Supporting Information

Porous Nanoparticle Supported Lipid Bilayers (Protocells) as Delivery Vehicles

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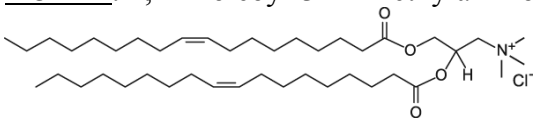
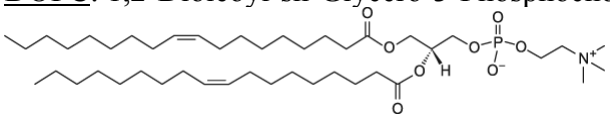
Materials: All phospholipids were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Cholesterol was purchased from Sigma. Texas Red-labeled DHPE lipid and fluorescein isothiocyanate (FITC) were purchased from Invitrogen (Carlsbad, CA). All the silanes and calcein were purchased from Aldrich. Chinese Hamster Ovary (CHO) cells and cell culture related chemicals and media were purchased from American Type Culture Collection (ATCC).

The structures and full names of lipids used in this study are listed below. The structures were adapted from the website of Avanti Polar Lipids Inc. and Invitrogen. All UV-vis absorption data were collected on a Perkin-Elmer spectrophotometer; all fluorescence data were obtained on a Horiba Jobin Yvon Fluoromax-4 fluorometer; and all light scattering data were collected on a Zetasizer Nano dynamic light scattering instrument (Malvern).

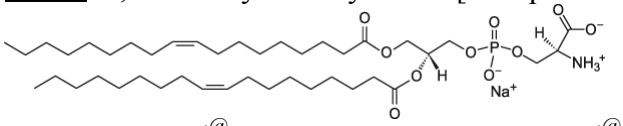
DOTAP: 1,2-Dioleoyl-3-Trimethylammonium-Propane (Chloride Salt)



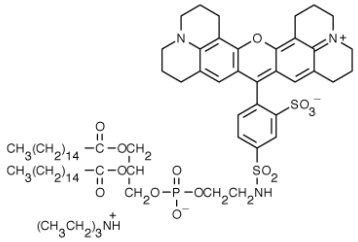
DOPC: 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine



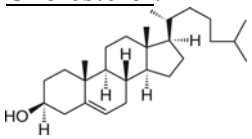
DOPS: 1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine] (Sodium Salt)



Texas Red@DHPE: Texas Red@1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt



Cholesterol:



Preparation of mesoporous silica nanoparticles:
The aerosol-assisted self-assembly method for preparing mesoporous silica nanoparticles has been described in previous publications and will only be briefly mentioned.[1] Silica/surfactant aerosols were generated using a commercial atomizer (Model 9302A, TSI, Inc., St Paul, MN) operated with nitrogen as a carrier/atomization gas.[2] The reaction was started with a homogeneous solution of soluble silica precursor (TEOS), HCl, and surfactant prepared in an ethanol/water solution with an initial surfactant concentration c_0 much less than the critical micelle concentration, c.m.c. The pressure drop at the pinhole was 20 psi. The temperature for the heating zones was kept at 400°C. Particles were collected on a Durapore Membrane Filter maintained at 80°C. Cetyltrimethylamonium bromide (CTAB) was selected as the structure directing template. In a typical synthesis, 55.9 mL H_2O, 43 mL ethanol, 1.10 mL 1N HCl, 4.0g Cetyl trimethylammonium bromide (CTAB), and 10.32g tetraethyl orthosilicate (TEOS) were mixed. To prepare FITC-labeled particles, 18 mg FITC and 100 μL 3-aminopropyltriethoxysilane (APTES) were reacted in 1 mL 200 proof ethanol in dark for four hrs. The reaction equation is presented below. 36 μL of 12N HCl was then added and the whole solution was added to the water/ethanol/HCl/CTAB/TEOS mixture described previously to make FITC-labeled particles.

