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Self-healable and reversible liposome leakage by citrate-capped gold nanoparticles probing initial adsorption/desorption induced lipid phase transition

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We herein report adsorption/desorption of citrate-capped gold nanoparticles (AuNPs) transiently leaks fluid phase DOPC liposomes, while the liposomes are not leaked by AuNPs capped with mercaptopropionic acid (MPA). Leakage also fails to occur for gel phase DPPC liposomes. Citrate-capped (but not MPA-capped) AuNPs raise the phase transition temperature of DPPC. We conclude that citrate-capped AuNPs interact with the PC liposomes very strongly inducing local fluid-to-gel lipid phase transition for DOPC. Leakage takes place during this transition, and membrane integrity is resumed after the transition. Citrate-capped AuNPs allow a stronger van der Waals force than MPA-capped AuNPs with PC liposomes, since the latter is separated from the liposome surface by the ~0.3 nm MPA layer.

The interplay between nanomaterials and lipid membranes has profound implications in physical sciences,[1] as well as applications in nanomedicine, biomaterials and bioanalytical chemistry.[2-8] For example, a number of nanoparticles are adsorbed by zwitterionic liposomes,[9-17] and some are reported to stabilize lipid membranes. Such systems have been used for drug delivery.[18,19]

Among different types of materials, gold nanoparticles (AuNPs) are particularly attractive.[20-22] The surface chemistry of gold can be conveniently controlled via adsorption of thiolated molecules or polymers.[23] AuNPs also have very high inter-particle van der Waals forces (e.g. large Hamaker constant), yielding low colloidal stability.[24] Finally, its unique color makes optical monitoring easy. Many studies were carried out on AuNP-decorated liposomes. For example, 10 nm AuNPs were mixed with zwitterionic liposomes, where leakage was observed only with cationic modifications.[25] Polymer and surfactant coated AuNPs of various sizes were also tested.[26-30] In addition, a few papers reported radiation-induced liposome leakage sensitized by AuNPs.[31-39]

In most previous work, the AuNP surface was capped by a strong ligand or polymer to improve their colloidal stability and to tailor surface property. These ligands also prevent a direct contact between the gold core and liposomes, hindering very short-ranged interactions. When prepared in an aqueous solution, most AuNPs are capped by citrate. Citrate is a weak ligand and it is generally accepted that it can be readily displaced. Therefore, citrate-capped AuNPs are considered to be 'naked', and strong short-ranged forces may emerge using naked AuNPs. Indeed, we herein report a very different behavior of citrate-capped AuNPs, inducing transient leakage of fluid phase liposomes upon adsorption or desorption. Unlike most previous work studying equilibrium conditions, citrate-AuNPs probe the initial adsorption/desorption and accompanying phase transition process.

The property of lipids is considerably affected by its headgroup chemistry. We are interested in phosphocholine (PC) lipids, because their zwitterionic headgroup avoids strong electrostatic interaction, facilitating observation of other forces.[10,11,15] To study adsorption, DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine, see Figure 1A) liposomes (~100 nm, Figure S1 for size) with 1% (weight percent) rhodamine (Rh)-DOPE label in the headgroup were mixed with 13 nm citrate-capped AuNPs (AuNP:DOPC = 50:1, particle molar ratio). After centrifugation, the supernatant was non-fluorescent (inset of Figure 1B), suggesting complete adsorption (note free liposomes cannot be spun down). The adsorption kinetics were very fast (Figure 1B), where fluorescence quenched rapidly upon mixing (within 1 min). To rule out adsorption through the Rh dye, we also employed lipids with an NBD label on the tail, where nearly 100% adsorption was achieved as well (the black bar in the first group, Figure 1C).

To probe the effect of surface chemistry, AuNPs were capped with MPA and GSH, respectively (see Figure 1A for structure). MPA-capped AuNPs are completely adsorbed by DOPC (Figure 1C, red bar), which is expected since carboxyl-terminated latex beads can also be adsorbed.[13] On the other hand, GSH-capped AuNPs are adsorbed to a lower extent (green bar). This might be related to its larger molecular size, separating the gold core from the lipid surface more, which may reduce the van der Waals force.

