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Phase Coexistence in Single-Lipid Membranes Induced by Buffering Agents

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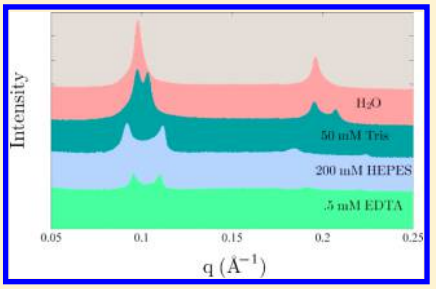
ABSTRACT: Recent literature has shown that buffers affect the interaction between lipid bilayers through a mechanism that involves van der Waals forces, electrostatics, hydration forces and membrane bending rigidity. This letter shows an additional peculiar effect of buffers on the mixed chain 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipid bilayers, namely phase coexistence similar to what was reported by Rappolt et al. for alkali chlorides. The data presented suggest that one phase appears to dehydrate below the value in pure water, while the other phase swells as the concentration of buffer is increased. However, since the two phases must be in osmotic equilibrium with one another, this behavior challenges theoretical models of lipid interactions.



INTRODUCTION

Buffer solutions are used in biomolecular research to electrostatically stabilize titratable molecular groups such as polar amino acid side chains in proteins and charged lipid head groups. Effects of pH buffers on membrane physical properties are generally neglected except in a few recent reports.[1−3] Zwitterionic buffers belonging to Good's series were shown to affect the interactions between neighboring membranes[2] and possibly alter membrane bending rigidity.[3] Membranes made of phosphatidylcholine (PC) lipids tend to form multilamellar lipid vesicles (MLVs) where the equilibrium repeat spacing (*D*-spacing) is set by a balance of attractive and repulsive forces. These forces include van der Waals (vdW) attraction, hydration repulsion, fluctuation repulsion, and electrostatics.[2] Zwitterionic buffers such as 2-(*N*-morpholino)ethanesulfonic acid (MES), 3-morpholinopropane-1-sulfonic acid (MOPS), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) have been shown to swell MLVs by modifying membrane interactions in at least three ways similar to swelling in salt: (1) reduction of vdW attraction due to dielectric properties,[2] (2) addition of electrostatic repulsions due to binding the lipid−water interface,[2] and (3) alteration of membrane bending rigidity possibly by inserting into the lipid bilayer.[1,3]

Work presented in this letter shows that this combination of effects can lead to phase coexistence in the case of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayers. Similar phase coexistence was reported by Rappolt et al. in the presence of alkali chloride salts, notably in the presence of LiCl.[4,5] No such phase coexistence was detected for the symmetric shorter-chain DLPC in either monovalent salt[6,7] or buffer solutions.[2] Data presented in this work show that HEPES, tris(hydroxymethyl)aminomethane (Tris), and ethylenediaminetetraacetic acid (EDTA), which are standard buffering agents used to stabilize proteins[8−11] induce phase separation in POPC in which the difference in *D*-spacing between the two phases grows with increasing buffer concentration. Specifically, HEPES was studied systematically incorporating a range of concentrations that includes those seen in studies, which stabilize membrane proteins in assays in the presence of synthetic lipid systems.[12−17]

In addition, the relative fraction of the two phases correlates with the pH of the buffered solution. HEPES has two titratable groups and therefore two pK_a values: a tertiary amine group (pK_{a1} = 7.5) and a sulfonic acid groups (pK_{a2} = 3.0), which confers a range of electrostatic properties depending on pH.[18] In addition to HEPES, typical buffer solutions can also contain other components such as Tris, which has a primary amine (pK_a ≈ 7.5) that stabilizes proteins and DNA,[19] and chelating agents such as EDTA, whose two amines (pK_a ≈ 6.1, 10.34)[20] and four carboxylates (pK_a ≈ 0.0, 1.5, 2.0, and 2.7)[21] seek to bind and sequester metal ions such as Ca^{2+} and Fe^{3+}. Hence, small-angle X-ray scattering data of POPC in the presence of HEPES solutions, with and without these other buffering agents, are presented to document their effects on intermembrane interactions.