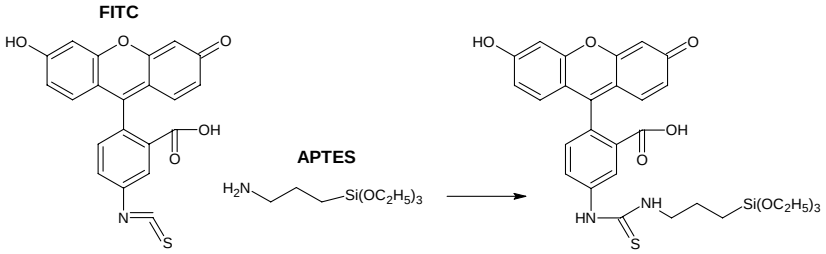


Figure S1. Reaction of FITC with APTES to form silane covalently labeled with fluorescein.

Characterization of mesoporous silica nanoparticles: The nitrogen sorption isotherm of the nanostructured silica particles shown in Figure S2 is a type IV isotherm with a very narrow hysteresis loop, typical of 2D hexagonal MCM-41 like mesoporous silica. The pore diameter was determined to be 1.95 nm with a narrow distribution using the Barrett–Joyner–Halenda (BJH) model (Figure S3). The Brunauer–Emmett–Teller (BET) surface area is 935m^2/g. The pore volume is 0.48 cm^3/g, and the porosity is 0.52.

Preparation of liposomes: Phospholipids were dissolved in chloroform at concentrations of 10–25 mg/mL. Aliquots were dispensed into scintillation vials so that each vial contained 2.5 mg lipids. For mixed lipids, the total amount of lipids was also controlled to be 2.5 mg per vial, and the percentage described in the paper was by mass. Some lipids were mixed with a small fraction (2-5%) of Texas Red-labeled DHPE. The chloroform in the vials was evaporated under a nitrogen flow in a fume hood and lipid films were formed. The vials were then stored in a vacuum oven at room temperature overnight to remove any residual chloroform. The samples were frozen at -20^{o}C before use.

To prepare liposomes, the vials were brought to room temperature and rehydrated by adding 1 mL of 0.5X PBS with occasional shaking for at least 1 hr, forming a cloudy lipid suspension. The suspension was extruded with a mini-extruder purchased from Ananti Polar Lipids. A membrane with pore diameter of 100 nm was used and at least ten extrusion cycles were performed. The resulting clear liposomes were stored in a new vial at 4 ^{o}C. Light scattering experiments showed that the as prepared liposomes have a mean hydrodynamic diameter of ~140 nm and the size distribution did not change after storing at 4 ^{o}C for a week.

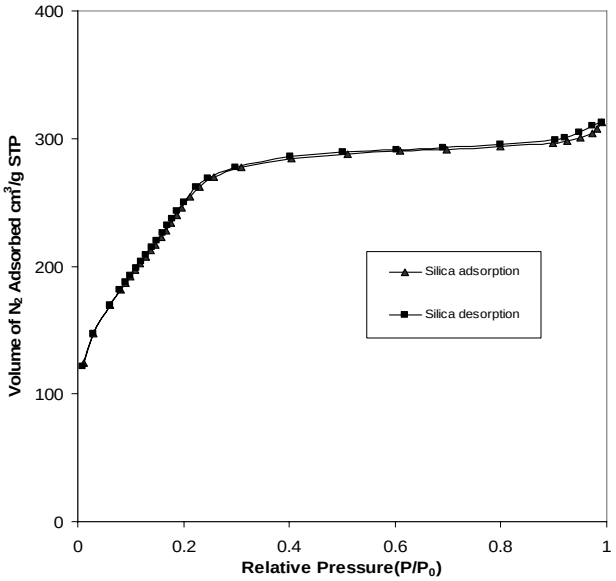


Figure S2. The nitrogen sorption isotherm of the mesoporous silica nanoparticles.