To have a complete understanding, we further studied the effect of lipid charge. Anionic DOPG did not adsorb any of these AuNPs,

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while cationic DOTAP adsorbed all of them (Figure 1C), which can be explained by electrostatic interactions. These three types of AuNPs are all negatively charged (ζ-potential = ~-30 mV, Table S1). In this study, we focus on DOPC to minimize strong charge-based interactions.

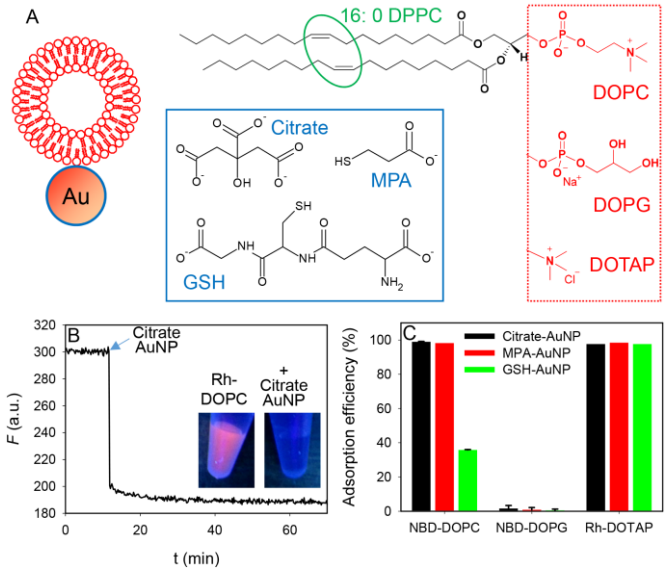

Figure 1. (A) A scheme showing AuNPs adsorption by a liposome, the surface ligands on AuNPs (blue box), different lipid head groups (red box) and hydrophobic tails. DPPC has 16 carbon saturated tails. (B) Kinetics of Rh-DOPC fluorescence change upon adding AuNPs (Rh-DOPC:AuNP=10:1 particle molar ratio). Inset: Fluorescence photographs of free Rh-DOPC and its mixture with citrate-AuNPs (AuNP:DOPC = 50:1, particle molar ratio) after centrifugation. (C) Quantification of liposomes adsorption of various AuNPs by measuring supernatant fluorescence after centrifugation.

To avoid salt-induced AuNP aggregation, our adsorption experiments were carried out in only 2 mM NaCl. Under this low salt condition, the citrate-capped AuNPs are stable (the first tube, Figure 2A). Interestingly, when DOPC liposomes (non-labeled) were added, the color of AuNPs still changed to purple/blue. The color changed more with a lower liposome concentration (Figure 2A). This color change is attributed to AuNP adsorption on liposome surface, shortening inter-particle distance to enhance surface plasmon coupling.

For quantitative measurement, UV-vis spectroscopy was used. The kinetics of adsorption was monitored using the 600 nm peak (Figure 2E). All the three samples showed a small jump in the first 30 sec. After that, the color change slowed down and higher liposome concentrations produced slower color change. Interestingly, the color change continued even after 1 h for all the samples. Since adsorption of AuNPs is finished in the first minute (Figure 1B), the slow color change is attributed to adsorbed AuNPs merging into larger aggregates by diffusion.

To further study this, cryo-TEM experiments were carried out at three AuNP:DOPC ratios (ratio between the number of AuNPs and the number of liposomes) after overnight incubation (Figure 2C, D). In each case, the liposome shape is maintained without membrane rupture. We did not observe local bending of the lipid membrane as in the case of adsorption of small silica NPs.[16,40] This indicates that the interaction force might be different for AuNPs and small silica NPs when interacting with PC lipids. In addition, AuNPs are not uniformly distributed on liposome surface. Even at 1:1 ratio, AuNPs still formed clusters, consistent with the color change. The aggregates are larger at lower liposome concentration to give more color change. The aggregation can be explained by the strong inter-AuNP van der Waals force (AuNPs are known to aggregate easily).

