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# Headgroup Inversed Liposomes: Biointerfaces, Supported Bilayers and Applications

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**Abstract**

Phospholipids are a major component of the cell membrane. In most natural phospholipids, the phosphate acts as a bridge, connecting the other portion of the polar headgroup with the hydrophobic tails. Such bridging phosphate is chemically quite inert. Synthetic lipids inversing the headgroup polarity of phosphocholine (PC) have been recently reported, and these are named CP lipids with a terminal phosphate, or CPe with the terminal phosphate capped by an ethyl group. This Feature Article summarizes the properties and applications of such inversed lipids. First, CPe liposomes were found to be highly resistant to protein adsorption with an even longer blood circulation time than PC liposomes allowing for enhanced accumulation in tumor sites. CPe liposomes do not interact with PC liposomes either, and this observation was different from that reported using CP polymers, which adhere strongly to cells. Second, CP liposomes interact strongly with many metal oxide nanoparticles (but not silica) forming supported lipid bilayers, while PC liposomes only form supported bilayers on silica. Finally, CP liposomes are good metal ligands based on its exposed terminal phosphate. $Zn^{2+}$ binds to CP liposomes so strongly that $Zn^{2+}$ sandwiched multi-layered lipid structures were observed. Aside from these fundamental aspects, the potential applications of these headgroup inversed lipids in drug delivery and biosensor development have also been described, which in turn promoted fundamental biointerface insights.

## Introduction

Phospholipids are a main component of the cell membrane. A typical phospholipid contains two long hydrophobic tails and a polar headgroup, which self-assembles into a bilayer structure. A few headgroups such as phosphocholine (PC), phosphoserine (PS), and phosphoethanolamine (PE) are quite common in the cell membrane, all containing a phosphate as a key building block. Aside from fundamental biological studies, various lipid vesicles or liposomes have been prepared for drug delivery,[1] biosensor development,[2] building biocompatible interfaces,[3-7] and model cell membrane studies.[8]

Through chemical synthesis, researchers can access many non-natural lipid headgroups, further broadening the range of application and providing new fundamental insights.[9] For example, cationic lipids such as DOTAP (with a trimethylammonium-propane headgroup) have been widely used for delivery of nucleic acids into cells.[10] By covalently linking two headgroups, Gemini surfactants/lipids have been prepared for enhanced gene delivery.[11]

In the last six years, another type of engineered lipids was designed by simply inversing the lipid headgroup structure.[12] Such lipids can also form liposomes but they have not been reported in natural biological systems. Research on such lipids has raised many interesting questions such as why nature has chosen to use the current lipid structure.[13] At the same time, they provide new building blocks for materials and have interesting adsorption properties for various proteins, inorganic surfaces, and metal ions. These surface properties also enabled their applications in drug delivery and anti-fouling.

Given these developments, it is now a good time to summarize the current state of the art, and to plan for the next stage of research. In this Feature Article, we describe the research on headgroup inversed lipids from both fundamental biointerface science and applications. Parallel to the lipid work, the same inversed headgroups have also been incorporated into various polymers and dendrimers.[13] Intriguingly, these polymers and lipids have very different surface properties in terms of interaction with cells. A

comparison is made on these two system when appropriate, and future research opportunities are discussed in the end.

**PC, CP and CPe lipids** The inversed lipids reported so far are all related to the PC headgroup.[12] The structure of a DOPC lipid is shown in Figure 1A. The zwitterionic PC has a positively charged choline and a negatively charged phosphate. The p$K_a$ of this phosphate is below 1,[14] and thus it is negatively charged at neutral pH, resulting in an overall charge of zero. PC lipids are the most abundant in the outer membrane of eukaryotic cells.[15] Similar to many other zwitterionic surfaces, PC lipid bilayers are highly resistant to protein adsorption (e.g. anti-fouling).[16] In this section, we compare PC with two headgroup inversed lipids: CP and CPe.

*Surface charge*. By simply switching the position of the phosphate and choline groups, the resulting lipid is named DOCP (Figure 1B). Regarding its charge, it is easy to think about phosphatidic acid (e.g. DOPA) that also has a terminal phosphate group with two p$K_a$ values (~3.0 and 8.0).[17] However, experimental data have indicated that both p$K_a$'s of DOCP were below 3.0.[12] Therefore, at neutral pH, this phosphate carries two negative charges. With just one positive charge, the CP has one net negative charge. The p$K_a$ difference between DOPA and DOCP is attributable to the positive charge from the choline that stabilizes the negative charges on the phosphate. In addition to the negative charge, the exposed terminal phosphate with three oxygen atoms available for coordination has a much stronger metal binding affinity.[18] The charge and metal binding properties of DOCP strongly affect its surface interactions.

To neutralize the extra negative charge, an ethyl group can be used to cap the phosphate, resulting in a lipid called DOCPe (Figure 1C). With a careful pH titration, its remaining phosphate p$K_a$ was estimated to be below 2-3.[12] Therefore, at neutral pH, DOCPe is charge neutral. DOCPe and DOPC mainly differ by the orientation of the charge dipole. Under the same experimental condition, the zeta-potential of DOCPe liposomes was nearly -20 mV at pH 7.0, more negative than DOPC (~-10 mV).[12]

Note these zeta-potential measurements were carried out in a buffer with a very low ionic strength (5 mM Tris, 5 mM NaCl). At physiological salt concentrations, these lipids are more close to charge neutral (still slightly negatively charged).[19] All these three lipids can form single-component liposomes and their representative cryo-TEM micrographs are shown in Figure 1G-I. With these three lipids, systematic comparisons have been made for deeper insights.

