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Electronic Supplementary Information

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

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Materials and Methods

Chemicals. All the phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Disodium calcein, $HAuCl_4$, glutathione (GSH), 3-mercaptopropionic acid (MPA), Triton X-100 were from Sigma-Aldrich (St Louis, MO). AuNPs (13 nm) were synthesized on the basis of the standard citrate reduction procedures and their concentration was estimated to be ~10 nM. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and NaCl were from Mandel Scientific (Guelph, ON, Canada). DNA (3'-SH-AAAAAAAAAAAAATGCGTAGACACT-5') was purchased from Integrated DNA Technologies (Coralville, IA) and purified by standard desalting. Milli-Q water was used to prepare all the buffers and solution. All other reagents and solvents were of analytical grade and used as received.

Preparation of liposomes. Liposomes were prepared using the standard extrusion with a mini-extruder from Avanti Polar Lipids. DOPC, DOPG, DOPC/DOTAP (1:1, w/w) or DPPC with a total mass of 2.5 mg was respectively dissolved in chloroform. Rh-labeled liposomes were prepared by including 1% (weight fraction) Rh-PE (2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissaminerhodamine B sulfonyl) (ammonium salt) in chloroform. 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) labeled liposomes were prepared by including 1% NBD-PC and 99% (weight fraction) DOPC or other lipids in chloroform. Chloroform was then removed under a gentle N_2 flow in a fume hood and trace amounts of residual chloroform was removed by storing the samples in a vacuum oven overnight at room temperature. The dried lipid films were kept under a N_2 environment and then stored at -20 °C prior to use. To prepare liposomes, the lipids except DPPC were hydrated with 0.5 mL buffer A (100 mM NaCl, 10 mM HEPES, pH 7.6) at room temperature with occasional sonication for at least 2 h. Therefore, the concentration of these three lipids were 5 mg mL^{-1}. The resulting cloudy suspension was extruded through two stacked polycarbonate membrane (pore size = 100 nm) for 21 times. After extrusion, the lipid solution appeared to be transparent, indicating downsize of the lipid structures and formation of liposomes. Since DPPC has a phase transition temperature of 41 °C, a 60 °C water bath was needed to hydrate DPPC with same volume of buffer A. Before extrusion, the extruder was also heated to 60 °C. Then the hydrated DPPC lipids were extruded at 60 °C for the whole extrusion process. Other procedures were the same as mentioned above.

Preparation of calcein-loaded liposomes. To encapsulate calcein, the above dried DOPC lipid film was hydrated with 100 mM disodium calcein solution overnight with occasional sonication to disperse the lipid. DOPC liposomes were extruded using 100 nm pore sized membrane for 21 times at room temperature. In the case of DPPC liposomes, the hydration and extrusion temperature was 65 °C, which

was above the T_m (approximately 41°C). Free calcein was removed by passing the samples (30 μL) through a Pd-10 column using buffer A for elution, where the first 600 μL of the fluorescent fraction was collected.

Preparation of AuNPs. Citrate-capped 13 nm gold nanoparticles were prepared by chemical reduction of $HAuCl_4$ using citrate as the reducing agent and stabilizer.[S1] $HAuCl_4$ (100 mL, 1 mM) was added to an aqua regia cleaned three-neck flask. When the solution began to reflux, 10 mL of 38.8 mM sodium citrate was added. The color changed from pale yellow to deep red and the system was allowed to reflux for another 15 min before heating was turned off. The diameter of AuNPs is ~13 nm. The concentration of the as-synthesized AuNPs is ~10 nM. AuNPs reduced by NaBH4 are prepared using our previously reported protocol.[S2]

