Interfacing Zwitterionic Liposomes with Inorganic Nanomaterials: Surface Forces, Membrane Integrity and Applications

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ABSTRACT

Zwitterionic phosphocholine (PC) lipids are the main constituent of the mammalian cell membrane. PC bilayers are known for its anti-fouling property, yet it is adsorbed by all tested inorganic nanoparticles. This Feature Article is focused on the developments in my lab in the past few years on this topic. The main experimental techniques include fluorescence-based liposome leakage assays, adsorption, and desorption, and cryo-TEM. Different materials interact with PC liposomes differently. PC liposomes adsorb on SiO_2 followed by membrane fusion with the surface forming supported lipid bilayers. TiO_2 and other metal oxides only adsorb intact PC liposomes via the lipid phosphate bonding; the steric effect from the choline group hinders liposome fusion onto the particles. Citrate-capped AuNPs are adsorbed very strongly via van der Waals force, inducing a local gelation. The consequence is a transient liposome leakage upon AuNP adsorption or desorption, and AuNP aggregation on the liposome surface. In the AuNP system, the lipid membrane fluidity is critical. All the carbon-based nanomaterials (graphene oxides, carbon nanotubes and nanodiamond) are adsorbed mainly via hydrogen bonding. The oxidation level of graphene oxide strongly influences the outcome of the final hybrid material. In the context of inorganic nanoparticle adsorption, insights are given regarding the lack of protein adsorption by PC bilayers. These inorganic/lipid hybrid materials can be used for controlled release, drug delivery, and fundamental studies. A few examples of application are covered towards the end and future perspectives are speculated.

1. INTRODUCTION

Interfacing lipid vesicles (liposomes) with inorganic surfaces results in interesting hybrid materials.[1, 2] Liposomes allow drug containment, provide a biocompatible interface, and serve as a model for the cell membrane, while inorganic materials possess optical, electric, magnetic and catalytic properties. Their hybrids are thus promising candidates for drug delivery, imaging, and biosensor development. Liposomes can be attached to surfaces via covalent linkages, specific bio-interactions (e.g. via DNA hybridization or biotin-avidin interactions), or simple physisorption. We are interested in studying adsorption since it is more cost-effective and readily available to most researchers.

Depending on the composition, size, and surface chemistry of both the liposomes and inorganic surfaces, various interaction mechanisms are possible. Instead of bulk planar surfaces, we focus on inorganic nanoparticles (NPs) for our discussion. With strong interactions, we can expect either lipid bilayer wrapping around NPs (Figure 1A), or NPs decorating the liposome surface (Figure 1B). For small NPs (i.e. below 5 nm) with a hydrophobic surface, they might be embedded between the bilayer (Figure 1C), while larger hydrophobic particles may be wrapped by a lipid monolayer (Figure 1D). It is still possible to trap non-interacting NPs inside (Figure 1E), or such NPs are repelled by the lipid surface from the outside (Figure 1F). These interaction formats correspond to various hybrid materials for different applications. While theoretical calculations have predicted these interactions,[3] they often ignored the specific *chemical* nature of different materials.

Various inorganic materials have different properties. For example, gold has plasmonic property, iron oxide is magnetic, TiO_2 is a photocatalyst, and ZnO absorbs UV light. For practical applications, these nanomaterials are often capped by strong ligands to achieve high

colloidal stability. These surface ligands, however, mask the chemical differences of each type of particle in terms of charge, hydration state, and specific chemical groups. We are interested in exploring the 'naked' particle surfaces without strong ligands and their interactions with PC liposomes.

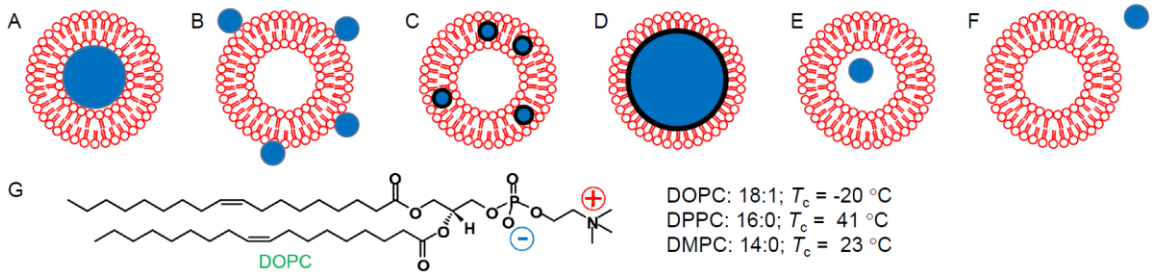

Figure 1. Different interactions between liposomes and inorganic NPs: (A) supported lipid bilayer formation; (B) NP adsorption; (C) small hydrophobic NPs embedded in the bilayer region; (D) large hydrophobic NPs wrapped by a lipid monolayer; and non-interacting NPs entrapped (E) or repelled (F) by the liposome. (G) The structure of a DOPC lipid with two 18-carbon tails each containing a double bond (18:1). Other tail structures give different phase transition temperature (T_c) values.

2. ZWITTERIONIC PC LIPIDS.

Many types of lipids have been identified in nature and more are available through chemical synthesis. The typical structure of a lipid contains a polar headgroup and two hydrophobic tails. Phosphocholine (PC) lipids represent a major component of the eukaryotic cell outer membrane.[4] This zwitterionic headgroup (Figure 1G) is known for its anti-fouling property (i.e. resistant to protein adsorption), and similar surface chemistry has been artificially engineered for various

biocompatible coatings.[5] The PC headgroup contains a choline and a phosphate. Choline is a quaternary ammonium cation and is always positively charged. Phosphate has a pK_a below 2; in the pH range concerned with most of the experiments, the phosphate is deprotonated. Therefore, the PC headgroup has a net charge of zero. The PC headgroup is heavily hydrated. The number of water molecules associated with each PC was calculated to be 23 in one study.[6] It is, however, concluded that the water structure surrounding a zwitterion is unperturbed, similar to that of the bulk water.[5] Therefore, water release related entropy change on the liposome surface does not contribute much thermodynamically to its adsorption. While PC bilayers are anti-fouling, they adsorb all tested inorganic NPs as will be described in this article. Therefore, we can deduce that inorganic NPs use different interaction mechanisms to achieve adsorption.

The hydrophobic tails of lipids can take many different forms. If the headgroup structure is fixed, the tails govern the phase of the lipid bilayer. Figure 2G shows the structure of a 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) lipid, where the two double bonds kink the packing of the tails, leading to a low phase transition temperature (T_c) of -20 °C. The lipids are in the gel phase below T_c but in the fluid phase above it. Raising T_c can be achieved by eliminating the unsaturated bonds, or by increasing the chain length. For example, DMPC and DPPC have T_c's at 23 °C and 41 °C, respectively.

Liposomes can interact with inorganic surfaces through a number of forces. Electrostatic interaction is probably the most commonly used, where oppositely charged liposomes and NPs are mixed. While electrostatic attraction is simple to achieve and effective, the cationic component is cytotoxic and cationic NPs can make pores on lipid bilayers.[7] In addition, electrostatic interactions have been extensively studied in general and thus will not be discussed here. Hydrophobic interactions are another force commonly associated with lipids. In the context

of interacting with inorganic NPs, this usually requires coating NPs with a hydrophobic ligand shell. As a result, these NPs have to be initially dispersed in an organic solvent, and the hydrophobic ligands mask the native surface property of NPs. We are interested in the interfacial chemistry directly on the particle surface (e.g. the naked surface) instead of those mediated by a ligand shell. In this case, hydrophobic interactions are also insignificant.