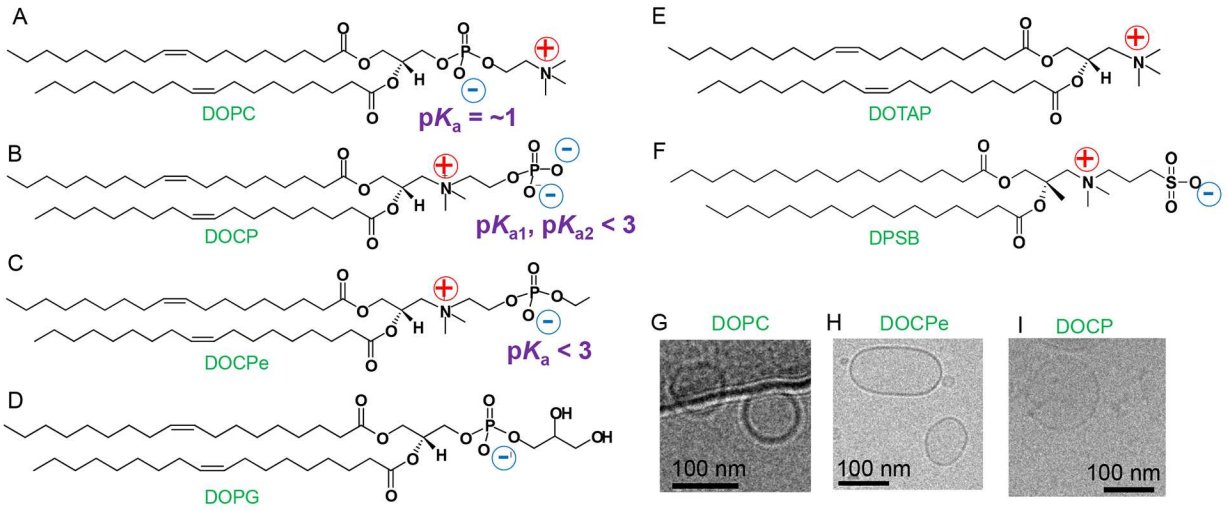


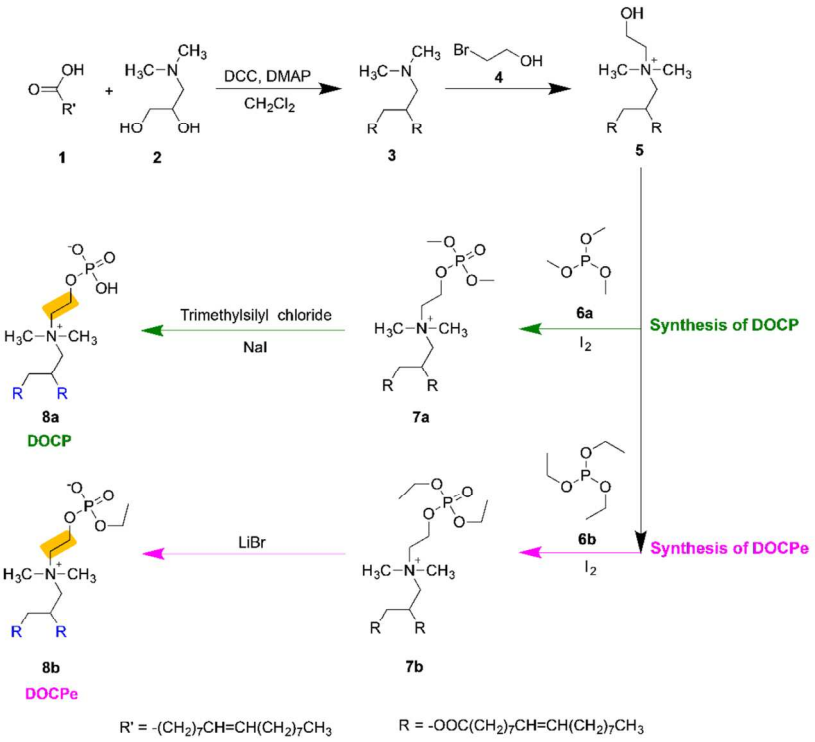

**Figure 1**. The structures of some phospholipids described in this article: (A) DOPC; (B) DOCP; (C) DOCPe; (D) DOPG; (E) DOTAP; and (F) DPSB. The p$K_a$ values of some phosphate groups are marked. Cryo-TEM micrographs of (G) DOPC; (H) DOCPe; and (I) DOCP liposomes. Figures adapted from Ref.[19] with permission. Copyright ® 2017 American Chemical Society.

*Lipid phase transition temperature*. In addition to charge, another important property of liposomes is phase transition temperature ($T_c$). Below $T_c$, lipids are packed in the gel phase with a low diffusion coefficient, while above $T_c$, lipids are in the fluid phase with faster lateral diffusion. Typically liposomes need to be prepared at a few degrees above the $T_c$ of the lipid. With the same headgroup, the $T_c$ of a lipid is determined by the length and structure of the tails. Compared to PC lipids, the CPe lipids with the same tail structure have very similar $T_c$'s (within 3 °C).[12] Szoka and coworkers further synthesized sulfobetaine

(Figure 1F),[20] and betaine-like lipids,[21] which are similar to CP in terms of orientation of the charge dipole. These lipids however have much higher $T_c$ values (e.g. up to 27 °C higher). In addition, these betaine lipids alone cannot form liposomes at physiological conditions unless with a very high salt concentration. The increased $T_c$ and failure in vesicle formation of the betaine lipids were attributed to strong ion-pairing interactions between adjacent headgroups. In turn, these results suggest that CP or CPe lipids do not form such strong ion pairs, which is more similar to PC. The charges must be better screened in the PC/CP type lipids than in betaine lipids by counter ions and the hydration shell.[20]

*Synthesis*. The synthesis of CP and CPe lipids was first described by Szoka and coworkers, and their procedures are briefly illustrated in Scheme 1.[12] The synthesis started with the Steglich esterification between oleic acid (**1**) and 3-dimethylamino-1,2-propanediol (**2**). Then the choline group was generated by quaternization of tertiary amine in structure **3** through nucleophilic substitution using 2-bromoethanol (**4**). Finally, DOCP (**8a**) or DOCPe (**8b**) was directly obtained by the reaction between structure **5** and phosphite (**6**) in the presence of $I_2$ followed by selectively removing the alkyl group from structure **7**. In the process, the ethyl (C2) linker (Scheme 1, highlighted in yellow) between choline and phosphate group can be easily changed to a C3 linker by using 3-bromopropanol in place of 2-bromoethanol (**4**). Although only an ethyl cap for the phosphate was demonstrated in this synthesis, a methyl or other capping groups can be attached using the same chemistry. In addition, lipids with saturated tails were synthesized for example, DMCPe, DPCPe and DSCPe with C13, C15 and C17 tails, respectively. Through these examples, it is clear that the structures of headgroup inversed lipids can be tailored, offering opportunities for systematic investigation.

**Scheme 1**. Synthesis of DOCP/DOCPe lipids based on the method reported by Szoka and coworkers.[12]




**DOCPe liposomes: interactions with PC membranes and proteins, and a comparison with CPm polymers**

The headgroup inversed lipids were first prepared by Szoka and coworkers in 2012 largely out of academic curiosity.[12] Interestingly, in the same year, Brooks and coworkers reported a dendrimer displaying a CPm headgroup (with a methyl cap, Figure 2A).[13] Since then, many related polymers (Figure 2B), copolymers (Figure 2C, 2E and 2F), and modified surfaces (Figure 2D) have been reported.[22, 23] In addition, a few capping groups such as methyl, butyl (Figure 2G) and unsaturated groups (Figure 2H) have been synthesized.[24] [25] [26] While the headgroup chemistry was similar, lipids and polymers are different in many ways. For example, the lipid headgroups are densely packed in a lipid bilayer, while the headgroups in the polymers are more flexible with a lower density.

With excellent biocompatibility, PC lipids have been widely used for drug delivery and a few commercial products such as Doxil are already in the market.[27] Given this success, a logic application of CPe liposomes is also drug containment and delivery. Delivery vehicles are likely to interact with proteins in blood, and also with the cell membrane. We discuss fundamental research on the interaction between CPe liposomes with cell membranes and proteins. A comparison was made with CPm polymers when appropriate. Interestingly and surprisingly, DOCPe liposomes and CPm polymers have drastically different properties.