Adsorption of AuNPs. To understand the adsorption of the citrate-capped AuNPs to zwitterionic DOPC, 2 μL Rh-labeled DOPC (10 nM, 1.08 mg mL^{-1}) was added to 100 μL of AuNPs (10 nM) and allowed for 15 min incubation. After centrifugation at 15,000 rpm to precipitate DOPC/AuNP conjugates, the supernatant fluorescence of the samples was photographed with a handheld UV lamp in a dark room using a digital camera (Canon PowerShot SD 1200 IS). Note that free liposomes cannot be precipitated at this centrifugation speed. We also tested the adsorption of the citrate-capped AuNPs to zwitterionic DOPC using a Varian Eclipse fluorometer. The fluorescence intensity of 600 μL Rh-labeled DOPC (0.1 nM) was monitored for ~5 min in a quartz cuvette before 6 μL AuNPs (100 nM) were added. The fluorescence intensity was monitored for 1 h to test adsorption of the citrate-capped AuNPs by Rh-DOPC. In order to avoid potential artifacts caused by the Rh dye, AuNPs were mixed with non-fluorophore labeled DOPC in the same conditions as mentioned above. In this case, adsorption is reflected from the color change of AuNPs. To study the adsorption efficiency of various charged liposomes by different kinds of AuNPs, NBD-PC labeled liposomes were also employed to avoid direct dye contact with AuNPs surface. NBD-DOPC, NBD-DOPG, NBD-DOTAP liposomes (2 μL, 10 nM, 1.08 mg mL^{-1}) were added to 100 μL citrate, MPA or GSH capped AuNPs (10 nM). After 15 min incubation, the mixtures were centrifuged at 15,000 rpm for 10 min. 10 μL supernatant was mixed with 90 μL HEPES (5 mM) and the fluorescence of these supernatant was monitored using the microplate reader by exciting at 485 nm (Infinite F200 Pro, Tecan).

Adsorption kinetics. To investigate concentration dependent relationship between liposome and AuNPs, we fixed the concentration of AuNPs at 5 nM and added different amounts of DOPC to reach the molar ratio between AuNPs and DOPC to be 1, 5 and 50, respectively (particle number ratio). The

color of the samples was photographed and UV-vis measurement was also carried out for up to 60 min on an Agilent 8453A instrument. Also citrate-capped AuNPs with 100 mM NaCl was used as a control.

Liposome integrity test and self-healing. To test liposome leakage induced by AuNPs, 5 μL Pd-10 column purified calcein-loaded DOPC liposome (~0.3 nM) were added to 595 μL buffer A in a quartz cuvette. The fluorescence intensity was monitored for ~5 min before 6 μL citrate-AuNPs, MPA-AuNPs or GSH-AuNPs (10 nM) were added. The fluorescence intensity was monitored for another 15 min before 10 μL 5% Triton X-100 was added. Calcein was excited at 485 nm and the emission fluorescence was monitored at 525 nm using a Varian Eclipse fluorometer. To test the effect of AuNP concentration, 6 μL different concentrations of AuNPs (0.5, 1, 2.5, 5, 10 nM) were added to the calcein-loaded DOPC liposome. To test self-healing property, two doses of 6 μL AuNPs (10 nM) were added. In another experiment, 20 μL of calcein-loaded DOPC liposome (~0.3 nM) was mixed with 20 μL AuNPs (5 nM). After incubating for 10 min, the sample was centrifuged at 10,000 rpm to precipitate liposome/AuNPs. The precipitation was dispersed in 20 μL Milli-Q water again. Then 10 μL liposome/AuNPs solution was added to 590 μL buffer A in a quartz cuvette. The fluorescence intensity was monitored for ~10 min before 6 μL AuNPs (10 nM) were added. To study the effect of dissolving adsorbed AuNPs, 6 μL KCN (1 M) was added to the mixture and fluorescence was monitored for another 20 min before 10 μL 5% Triton X-100 was added. Alternatively, 6 μL GSH (10 mM) was added to remove AuNPs from DOPC. Finally, DPPC was tested in a similar way.