The focus of this article is the anti-fouling PC lipids. While PC liposomes resist protein adsorption, they are adsorbed by all the tested inorganic NPs. The presented data is mainly from recently published papers in my lab, emphasizing the non-electrostatic and non-hydrophobic interactions occurring with a few types of native inorganic NP surfaces. Simulation and experimental work from other labs will also be included when appropriate, but this article is not intended to be a comprehensive review of this field in general. I hope to articulate the importance and distinction of each inorganic surface. Towards the end, some applications will be briefly outlined followed by future perspectives.

3. ASSAY METHODS.

Liposome interacting with planar surfaces is often studied by quartz crystal microbalance with dissipation (QCM-D), atomic force microscopy (AFM), and fluorescence microscopy. They probe liposome adsorption, fusion with surface, lipid organization on surface, and fluidity. We are interested in solution phase colloidal interactions using NPs, and none of the above tools are directly applicable. Instead, a few other assays were employed for the solution system. 1) Calcein leakage test is a commonly used assay for probing membrane integrity in biochemistry. We typically encapsulate 100 mM disodium calcein to hydrate PC lipid films, and remove the free calcein using a simple Pd-10 column. The calcein trapped inside the liposome is self-

quenched, and enhanced fluorescence is observed upon liposome leakage or rupture. At the end of each experiment, full rupture is achieved by adding a surfactant such as Triton X-100 to quantify the amount of leakage. Aside from calcein, other dyes can also be loaded. 2) Adsorption can be measured using rhodamine (Rh), nitrobenzoxadiazol (NBD) or other fluorophore-labeled liposomes. Most inorganic NPs can be precipitated using a common benchtop centrifuge, but free liposomes cannot. By quantify the fluorescence in the supernatant, the amount of liposome adsorption can be measured as a function of buffer composition. Typically NBD is labeled in a lipid tail and thus it does not interfere with liposome adsorption, but NBD has low fluorescence quantum yield and is easily bleached. Rh are Texas Red are much brighter dyes typically labeled on the lipid headgroup, and control experiments are needed to ensure that adsorption is not due to the properties of the dyes. 3) Cryo-TEM is a powerful technique to measure the morphology of liposomes after adsorption. Samples are prepared by a quick freezing in liquid ethane and both intact liposomes and NP-supported bilayers can be well resolved. 4) Cell uptake studies are also useful. Free PC liposomes are not internalized by cells due to their anti-fouling property, while liposome/NP complexes are often taken by cells. This can be an initial study for drug delivery and it also confirms the NP adsorption reaction. 5) Dynamic light scattering (DLS) is used for studying the size and surface charge of liposomes, NPs, and their complexes. 6) Differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) are powerful thermal chemistry methods for probing lipid phase transition and ligand binding thermodynamics, respectively. Due to the large surface area of NPs, such methods can be better applied on the NP system than on planar surfaces. 7) Chemical probing extracts the mechanism of adsorption by changing buffer salt, pH, adding urea or other specific ions and chemicals. Some of these assays provide information that can be hardly obtained from the traditional surface science

measurements. Overall, most of these solution phase measurements can be carried out conveniently. Below, we discuss a few representative inorganic NPs using these assays.

4. SILICA. Silica (SiO_2) or glass is the most extensively studied surface for lipid interaction, and a large body of literature exists on this topic.[8, 9] Both simple PC liposome adsorption and formation of supported lipid bilayers (SLBs) are possible.[1, 10] The early theoretical work indicates the balance of liposome adhesion energy and the curvature energy to drive the liposome deformation and fusion onto the silica surface.[11] Later Richter and co-workers reported the importance of lipid lateral interaction, where DOPC liposomes are only adsorbed at low surface coverage, while fusion is facilitated at high surface coverage.[12] Ionic strength, pH, and divalent metals are able to modulate the SLB formation, which is attributed to an extra electrostatic contribution on top of the attractive van der Waals force.[13, 14] Additional charge or specific chemical interactions have also been harnessed to facilitate SLB formation using a broad range of lipids.[15] The bilayer fluidity, temperature, liposome size and concentration are also found to be important for PC liposome fusion with silica.[8, 9] A thin water layer of ~ 1 nm separates the lipid headgroup from the silica surface,[16] allowing the SLB to retain many properties of free-standing membranes. While most experiments were performed on planar silica surfaces, silica NPs were also studied.[17-22] These studies set a solid basis for exploring the interaction between PC liposomes with other types of inorganic NPs.

5. METAL OXIDES. Metal oxides encompass a diverse range of materials with very useful electric, optical, magnetic and catalytic properties. While it is well-established that PC liposomes fuse onto silica NPs, we are interested in other oxides (strictly speaking, silica is not a metal

oxide). Previous research on this topic has been mainly carried out on TiO_2. For example, PC liposomes were reported to adsorb on TiO_2 without forming SLBs even in the presence of Ca^{2+}.[23, 24] On the other hand, with a high liposome concentration and a long incubation time, Tero et al claimed that PC liposomes can form similar bilayers on TiO_2 (100) as that on SiO_2 based on van der Waals interaction, and the attraction potential is 20-fold larger on TiO_2.[25] Others reported that low pH, or incorporation of anionic lipids plus Ca^{2+} is needed to form SLBs on TiO_2.[26-28]

The above studies mainly employed bulk planar TiO_2 surfaces, and the overall impression is that it is more difficult to form PC SLBs on TiO_2. We instead carried our studies in the solution phase.[29] Most of our experiments were in a pH 6-7 buffer containing 10-100 mM Na^+ (pH and salt were often systematically studied). From cryo-TEM, we noticed that TiO_2, Fe_3O_4 and ZnO NPs adsorb spherical DOPC liposomes with no sign of SLBs (Figure 2B-D). For comparison, SLBs on SiO_2 NPs are also shown (Figure 2A). Aside from the drastically different TEM micrographs, we found a few additional differences between SiO_2 and these metal oxides. First, PC liposome adsorption is completely inhibited at high pH (e.g. pH 11) on the metal oxides (the example of TiO_2 shown in Figure 2E), but silica still maintains a high adsorption efficiency. Once adsorbed, however, raising pH cannot wash DOPC off from the TiO_2 NPs, indicating high pH only posed a kinetic barrier for adsorption. Second, free phosphate ions inhibited PC liposome adsorption by these metal oxides but not by silica (Figure 2F).

Based on these observations, we proposed that the metal oxides use a different type of interaction force for binding PC liposomes. Metal oxides can bind to the phosphate groups of the liposome via a nucleophilic reaction, and this bonding interaction is very strong (e.g. chemisorption, Figure 2G). At high pH, the Ti center is negatively charged with the deprotonated

hydroxyl being a poor leaving group, which is unfavorable for the nucleophilic attack by the lipid phosphate and explains the pH effect. The phosphate/TiO_2 bonding was also studied using IR spectroscopy. The lack of fusion of DOPC liposome on TiO_2 is attributed to the steric effect from the choline group. Adsorption of a few lipid molecules can be accommodated but fusion to form a whole bilayer is less likely. The role of lipid phosphate was recently noticed also by other researchers.[30]

Adsorption of PC liposomes onto silica is known to take place via van der Waal forces with a thin layer of water separating these two surfaces, resulting in no steric effects (Figure 2H). While the TiO_2 surface is initially also hydrated, local dehydration at the point of contact is expected to establish the direct lipid phosphate bonding. Molecular dynamics simulation was carried out by Fortunelli and Monti, and they pointed out the importance of the hydration state of the TiO_2 surface and direct phosphate interaction.[31] It is, however, still unclear if the lack of SiO_2 type of liposome fusion on TiO_2 is caused by the lipid phosphate bonding, by the hydration difference on these two oxide surfaces, or by their different van der Waals force interactions occurring with the lipid bilayers. The role of surface water in this system will be a topic of future studies.