EXPERIMENTAL SECTION

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) were purchased from Avanti Polar Lipids (Alabaster, AL). All buffer components were

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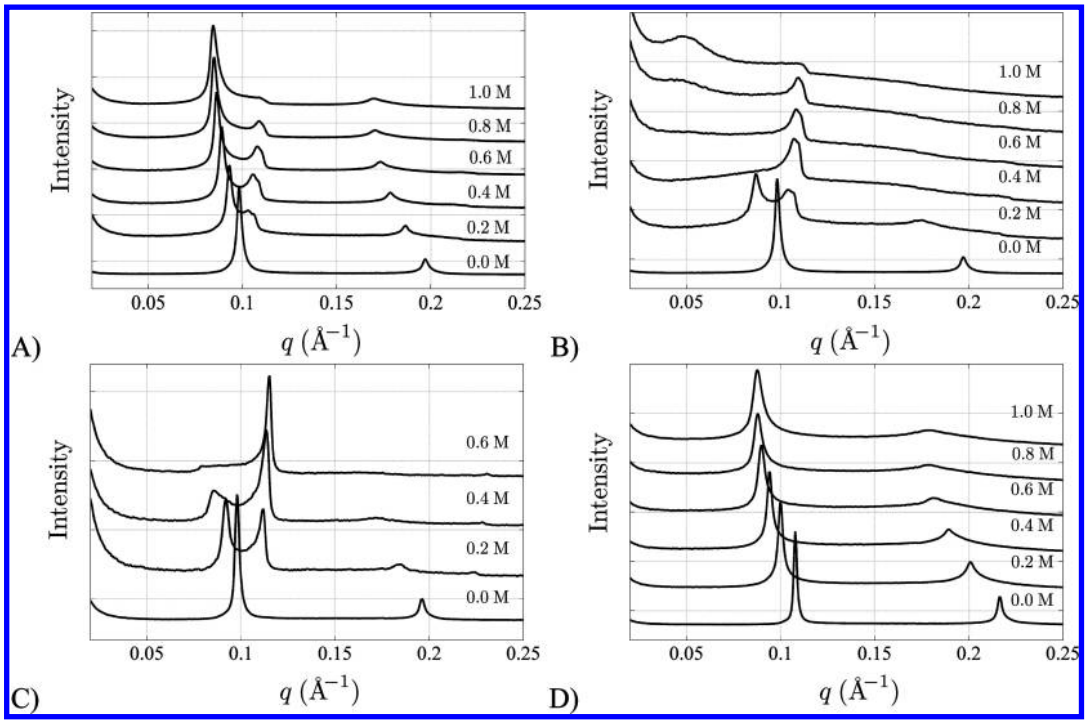

Figure 1. SAXS intensity profiles of POPC in various concentrations of HEPES solutions at pH 4 (A), pH 7 (B), and pH 8 (C). SAXS intensity plots of DLPC in HEPES solutions at pH 4 (D).