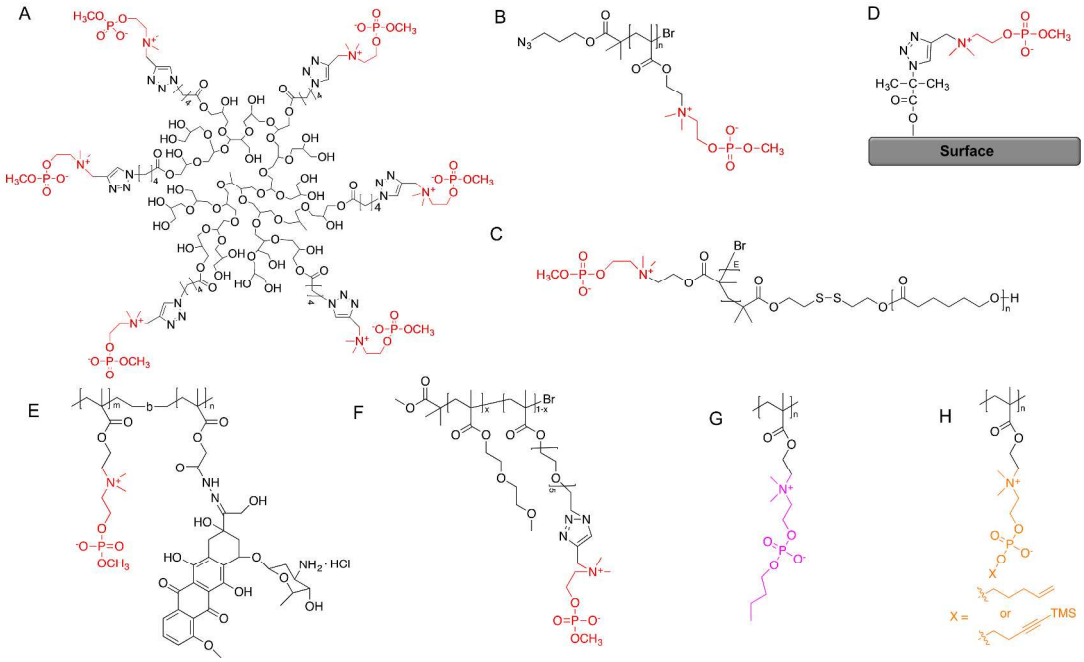



**Figure 2**. Structures of literature reported CPm terminated (A) dendrimers;[13] (B) polymers;[28] (C) polymer micelles;[29] and (D) surfaces.[30] CPm block copolymers containing a covalently attached (E) DOX,[22] and (F) thermal responsive block.[23] CP polymers (G) caped with butyl groups,[24 25] and (H) functionalized with different unsaturated groups (X can be alkenes or alkynes).[26]

*DOCPe liposomes: membrane interactions*. Before discussing liposomes, it is worthwhile mentioning CPm polymers. The specific binding between a CPm dendrimer (Figure 2A) and the PC headgroup in the

cell membrane was first proposed by Brooks and coworkers.[13] They observed adhesion of their CPm dendrimer to red blood cells (RBCs) causing cell aggregation, while the same dendrimer with a PC headgroup did not show any measurable adhesion ($K_a$ at least 50 times higher for the CPm). Following the initial dendrimer design, the group then synthesized CPm polymers (Figure 2B), also inducing cell aggregation. For example, Figure 3F shows an SEM micrograph of well-dispersed RBCs. After adding the CPm polymer (1 mg/mL), the cells aggregated extensively (Figure 3G). Similar cell adhesion was also reported by Li and coworkers by displaying CPm on a silica surface (Figure 2D).

To explain such strong cell adhesion, Brooks and coworkers proposed that the PC dipole can interact with the CPm dipole forming a quadrupole (Figure 3A). This explanation has also been adapted by other researchers working on similar polymers. In this model, the phosphate and choline in the PC lipid membrane interact respectively with the choline and phosphate groups in the CPm by electrostatic attraction. Brooks and coworkers demonstrated stronger dendrimer adhesion to cells by decreasing salt concentration, supporting electrostatic mediate attraction.[13] A binding assay was performed using flow cytometry with DPPC/cholesterol liposomes as model cell membranes. To further support this model, extensive efforts have been made to directly observe such interactions using $^{31}$P NMR and FT-IR spectroscopy. However, neither method produced the expected evidence of interaction. Using prop-2-ynyle choline phosphate (p-CP) as a simplified CP group, the authors mixed it with DPPC lipid and analyzed the mixture with electrospray mass spectrometry. The mass corresponding to the sum of these two molecules was obtained in this experiment. Note that p-CP does not have the polyvalent advantage of the original dendrimer and its binding to PC should be weaker. In addition, mass spectrometry probes gas phase interactions, which might be different from reactions in aqueous solutions. Finally, the association of these two does not necessarily support the proposed molecular arrangement. Therefore, direct experimental evidence for the proposed model has yet to be demonstrated.

In a *Langmuir* Feature Article in 2014, Schlenoff raised an interesting point that if PC/CPm can have quadrupole interactions, then PC/PC might also have similar interactions.[16] However, it is well

known that PC lipid membranes do not stick to each other. We draw the simplified interaction schemes in Figure 3B-E. It is interesting to note that if the lipid headgroups are oriented vertically respect to the bilayer plane (represented by the dashed arc), the PC/CP interaction is more stained (Figure 3B), while the PC/PC interaction is more favorable (Figure 3C). In this case, we would expect even better adhesion between PC membranes. Based on the literature, the PC headgroup is oriented near parallel to the lipid surface,[31] and in this case the interaction of PC/CP and PC/PC should be similar (Figure 3D, E). A molecular model showing this parallel PC headgroup is included in Figure 3E, although how does a CP or CPe headgroup orients remains unclear. Therefore, we discussed the two extreme cases of headgroup orientation. Overall, from simple theoretic arguments, CP seems to have no advantage of interacting with PC. The dipole-dipole interaction proposed in Figure 3A is reminiscent of the strong ion-pairing interactions in the betaine lipids described by Szoka and coworkers.[20, 21] When such interactions are strong, they cannot form liposomes on their own unless charge interactions are screened by a very high salt concentration.

To experimentally understand the binding between CPe and PC membranes, we performed dynamic light scattering (DLS) and isothermal titration calorimetry (ITC) studies using liposomes. If strong adhesion occurs, we expect that mixing DOPC and DOCPe liposomes should form large agglomerates that can be detected by DLS. In addition, electrostatic based interactions should release heat. In both experiments, however, no evidence of interaction was observed (Figure 3H, I), while our positive controls of mixing anionic DOPG liposomes (Figure 1D) with cationic DOTAP liposomes (Figure 1E) produced strong signal of adhesion.

Taken together, although strong evidence exists for interactions between CPm polymers and cell membranes, the proposed binding model in Figure 3A has yet to be fully substantiated. Based on the simple model liposome system, little evidence supports such interactions. Brooks and coworkers have articulated the requirement of flexibility in the CPm chains to achieve the intended CPm/PC interactions.

It is understandable that such flexibility is better available in polymers or dendrimers than in liposomes. It could be that the chain flexibility is key for the observed difference between CP polymers and liposomes.