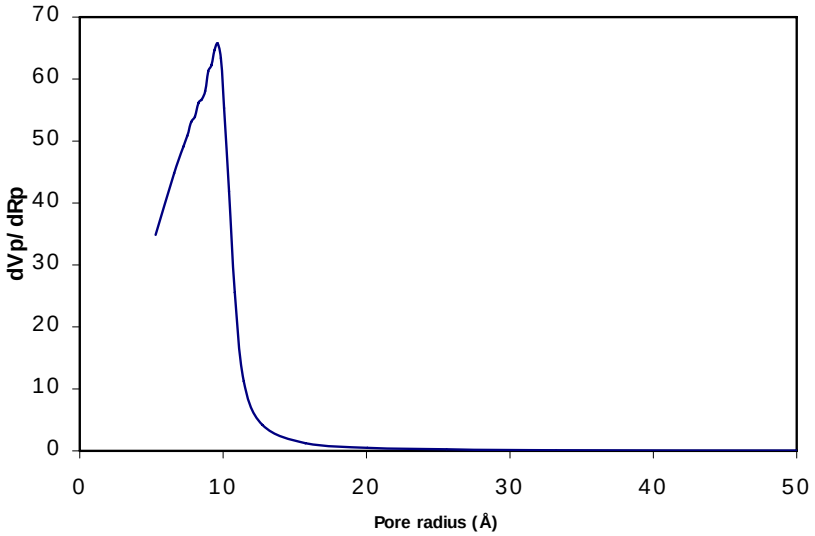


Figure S3. The determination of pore size with the Barrett–Joyner–Halenda (BJH) model.

Preparation of supported bilayers (protocells): The silica nanoparticles were weighed (usually 25 to 50 mg) and transferred into a scintillation vial. 20 mL of 200 proof ethanol with 1% HCl was added and the solution was sonicated for at least 30 min to extract the CTAB surfactant from the pores. The particles were collected by centrifugation and removal of the supernatants. The washing process was repeated twice with ethanol and twice with water. To make the surface more hydrophilic, the particles were then treated with 4% ammonium hydroxide and 4% hydrogen peroxide at 80 ^{o}C for 10 min. After washing with water, the particles were further treated with 0.4 M HCl and 4% hydrogen peroxide at 80 ^{o}C for 10 min and washed with water.[3] The final concentration of silica nanoparticles were made to be 25 mg/mL in water.

Equal volumes (usually 50 μL) of the above prepared silica nanoparticles and liposomes (2.5 mg/mL) were mixed by pipetting the mixture several times. The mixture was allowed to sit at room temperature for 20 min with occasional agitation. Extra lipids were removed by centrifugation of the mixture at 4000 rpm for 1 min and removal of the supernatant. The supported bilayers were subsequently washed with 200 μL of 0.25X PBS and finally dispersed in 200 μL 0.25X PBS. To prepare supported bilayers that encapsulate calcein, the silica nanoparticles were first mixed with 250 μM calcein and liposomes were subsequently added. The remaining procedures were the same. Because DOTAP showed the highest calcein encapsulation efficiency, most supported bilayers were prepared with DOTAP lipids.

Cell culture: Chinese Hamster Ovary cells (CHO) were obtained from the American Type Culture Collection (ATCC) and maintained in K-12 media supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin. The media were changed every two to three days and the cells were passaged by trypsinization. To prepare samples for confocal imaging, round glass cover slips were used for cell growth. The glass slides were treated with 0.1M KOH for at least 24 hrs before use. Cells in the media were dropped onto the cover slips and the slips were kept in Petri dishes. The cells were kept in an incubator at 37 ^{o}C with 5% CO_2 and 95% humidity.

Cell uptake of supported bilayers (protocells): 1 mL of serum free media was warmed to 37 ^{o}C and 10 μL of the above prepared supported bilayers were added and vortexed. To study the uptake of supported bilayers by CHO cells, the cells were grown to ~70% confluence. The old media was removed and fresh media with supported bilayers were introduced. The cells were incubated for four hrs at 37 ^{o}C and free particles were washed away with PBS and media before imaging.

