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Inverse-Phosphocholine Lipids: A Remix of a Common Phospholipid

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Supporting Information

ABSTRACT: Zwitterionic inverse-phosphocholine (iPC) lipids contain headgroups with an inverted charge orientation relative to phosphocholine (PC) lipids. The iPC lipid headgroup has a quaternary amine adjacent to the bilayer interface and a phosphate that extends into the aqueous phase. Neutral iPC lipids with ethylated phosphate groups (CPe) and anionic iPC lipids non-ethylated phosphate groups (CP) were synthesized. The surface potential of CPe liposomes remains negative across a broad pH range and in the presence of up to 10 mM Ca^{2+}. CP liposomes aggregate in the presence of Ca^{2+}, but at a slower rate than other anionic lipids. Hydrolysis of CP lipids by alkaline phosphatases generates a cationic lipid. CPe liposomes release encapsulated anionic carboxyfluorescein (CF) 20 times faster than PC liposomes and release uncharged glucose twice as fast as PC liposomes. As such, iPC lipids afford a unique opportunity to investigate the biophysical and bioactivity-related ramifications of a charge inversion at the bilayer surface.

Zwitterionic phosphatidylcholine (PC) lipids are a primary component of cellular bilayer membranes that compartmentalize cells. The PC headgroup is exceptionally well hydrated, maintains its zwitterionic character over a broad pH range, and interacts weakly with divalent ions, all properties that make it ideal for its central role in cell membranes. In this report, we investigate how an inversion of the PC headgroup—moving the positively charged quaternary amine adjacent to the bilayer, and extending the anionic phosphate into aqueous interfacial region—alters the chemical and physical properties of the bilayer. We synthesized a family of inverse phosphocholine (iPC) lipids (Figure 1a) and characterized their pH dependence, interactions with calcium, and permeability to an anionic water-soluble reporter, carboxyfluorescein (CF).

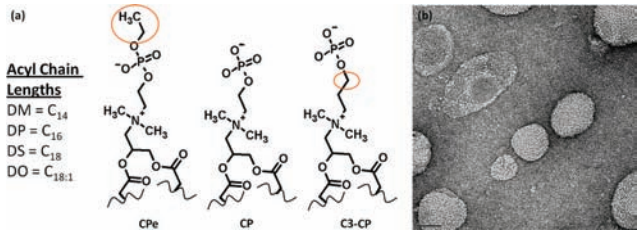

Figure 1. (a) General structure of PC, CP, and CPe lipids; (b) TEM image of DPCPe/Cholesterol (6:4) liposomes, 65 nm in diameter by light scattering, scale bar = 50 nm.

Lipids have three primary regions for potential modification: the hydrophobic tails, hydrophilic headgroup, and linker region between the headgroup and tails (Figure 2). Changes to PC

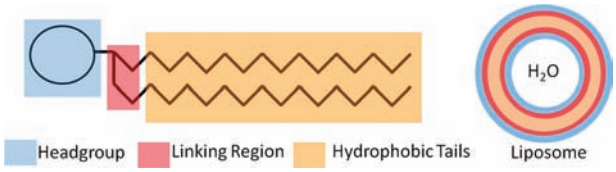

Figure 2. Generic lipid and lipid vesicle (liposome) structures.

lipids at these three sites have been explored by many groups;[1] however, to our knowledge, the inversion of the charged moieties in the PC headgroup. Further, this inverted charge orientation is not observed in naturally occurring lipids. Generally, naturally occurring lipids with anionic headgroups, such as phosphatidic acid (PA), phosphatidylserine (PS), phosphotidyl inositol (PI), sphingosine-1-phosphate (S1P), and ceramide-1-phosphate (C1P) play more than a just a structural role. PA, PI, S1P, and C1P have been identified as key bioactive agents in a variety of signaling pathways[2] such as cell growth/apoptosis,[3] lymphocyte trafficking,[4] chemotaxis,[5] and calcium release.[3b] On the basis of the biological roles of these naturally occurring lipids, it is possible that either the CP or the CPe lipids could exhibit similar activities, allowing them to adopt a therapeutic role as well as serving as a structural component in a liposome bilayer. In addition to their potential for biological activity, iPC lipids are also an exceptionally useful tool for exploring the role of charge-orientation at the bilayer−water interface.