Another difference between the lipid and polymer systems is the length of the capping ligand. The commercially available lipid is CPe (capped with an ethyl group), while most synthetic polymers/dendrimers contained CPm (a methyl cap). Here, we also review the effect of capping group in the literature. Fujii et al synthesized a special CPm lipid with four CPm groups based on a calix[4]arene structure.[32] This lipid formed small micelles that were effectively internalized by A549 cells at 37 °C but no binding was detected at 4 °C. This work suggested that the micelles were internalized by endocytosis, but the micelle did not adhere to the cells when energy-dependent endocytosis was inhibited at low temperature. By capping CP with a butyl group, Emrick and coworkers reported the synthesis of CPb polymers, which failed to adhere to RBCs since no aggregation was observed.[24] Note this is a polymer instead of liposome. Taken together, no lipids were reported to adhere to cells with methyl or ethyl groups, and a butyl capped CP polymer also failed to adhere. A direct comparison of a CPe (or CPm) polymer with liposomes displaying the exact headgroup is still needed to fully test the hypothesis of chain flexibility. From application standpoint, both adhesion and non-adhesion materials are useful for drug delivery (*vide infra*).

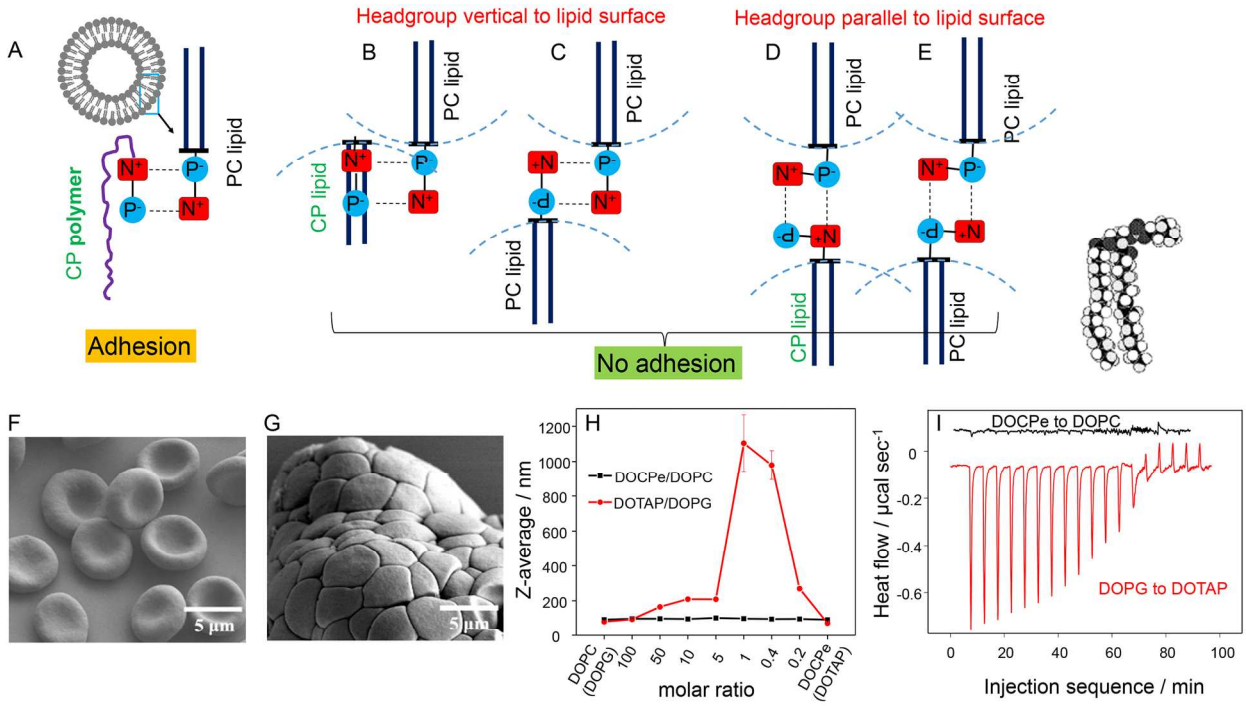



**Figure 3**. (A) Schematic presentation of the proposed CPm polymer interacting with the PC lipid via dipole-dipole interactions. Schematics of (B, D) CP/PC and (C, E) PC/PC lipid interactions when the lipid headgroups are aligned (B, C) vertically; or (D, E) parallel to the lipid bilayer surface. The dashed arcs represent the cross-sections of lipid surfaces. The lipid model structure in (E) is adapted from Ref.[31] with permission. Copyright ® 1999 Elsevier. In all these CP polymers or lipids, the capping methyl or ethyl groups were omitted for clarity of the figures. SEM micrographs of (F) free RBCs; and (G) after adding a CPm polymer adapted from Ref.[28] with permission. Copyright ® 2013 Royal Society of Chemistry. Studying the interaction between DOPC and DOCPe liposomes using (H) DLS and (I) ITC, and DOTAP/DOPG liposomes were used as positive controls. Figure adapted from Ref.[19] with permission. Copyright ® 2017 American Chemical Society.

*DOCPe liposomes: protein adsorption*. In addition to binding to the cell membrane, another aspect of drug delivery is protein adsorption. Right after injecting liposomes into an animal or even just adding liposomes in a cell culture, they are expected to encounter proteins. Protein adsorption is critical for

cellular uptake of nanoparticles. For example, highly negatively charged DNA-functionalized gold nanoparticles can be efficiently internalized by cells,[33] and this was attributed to the adsorbed protein layer interacting with cell surface receptors.[34] Zwitterionic PC lipids are highly resistant to protein adsorption (anti-fouling) and its fundamental reason has been nicely summarized by Schlenoff.[16] The lack of protein adsorption can also explain the resistance of cellular uptake of PC liposomes. Similarly, it is not surprising that DOCPe liposomes are also resistant to protein adsorption.

Li and coworkers used a click reaction to immobilize CPm on a silica surface. Compared to the contact angle of their bare silica surface (60.3 °), the CPm terminated surface (53.7 °) was slightly more hydrophilic. While this change in contact angle suggested modification of the surface, their contact angle of the bare silica surface was much higher than typically expected for a clean surface. Compared to the bare silica, their CPm capped surface was more resistant to protein adsorption for all the proteins tested, including 100% serum (Figure 4A).[30, 35] However, no comparison was made between CPm and PC surfaces in this study.

We measured protein adsorption by DOPC and DOCPe liposomes using ITC, and neither one showed detectable heat (Figure 4B-C). Therefore, both liposomes are anti-fouling and this is consistent with their zwitterionic properties. To evaluate which one is even more resistant to protein adsorption, a further comparison was made using cellular uptake experiments.[19] When fluorophore-labeled liposomes were incubated with RAW264.7 macrophage cells, a slight uptake was still observed with the DOPC liposomes but much less with the DOCPe liposomes as confirmed by both confocal fluorescence microscopy and flow cytometry (Figure 4D).

The above studies relied on physisorption of proteins. We also incorporated 5% MPB-PE lipid (containing a thiol reactive maleimide group) into our DOCPe formulation. If we pre-reacted this liposome with proteins containing cysteines (e.g. chemisorption of proteins), the liposome was efficiently internalized (the red fluorescence in Figure 4F compared to that in Figure 4E). If we cap the MPB group with beta-mercaptoethanol before adding proteins, the uptake was then inhibited again (Figure 4G). Given

the importance of protein adsorption on cellular uptake, this result suggested that DOCPe might be even more resistant to protein adsorption than DOPC. The reason for this difference has yet to be fully understood. Given the vast number of proteins with different hydrophobicity, charge and other properties, it is hard to conclude that DOCPe is more resistant to protein adsorption in each case. A full understanding on this topic might be more of theoretical importance. For drug delivery applications, the better resistance in serum is of more practical relevance. Although a systematic comparison between DOCPe and DOCP liposomes for protein adsorption has not yet been reported, one can predict that negatively charged DOCP might be much better at adsorbing proteins due to electrostatic attraction with cationic domains on proteins.