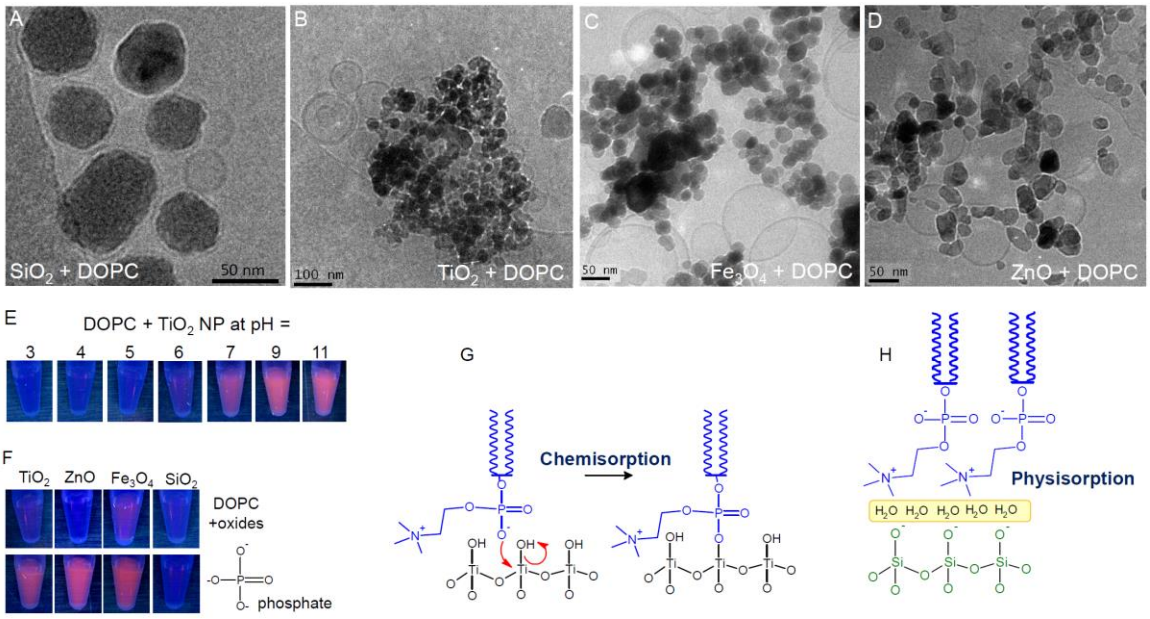

Figure 2. Cryo-TEM micrographs of DOPC liposomes mixed with (A) SiO_2; (B) TiO_2; (C) Fe_3O_4; and (D) ZnO NPs. SLBs are formed on SiO_2, while the other NPs only adsorbed the DOPC liposomes. (E) DOPC adsorption by TiO_2 NPs is inhibited at high pH, as indicated by the strong supernatant fluorescence from the Rh label. (F) Free phosphate ions inhibit DOPC adsorption by the metal oxides but not by SiO_2. (G) A proposed mechanism of DOPC phosphate forming a covalent bond with the TiO_2 surface based on a nucleophilic reaction. The steric effect from the choline group prevents liposome fusion onto the surface. (H) SLB on SiO_2 based on the van der Waals force and a thin layer of water separates the two surfaces. Reprinted with permission from reference [29]. Copyright 2014 John Wiley & Sons, Inc.

Shortly after publication of our TiO_2 work,[29] I attended a Gordon Research Conference in June 2014 at Newport, RI, where I was chatting with Paul Cremer about our model of TiO_2 adsorption. Paul suggested that if this model is correct, we should see liposome fusion by flipping the polarity of the PC headgroup and directly exposing the phosphate. This way, the

steric effect is eliminated. Szoka and co-workers first reported this inverse PC lipid and named it DOCP.[32] Using calcein-loaded DOPC liposomes, we did not observe leakage upon adding TiO_2 (Figure 3C), which is consistent with the cryo-TEM data of simple adsorption. On the other hand, DOCP liposomes leaked upon mixing (Figure 3D), suggesting fusion might take place. Using cryo-TEM, we indeed identified features of supported DOCP bilayers surrounding the TiO_2 surface (Figure 3E), yet the control experiment still observed intact DOPC liposomes (Figure 3F). Based on these, we summarized the interaction between TiO_2 NPs and these two types of liposomes in Figure 3A. Interestingly, SiO_2 behaves completely oppositely (Figure 3B).

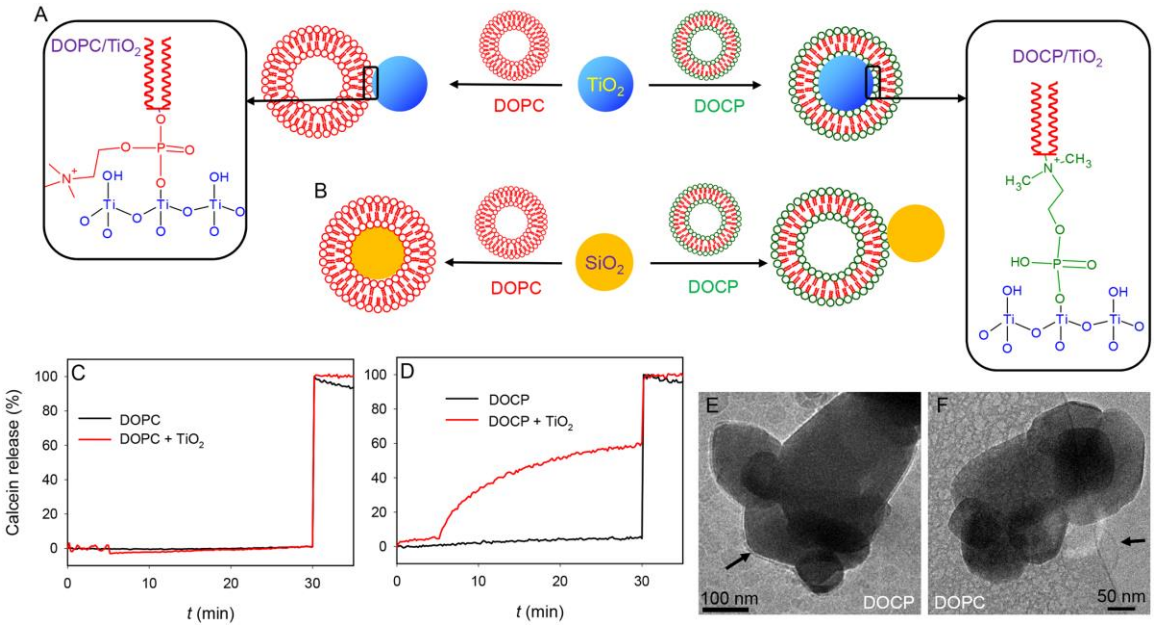

Figure 3. (A) Schematics of TiO_2 NP interacting with DOPC and DOCP liposomes. Both liposomes bind via the phosphate, but DOCP does not have the steric hindrance from the choline group. (B) Interaction of the two liposomes with SiO_2 NPs. DOCP liposomes are only adsorbed without fusion due to charge repulsion. Note that DOCP carries a net negative charge. Leakage

assays using (C) DOPC and (D) DOCP liposomes loaded with calcein. Cryo-TEM micrographs of TiO_2 NPs (E) forming supported bilayers with DOCP liposomes and (F) adsorbing intact DOPC liposomes. Reprinted with permission from reference [33]. Copyright 2015 American Chemical Society.