At the transition temperature (T_m), PC lipids transition from an ordered, crystalline state to a more disordered, fluid state.[6] This transition temperature is often dictated by the length and degree of saturation of the hydrophobic tails. The T_m for the three saturated chain CPe lipids (DMCPe, DPCPe, and DSCPe) were measured by differential scanning calorimetry following hydration from a thin lipid film and dispersion by brief periods of vortexing. Their T_m's were found to be 21.4, 41.2, and 53.7 °C for DMCPe, DPCPe, and DSCPe, respectively (see Supporting Information (SI) Table S1). These values are similar to the T_m of their PC-lipid analogues (DMPC, DPPC, and DSPC) which are 24.2, 41.7, and 55.3 °C, respectively.[6] Interestingly, sulfobetaine (SB)[7] lipids and

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betaine-like (BL)[8] lipids, which also have headgroups with a positively charged group adjacent to the hydrophobic region and a negatively charged group that extends away from the bilayer interface, have substantially elevated T_m relative to CP and PC lipids. SB lipids have T_m of 46 °C (C_{14}), 59 °C (C_{16}), and 68 °C (C_{18}) and BL lipids have T_m of 49 °C (C_{14}), 58 °C (C_{16}), and 67 °C (C_{18}) when measured under the same conditions as the CP lipids. Additionally, the SB and BL lipids do not form liposomes in an aqueous solution with 150 mM NaCl, even above their T_m, whereas dry films of CPe lipids hydrated easily and formed small liposomes upon sonication above their T_m (Figure 1b). The increased T_m and difficulty in vesicle formation of the SB and BL lipids were attributed to the ion-pairing between adjacent headgroups. The absence of these properties in the CPe lipids indicates that ion-pairing in the headgroups observed in the SB liposomes is not present in CPe liposomes.

We investigated how the charge inversion (switching the locations of the quaternary amine and the phosphate relative to PC) of the iPC headgroups would affect the overall surface potential of the liposomes they formed (Figure 3). The neutral,

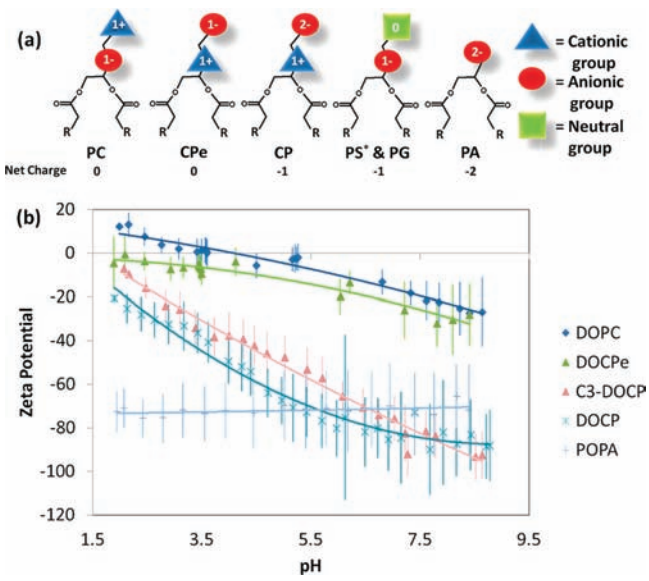

Figure 3. (a) Illustration of the charge orientation and net charge of naturally occurring phospholipid headgroups (PC, PS, PG, PA) and the CP lipid headgroups (CPe and CP). *The neutral block of the PS headgroup contains a primary amine and carboxylate that are zwitter-neutral at physiological pH. (b) Liposome zeta-potential as a function of solution pH.

zwitterionic lipids (DOPC [1,2-dioleoyl-glycero-3-phosphocholine] and DOCPe) had surface potentials that were negative at high pH and became positive (DOPC) or neutral (DOCPe) near pH 2. At low pH, DOPC is a slightly more positive than DOCPe, which suggests that the pK_a for the phosphate of DOCPe may be lower than for DOPC (typically between 2 and 3).[9]

The surface potentials for all three liposome solutions made from anionic lipids (POPA [1-palmitoyl-2-oleoyl-glycero-3-phosphatidic acid], DOCP, and C3-DOCP) start off highly negative and do not differ significantly above pH 5.5. The surface potential of the POPA liposomes, which do not contain a cationic moiety in the headgroup (Figure 3a), remains negative and constant as the pH decreases. However, because the CP liposomes contain a cationic group, their surface potential should approach neutrality when significantly more than 50% of the phosphate groups have been protonated. The first protonation event in PA headgroups occurs at pK_{a2} ~ 8,[10] but the surface potential for the CP liposomes remains negative well below pH 8, which suggests that the pK_{a2} of the phosphate in the CP is lower than the pK_{a2} of the phosphate in PA. The second protonation event for the phosphate on the CP lipids may occur at a pH similar to the phosphate in DOCPe .