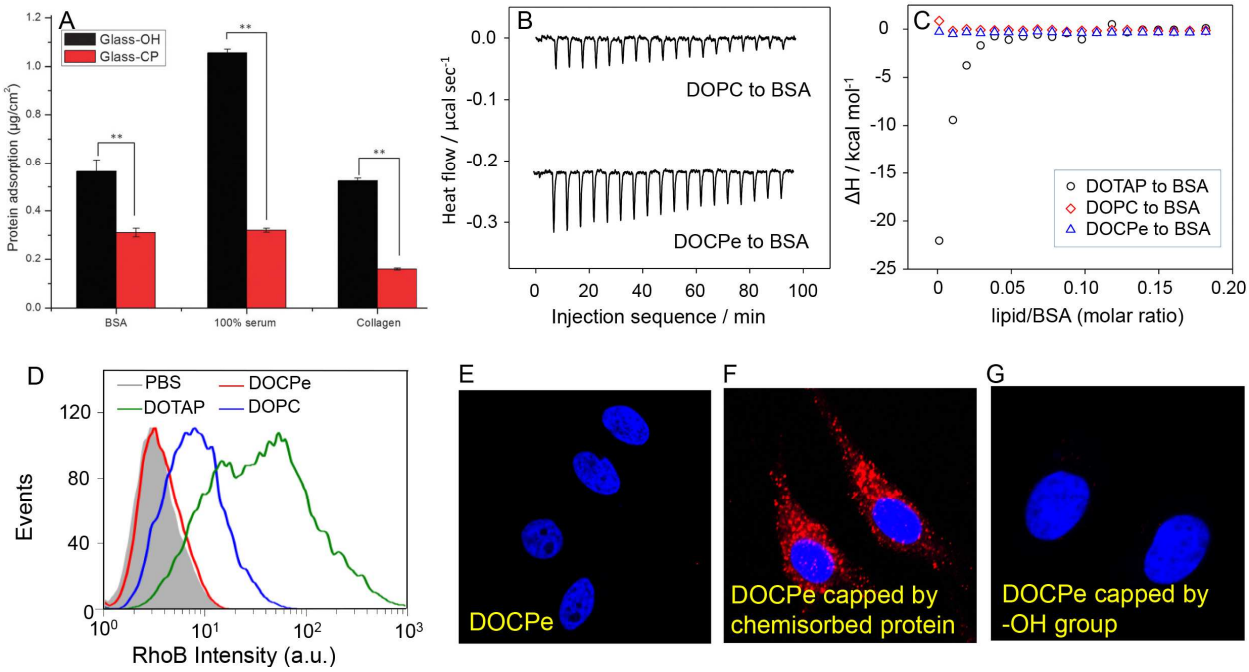



**Figure 4**. CPm or CPe surfaces resistant to protein adsorption. (A) A comparison of CPm and silica surfaces for protein adsorption adapted from Ref.[30] with permission. Copyright ® 2015 Royal Society of Chemistry. (B) ITC traces and (C) integrated heat of titrating various liposomes to BSA. (C) Flow cytometry histograms of RAW264.7 cells incubated with various fluorophore-labeled liposomes. DOTAP was used as a positive control. The uptake of DOCPe liposomes was even less than that of DOPC liposomes. (E-G) Test of proteins chemisorbed on DOCPe liposomes. (E) Free rhodamine-labeled (Rh)

DOCPe liposomes incubated with HeLa cells. (F) After reacting MPB-labeled DOCPe liposomes with proteins to achieve chemisorption, cellular uptake was achieved indicated by the red fluorescence. (G) Capping the MPB-DOCPe liposomes with beta-mercaptoethanol inhibited protein adsorption and cellular uptake. Figures adapted from Ref.[19] with permission. Copyright ® 2017 American Chemical Society.

*Drug delivery applications of DOCPe liposomes*. Given the properties of CPe liposomes (e.g. resistant to protein and lipid adsorption), they have been used for drug delivery applications in a few different ways. For comparison, we also briefly review the drug delivery work involving CPm polymers.

Since DOCPe liposomes do not adhere to DOPC liposomes, cells or proteins, we hypothesized that DOCPe might be able to circulate a long time in vivo. We then followed the circulation of DOCPe liposomes after injecting it in the tail vein of mice. Surprisingly, the DOCPe liposomes circulated longer even than DOPC liposomes (Figure 5A). The DOCPe concentration in blood was more than twice of that of DOPC from 30 min to 8 h after injection, consistent with the greater resistance of DOCPe to uptake by the macrophages described above. Such longer circulation allows better accumulation of DOCPe liposomes in the tumor based on the enhanced permeability and retention (EPR) effect (Figure 5B). By adding targeting ligands such as peptides, antibodies, it might also achieve active targeting.[19] Negatively charged DOCP lipid was incorporated in liposome formulations by Szoka and coworkers with targeting peptides for drug delivery. In that case, the role of DOCP was mainly to provide negative charges to the liposome.[36]

In sharp contrast to the resisted cellular uptake of DOCPe liposomes, the CPm polymers were reported to have strong cell adhesion, which promoted cellular uptake. Using the CPm block copolymers shown in Figure 2C [29] and Figure 2E, Yu and coworkers demonstrated the delivery of doxorubicin (DOX) loaded in their micelles, while similar micelles with a PC headgroup had very poor cellular uptake. Similarly, pH and thermal responsive CPm polymers (Figure 2F) were prepared for drug delivery

applications.[23, 37, 38] In addition, CPm-functionalized cellulose was used to capture RBCs, while the control PC surface failed to do it.[39]

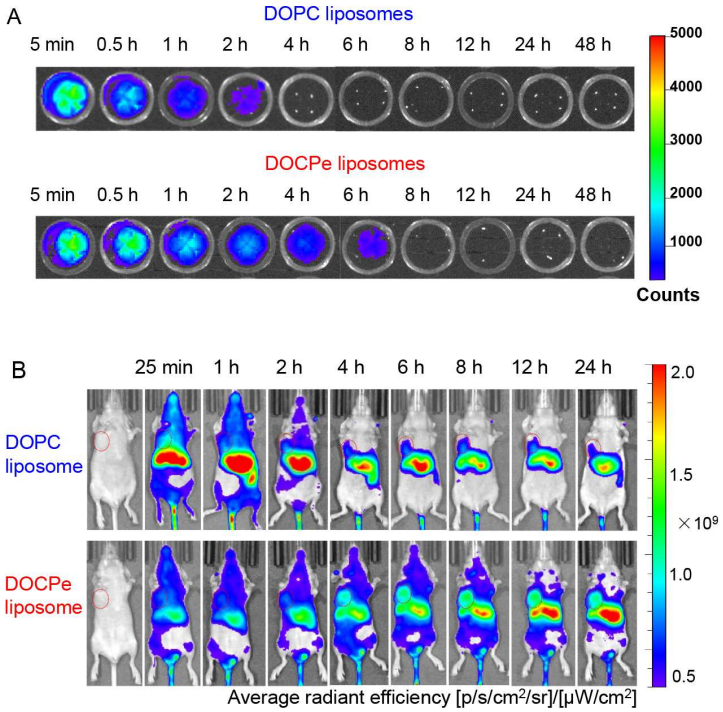



**Figure 5**. (A) In vitro fluorescence images of the plasma extracted at different time points after injecting the two liposomes labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine (DiR, a near IR fluorophore) indicating that the blood circulation time of the DOCPe liposomes was longer than that of DOPC liposomes. (B) Better accumulation of dye-labeled DOCPe liposomes in the tumor site indicated by a red circle. Figures adapted from Ref.[19] with permission. Copyright ® 2017 American Chemical Society.

