regions may thus hold an important key for elucidating the molecular mechanisms of calcium-induced cross-linking of vesicles and membrane fusion.

Methods

Simulations

MD simulations were carried out for solvated lipid bilayer systems composed of POPC and POPE. Details of the molecular composition for all modeled systems are provided in SI Table S1. For MD simulations of POPC and POPE lipids SLIPIDS [23,24]

parameters were used with a compatible TIP3P water model[25]. The charges of the salt ions (450 mM $CaCl_2$ and 150 mM NaCl) were scaled by a factor of 0.75 in order to account for electronic polarization effects in a mean field way. [26,27] All simulations were carried out with an isothermal-isobaric ensemble using the GROMACS 4.6.7 simulation package.[28] The Particle Mesh Ewald method (PME) was used for the long-range electrostatic interactions, with a real space cutoff of 1 nm. In all three dimensions periodic boundary conditions with the minimum image convention were used. All simulations were carried out at ambient pressure of 1 bar and temperature of 300 K. Constant temperature conditions were maintained using the Nosé-Hoover thermostat [29,30] with the membrane and solvent coupled to separate heat baths. The isobaric conditions were maintained using a semi-isotropic Parinello-Rahman Barostat[31]. The lengths of all covalent bonds to hydrogen atoms was held fixed using the LINCS algorithm. [32] The timestep for all simulations was set to 2 fs. [33]

For the curved bilayer systems an initial run of 70 ns was performed to generate curvature without applying any position restraints, followed by a simulations with position restrains applied in the x and y directions on the central glycerol carbons of the POPE lipids in order to stop their lateral diffusion. Note that in the absence of position restraints mixing of POPC and POPE leading to loss of curvature is observed at the sub-microsecond timescale. The bilayer was then equilibrated for 200 ns after which the salt ions were added to the system and whole system was simulated for 400 ns, the last 200 ns of which were considered for analysis. For the planar system the bilayer was constructed purely with POPC and equilibrated for 50 ns before adding the salt ions. Simulation was then run for 300 ns with the last 100 ns used for analysis.

Free energy calculations employing the umbrella sampling method were performed. The umbrella windows were obtained using a pull simulation starting from

the last frame of the equilibration run. A pulling force of 10 kJmol^{-1}Å $^{-2}$ was employed, with the reaction coordinate being the nearest distance of the membrane bilayer from the calcium ion. The simulation of each of the umbrella windows was 100 ns long. The free-energy profiles were then reconstructed using the weighted histogram analysis method (WHAM) [34].

Experiments

A brief description of the experimental setup given below is further expanded in the SI. POPC liposomes formed in water (with 0.1 mM EDTA to chelate any traces of calcium) or in 450 mM solution of $CaCl_2$ were either extruded through polycarbonate filters (nominal pore diameter of 0.2 μm) or sonicated using tip ultrasonicator (Sonopuls HD 2070, Bandelin electronic GmbH, Germany), yielding LUVs and SUVs, respectively. Fluorescent polarity probes, Laurdan or Dtmac, were added to liposome dispersions (0.5 mM lipid) in a methanol solution while vortexing. The fluorescence probe to lipid molar ratio was ~1 : 400 with methanol content < 0.5%. Samples were further vortexed for 15 min, transferred to spectroscopic cuvettes and measured immediately. DLS was measured at 298 K using the Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). TDFS method was performed using Fluorolog-3 spectrofluorimeter (model FL3-11, JobinYvon Inc., Edison, NJ, USA) and a time-correlated single-photon counting spectrometer (5000 U SPC, equipped with a 370 nm NanoLED 11 laser diode and a cooled Hamamatsu R3809U-50 microchannel plate photomultiplier, IBH, Glasgow, UK). Fluorescence decays measured at multiple emission wavelengths (400-540 nm for Laurdan, and 410-550 nm for Dtmac; all with 10 nm steps) were used to reconstruct time-resolved emission spectra (TRES), which carry information on the dipolar relaxation at a probe location. Total TRES shift, $\Delta\nu$, reports on membrane polarity/hydration, and integrated relaxation time, τ_r, reflects local

mobility of hydrated phospholipids. Changes in local lipid mobility were calculated as $\%mob = (1 - \tau_{Ca}/\tau_{H2O}) \cdot 100\%$, where Ca and H_2O subscripts indicate relaxation times obtained in the presence and absence of 450 mM $CaCl_2$, respectively. The principles of the method are described in recent reviews [19], [35] and the details of the setup were identical with those recently published [36,15].

ASSOCIATED CONTENT

Additional figures and details of the simulation systems compositions are provided in the Supporting Information.

Supporting_Information.pdf

AUTHOR INFORMATION

The authors declare no competing financial interests.