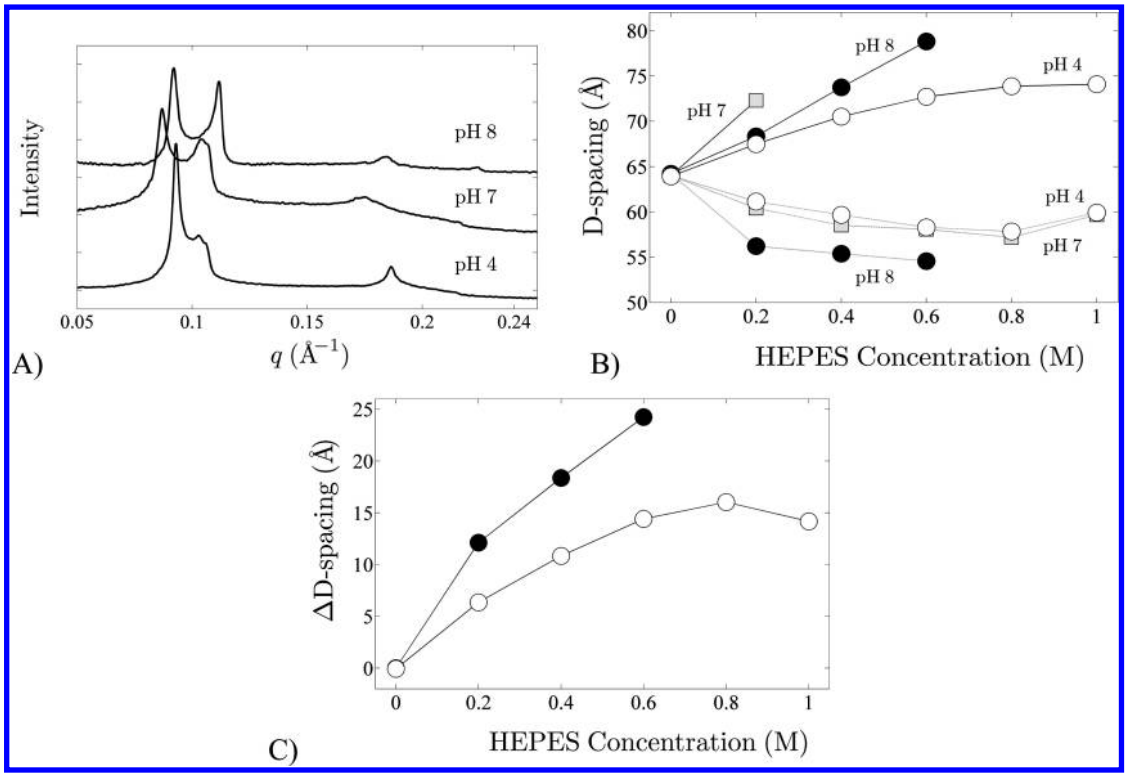

Figure 2. A comparison of SAXS profiles of POPC in 200 mM HEPES solutions at pH 4, 7, and 8 (A). *D*-spacing versus HEPES concentration for POPC multilayers at pH 4, 7, and 8 denoted by ○, □, and ●, respectively (B). The difference in *D*-spacing between POPC phase I and phase II (Δ*D*-spacing) versus HEPES concentration at pH 4 and 8, denoted by ○ and ●, respectively (C).

purchased from Fisher Scientific (Pittsburgh, PA). Conductivity measurements of buffer solutions were conducted using a GE conductivity cell (Cat. 18-1111-05). Following previously described methods,[2] MLV liposomes were prepared by hydrating between 10 and 60 mg of lyophilized lipid powder in 1 to 6 mL buffer solution to a final lipid concentration of 10 mM. Solutions were put through 3-freeze/thaw cycles and were then allowed to equilibrate at room temperature for more than 3-5 days. Lipids were tested for sample deterioration by SAXS on two different occasions within a 4-month time span and had negligible changes in their scattering profiles. Small