## PC and CP lipids for supported bilayers

The above discussed interaction of headgroup inversed liposomes with PC lipid bilayers and proteins are made in the context of drug delivery. Another important aspect is their interaction with inorganic surfaces. In this regard, PC liposomes have been the most extensively studied, especially its adsorption and

spreading on silica surfaces.[3, 40, 41] A PC liposome can spontaneously break on a clean silica surface forming a supported lipid bilayer.[41, 42] It has been generally accepted that PC bilayers are supported on silica via van der Waals interactions with a thin water layer (e.g. <1 nm) between the two surfaces. However, PC liposomes do not form supported bilayers with most other metal oxides, such as $TiO_2$, ZnO and $Fe_3O_4$.[5, 43, 44]

Phosphate can interact strongly with these metal oxides (excluding $SiO_2$), and we found that the phosphate group in PC lipids can also interact with these oxide surfaces.[43] However, the choline group might induce a steric effect to prevent full fusion of the liposomes. With this hypothesis, if we inverse the lipid headgroup using DOCP, the phosphate is fully exposed and this may lead to supported bilayer formation on these oxides.[45] We first used a leakage assay by encapsulating ~100 mM calcein dye in DOPC and DOCP liposomes. Supported bilayer formation is accompanied with calcein leakage and fluorescence enhancement, while simple adsorption would not have such an effect. Most oxides leaked DOCP liposomes but not DOPC ones (Figure 6A), suggesting formation of supported DOCP bilayers.[46] The only exception was silica, which leaked DOPC instead (Figure 6B). ZnO and NiO leaked both liposomes (Figure 6C), and this was attributed to their cationic surface damaging lipid membranes (Figure 6D). Pore formation on supported bilayers was reported with various cationic nanoparticles.[47]

We performed most of the above work at neutral pH without $Ca^{2+}$. It was reported that at very acidic pH, supported PC bilayers could form on $TiO_2$ and $Al_2O_3$.[48-50] Such PC bilayers on $Al_2O_3$ were found to also have a water layer even thicker than that on $SiO_2$.[50] The need for acidic pH was attributed to overcoming the repulsive force related to the interfacial water. Sapphire, a type of doped $\alpha$-$Al_2O_3$, however, could spontaneous fuse with PC lipids under physiological conditions, although $Ca^{2+}$ was included in the buffer.[51, 52] Silicates such as mica can also form supported PC bilayers in a way similar to silica, although $Ca^{2+}$ was found to be important.[53] Mica have yet to be tested with CP lipids but we expect that they might behave more like $SiO_2$.

It needs to be pointed out here that DOCP bilayers formed on metal oxides (e.g. $TiO_2$) has several fundamental differences from DOPC bilayers on $SiO_2$. It is expected that the lower leaflet of DOCP bilayers should be strongly adsorbed to $TiO_2$ and thus quite immobile. This is reminiscent of hybrid bilayer membranes like thiol monolayers on gold with a lipid layer above.[54] Such bilayers are more stably adsorbed and the top layer could have a different lipid composition. However, they are less mobile and can hardly accommodate a transmembrane species.

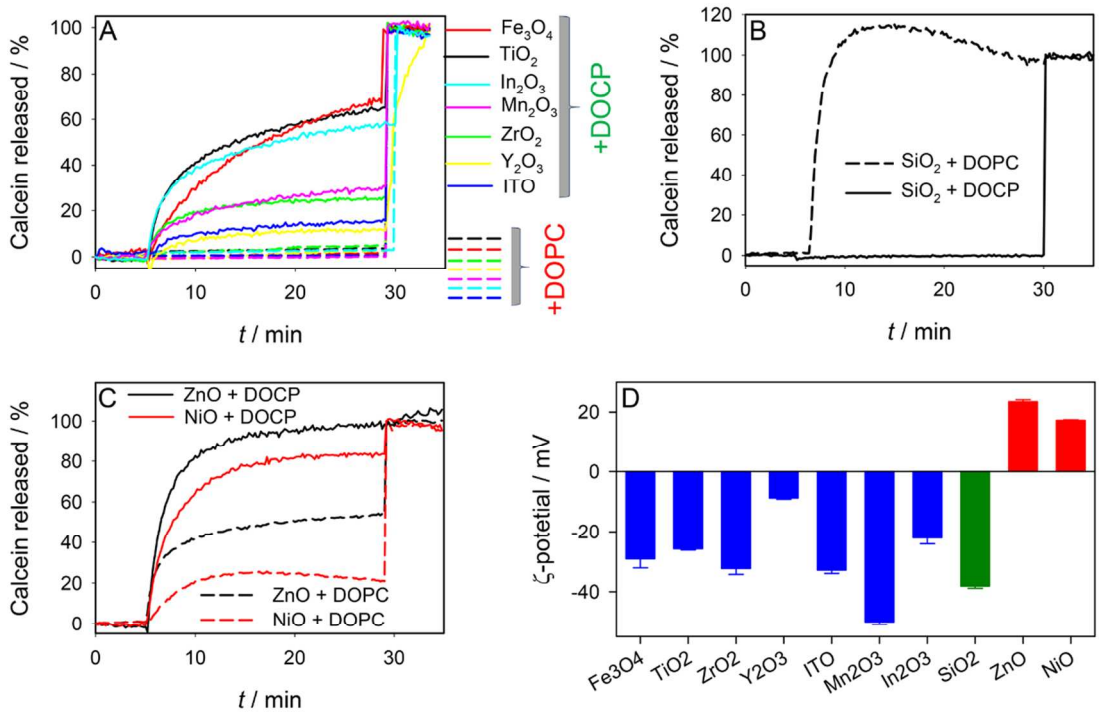



**Figure 6**. (A) Seven different metal oxide nanoparticles leaked calcein encapsulated DOCP liposomes but not DOPC liposomes. (B) Silica nanoparticles leaked DOPC but not DOCP liposomes. (C) NiO and ZnO leaked both liposomes attributable to their cationic surface. (D) The zeta-potential of these metal oxides. Figure adapted from Ref.[46] with permission. Copyright ® 2016 John Wiley and Sons.

In addition to the calcein leakage assay, cryo-TEM was also performed to confirm the interactions. Indeed, with $Fe_3O_4$ we observed supported DOCP bilayers (Figure 7F) but adsorbed DOPC liposomes (Figure 7G). With silica, we observed supported DOPC bilayers (Figure 7I), while DOCP liposomes barely interacted with silica likely due to electrostatic repulsion (Figure 7H). ZnO appeared fully disrupted the DOCP membranes (Figure 7J) and they were adsorbed around DOPC liposomes (Figure 7K). With these two lipids (Figure 7A, B), we classified metal oxides into three types (Figure 7C-E). Most metal oxides are similar to $TiO_2$ and $Fe_3O_4$, and they can leak DOCP liposomes suggesting formation of supported bilayers. Silica behaved oppositely, leaking DOPC but not DOCP. Mica and some $Al_2O_3$ might also belong to this type. Such oxide supported CP liposomes are useful for bioconjugation, device fabrication, drug delivery and sensing.