ACKNOWLEDGMENT

P.Jun. acknowledges support from the Czech Science Foundation (grant no. 16-01074S) and from the Academy of Finland via the FiDiPro award. A.M. acknowledges Magnus Ehrnrooth foundation, Finland for funding. P.Jur. and M.H. acknowledge the Czech Science Foundation (P208/12/G016). M.H thanks Czech Academy of Sciences for the Praemium Academiae award. C.A. thanks the German Academic Exchange Service (DAAD) for support via a P.R.I.M.E. fellowship. Allocation of computer time from the CSC Finland is highly appreciated.

References:

(1) McMahon, H. T.; Gallop, J. L. Membrane Curvature and Mechanisms of Dynamic Cell Membrane Remodelling. *Nature* **2005**, *438* (7068), 590–596.

(2) Różycki, B.; Lipowsky, R. Spontaneous Curvature of Bilayer Membranes From Molecular Simulations: Asymmetric Lipid Densities and Asymmetric Adsorption. *J. Chem. Phys.* **2015**, *142* (5), 054101.

(3) Faucon, J. F.; Mitov, M. D.; Méléard, P.; Bivas, I.; Bothorel, P. Bending Elasticity and Thermal Fluctuations of Lipid Membranes. Theoretical and Experimental Requirements. *Journal de Physique* **1989**, *50* (17), 2389–2414.

(4) Evans, E.; Rawicz, W. Entropy-Driven Tension and Bending Elasticity in Condensed-Fluid Membranes. *Phys. Rev. Lett.* **1990**, *64* (17), 2094–2097.

(5) Rawicz, W.; Olbrich, K. C.; McIntosh, T.; Needham, D.; Evans, E. Effect of Chain Length and Unsaturation on Elasticity of Lipid Bilayers. *Biophysical Journal* **2000**, *79* (1), 328–339.

(6) Cuvelier, D.; Derényi, I.; Bassereau, P.; Nassoy, P. Coalescence of Membrane Tethers: Experiments, Theory, and Applications. *Biophysical Journal* **2005**, *88* (4), 2714–2726.

(7) Cooke, I. R.; Deserno, M. Solvent-Free Model for Self-Assembling Fluid Bilayer Membranes: Stabilization of the Fluid Phase Based on Broad Attractive Tail Potentials. *J. Chem. Phys.* **2005**, *123* (22), 224710.

(8) Goetz, R.; Gompper, G.; Lipowsky, R. Mobility and Elasticity of Self-Assembled Membranes. *Phys. Rev. Lett.* **1999**, *82* (1), 221–224.

(9) Harmandaris, V. A.; Deserno, M. A Novel Method for Measuring the Bending Rigidity of Model Lipid Membranes by Simulating Tethers. *The Journal of Chemical Physics* **2006**, *125* (20), 204905.

(10) Lindahl, E.; Edholm, O. Mesoscopic Undulations and Thickness Fluctuations in Lipid Bilayers From Molecular Dynamics Simulations. *Biophysical Journal* **2000**, *79* (1), 426–433.

(11) Marrink, S. J.; Mark, A. E. Effect of Undulations on Surface Tension in Simulated Bilayers. *J. Phys Chem. B* **2001**, *105* (26), 6122–6127.

(12) Kavalali, E. T. The Mechanisms and Functions of Spontaneous Neurotransmitter Release. *Nat. Rev. Neurosci.* **2015**, *16* (1), 5–16.

(13) Kumar, V. V. Complementary Molecular Shapes and Additivity of the Packing Parameter of Lipids. *Proceedings of the National Academy of Sciences* **1991**, *88* (2), 444–448.

(14) Vanni, S.; Hirose, H.; Barelli, H. E. L. E. N.; Gautier, R.; Antonny, B. A Sub-Nanometre View of How Membrane Curvature and Composition Modulate Lipid Packing and Protein Recruitment. *Nat Commun* **2014**, *5*, 1–10.

(15) Melcrová, A.; Pokorna, S.; Pullanchery, S.; Kohagen, M.; Jurkiewicz, P.; Hof, M.; Jungwirth, P.; Cremer, P. S.; Cwiklik, L. The Complex Nature of Calcium Cation Interactions with Phospholipid Bilayers. *Sci Rep* **2016**, *6*, 38035.

(16) Jungwirth, P. Ions at Biological Interfaces. In *Encyclopedia of Applied Electrochemistry*; Springer New York: New York, NY, 2014; pp 1131–1135.

(17) Magarkar, A.; Karakas, E.; Stepniewski, M.; Róg, T.; Bunker, A. Molecular Dynamics Simulation of PEGylated Bilayer Interacting with Salt Ions: a Model of the Liposome Surface in the Bloodstream. *J. Phys Chem. B* **2012**, *116* (14), 4212–4219.