6. GOLD NANOPARTICLES.

Gold nanoparticles (AuNPs) are particularly important for nanotechnology due to its strong and distance-dependent color, high stability, tunable surface chemistry, and low toxicity.[34, 35] Interfacing AuNPs with lipid bilayers has been extensively studied. Most of the previous work employed AuNPs capped by either polymers or thiolated ligands, and focused on applications including controlled release, drug delivery, and toxicity studied.[36-38] A coarse-grained molecular dynamics simulation suggests the effect of ligand charge and density in penetration of AuNPs cross the bilayer membrane; cationic AuNPs might disrupt the membrane, while anionic and neutral particles are covered by a lipid bilayer upon crossing the bilayer, similar to endocytosis.[39] Free energy calculations were also made regarding surface-capped AuNP penetration, and hydrophobic effects were highlighted.[40] These simulation efforts, however, emphasize mainly the surface ligands without addressing the role of the gold core. Most AuNPs prepared in aqueous solutions are loosely capped by citrate, yielding a moderate electrostatic protection. Citrate-capped AuNPs have low colloidal stability; addition of ~20 mM NaCl can induce AuNP aggregation. This is also related to the very large Hamaker constant of gold (e.g. nearly 70 times larger than that of latex beads), meaning AuNPs experience much stronger attractive van der Waals force at the same distance.[41]

While surface capping improves colloidal stability, the native AuNP surface is masked. To have a baseline understanding without strong capping ligands, we decided to start with citrate-capped AuNPs, which are often considered to be 'naked' since the surface citrate can be easily displaced. Again, we focus on zwitterionic PC liposomes to avoid strong electrostatic interactions.

6.1. Visual observation. When citrate-capped 13 nm AuNPs were mixed with DOPC liposomes, we immediately observed the color AuNPs changing from red to purple or blue, suggesting aggregation of AuNPs. Only dilute buffers were used to maintain a neutral pH without additional salt to ensure a low ionic strength and avoid non-specific AuNP aggregation. The extent of color change is inversely proportional to the amount of DOPC liposome added; the largest color change occurs at the lowest liposome concentration (Figure 4A).[42] Without the liposome, AuNPs remain dispersed under the same buffer conditions. This indicates that AuNPs can be quickly adsorbed by DOPC liposomes and aggregate on its surface. Our cryo-TEM data further support the tendency of AuNPs to form clusters on the liposome surface (Figure 4B, C). Even at a 1:1 ratio between the number of AuNPs and liposomes, AuNPs still aggregated, leaving many liposomes without AuNP attached. The overall shape of the liposomes was maintained and they were not ruptured by AuNPs. Unlike metal oxides that can strongly interact with the phosphate group of the lipid, AuNPs do not interact with phosphate based on the soft-hard-acid-base theory.

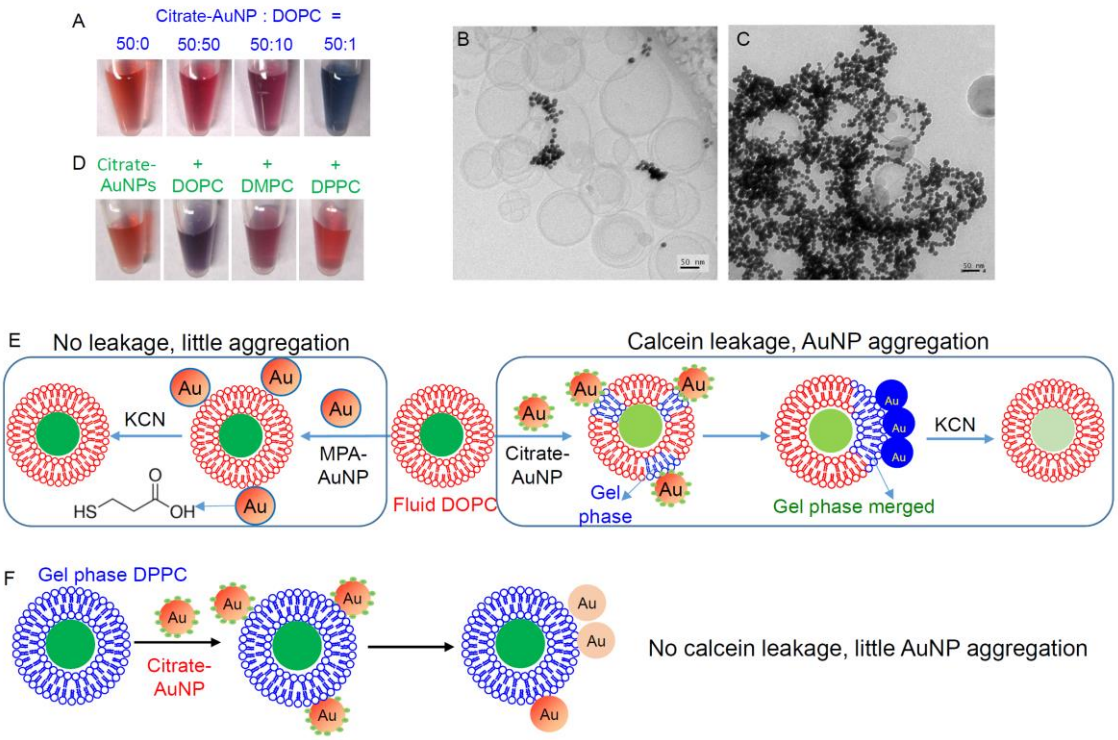

Figure 4. (A) Photograph of citrate-capped AuNPs mixed with DOPC liposomes at various liposome concentrations. AuNPs = 10 nM. Cryo-TEM micrographs of citrate-capped AuNPs mixed with DOPC at (B) 1:1, and (C) 50:1 particle number ratio. The overall spherical shape of the liposomes is maintained. (D) Photograph of citrate-capped AuNPs mixed with PC liposomes of different tail structures and thus T_c values. (E) Schematics of the interaction mechanism between citrate-capped and MPA-capped AuNPs with DOPC liposomes. The strong local phase transition with citrate-AuNP induces AuNP aggregation to eliminate gel/fluid phase boundaries and also causes leakage. MAP-capped AuNPs interact with the liposome surface less strongly and the increase of T_c is less. (F) Schematics of citrate-AuNPs adsorption on gel phase DPPC liposomes. The liposome remains in the gel phase and AuNPs are not extensively aggregated. Reproduced from reference [42] with permission from The Royal Society of Chemistry.

6.2. AuNP adsorption induced local lipid phase transition. To study liposome integrity, the calcein leakage test was performed. Addition of citrate-capped AuNPs to calcein-loaded DOPC liposomes resulted in a quick fluorescence enhancement (Figure 5A, black trace). Since the overall liposome integrity is maintained from the cryo-TEM data, this fluorescence enhancement is attributed to liposome leakage instead of rupture. Leakage occurred only with citrate-capped AuNPs, while AuNPs capped by strong ligands such as mercaptopropionic acid (MPA) or glutathione (GSH) did not leak. Our control experiments with $NaHB_4$ reduced AuNPs (also easily displaceable ligand) also leaked AuNPs. Therefore, citrate is not required for leakage; it is important for the native AuNP surface to directly contact the liposome surface. After the addition of AuNPs, the fluorescence signal then stabilized in ~2 min, and more AuNPs can induce more leakage (Figure 5B). Furthermore, we centrifuged the DOPC/AuNP conjugate, washed it to remove free liposomes, and then re-dispersed the sample in a fresh buffer. Adding more AuNPs still resulted in further leakage (Figure 5C). This suggests that leakage is transient and after a brief leakage, the leaking sites are sealed.