Test the effect of supported bilayers on CHO cell viability: CHO cells were incubated with supported bilayers as described above. The media was removed and 300 μL viability dyes (0.5 μL calcein-AM and 2 μL ethidium homodimer dissolved in 2 mL serum free media) were added. The cells were incubated at 37 ^{o}C for 30 min. Fluorescence under an inverted fluorescence microscope was monitored. Viability assays indicated that >97% of the cells were viable.

Confocal fluorescence microscopy: A Bio-Rad Radiance 2100 confocal fluorescence microscope system was used for imaging cells. Argon 488 nm line was used for imaging FITC and calcein; green HeNe (543 nm) was used for imaging Texas Red; and Red Diode (633 nm) –was used for DIC imaging. All images were collected with a 60X oil immersion objective. To image supported lipid bilayers, 3 μL of dilute protocells were spotted on a glass slide and sealed with a cover slip by super glue.

Quantification of calcein encapsulated by different lipids: Mesoporous silica nanoparticles (50 mg, no FITC modification) were dispersed in 2 mL water. 5 μL of 100 mM calcein was added and so the final dye concentration was ~250 μM. The solution was divided into 50 μL aliquots and equal volumes of liposomes of different compositions were mixed to form supported bilayers. The supported bilayers were centrifuged and washed three times with 200 μL of 0.25X PBS to remove free calcein. Finally 50 μL of 1% SDS was added to the precipitated supported bilayers to disrupt the membrane and release the calcein dye. 150 μL of 0.25X PBS was then added to make the final volume to be 200 μL and the tubes were centrifuged at 15000 rpm for 2 min to precipitate silica nanoparticles. 10 μL from the supernatant was transferred into a quartz microcuvette with a path length of 1 cm to measure the absorbance at 500 nm, which should be proportional to the amount of calcein dye retained in the mesoporous silica nanoparticle.

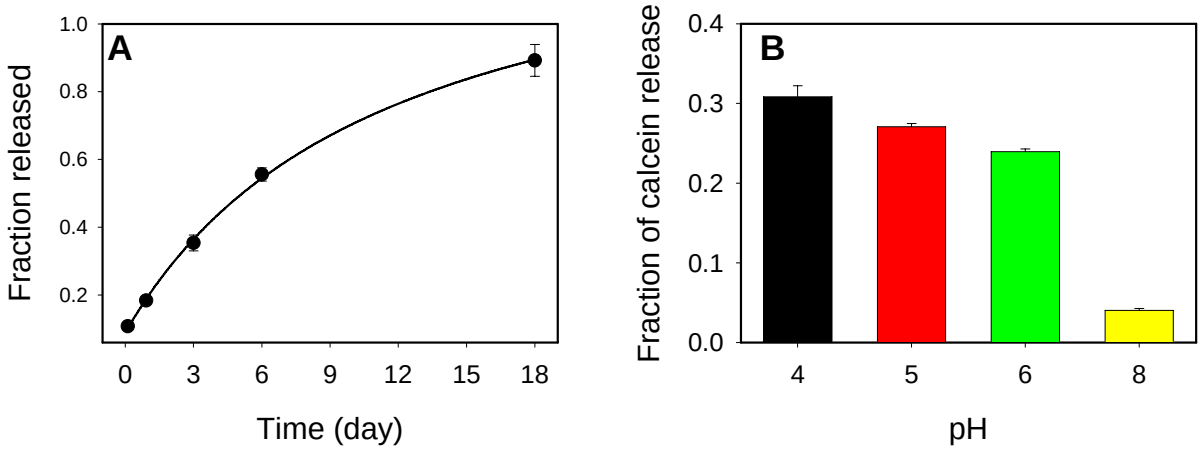


Figure S4. Release profile of DOTAP lipid and mesoporous silica nanoparticle encapsulated calcein as a function of time (A) and as a function of pH (B).