Cryo-TEM. The DOPC/AuNPs (particle molar ratio 1:50) were prepared by adding DOPC (2 μL, 10 nM) to AuNPs (100 μL, 10 nM). The molar concentration of AuNPs and liposomes refer to the particle concentrations in this paper (instead of the atomic or individual lipid concentrations) unless indicated otherwise. Samples at other ratios were prepared in a similar way. The samples were then allowed to sit at room temperature overnight to allow complete adsorption and to reach equilibrium. Cryo-TEM experiment was performed by spotting the DOPC/AuNPs (5 μL) on a carbon-coated copper TEM grid (treated with plasma to ensure surface was hydrophilic) in a humidity controlled chamber (FEI Vitrobot). The humidity was set to be 95 to 100% during this operation. The grid was blotted with two filter papers for 2 sec and quickly plunged into liquid ethane. The sample was then loaded to a liquid N_2 cooled cold stage and loaded into a 200 kV field emission TEM (FEI Tecnai G2 F20). The samples were imaged when the temperature was stabilized at -175 °C.

Surface modification of AuNPs. To prepare surface modified AuNPs, 10 μL MPA (25 mM) or GSH (25 mM) was added to 990 μL AuNPs (10 nM) and incubated overnight. The final concentration of

MPA or GSH was 250 μM. DNA-functionalized AuNPs (13 nm) were synthesized following the literature procedure.[S3]

Phosphate or choline inhibition studies. Phosphate buffer (1 μL, 100 mM, pH 7.6) or choline chloride (1 μL, 100 mM) was added to 100 μL of citrate-capped AuNPs (10 nM). And the final concentration of phosphate buffers, choline chloride or HEPES was 1 mM. Then the mixture was incubated for 10 min. 2 μL of 10 nM DOPC liposomes (1.08 mg mL^{-1}) were added to the mixture and incubated for another 10 min. The samples was photographed using a digital camera.

DSC. To test the phase transition temperature of DOPC/AuNPs, 20 μL DPPC (46 nM, 5 mg mL^{-1}) was mixed with 980 μL 5 or 10 nM AuNPs (molar ratio 1:5 or 1:10) for 60 min. DPPC/MPA-AuNPs (molar ratio 1:10) or free DPPC liposome were also tested. DPPC concentration for each mixture is 1 nM. The phase transition temperature was investigated using a differential scanning calorimeter (MicroCal VP-DSC). For the DSC measurement, the sample above was degassed and introduced into DSC sample cell, while the reference cell was filled with Milli-Q water. The sample solutions were scanned from 25 to 60 °C for DPPC/AuNPs and 4 to 45 °C for DOPC/AuNPs at a heating rate of 1 °C min^{-1}.

Temperature-dependent calcein leakage. Calcein leakage from calcein loaded DPPC was related to the phase transition temperature. 3 μL of purified calcein loaded DPPC was added to 100 μL buffer A. Then 20 μL of the samples were loaded into real-time PCR tubes (replicate of 4) and the temperature was increased by 1 °C from 10 °C to 84 °C using a real-time PCR thermocycler (CFX96, Bio-Rad). The incubation time at each temperature was 20 sec and fluorescence in the FAM channel was read at each temperature. The first derivative of the fluorescence increase curve was plotted.

Liposome characterization. The size of our liposome samples was characterized using DLS (Figure S1). To understand the effect of surface charge, we also measured the ζ-potential of different types of AuNPs and liposomes. AuNPs, MPA-AuNPs, GSH-AuNPs, DNA-AuNPs (5 nM) or liposomes (50 μg mL^{-1}) were used for ζ-potential measurement on a Malvern Zetasizer Nano ZS90 with a He-Ne laser (633 nm) at 90° collecting optics. The data were analyzed by Malvern Dispersion Technology Software 4.20.All the liposomes are around 100 nm, consistent with the membrane pore size used for extrusion. Their surface charge and ζ-potential of all kinds of AuNPs we used were also measured shown in the Table S1. The measurements were carried out in 10 mM HEPES buffer at 25 °C.