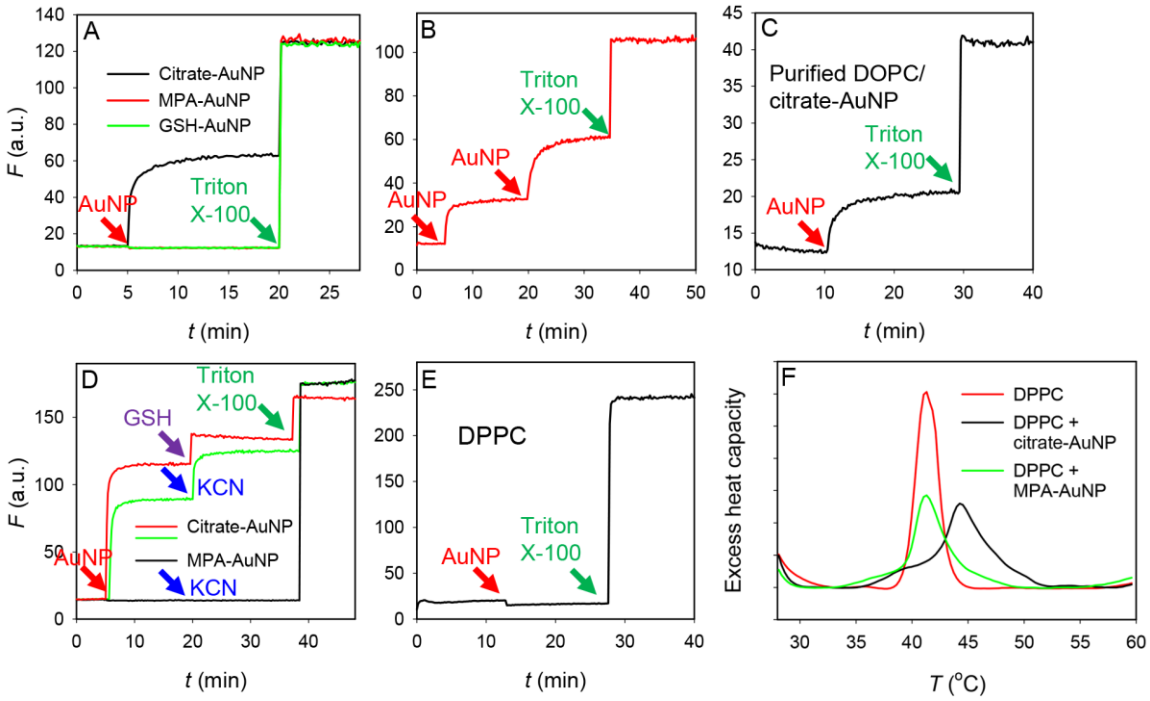

Figure 5. AuNP-induced liposome leakage test. AuNPs are capped by citrate unless otherwise indicated. (A) DOPC liposome leakage by AuNPs with different surface ligands. (B) Leakage induced by adding AuNPs in steps. (C) The initial AuNP/DOPC complex is prepared and centrifuged to remove free liposomes. Adding more AuNPs induces further leakage. (D) Adding KCN (10 mM) and GSH (0.1 mM) to citrate-AuNP/DOPC complex induces further leakage, but no leakage for MPA-AuNP when KCN is added. (E) No leakage occurs for DPPC liposomes with citrate-AuNPs. (F) DSC traces of DPPC in the presence of AuNPs without different surface ligands. Reproduced from reference [42] with permission from The Royal Society of Chemistry.

This is the first report of small NP induced PC liposome leakage without causing an overall rupture (e.g. silica is an example of rupturing PC liposomes). The Granick group previously reported stabilization of PC liposomes by adsorbed small latex beads,[43] and other

types of nanoparticles.[44] In other words, these particles reduced liposome leakage. They attributed it to a local gelation of the liposome at the sites of NP adsorption.[45] Protein adsorption was also reported.[46] We studied graphene oxide, nanodiamond, a few metal oxides, and here MPA and GSH-capped AuNPs.[43, 44, 47] None of them leaked PC liposomes.[48] Therefore, citrate-capped AuNPs are unique in its ability to leak DOPC liposomes. After the initial leakage, whether the adsorbed citrate-AuNPs can further stabilize the liposomes remains to be determined.

What's also striking is that when AuNPs are desorbed from the liposome surface by adding GSH, or when AuNPs are dissolved by adding KCN, leakage also occurs (Figure 5D). Without pore formation, liposomes leak its content the fastest at its T_c, where the fluid/gel phase transition occurs. The rapid conversion of lipid packing between the two states compromises membrane integrity.[49] We reason DOPC undergoes a fluid-to-gel phase transition at the sites of citrate-AuNP adsorption; leakage occurs during this transition. Once reaching the gel state, leakage stops. Upon removing the AuNPs, the same sites undergo the gel-to-fluid transition, also resulted in a transient leakage. When capped by MPA or GSH, the AuNPs are slightly farther away from the liposome surface, leading to weaker van der Waals force. This adsorption-induced increase of T_c is likely true for other types of nanomaterials as well. However, they either have smaller van der Waals force or are positioned slightly away from the surface, and the extent of phase transition needed cannot be reached at room temperature to induce leakage. Instead, they only exert the stabilization effect.[43, 45]

If this model is true, no leakage should occur for a liposome already in the gel phase. Indeed, DPPC liposomes (T_c = 41 °C) failed to leak with citrate-capped AuNPs (Figure 5E). Our hypothesis is also supported by the DSC measurement. After mixing DPPC liposomes with

citrate-AuNPs, we observed a 4 °C shift and significant broadening of the phase transition profile, while MPA-capped AuNPs caused no shift (Figure 5F). For comparison, adsorption of 5 nm silica raised the T_c by only 0.7 °C.[50] This also supports a much stronger interaction between PC lipids and citrate-AuNPs.

6.3. Effect of liposome fluidity. Another piece of evidence of AuNP-induced lipid phase transition comes from the aggregation of AuNPs in the presence of liposomes of different T_c values (Figure 4D). Interestingly, if we replace the fluid DOPC liposomes with the DMPC and DPPC liposomes, the extent of color change decreased significantly.[51] Gel phased lipids have lower lateral diffusion coefficients. At the first glance, the observation might be attributed to the slower diffusion of individual DPPC lipids in the bilayer and thus cannot carry the adsorb AuNPs as quickly. However, this is not a pure kinetic effect, since even after a long time, the color of AuNPs remained similar, and the diffusion coefficients of fluid and gel phase lipids differ only by ~5-fold. We proposed a model of merging of the lipid fluid/gel interfaces (Figure 4E). Such interfaces are associated with high interfacial energy, and thus there is a thermodynamic driving force for AuNPs to cluster and eliminate such interfaces. Otherwise, AuNPs would not aggregate under such a low salt concentration (~2 mM Na^+). Again, when AuNPs are capped by MPA, such color change was not observed. Citrate-AuNPs do not aggregate much on DPPC (already in gel phase before AuNP adsorption) due to a lack of further thermodynamic driving force (Figure 4F).[51] Very recently, similar observations were made in the context of assembly AuNPs on liposomes by tuning temperature by Sugikawa et al.[52]

7. CARBON NANOMATERIALS.

Carbon-based nanomaterials have fueled the growth of the nanotechnology field tremendously. Carbon nanotubes, graphene, and nanodiamonds are important examples, and they are widely used for device fabrication, sensing, and drug delivery.[53, 54] Their interactions with lipids are also important for understanding nano-toxicology.[55] Graphene is a single layer of graphite, and its interaction with lipids has attracted a lot of interest. Since graphene cannot be easily dispersed in water, most experiments in solution used graphene oxide (GO), while simulation work often employed graphene.