(18) Yang, J.; Calero, C.; Bonomi, M.; Martí, J. Specific Ion Binding at Phospholipid Membrane Surfaces. *J Chem Theory Comput* **2015**, *11* (9), 4495–4499.

(19) Jurkiewicz, P.; Sýkora, J.; Olzyńska, A.; Humpolícková, J.; Hof, M. Solvent Relaxation in Phospholipid Bilayers: Principles and Recent Applications. *J*

Fluoresc **2005**, *15* (6), 883–894.

(20) Sýkora, J.; Jurkiewicz, P.; Epand, R. M.; Kraayenhof, R.; Langner, M.; Hof, M. Influence of the Curvature on the Water Structure in the Headgroup Region of Phospholipid Bilayer Studied by the Solvent Relaxation Technique. *Chem. Phys. Lipids* **2005**, *135* (2), 213–221.

(21) Jurkiewicz, P.; Cwiklik, L.; Jungwirth, P.; Hof, M. Lipid Hydration and Mobility: an Interplay Between Fluorescence Solvent Relaxation Experiments and Molecular Dynamics Simulations. *Biochimie* **2012**, *94* (1), 26–32.

(22) Simunovic, M.; Lee, K. Y. C.; Bassereau, P. Celebrating Soft Matter's 10th Anniversary: Screening of the Calcium-Induced Spontaneous Curvature of Lipid Membranes. *Soft Matter* **2015**, *11* (25), 5030–5036.

(23) Jämbeck, J. P. M.; Lyubartsev, A. P. Derivation and Systematic Validation of a Refined All-Atom Force Field for Phosphatidylcholine Lipids. *J. Phys Chem. B* **2012**, *116* (10), 3164–3179.

(24) Jämbeck, J. P. M.; Lyubartsev, A. P. An Extension and Further Validation of an All-Atomistic Force Field for Biological Membranes. *J Chem Theory Comput* **2012**, *8* (8), 2938–2948.

(25) Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W.; Klein, M. L. Comparison of Simple Potential Functions for Simulating Liquid Water. *The Journal of Chemical Physics* **1983**, *79* (2), 926–935.

(26) Kohagen, M.; Mason, P. E.; Jungwirth, P. Accurate Description of Calcium Solvation in Concentrated Aqueous Solutions. *J. Phys Chem. B* **2014**, *118* (28), 7902–7909.

(27) Kohagen, M.; Mason, P. E.; Jungwirth, P. Accounting for Electronic

Polarization Effects in Aqueous Sodium Chloride via Molecular Dynamics Aided by Neutron Scattering. *J. Phys Chem. B* **2016**, *120* (8), 1454–1460.

(28) Pronk, S.; Páll, S.; Schulz, R.; Larsson, P.; Bjelkmar, P.; Apostolov, R.; Shirts, M. R.; Smith, J. C.; Kasson, P. M.; van der Spoel, D.; et al. GROMACS 4.5: a High-Throughput and Highly Parallel Open Source Molecular Simulation Toolkit. *Bioinformatics* **2013**, *29* (7), 845–854.

(29) Nosé, S. A Unified Formulation of the Constant Temperature Molecular Dynamics Methods. *The Journal of Chemical Physics* **1984**, *81* (1), 511–519.

(30) Hoover, W. G. Canonical Dynamics: Equilibrium Phase-Space Distributions. *Phys. Rev. A* **1985**, *31* (3), 1695–1697.

(31) Parrinello, M.; Rahman, A. Polymorphic Transitions in Single Crystals: a New Molecular Dynamics Method. *Journal of Applied Physics* **1981**, *52* (12), 7182–7190.

(32) Hess, B.; Bekker, H.; Berendsen, H. LINCS: a Linear Constraint Solver for Molecular Simulations. *Journal of computational …* **1997**.

(33) Essmann, U.; Perera, L.; Berkowitz, M. L.; Darden, T.; Lee, H.; Pedersen, L. G. A Smooth Particle Mesh Ewald Method. *The Journal of Chemical Physics* **1995**, *103* (19), 8577–8593.

(34) Hub, J. S.; de Groot, B. L.; van der Spoel, D. G_Wham—a Free Weighted Histogram Analysis Implementation Including Robust Error and Autocorrelation Estimates. *J Chem Theory Comput* **2010**, *6* (12), 3713–3720.

(35) Amaro, M.; Šachl, R.; Jurkiewicz, P.; Coutinho, A.; Prieto, M.; Hof, M. Time-Resolved Fluorescence in Lipid Bilayers: Selected Applications and Advantages Over Steady State. *Biophys. J.* **2014**, *107* (12), 2751–2760.

(36) Khandelia, H.; Loubet, B.; Olzyńska, A.; Jurkiewicz, P.; Hof, M. Pairing of

Cholesterol with Oxidized Phospholipid Species in Lipid Bilayers. *Soft Matter* **2014**, *10* (4), 639–647.