The surface potentials of the two anionic CP liposomes (DOCP and C3-DOCP) do not differ significantly for the majority of the titration, indicating that separating the cationic amine from the phosphate by an additional $-CH_2-$ unit does not change the phosphate pK_a's significantly.

The surface potential measurements revealed subtle differences between the CPe and PC headgroups, but significant differences in the charge properties of CP and PA. Lipids with anionic headgroup, such as PA and PS, coordinate with divalent cations such as Ca^{2+}, resulting in the aggregation and/or fusion of liposomes containing these anionic lipids.[11] Calcium-induced aggregation occurs when Ca^{2+} ions form bridges between the outer membranes of two liposomes.[12] The rate of bridge formation is affected by the rate of association/dissociation of the Ca^{2+} ions with the phosphate of the lipid headgroup, as well as the rate of dimerization of the two liposomes.[13] The association/dissociation rates and the Ca^{2+} concentration dictate the amount of Ca^{2+} bound to the liposome surface at any given time. The aggregation rate will increase as the Ca^{2+}–phosphate interaction increases and as the Ca^{2+} concentration increases. To determine how the altered electronic properties of the iPC liposomes impact their behavior in the presence of Ca^{2+}, we compared the aggregation of the CPe and CP lipids to traditional zwitterionic and anionic lipids (Figure 4).

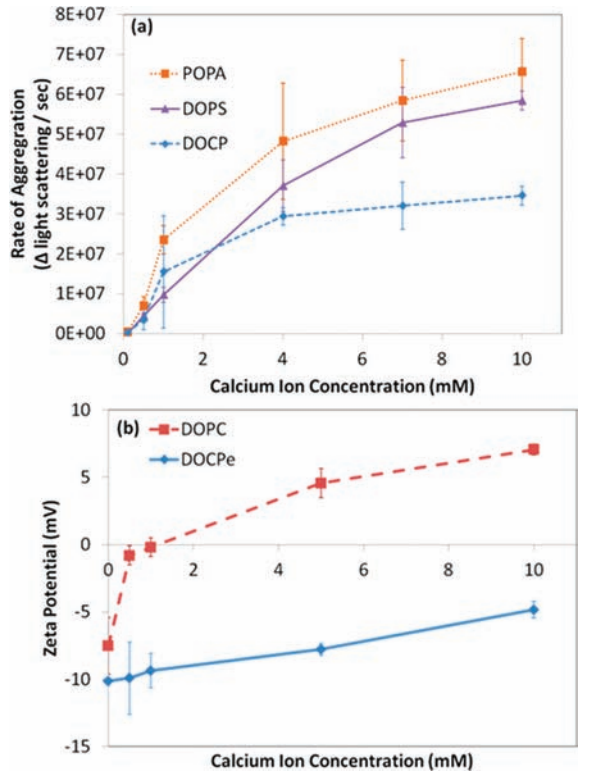

Figure 4. (a) Calcium-induced liposome aggregation rates. (b) Shift in zeta potential in the presence of Ca^{2+}.

CP liposomes aggregate in the presence of calcium (Figure 4a and SI Figure S2). The aggregation rates of DOCP liposomes

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differ from what is observed using DOPS (1,2-dioleoyl-glycero-3-phosphoserine) or DOPA (Figure 4a) in that the rate does not significantly increase above 4 mM Ca^{2+}. The increased aggregation rates for DOPS and POPA relative to DOCP at higher Ca^{2+} concentrations and the plateau effect for DOCP suggest some sort of saturation of the Ca^{2+}–DOCP interaction that is not observed for POPA or DOPS and could result from the presence of a formal positive charge in the DOCP headgroup.