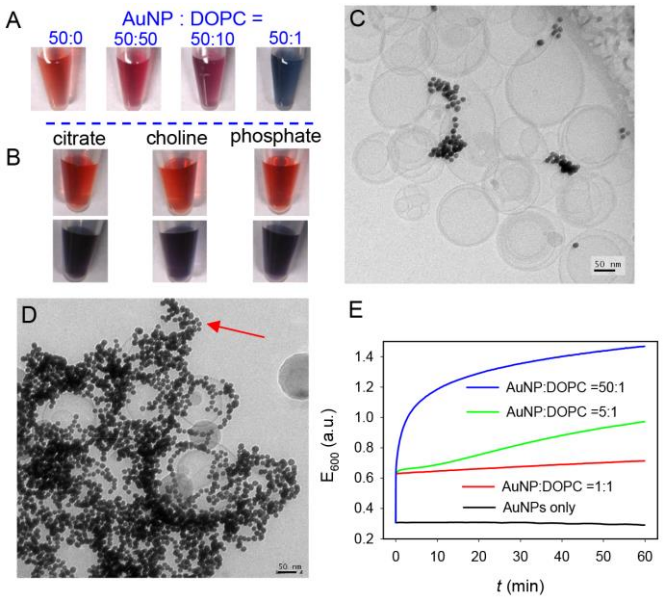

Figure 2. (A) Photograph of citrate-capped AuNPs mixed with DOPC liposomes at various liposome concentrations. AuNPs = 10 nM. (B) Pre-incubation of citrate-capped AuNPs with choline chloride or phosphate (1 mM) (the top row) followed by adding DOPC (the bottom row). All the samples changed color indicating no inhibition of adsorption. Cryo-TEM micrographs of citrate-capped AuNPs mixed with DOPC at 1:1 (C), and 50:1 (D) particle molar ratio. The overall structure of the liposomes are maintained. (E) Kinetics of color change monitored by the absorbance at 600 nm.

Taken the fluorescence, UV-vis and cryo-TEM data together, citrate-AuNPs are quickly adsorbed (within 1 min) by DOPC liposomes. After that, AuNPs undergo aggregation on the liposome surface since they have strong inter-particle van der Waals force. In some cases, AuNP aggregation occurred even outside the liposome template (Figure 2D, by the arrowhead). AuNPs also seem to extend beyond a monolayer on the liposome surface. This may be explained by that once some larger clusters are formed, they can strongly attract individual AuNPs with their collective van der Waals force.

After confirming adsorption and the overall liposome shape, liposome stability was studied next. Calcein dye at a self-quenching concentration (100 mM calcein) was encapsulated into DOPC liposomes. Liposome leakage is thus reflected by fluorescence enhancement. The liposome was dispersed in a buffer containing 100 mM NaCl to balance osmotic pressure cross the liposome membrane. The background fluorescence of this liposome was stable in the absence of AuNPs, while addition of citrate-capped AuNPs resulted in a quick fluorescence enhancement (Figure 3A, black trace). Since the overall liposome integrity is maintained as

indicated by cryo-TEM data, this enhancement is attributed to liposome leakage. We monitored leakage for 20 h and leakage indeed only took place in the first few minutes (Figure S2). The fluorescence signal stabilized after ~2 min. The kinetics of fluorescence increase here is similar to that measured using Rh-DOPC (Figure 1B), but much faster than that of color change (Figure 2E). Therefore, liposome leakage is related only to the initial AuNP adsorption but not subsequent AuNP aggregation. Note this is the only experiment with 100 mM NaCl, and all other experiments were carried out with 2 mM NaCl. To ensure that leakage also takes place at lower salt concentrations, we repeated the measurement as a function of NaCl concentration (Figure S3). Indeed, leakage was observed for all the samples.

Since a fraction of dye is still trapped by the liposomes (Triton X-100 induced further dye release), leakage is only related to local and transient perturbation or defects. Leakage also depends on AuNP concentration; higher concentration AuNPs leaked more (Figure 3B). Adding more AuNPs induced further leakage (Figure 3C). One possibility is that liposomes with AuNPs are fully leaked, and further leakage is from free liposomes that interact with new incoming AuNPs. To eliminate free liposomes, we harvested the AuNP/calcein-DOPC complex by centrifugation. Note that free DOPC liposomes cannot be precipitated at this low centrifugation speed (15,000 rpm). For this complex, a fraction of the dye already leaked. A stable fluorescence was observed until more free AuNPs were added (Figure 3D). These data suggest that leakage is transient and the leakage sites can self-heal.