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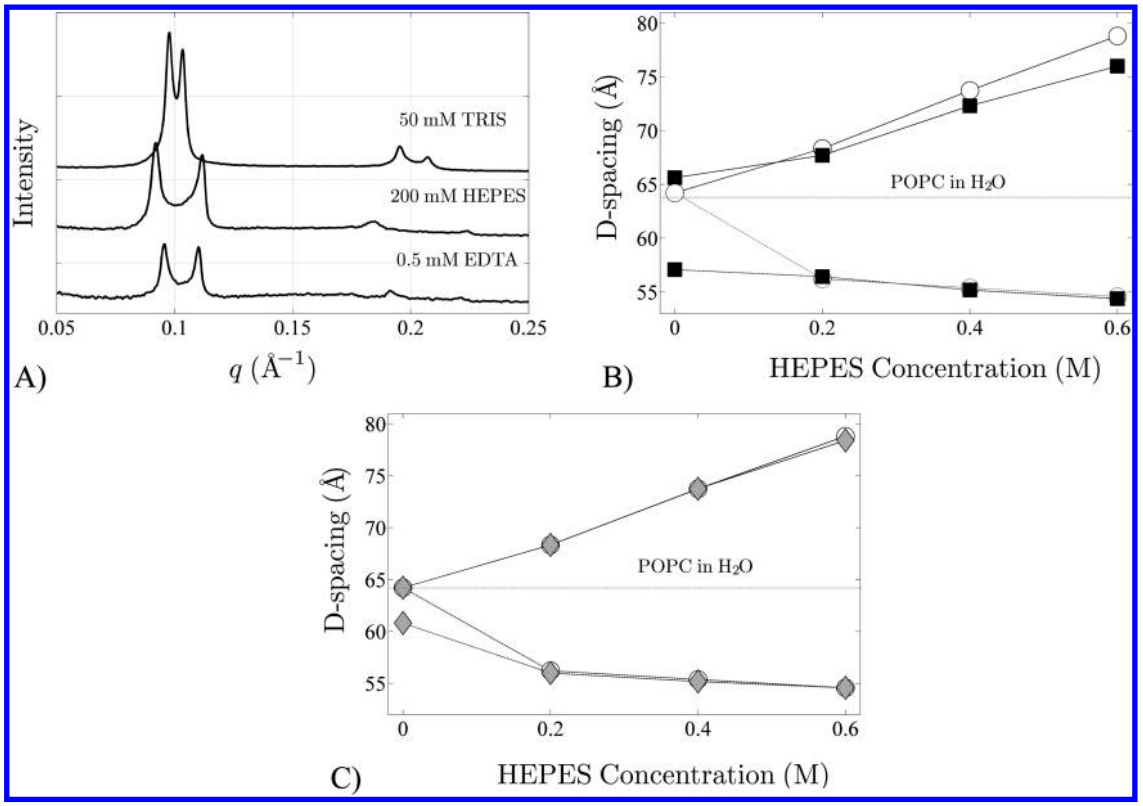

Figure 3. Comparison of SAXS intensity profiles of POPC in EDTA, HEPES, and Tris at pH 8 (A). Comparison of *D*-spacing of POPC multilayers in HEPES concentration at pH 8 (○) versus that in the presence of 0.5 mM EDTA (B) and 50 mM Tris (C) denoted by ■ and ◊ respectively. The horizontal dotted lines in panels B and C indicate the *D*-spacing of POPC in pure water.

angle X-ray scattering (SAXS) measurements were performed at the Advanced Photon Source beamline 12-ID-B and 12-ID-C. The energy at beamline 12-ID-B was fixed at 14 keV, with data collected using a 2 M Pilatus detector set for a sample-detector distance of ∼1900 mm. The overall beam flux was approximately 2×10^{12} photons/second. The pinhole setup at 12-ID-C used a photon energy of 12 keV, using a 4-quadrant mosaic X-ray CCD camera Platinum detector built in house (1024 × 1024 pixel). The sample-detector distance was ∼2200 mm and had a flux of approximately 5×10^{12} photons/second. Lipid samples were X-rayed either in glass capillaries or suspended in droplet form in the X-ray beam path at ∼23 °C for 0.1 s. Two-dimensional (2D) scattering data for five shots were averaged and integrated over the χ angle to obtain intensity versus q (Å^{-1}). A custom Matlab script was used in fitting Lorentzian curves to the center portion of the scattering peaks and to calculate the *D*-space values.

■ RESULTS AND DISCUSSION

Figure 1 shows SAXS measurements conducted at ambient room temperature of POPC lipid vesicles for various HEPES concentrations at pH 4, 7, and 8. For comparison, measurements for the symmetric shorter-chain DLPC in HEPES at pH 4 are also included (Figure 1D). While the scattering peaks index for a single phase in the case of DLPC, the scattering from POPC indicates the coexistence of distinct phases. POPC in the presence of HEPES presents a pair of scattering peaks that shifts to lower q values (smaller scattering angles) similar to DLPC and another that shifts to larger q values (larger scattering angles) as the HEPES concentration is increased. Upon close inspection, the peaks shifting to high q values are closely spaced doublet peaks at pH 4 and 7 but not at pH 8. In all of the following discussion, the doublet will be treated as a single phase and reported as an average *D*-spacing.