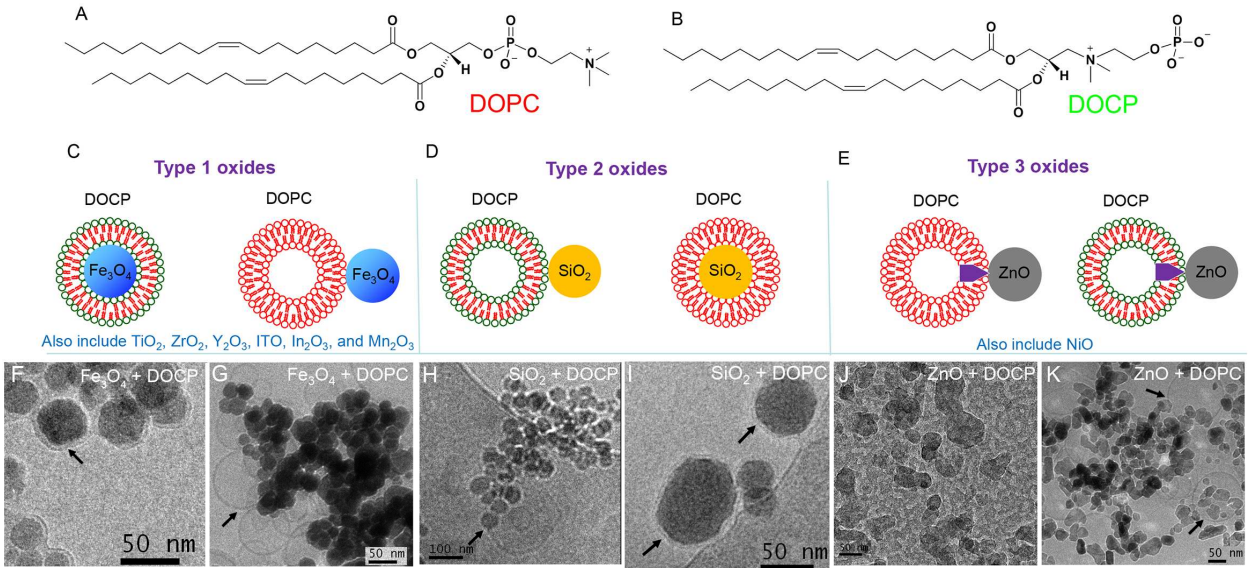



**Figure 7**. The structures of (A) DOPC and (B) DOCP lipids. Based on the interactions with these two liposomes, metal oxides are classified into three types. (C) Type 1 oxides are adsorbed by DOPC, but form supported bilayers with DOCP. (D) Type 2 forms supported bilayers with DOPC. (E) Type 3 includes cationic ZnO and NiO, damaging the membranes (pores indicated by the purple color). Full membrane disruption by ZnO occurred to DOCP liposomes. Cryo-TEM micrographs of (F, G) $Fe_3O_4$, (H, I) $SiO_2$, and (J, K) ZnO nanoparticles mixed with (F, H, J) DOCP or (G, I, K) DOPC liposomes. The

arrowheads point at the lipid features. Adapted from Ref.[46] with permission. Copyright ® 2016 John Wiley and Sons.

**CP liposomes interacting with metal ions**

In recent years, the interaction between metal ions and lipids has attracted more and more attention.[55-60] The role of $Ca^{2+}$ in promoting liposome fusion has been long been documented.[61, 62] Cremer and coworkers studied metal binding to phosphoserine (PS) and phosphorylethanolamine (PE) lipids.[57, 58, 60, 63] They found that PS lipids bind to $Cu^{2+}$ reversibly with an extremely high affinity, while PE lipids bind to $Cu^{2+}$ enhanced the oxidation of a lipid associated dye.[57, 60] Recently they demonstrated that $Ni^{2+}$ binding to PE lipids was thousand-fold tighter than to PC lipids.[64] $Hg^{2+}$ strongly binds to PE lipids resulting in leakage of the liposomes with a high PE content.[56] Synthetic metal ligands were also introduced to liposomes.[65, 66] Most of the above studies focused on serine, amine and carboxyl groups for metal binding. Based on our studies of liposome adsorption on metal oxides,[45, 46] phosphate could also be a useful metal ligand. We reason that CP might be a stronger ligand than PC. In general, terminal phosphate has stronger metal binding affinity than bridging phosphate.[18]

Szoka et al studied the aggregation rate of DOCP, DOPS and DOPA liposomes induced by $Ca^{2+}$. These are all negatively charged lipids. DOPS and DOPA liposomes aggregated faster with increasing $Ca^{2+}$ concentration up to 10 mM, but the aggregation of DOCP liposome reached a plateau at 4 mM $Ca^{2+}$. This suggests the importance of chemical groups on the lipid beyond simple electrostatic interactions.[9] Not surprisingly, neither neutral DOCPe nor DOPC liposomes aggregated with 10 mM $Ca^{2+}$. The zeta-potential of DOPC liposome increased dramatically with added $Ca^{2+}$, and it became positive at 10 mM $Ca^{2+}$. In contrast, the zeta-potential of DOCPe liposomes was less affected by $Ca^{2+}$, and it remained negative with up to 10 mM $Ca^{2+}$, suggesting a weaker interaction of $Ca^{2+}$ with DOCPe compared to DOPC (note both are charge neutral). This difference was rationalized by the location of the phosphate in

these lipids. In DOPC liposomes, the phosphate groups are located at the bilayer interface, which are better oriented and more ordered facilitating $Ca^{2+}$ binding.[12] However, in DOCPe, the phosphate groups extend to the aqueous phase with a higher freedom of motion. Therefore, binding of metal ions to lipids could also be affected by subtle differences.

In addition to $Ca^{2+}$, $Zn^{2+}$ is also an abundant metal in biological systems. $Zn^{2+}$ could bind to lipid membranes via both electrostatic interactions and Lewis acid/base interactions.[67, 68] Being a strong Lewis acid, $Zn^{2+}$ could interact strongly with phosphate. Recently, we studied the binding of $Zn^{2+}$ to DOPC and DOCP liposomes, and $Zn^{2+}$ interacted with these two differently.[69] $Zn^{2+}$ induce aggregation of DOCP liposomes at various pH's, and this is reflected from dynamic light scattering (DLS, Figure 8A), while no size change was observed for DOPC liposomes. Note that $Zn^{2+}$-induced DOCP aggregation occurred with sub-mM $Zn^{2+}$, suggesting a higher $Zn^{2+}$ binding affinity than $Ca^{2+}$ binding. Furthermore, $Zn^{2+}$ could leak DOCP liposomes but not DOPC liposomes based on our calcein leakage assays (Figure 8B). Finally, from TEM, we observed aggregated DOCP liposomes in the presence of $Zn^{2+}$ with different morphologies, e.g. multilamellar liposome structures (Figure 8C) and multilayered ribbon-like structures of micrometer length (Figure 8D). The formation of both structures from unilamellar liposomes would leak the liposomes. This work revealed CP to be a high affinity ligand for $Zn^{2+}$, and various interesting nanoscale lipid structures could be produced from such interactions.