Interestingly, leakage occurred only with citrate-AuNPs, while no leakage was observed with MPA or GSH-capped AuNPs (Figure 3A, red and green lines), although MPA-capped can also be efficiently adsorbed by DOPC liposomes (Figure 1C). Control experiments indicate that related small molecules (citrate, MPA, GSH) cannot leak the DOPC liposome, and AuNPs have to be involved (Figure S4).

In the cryo-TEM data, we observed AuNP clustering on the liposome surface (after overnight incubation). A question is whether such clustering is required for leakage or not. The time scale of leakage is ~ 1 min based on the data in Figure 3, this is close to the rate of initial AuNP adsorption measured by fluorescence-based assay. On the other hand, AuNP aggregation on liposome surface takes place at a much longer time scale. As shown in Figure 2E, color change continued over 1 h for all the measured ratios (compare this kinetics with that in Figure 3B). Since sufficient amount of calcein is still inside DOPC that can be further leaked by additional AuNPs, aggregation of AuNPs do not cause additional leakage.

The Granick group previously reported stabilization of PC liposomes by adsorbed small latex beads.[13] Protein adsorption was also studied.[41] We studied graphene oxide, nanodiamond, a few metal oxides, and here MPA and GSH-capped AuNPs.[9,12,13] No leakage was observed for any of them (large silica NPs disrupt liposomes via liposome fusion). Therefore, citrate-capped AuNPs are the first negatively charged nanoparticle to leak PC liposomes without undergoing liposome fusion. It was proposed that nanoparticle adsorption can induce a local lipid surface reconstruction, where the underlying fluid lipids turn to gel phase.[14] It is known that without pore formation, a liposome leaks its content the fastest at its T_c, or when there is a fluid/gel phase transition. This transition does not have to take place on the whole liposome, and can happen locally. For example, when we monitored leakage of calcein loaded DPPC liposomes (phase transition temperature, T_c = 41 °C) as a function of temperature, leakage occurred the most at 40 °C (Figure 4D). This is attributed to the rapid conversion of lipid packing between fluid and gel state nearly T_c, thus diminishing membrane integrity. Therefore, we reason that for DOPC, if a well-defined phase transition occurs, leakage can be explained.

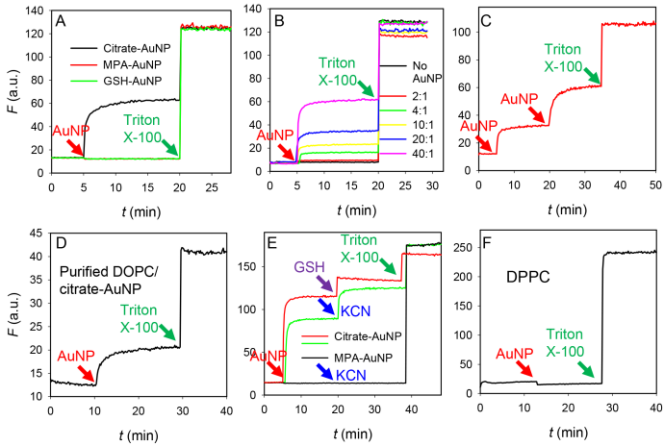

Figure 3. Liposome leakage test. AuNPs are capped by citrate unless otherwise indicated. (A) DOPC leakage by AuNPs with different surface ligands. (B) Effect of AuNP concentration. (C) Leakage induced by adding AuNPs in steps. (D) The initially AuNP/DOPC complex was prepared and centrifuged to remove free liposomes. Adding more AuNPs induced further leakage. (E) 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 was added. (F) No leakage occurs for DPPC liposomes with citrate-AuNPs.