The *D*-spacing for the two POPC phases are plotted versus HEPES concentration in Figure 2, where the average *D*-spacing is plotted in the case of doublets. Phase I, which swells with the addition of HEPES, behaves similarly to DLPC while phase II appears to dehydrate. This phase coexistence is robust to pH changes below and above the larger pK_a value of HEPES indicating that the mechanism responsible for this phase coexistence is present regardless of the protonation state of the buffer. However, pH values affect both the *D*-spacing (Figure 2B) and the relative intensity of the scattering peaks (Figure 2A). For example, the steeper increase of *D*-spacing for phase I is observed at pH 7, while the lowest *D*-spacing values for phase II are observed at pH 8. At pH 7, HEPES is predominantly zwitterionic (plus a negatively charged fraction), while at pH 8 it is predominantly negative (plus a zwitterionic fraction). Although it is tempting to speculate that the two MLV phases correspond to the two different titrated forms of HEPES, it is not immediately clear how such demixing can occur on the length scale of MLVs. However, complete demixing of the two buffer forms might not be required. It is conceivable that the ratio between protonated and unprotonated buffer is different in the two phases and may be sufficient to drive phase separation.

The observed phase coexistence is also robust to various buffering agents. Figure 3A compares scattering profiles of POPC in three different buffers at pH 8: 200 mM HEPES, 50 mM Tris, and 0.5 mM EDTA. At pH 8 the amine groups of Tris have been deprotonated, while EDTA is mostly zwitterionic. A phase coexistence is present in all cases, and interestingly, 0.5 mM EDTA has a comparable effect to that of 200 mM HEPES suggesting a correlation between the ionic

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strength and phase *D*-spacing (Figure 2A). In mixtures, however, the effect of HEPES is dominant over that of additives for HEPES concentrations higher than 200 mM, as shown in Figure 3B,C.

The presence of two (or more) *D*-spacing values for a MLV sample in equilibrium creates an interesting and challenging theoretical question on intermembrane interactions. It is unlikely that the observed phase coexistence is an artifact of sample preparation or history. In addition, the total number of components in the system increases as buffering agents are included, allowing an expansion in the number of possible phases under the Gibbs phase rule.[22,23] This is demonstrated by the systematic changes in *D*-spacing in Figure 2 and by the robustness to pH values and additives. In addition, sample preparation and equilibration followed tested and established procedures used in previous similar studies of lipids in salt[6,7] and buffer solutions.[2] The measurements reported here were conducted at room temperature (≈ 23 °C) and in the presence of excess buffer solution. These conditions are far from the phase boundaries of the POPC/water phase diagram so artifacts due to proximity to phase boundaries are unlikely. However, the phase separations induced by these buffers have characteristics similar to those previously observed with multicomponent lipid membrane systems.[24]

For a given pH value, the gap in *D*-spacing values between the two phases increases with HEPES concentration as shown in Figure 2C. Since differences in *D*-spacings are as high as 15 Å, the observed phenomena cannot be due solely to membrane thickness variations but rather due to changes in the interlamellar water space. The interesting behavior observed here is that phase II appears to dehydrate below the value in pure water, while phase I swells. However, the two phases must be in osmotic equilibrium with one another and this is a peculiar feature that should be accounted for in theoretical models of lipid interaction. Such models should include a charge regulation mechanism to account for electrostatic effects[6,25] as well as account for the screening of vdW attraction[7] and possible changes in the bilayer elasticity.[1,3]

The behavior of phase I can be explained by a reduction in vdW attraction and possibly electrostatic charging.[2,7] The explanation for the phase II behavior is not immediately obvious, but one important observation is the qualitatively different *D*-spacing variations for the two phases. For phase II, the *D*-spacing shows a relatively flat region after an initial drop at low buffer concentration, while phase I increases steadily. The simplest mechanism that can explain this behavior is a suppression of the undulation repulsion for phase II. It has been shown that bilayer undulations can add on the order of 7-12 Å to the *D*-spacing,[26,27] which is the range of values observed here. Once membrane undulations are suppressed at low buffer concentration, addition of more buffer cannot further modify the *D*-spacing of phase II reaching a minimum as observed. It is important to note that this minimum is still higher than typical values for POPC under osmotic stress.[4,5,28,29] This means that the observed phase is not simply due to dehydration but to a change of membrane interaction parameters.