Table S1. ζ-potential of the materials used in this work. The experiments were carried out in 10 mM HEPES buffer (pH 7.6) at 25 °C.

	Citrate-AuNPs	MPA-AuNPs	GSH-AuNPs	DNA-AuNPs	DOPC	DOPG	DOTAP	DPPC
ζ-potential (mV)	-35.1	-25.9	-27.5	-47.6	-4.54	-40.2	61.5	-0.723

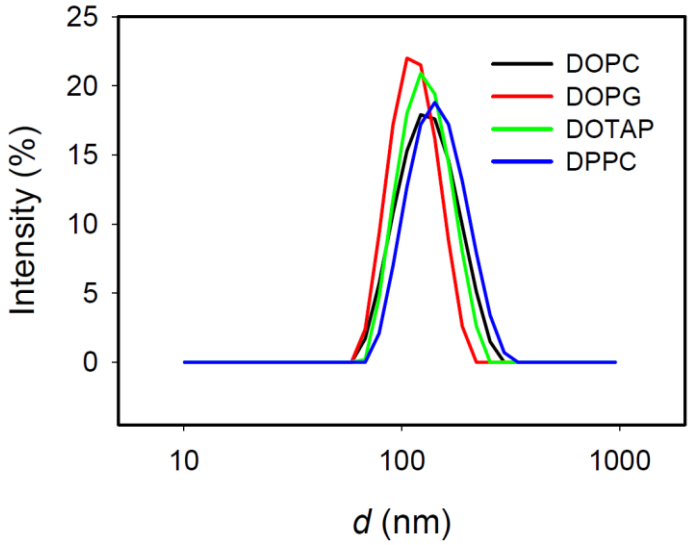

Figure S1. DLS of the four types of liposomes (DOPC, DOPG, DOTAP and DPPC).

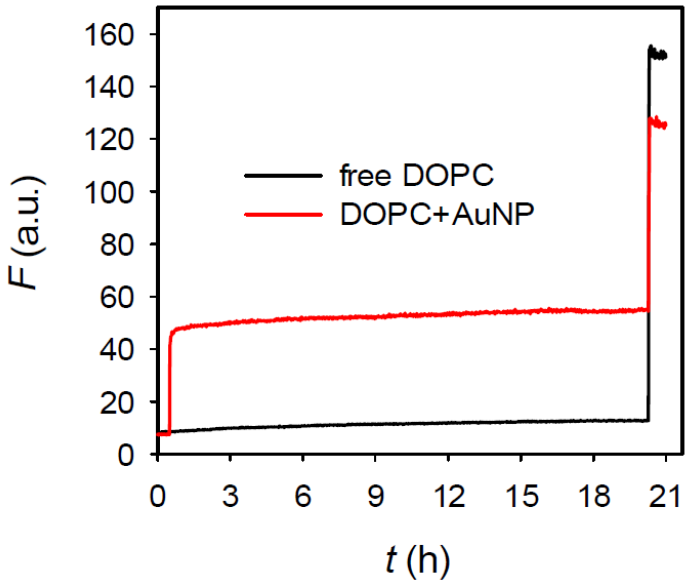

Figure S2. DOPC Leakage by citrate-capped AuNPs monitored over a long time course. AuNP:DOPC =40:1.