If this hypothesis is true, removing citrate-capped AuNPs should induce a gel-to-fluid transition, which should also cause leakage. To test reversibility, we added KCN to dissolve AuNPs, which indeed leaked citrate-capped but not MPA-capped AuNPs (Figure 3E). KCN itself does not leak the liposome (Figure S4). When GSH was added, an even sharper leakage kinetics were observed. GSH capping also weakens the AuNP/DOPC interaction, leading to AuNP desorption. This capping reaction is faster than KCN-induced AuNP dissolution, explaining the faster kinetics. GSH is an abundant intracellular thiol, and this GSH induced leakage might be useful for controlled release applications. We further hypothesize that no leakage should occur for a liposome that is already in the gel phase. To test it, calcein-loaded gel phase DPPC liposomes (T_c = 41 °C) were used. Indeed, no leakage was detected with citrate-capped AuNPs (Figure 3F).

It has recently been reported that citrate might organize on AuNPs via intramolecular hydrogen bonding and other weak inter-molecular forces.[42] Such a special surface ligand organization might also contribute to leakage. To test this, we also used AuNPs reduced by $NaBH_4$. The size of such AuNPs is ~5 nm. We measured DOPC leakage when mixed by $NaBH_4$ reduced AuNPs and leakage was also observed (Figure S5). Therefore, citrate is not required for leakage

to occur. As long as the surface ligand is weak and can be displaced, AuNPs are likely to cause leakage.

The nanoparticle adsorption induced fluid-to-gel transition was only previously probed indirectly using the Laurdan dye.[14] Herein, we aim to study it directly using differential scanning calorimetry (DSC). DOPC has a T_c of -20 °C, and is thus fluid at room temperature. No phase transition was observed after adding citrate-AuNPs (Figure S6), suggesting the final T_c was still below 8 °C. We then measured free DPPC, showing a T_c of 41 °C as expected (Figure 4B, black trace). Adding 5:1 molar ratio of AuNPs resulted in a slight shift in T_c to 42 °C, while adding a 10:1 ratio shifted T_c to 44 °C with significant peak broadening. Therefore, citrate-capped AuNPs influenced the packing of DPPC lipid to make it in an even stronger gel phase. On the other hand, MPA-capped AuNPs did not change the T_c of DPPC, causing only a moderate peak broadening (Figure 4C, green trace). To explain DOPC leakage using the phase transition theory would imply the T_c needs to be increased from -20 °C to above room temperature. This is clearly not observed by DSC since the T_c measured with DOPC/AuNP is below 8 °C. This is rationalized by the following. The reported T_c is only the peak temperature. By looking at the DPPC data, the transition peak is significantly broadened. Therefore, a small fraction of the lipid (e.g. those directed under AuNPs) might have a much higher transition temperature. In addition, in the time scale of DSC measurement (e.g. 1 °C/min), lipid molecules have sufficient time to diffuse around and the DSC measured T_c should be an averaged value. A small portion of lipids right under the AuNPs should have much higher T_c.

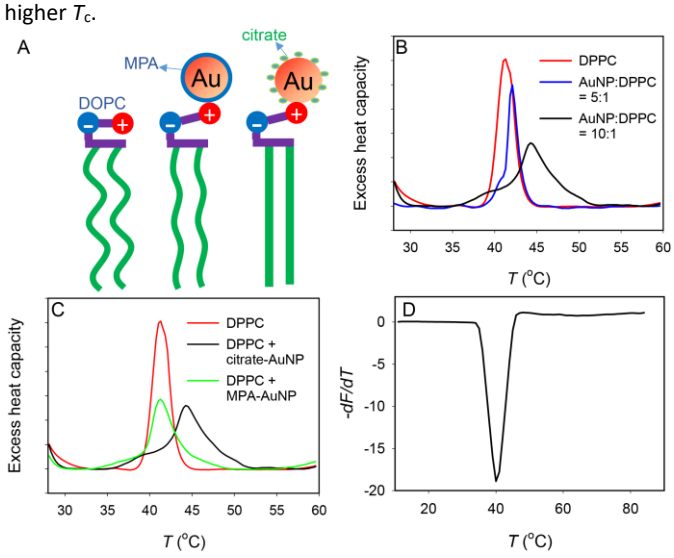

Figure 4. (A) A scheme of AuNP induced PC liposome leakage via phase transition due to a combination of electrostatic interaction and van der Waals force. DSC traces of DPPC as a function of citrate-capped AuNP concentration (B) and as a function of AuNP surface chemistry (C). (D) Calcein loaded DPPC leakage as a function of temperature.