Assuming that both phases are lamellar, the free energy of the system should present two or more minima when plotted versus interlamellar spacing. This is an unusual feature that needs to be added to existing models of membrane interactions.[2,6,7,30−32] The two distinct minima in the free energy profile required by the observed phase coexistence must also have comparable depths. In this respect, it would be interesting to quantify the fraction of lipid in the respective phases. While this could in principle be based on the relative intensity of scattering peaks there are complications in this procedure due to variations in form factors[33,34] as well as integration artifacts due to the long tails of the scattering peaks produced by bilayer undulation.[33] Nevertheless, peak intensities in Figure 1 indicate that phase I is dominant for POPC/HEPES at pH 4, while phase II is dominant at pH 7 and 8. In this respect, it is also interesting to note that a concentration of 0.5 mM EDTA (Figure 3) is sufficient to replicate the results seen with 200 mM HEPES at pH 8, while the effect of 50 mM Tris is commensurate to HEPES at the same concentration and pH. Buffer conductivity was used to further explore this behavior (Figure 4), where EDTA buffers yielded a higher conductivity

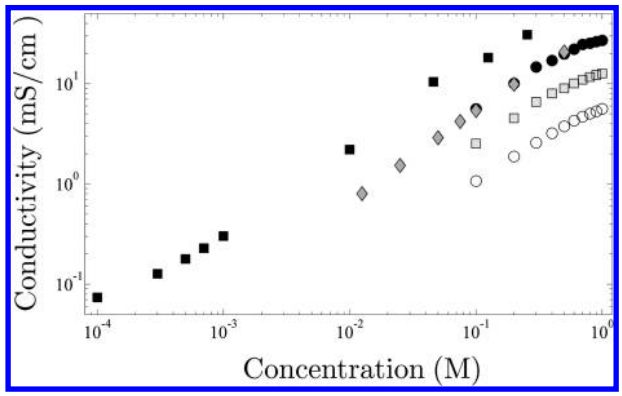

Figure 4. Conductivity of buffers reported as a function of pH and concentration for HEPES (pH 4, 7, and 8 denoted by ○, □, and ●, respectively), EDTA pH 8 (■), and Tris pH 8 (◊).

than those containing Tris and HEPES at pH 8. This suggests that ionic strength may play an important role in lipid organization and therefore phase separation.

As mentioned above, the swelling behavior of phase I can be explained by a combination of vdW forces reduction and added electrostatic repulsion.[2,7] Comparison of HEPES and HEPES with EDTA data in Figure 3B shows that addition of EDTA reduces the swelling of phase I caused by HEPES. This indicates that the electrostatic charging due to HEPES and EDTA must have opposing charges. Since EDTA is highly negative it follows that HEPES charges POPC membranes positively, a result that is consistent with those obtained with DLPC and MOPS.[2]

■ CONCLUSIONS

HEPES, Tris, and EDTA induce a clear phase separation of POPC multilayers in excess solution, with an increase of *D*-spacing in one phase and a reduction in the other. Since the two phases must be in osmotic equilibrium with each other and the excess solution, the different *D*-spacing behavior must be due to distinct mechanisms. The swelling of phase I with added buffer components can be explained by a reduction of vdW attraction forces and added electrostatic repulsion. In contrast, the *D*-spacing reduction measured for phase II may be explained by a suppression of bilayer undulation possibly due an increase of bilayer bending rigidity. The experimental results in this Letter provide a basis for further developing theoretical models to describe membrane interactions.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. A.K.-H. initiated and designed the study, performed experiments, data analysis and manuscript preparation; M.A.J. designed the study, performed experiments, data analysis, and manuscript preparation; S.S. acquired beam time, performed experiments and operated beamlines; H.I.P. initiated study, data analysis and manuscript preparation.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; SAXS, small angle X-ray scattering; vdW, van der Waals

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