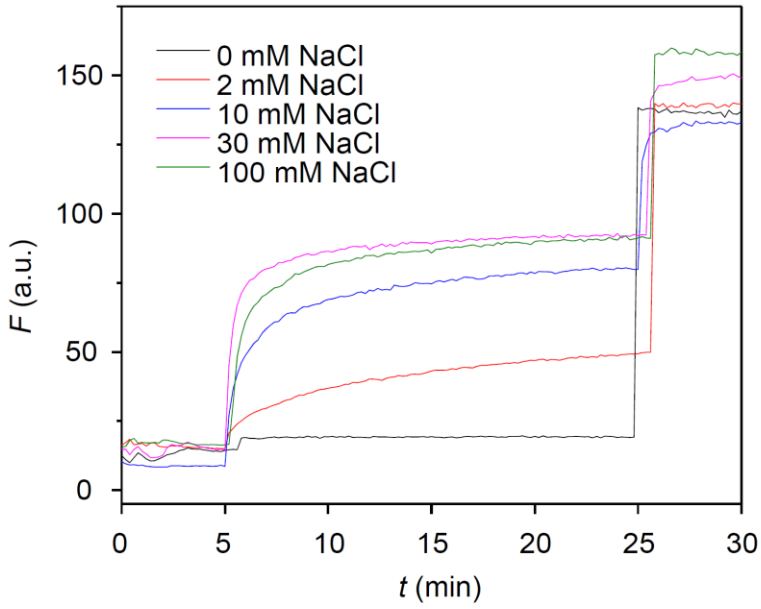

Figure S3. Citrate-capped AuNPs inducing leakage of calcein-loaded DOPC liposomes at different NaCl concentrations. Even 2 mM NaCl can induce liposome leakage. However, when NaCl concentration was zero, no leakage was observed, which might be due to the initial electrostatic repulsion between DOPC and AuNPs. Note that DOPC still carries a slight negative charge.

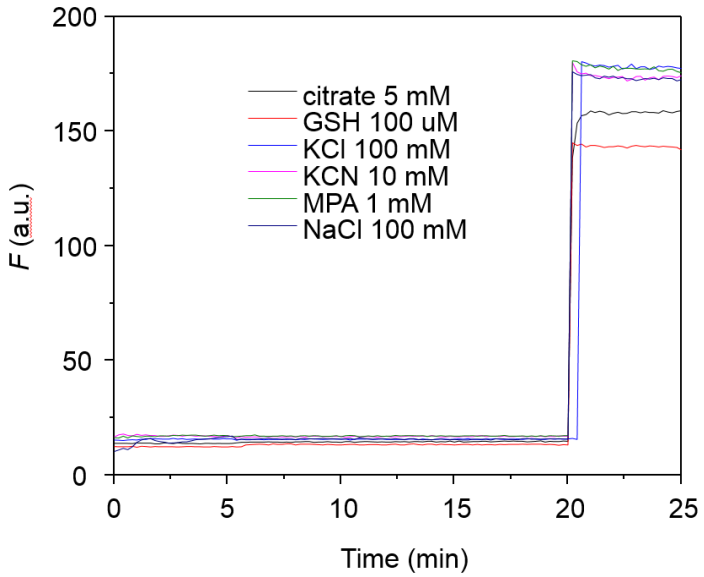

Figure S4. Leakage test of calcein encapsulated DOPC liposome by various chemicals. These chemicals were added at 5min and at 20 min, Triton X-100 was added.