Unlike the anionic lipids, DOCPe and DOPC liposomes did not aggregate at 10 mM Ca^{2+} (see SI Figure S2). To determine if Ca^{2+} interacts with DOCPe and DOPC differently, the surface potentials of the DOCPe and DOPC liposomes were measured in the presence of various concentrations of Ca^{2+} (Figure 4b). The surface potential of the DOPC liposomes becomes more positive as the Ca^{2+} concentration increases, which is in agreement with previous reports.[14] The surface potential of the DOCPe liposomes also becomes more positive as the Ca^{2+} concentration is increased; however, there is no immediate increase between 0 and 0.1 mM Ca^{2+} and the overall magnitude of the increase is less. Additionally, the surface potential of the DOCPe liposomes remains negative up to 10 mM Ca^{2+}. It is possible that Ca^{2+} interacts more strongly with the surface of the DOPC liposomes due to the location of the anionic group. We hypothesize that because the phosphates in the PC headgroups are located at the bilayer interface, they are better oriented and more ordered than the phosphates in the CPe headgroups which extend into the aqueous phase. Ca^{2+} could then bind more avidly to one or two phosphates in the DOPC liposomes than in the DOCPe liposomes, where the phosphate has a greater freedom of motion. Therefore, it is not the overall surface charge that determines the extent of the Ca^{2+} interaction in DOPC and DOCPe, but the charge of the headgroup moiety adjacent to the hydrophobic bilayer surface.

The Ca^{2+} aggregation and interaction studies demonstrate that the inverted charge orientation changes how the iPC liposomes interact with ions in solution compared to PC liposomes. Monovalent ions (Na^+ and Cl^-) can also coordinate to the bilayer interface, and for PC liposomes, Na^+ ions interact preferentially over Cl^- due to the electrostatic attraction between the Na^+ and the anionic phosphate.[15] This phenomenon, which attracts positively charged molecules or ions toward and sometimes into the bilayer, results in an increased permeability for Na^+ over Cl^- for PC liposomes.[16] In the case of the iPC lipids, the anionic phosphate is replaced with a positively charged quaternary amine, which should have the opposite effect by attracting anionic compounds to the surface and increasing their permeability relative to cations. Therefore, the release rate for an encapsulated anionic compound from an iPC liposome should be greater than for a PC liposome. We tested this hypothesis by measuring the release rates of an encapsulated model anionic compound, carboxyfluorescein (CF) and compared the results to the release of uncharged glucose (Figure 5).

As predicted, the DPCPe liposomes released CF 20 times faster than DPPC liposomes at 37 °C, while releasing glucose only twice as fast. The difference between these two rate ratios suggests that DPCPe liposomes are preferentially permeable to anions showing that the charge of the headgroup moiety adjacent to the bilayer plays a significant role in the release of charged molecules. Further biophysical studies are required to learn if this is a general phenomenon.

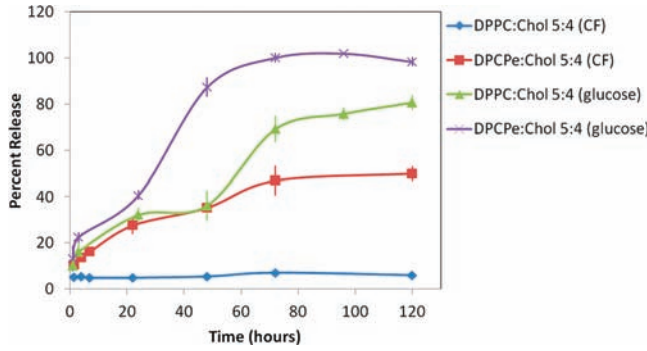

Figure 5. CF and glucose release profiles from DPPC and DPCPe liposomes.

If the permeability is dictated by the charge at the bilayer interface, then mixed DPCPe/DPPC liposomes could be used to create a liposome with an adjustable content release profile for charged compounds (SI Figure S4). Adjustable release kinetics for liposomal delivery could enhance both existing therapies and aid in the development of new approaches to liposomal drug delivery.

We have synthesized a new class of zwitterionic phospholipids, iPCs, with an inverted headgroup charge orientation relative to traditional PC lipids. Both CP and CPe liposomes have negative surface potentials across a broad pH range, but CPe liposomes do not appreciably interact with Ca^{2+}. CP lipids have a terminal phosphate group which is similar to that found in many biologically active lipids. Furthermore, alkaline phosphatase can remove the phosphate from CP lipids (see SI Table S2) to generate a cationic lipid. This can be exploited to make a biologically sensitive liposome which may be useful for cytoplasmic delivery of encapsulated contents. These iPC lipids provide additional opportunities to study the influence of overall headgroup charge versus headgroup structure (i.e., the locations of the anionic and cationic groups) on the intrinsic biological activity of anionic signaling lipids.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic protocol, materials, experimental details, DSC data, sample Ca^{2+} aggregation profiles. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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