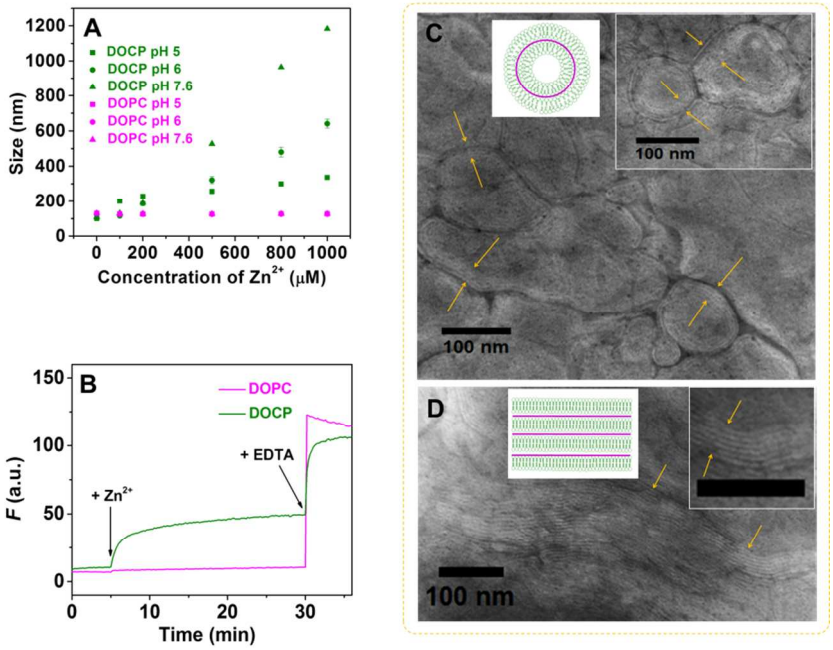



**Figure 8**. (A) Average hydrodynamic diameter of DOCP and DOPC liposomes as a function of $Zn^{2+}$ concentration at pH 5, pH 6, and pH 7.6. The size of DOPC remained constant while the size of DOCP increased. (B) Leakage tests of calcein-loaded DOCP and DOPC liposomes with $Zn^{2+}$ added at 5 min. Further addition of EDTA at 30 min to remove $Zn^{2+}$ induced more leakage. Negative stain TEM micrographs and schematic illustration of some products formed by mixing DOCP liposomes with 2 mM $Zn^{2+}$ such as (C) fused multi-layered liposomes and (D) lipid multi-layers sandwiching $Zn^{2+}$. Scale bars = 100 nm. Figures adapted from Ref.[69] with permission. Copyright ® 2017 American Chemical Society.

## Summary and future directions

In summary, we have reviewed the interactions of CP and CPe liposomes with PC bilayers, proteins, inorganic surfaces and metal ions. Compared to PC liposomes, CPe has even longer blood circulation time suggesting even better anti-fouling properties. CP liposomes are negatively charged with fully exposed terminal phosphate, which are better metal ligands for interacting with metal oxides and metal ions. Comparisons were also made with CPm dendrimers/polymers for adsorption of proteins, lipids and

cells. Interestingly, an opposite behavior was observed between liposomes and polymers in most cases, although these two are quite similar in chemistry. The difference could be attributed to the different ways of displacing the headgroups leading to different densities of the headgroup, and the hydration state of the molecules. Applications in drug delivery and formation of supported bilayers have also been described. Based on these progresses, we propose the following research opportunities.

*Fundamental studies*. The synthesis of CP lipids was reported only six years ago, and this is a quite young field. Many interesting observations have been made but their chemical basis are often unclear. For example, we do not have a good explanation for the longer circulation of CPe liposomes than PC liposomes. In addition, an intriguing aspect is the difference between CPe/CPm polymers and liposomes, and we still lack fundamental understandings to fully rationalize experimental observations. Other types of spectroscopic methods especially vibrational spectroscopy can be used to further probe proposed quadrupole interactions. Modeling is a powerful approach, and this should be a relatively simple and interesting system with experimental verification readily available. While the cell binding property of CPm polymers has been extensively demonstrated, more direct evidence of the proposed CPm/PC interaction is needed. Since the cell surface is very complex, model membranes are a better system for fundamental studies. Mixing PC and CP lipids can be easily achieved but studies on this front is quite limited, while such PC/CP mixed polymers have been reported with interesting solution properties.[25] Such relatively simple studies might offer useful insights as well.

*New synthesis*. A lot of development in synthesis has been made on the polymer front, forming various polymers, dendrimers, and micelles. Most of them were capped by a methyl group for the terminal phosphate. Recently, Hu and Emrick developed the chemistry to synthesize CP polymers with other capping groups on the phosphate, such as fluorophores, prodrugs, alkene and alkyne groups.[26] For most of the liposome work, only the commercially available lipids were used and their chemistry could also be significantly broadened by synthesis. It would be useful to produce different capping ligands so that the lipid and the methyl group capped polymers are more directly comparable. In addition, other

phospholipids might be inversed as well, and other zwitterionic headgroups can also be tested in the structure of lipids. Such development can further our fundamental understanding and also bring new molecules and materials for study.

*Exploring new applications*. These new lipid molecules may have unexpected applications. For example, we have already shown that CPe liposomes are longer circulating in blood than PC liposomes, and this alone has very interesting applications in developing drug delivery vehicles. In addition, PEG and targeting ligands can be added to achieve targeted delivery. To have a clinical impact, more systematic work is needed. In addition to drug delivery, forming supported CP bilayers on metal oxide surfaces can allow bioconjugation and device fabrication. Many metal oxides cannot be easily functionalized and having a lipid bilayer can solve this problem. Finally, using terminal phosphate as a metal ligand could open up many bioinorganic chemistry topics and such interactions may also be used for metal sensing and catalysis.

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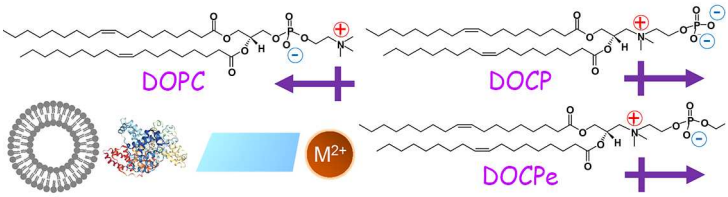
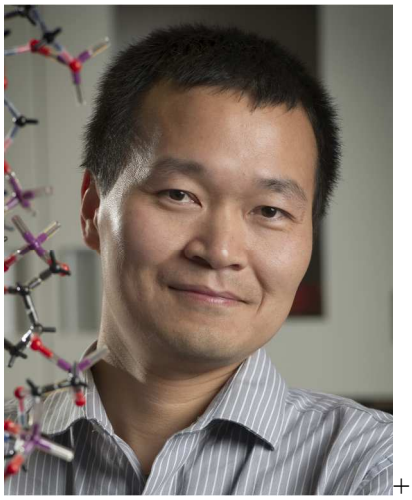



**Author biography**



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