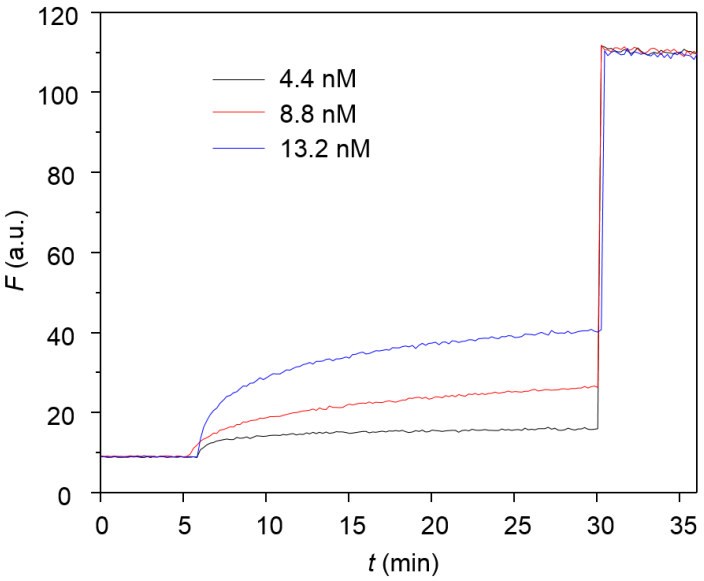

Figure S5. Test of calcein-loaded DOPC liposome leakage by adding AuNPs reduced by $NaBH_4$. 6 µL different concentrations (4.4 nM, 8.8 nM, 13.2 nM) of $NaBH_4$ reduced AuNPs were added to 594 µL liposome solution dispersed in 10 mM HEPES (pH 7.6), 100 mM NaCl. Leakage is observed in a AuNP concentration dependent manner. The slower leakage kinetics might be a reflection of the adsorption kinetics of smaller AuNPs. This study indicates that citrate ligand is not a requirement for AuNP-induced liposome leakage.

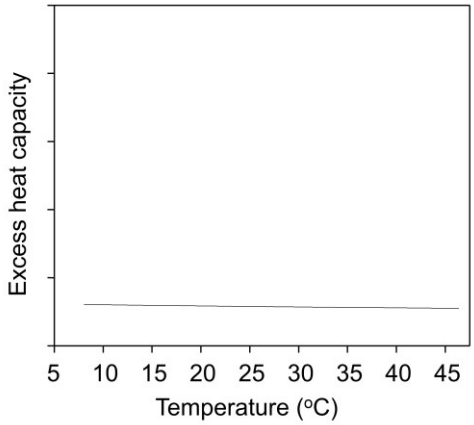

Figure S6. DSC trace of AuNP/DOPC scanned from 4 to 45 °C.

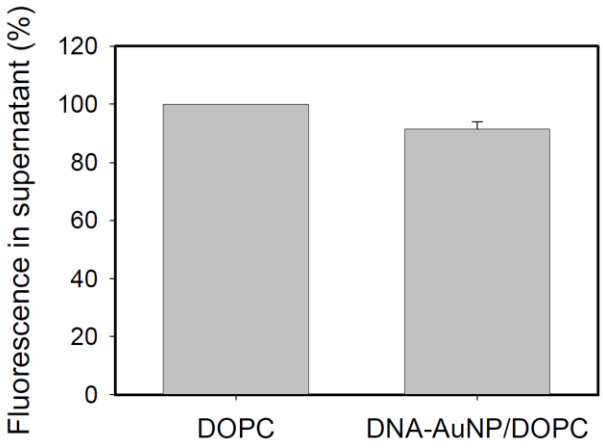

Figure S7. Fluorescence in the supernatant for adsorption of DNA-AuNP/Rh-DOPC. To confirm coating with even larger negatively charged molecules such as DNA can largely block AuNP adsorption, Rh-DOPC liposomes (2 μL, 10 nM, 1.08 mg mL^{-1}) were added to 100 μL DNA capped AuNPs (10 nM). After 15 min incubation, the mixtures were centrifuged at 15,000 rpm for 10 min. 10 μL supernatant was mixed with 90 μL HEPES (5 mM) and the fluorescence of these supernatant was monitored using the microplate reader by exciting at 535 nm (Infinite F200 Pro, Tecan). Over 90% DOPC liposomes were still in the supernatant compared to free NBD-DOPC with the same concentration.

Additional references

S1. J. J. Storhoff, R. Elghanian, R. C. Mucic, C. A. Mirkin and R. L. Letsinger, *J. Am. Chem. Soc.*, 1998, **120**, 1959-1964.

S2. N. J. Lang, B. Liu and J. Liu, *J. Colloid Interf. Sci.*, 2014, **428**, 78-83.

S3. X. Zhang, M. R. Servos and J. Liu, *Langmuir*, 2012, **28**, 3896-3902.