Since graphene is a well-defined material, a number of theoretical simulations have been reported. Kral and co-workers predicted the insertion of graphene into POPC lipid bilayers via hydrated micelles of graphene flakes (Figure 6A),[56] and that graphene interacts favorably with the hydrophobic lipid tails. Tu *et al* also simulated graphene/lipid interactions and proposed lipid extraction from the bilayer. They predicted that the lipid tails are parallel to the graphene surface (Figure 6B).[57] This lipid extraction mechanism was used to explain the toxicity of graphene to bacterial cells. In other studies, lipid interaction was also proposed to be a major reason of the anti-microbial activity of graphene-based materials.[58, 59] Li et al observed an edge-first uptake and internalization of graphene up to 10 μm lateral size by mammalian cells. After detailed molecular dynamics simulation, the authors proposed graphene entering into the lipid bilayer via corners or asperities (as opposed to enter via flat edges) to lower the energy barrier (Figure 6C).[60]

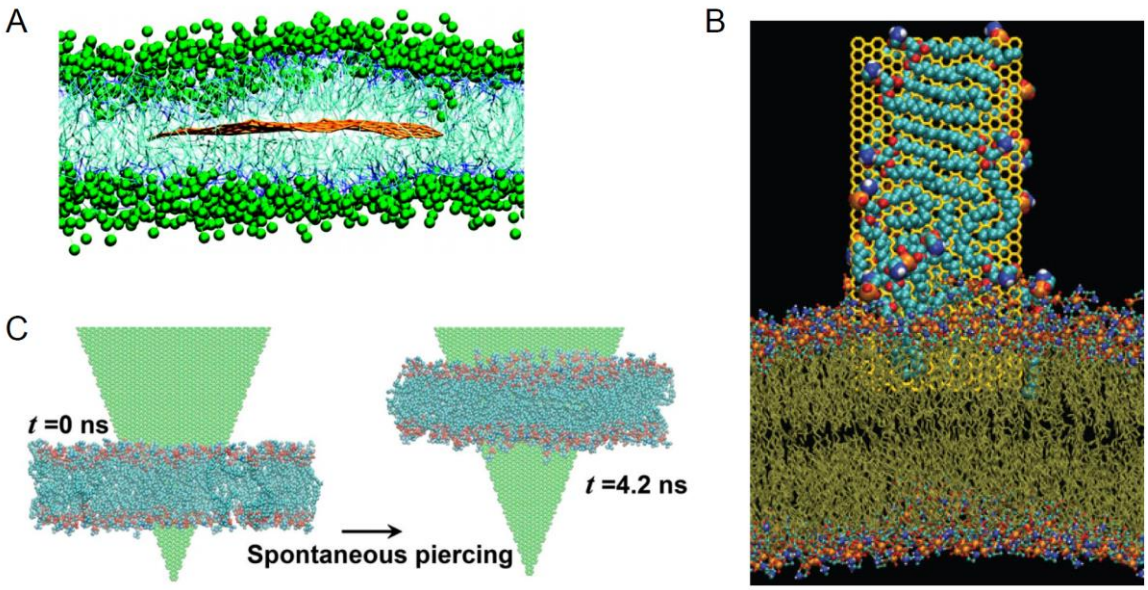

Figure 6. (A) An equilibrium structure of a nanoscale graphene piece inserted into the bilayer membrane of POPC. (B) A simulated image of a fully restrained graphene sheet docked at the surface of the outer leaflet of a pure POPE membrane. In this case, lipids are extracted from the membrane. (C) Molecular dynamics simulations of entering lipid bilayer via its corner piercing as a means of reducing the energy barrier. Reprinted respectively from reference [56], [57], and [60]. Copyright 2009 the American Chemical Society, 2013 Nature Publishing Group, and 2013 National Academy of Sciences.

In addition to simulation, experimental efforts have also been reported. Loh and co-workers employed graphene film prepared by chemical vapor deposition to study lipid interaction and device fabrication.[61] They tested liposomes of different charges and proposed SLB formation in a way similar to that on silica surface. In their model, a thin water layer separates the lipid headgroup and the graphene surface. Frost *et al* reported that GO with 20% oxygen content can rupture pre-adsorbed liposomes, leading to the formation of a lipid/GO

multilayer structure (alternating GO monolayers and lipid bilayers).[62] Charge interaction between cationic liposomes and anionic GO appeared to be important in this case. AFM was used in both papers, but it cannot resolve the lipid films clearly.

Since different research groups proposed different models, and AFM cannot provide conclusive microscopic images, we decided to carry out a systematic study using three types of graphene materials with different levels of oxidation: highly oxidized GO (~40% oxygen content), pristine graphene, and reduced GO (rGO) with an intermediate oxygen content.[63] We started our work by using Rh-labeled liposomes carrying different charges and mixed them with GO. After a brief centrifugation, GO and the associated lipids were precipitated (Figure 7A). As expected, cationic DOTAP liposomes adsorbed onto the negatively charged GO, while anionic DOPG liposomes did not adsorb. Interestingly, zwitterionic DOPC liposomes also adsorbed. The adsorption of DOPC does not rely on electrostatic interactions since stable adsorption is still achieved even in 1 M Na^+ (Figure 7B). We also studied nanodiamond (ND) and single-walled carbon nanotubes (CNT).[48] Their adsorption by DOPC was probed by urea (Figure 7D). Adsorption of DOPC was weakened by urea for all these materials, and the affinity ranking goes GO > ND > CNT. Urea disrupts hydrogen bonds and this experiment suggests that hydrogen bonding might be a major stabilizing force for PC liposomes to adsorb these carbon-based nanomaterials. In addition, DOPC adsorption is weakened at high pH (Figure 7C), also supporting that hydrogen bond might be important.

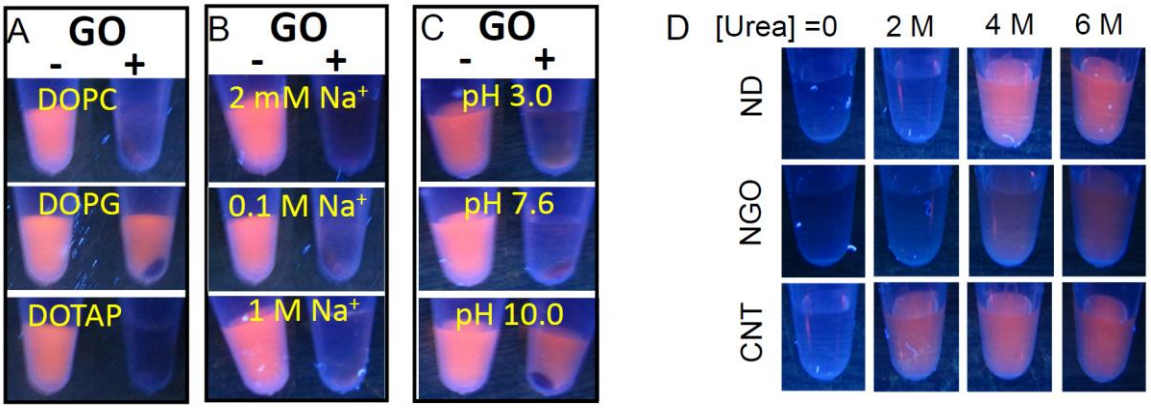

Figure 7. Fluorescent assays for probing the adsorption between GO and Rh-labeled liposomes. (A) Effect of liposome charge. Effect of (B) salt concentration and (C) pH for DOPC liposome adsorption. (D) Effect of urea on DOPC adsorption by three types of nanocarbons. All the samples were centrifuged, and a strong supernatant fluorescence indicates a lack of liposome adsorption. Reprinted with permission from reference [63], and [48]. Copyright 2013 John Wiley & Sons, Inc. and 2013 The Royal Society of Chemistry.

To understand the state of liposome adsorption, we further carried out cryo-TEM experiments. DOPC liposomes were adsorbed as intact liposomes on the edge of the GO sheets (Figure 8A). The edge of GO is rich in carboxyl groups.[64] Combined with the pH and urea probing data, we reason that the carboxyl group of GO interacts with the PC headgroup via hydrogen bonding. Recently, Jiang and co-workers used surface enhanced IR spectroscopy to study the interaction between PC lipids and GO.[65] They proposed a combination of electrostatic repulsion, electrostatic attraction, hydrophobic interaction, and hydrogen bonding. For hydrogen bonding, a water molecule was proposed to bridge the lipid phosphate and the carboxyl group on GO. From the pH range of 3 to 10, only GO can be (de)protonated. Based on our pH-dependent

study, the group on GO should serve as a hydrogen bond donor, since high pH inhibits adsorption. Our results suggest that hydrogen bonding is the dominating interaction force.