Taken together, there are two steps in the interaction process. Right after adsorption, the lipid undergoes a fluid-to-gel phase transition. Citrate-capped AuNPs leak DOPC liposome due to a strong interaction (so strong to shift the T_c). After finishing this phase transition, the liposome locally turns into the gel phase and stops leaking. The degree of gelation is different with different nanoparticles, and citrate-AuNP is the strongest among all tested. A key question is why citrate-capped AuNPs can interact more strongly with DOPC.

The PC headgroup is comprised a negatively charged phosphate and a positively charged choline, rendering overall charge neutral (Figure 1A). The PC head group is roughly parallel to the liposome surface,[43] occupying more space and making the hydrophobic tails loosely packed. In the presence of negatively charged nanoparticles, the headgroup may tilt to favor interacting via the positively charged choline as proposed by Granick and co-workers (Figure 4A).[14] However, this simple electrostatic model cannot explain the stronger effect of citrate-AuNPs, since all the tested AuNPs have a similar negative zeta-potential (Table S1).

Another possibility is chemical interactions (e.g. chemisorption). The involved chemical groups include citrate, phosphate, choline, and gold surface. If direct chemical interactions exist (e.g. covalent bonding or strong coordination interactions that cannot be quickly displaced), adding these free ions might block adsorption. For example, we previously blocked DOPC adsorption by TiO_2 NPs using free phosphate, suggesting direct chemical interaction between the lipid phosphate and the particle surface.[15] However, adsorption still took place even after pre-incubation of AuNPs with these free groups (1 mM each, higher than the corresponding molecular concentration on each surface, Figure 2B). Therefore, AuNPs do not form stable bonds with each individual groups. It is the collective interaction with the whole lipid bilayer that affords the strong van der Waals force. The leakage from GSH-induced AuNP desorption also argues against a strong covalent bond between gold and lipid, since GSH can quickly displace AuNPs from the liposome.

We reason that in addition to the electrostatic interaction, which may tilt the headgroup to a similar extent, citrate-capped AuNPs may exert stronger van der Waals force to result in a better packing of the lipid tail. First, we can compare adsorption strength. When AuNPs are coated with a small ligand MPA, it can still be effectively adsorbed. Coating with the more bulky GSH decreases adsorption (Figure 1C). Coating with even larger molecules such as DNA completely block AuNP adsorption (Figure S7). These surface ligands separate the AuNP core from the liposome surface, thus decreasing the van der Waals force contribution from the gold core. MPA is almost the shortest thiolated carboxyl molecule that can be put on AuNPs, and its size is only ~0.3 nm. Since even this small separation from the gold surface can make a difference, the interaction causing leakage must be very short ranged, and van der Waals force is a relatively short-ranged interaction. Its strength increases significantly as the distance is shortened. MPA-capped AuNPs already induced broadening of DSC spectrum. We reason that the extra van der Waals force from citrate-AuNPs made the phase transition needed for leakage. In most previous work, only the equilibrium state after adding nanoparticles was studied. We herein have a method to monitor the reaction from the onset of adsorption to the equilibrium state or from equilibrium to desorption.

In summary, our data indicate that the interaction between AuNPs and liposomes can be modulated by surface engineering to modulate surface forces. For example, 'naked' AuNPs transiently and reversibly leak liposomes. Leakage can be blocked by capping

AuNPs with a thiol monolayer. Right at the interface, even a thin molecular gap can make a large difference in interaction force. We probed the initial adsorption/desorption process, complementary to previous work studying the equilibrium state (e.g. measurements were done after mixing NPs with liposomes until signal stabilizes). Finally, this system can be used to create controlled release materials. For example, adsorbed AuNPs stabilize DOPC liposomes. If these AuNPs are displaced from the liposome surface under in vitro or in vivo conditions, liposome content release can be achieved. Knowing that naked AuNPs can be tightly adsorbed, further conjugation of targeting peptides or aptamers on gold is possible, thus creating multifunctional hybrid materials.

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