Interestingly, rGO showed a very high capacity for DOPC adsorption (Figure 8B). We can only resolve the liposomes on the edge, while in the plane, the liposomes are densely packed. The liposomes on the edge are also distorted, which is quite different from that on GO, where the spherical shape is retained. Finally, on pristine graphene, we cannot resolve even a single liposome structure (Figure 8C). Using the Rh-labeled liposomes, we know that lipids are adsorbed. Therefore, we reason that the liposome must have ruptured and the hydrophobic tail of the lipids are interacting with the graphene surface as shown in Figure 6A. Aside from graphene oxide, we also confirmed the adsorption of ND (Figure 8D) and CNT (Figure 8E) by intact DOPC liposomes using cryo-TEM.[48]

Using calcein-loaded liposomes, we probed liposome leakage (Figure 8F). No leakage was observed with GO, while ~20% leakage occurred with rGO. Pristine graphene induced ~30% leakage in the first 20 min and after a long time it reached ~70% leakage. This high leakage is also consistent with liposome rupture on graphene surface. Therefore, the interaction between graphene and PC liposomes strongly depends on the oxidation level of graphene. For pristine graphene, hydrophobic interaction is dominating, while for highly oxidized GO, hydrogen bonding is more important. ND and CNT do not leak PC liposomes based on our calcein leakage assays.[48]

We also studied the interaction between liposomes and GO using ITC. This technique measures the amount of heat during binding reactions. When DOPC liposomes are mixed with GO, heat is released. On the other hand, cationic DOTAP liposomes absorb heat, suggesting a strong hydrophobic interaction. The surface of GO is heterogeneous, containing both carbon-rich

more hydrophobic domains and highly oxidized hydrophilic domains.[66] The size of such domains is around a few nanometers. It is likely that more water molecules are released from the hydrophobic regions on GO upon interacting with DOTAP, and these regions may form supported DOTAP monolayers.[67] DOPC liposomes interact mainly with the highly oxidized GO edge, and the hydration on GO is less perturbed.

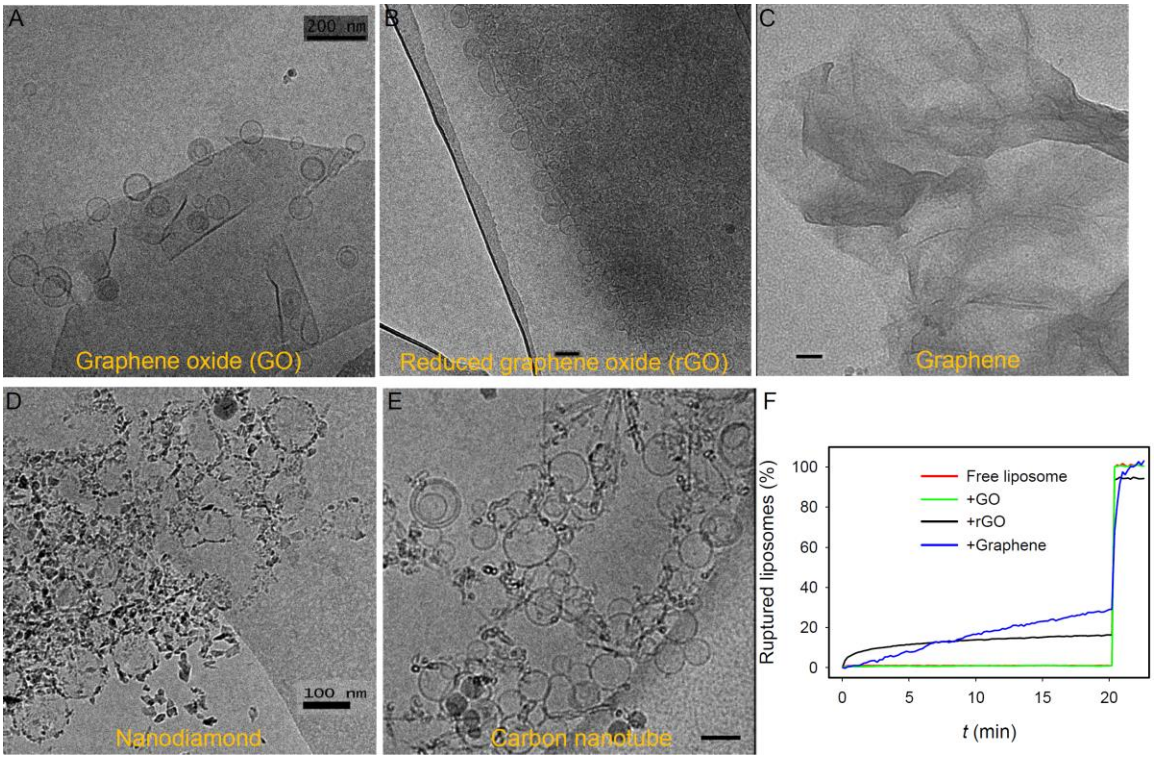

Figure 8. Cryo-TEM micrographs of DOPC liposomes mixed with (A) GO, (B) rGO, (C) graphene, (D) NDs, and (E) CNTs. The graphene sample (C) has no visible liposomes indicating liposome rupture. (F) Calcein leakage test of DPPC liposomes mixed with the three types of graphene. Triton X-100 was added at 20 min to fully rupture the liposomes. Reprinted with permission from reference [63], and [48]. Copyright 2013 John Wiley & Sons, Inc. and 2013 The Royal Society of Chemistry.

8. PROTEINS.

PC terminated surfaces are anti-fouling, meaning that they resist protein adsorption. Anti-fouling is attributed to the lack of ion pair formation between zwitterionic PC and proteins.[5] Proteins usually contain both positively and negatively charged domains. For a surface with a certain charge (i.e. non-zwitterionic surfaces), proteins adsorb using their oppositely charged domains forming ion pairs at the interface. Each ion pair would release two small counter ions, whose entropy is the thermodynamic basis for protein adsorption. However, zwitterionic surfaces (e.g. PC bilayers) do not form ion pairs, explaining their resistance to protein adsorption.

It is interesting to note that PC liposomes can adsorb all tested inorganic NPs, which are certainly not adsorbed by ion pairs. The general relationships among these three types of surfaces are shown in Figure 9A. When examined in more detail as summarized in this article, each type of inorganic NP uses a different mechanism to interact with PC liposomes: hydrogen bonding for GO, ND and CNT, phosphate bonding for metal oxides, and van der Waals force for citrate-capped AuNPs and SiO_2. The densely packed functional groups on inorganic NPs may result in polyvalent interactions, which further enhance adsorption affinity.

A consequence of lack of protein adsorption is that PC liposomes are not internalized by cells (Figure 9B). Since inorganic NPs can adsorb proteins, their liposome hybrids can be internalized (Figure 9C-G). In this regard, cellular uptake is another assay to confirm NP adsorption.

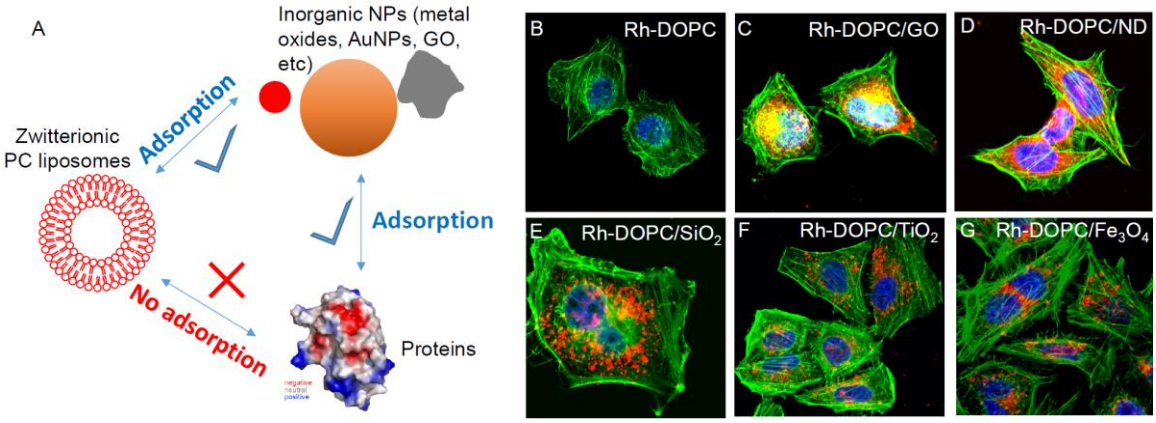

Figure 9. A scheme showing the adsorption interaction between PC liposomes, proteins, and inorganic NPs. PC liposomes resist protein adsorption but it can adsorb all tested NPs. Inorganic NPs can also effectively adsorb proteins. Confocal fluorescence micrographs of HeLa cells incubated with (A) free Rh-labeled DOPC liposomes, and the liposomes mixed with (C) GO, (D) ND, (E) SiO_2, (F) TiO_2, and (G) Fe_3O_4 NPs. Blue: cell nuclei; green: actin; red: liposome. Reprinted with permission from reference [29]. Copyright 2014 John Wiley & Sons, Inc.

9. APPLICATIONS.

Since this article mainly deals with fundamental interactions at the PC liposome interface, the applications of such hybrid materials are only briefly discussed here. Focused reviews for their drug delivery have been published,[68, 69] and we emphasize it from a conceptual level with a few examples. 1) SLBs allow bioconjugation via lapidated ligands (Figure 10A). This is useful for targeted drug delivery. The inorganic core can adsorb drugs (especially for porous core), and this solves the low loading efficiency problem of liposomes. The core can also provide magnetic and fluorescence property, depending on the core composition.[70-72] Wrapping a PC lipid layer on an inorganic NP can make the surface less sticky (anti-fouling), and thus increase biocompatibility

of the core. A good example incorporating all these features was reported by Brinker and co-workers (Figure 10B).[73] 2) For NPs stably adsorbed (Figure 10C), this system can be used for controlled release. We have demonstrated this with an IR laser heating the DPPC/GO complex,[63] and also UV irradiation of the DOPC/TiO_2 complex to induce lipid damage.[29] The confocal fluorescence micrographs of HeLa cells incubated with calcein-loaded DOPC/TiO_2 before and after UV irradiation is shown in Figure 10D, indicating the diffusion of calcein throughout the cell plasma after UV exposure, possibly due to membrane damage. Using AuNPs to control liposome leakage has been extensively studied.[37, 74] 3) Finally, these systems are useful for understanding fundamental interaction mechanisms of nanomaterials with biological membranes. PC lipids are the most abundant lipids of the cells' outer membrane. The fact that all the inorganic NPs can be adsorbed indicates a mechanism of potential toxicity.

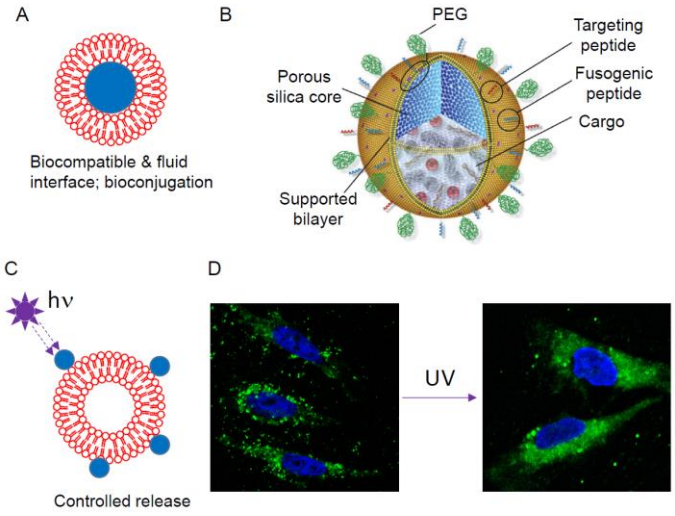

Figure 10. Cartoons of examples of (A) SLBs and (C) NPs adsorbed by liposomes that may allow controlled content release by light. (B) A scheme of a mesoporous SiO_2 NP enveloped by a lipid bilayer and the incorporation a diverse range of functional ligands for drug loading and targeted delivery. (D) Confocal fluorescence micrographs showing calcein loaded DOPC/TiO_2

uptake by HeLa cells. After UV treatment, calcein release is observed. Panel (B) reprinted from reference [73]. Copyright 2011 Nature Publishing Group.

CONCLUSIONS AND PERSPECTIVES

This article has focused mainly on our work in the past few years at the biointerface of PC liposomes and a few types of inorganic NPs. Most of the measurements were carried out with colloidally dispersed NPs and liposomes. So far, all tested inorganic NPs can be adsorbed by the PC liposomes. It is interesting to note that different NPs take different mechanisms for adsorption: van der Waals force for silica (with liposome fusion onto the surface) and AuNPs (but no liposome fusion); lipid phosphate interaction with most metal oxides, and hydrogen bonding with graphene oxide. It is also interesting to think about the difference between proteins and the inorganic NPs for adsorption by the PC liposomes. While most of the researchers use bulk planar surfaces and traditional surface science tools, we focused on NP dispersions. I herein list a few comparisons of these two approaches. 1) Different instruments are used, and our NPs can be studied with readily available spectrometers. 2) NPs have more surface area, but planar surfaces are more controllable (e.g. a specific crystal plane can be prepared). 3) For most biomedical applications and nano-toxicology studies, NPs are more directly relevant.

Future directions on this topic are likely to involve the following aspects. 1) Continue on fundamental studies and explore the surface forces. For example, the role of surface water on both the liposome and inorganic NPs. This can be potentially studied using ITC and various vibrational spectroscopy. Quantitative measurement of the interaction forces is also important, and molecular dynamics simulation may provide new insights into the biointerfaces (some applications of it are already shown in this article). 2) Explore the unique features of both

inorganic NPs and liposomes for practical applications. For example, the core can be used for drug containment, magnetic separation, imaging, and the lipid shell has fluidity, can incorporate targeting ligands, and ion channels. The membrane also allows insertion of ion channels and other cell mimicking features. By attaching affinity ligands, analytical applications can also be envisioned. 3) Finally, cross-membrane communication is another interesting aspect. In biological system, this is highly important and is accompanied by protein channels, protein assembly/conformational change, membrane potential, and membrane raft formation. This aspect of research has not been fully explored with a nanoparticle component. Overall, fundamental understandings are key to all applications.

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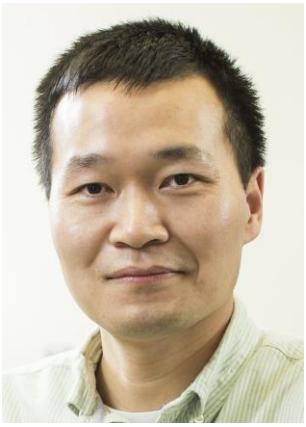
Author Biography



Juewen Liu received his BSc from the University of Science and Technology of China in 2000 and PhD in Chemistry from the University of Illinois at Urbana-Champaign in 2005. After postdoctoral research at the University of New Mexico and Sandia National Laboratories, he joined the Department of Chemistry of University of Waterloo in 2009, and now he is an Associate Professor there. He is interested in studying the adsorption of biomolecules such as DNA and lipids by nanomaterials both for fundamental understandings and for analytical and biomedical applications. He received an Early Researcher Award from the Ontario Ministry of Research and Innovation (2011) and the Fred Beamish Award from the Canadian Society for Chemistry (2014). He has authored over 150 papers, receiving nearly ten thousand citations.

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