Quantification of calcein release profile: With the method described above, 200 μL of supported lipid bilayers (DOTAP lipids) with calcein encapsulated inside were prepared. At designated time points, 20 μL aliquots were taken out into another tube and centrifuged at 15000 rpm for 2 min. 10 μL of the supernatant was taken out and transferred into another tube and its fluorescence intensity is denoted to be F_1. The fluorescence intensity of the remaining 10 μL is denoted to be F_2, which includes the other 10 μL of the supernatant and the silica precipitant. The fraction of release was calculated to be $2\times F_1/(F_1+F_2)$. To measure fluorescence, the dye was released by using 20 μL of 2.5% SDS and the solution was finally dispersed in 500 μL PBS and centrifuged at 15000 rpm for 5 min to precipitate all the silica nanoparticles. 400 μL of the supernatant was transferred into a fluorescence cuvette and the calcein fluorescence was measured by exciting at 467 nm and collecting emission at 517 nm. All experiments were run in triplicate.

As can be observed from Figure S4A, ~90% of the calcein dye was released in 18 days and the rate of releasing gradually decreased with time. A pH-dependent study was also performed and the release was measured after 12 hrs (Figure S4B). The fraction of release significantly increased at lower pH.

Estimation of the concentration of calcein inside mesoporous silica nanoparticles: When 100% DOTAP lipids were used to form supported bilayers, the retained calcein has an absorbance of 2.5 (Figure 2A), which corresponded to a concentration of 45 μM (the extinction coefficient of calcein is ~55,000 $M^{-1}cm^{-1}$ at 500 nm). Because the volume of this final solution was 200 μL, the retained calcein in silica was 9 nmol. The silica mass was 1.25 mg (50 μL of 25 mg/mL). The density of mesoporous silica nanoparticles was estimated to be 1.07 g/cm^3. Therefore, the volume of the silica was 1.17×10^{-3} cm^3, and the concentration of calcein inside silica was 7.7 mM.

The initial calcein concentration in solution was 250 μM and the final concentration was 70 μM, with the remaining calcein being inside the silica nanoparticles. Therefore, ~72% of the dye was encapsulated in the particles, and the concentration inside silica was ~110 times higher than that in solution.

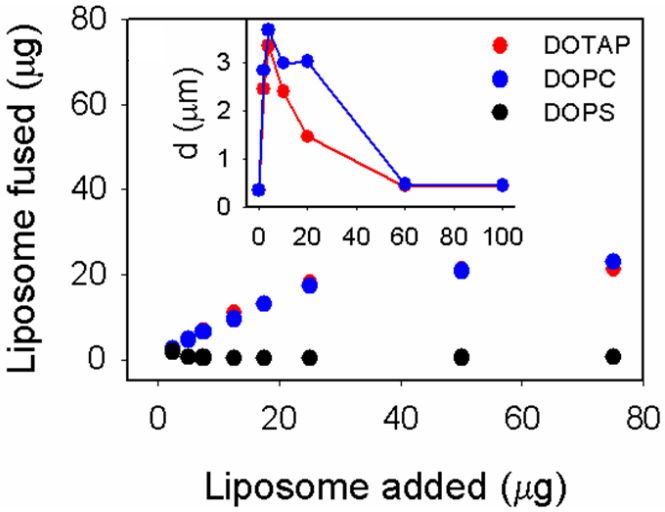


Figure S5. Lipid association with silica nanoparticles as a function of added liposome amount. Three kinds of liposomes were tested and each contained 5% Texas Red label. Inset: aggregation induced by low concentration of liposomes characterized by light scattering.

Lipid association with mesoporous silica nanoparticles: To measure the amount of lipid associated with silica nanoparticles as a function of lipid concentration, 20 μL aliquots of 25 mg/mL silica nanoparticles were mixed with 1, 2, 3, 5, 7, 10, 20, and 30 μL of 2.5 mg/mL lipids. The lipids tested included DOPC, DOPS and DOTAP, all containing 5% DHPE-Texas Red labels. The mixtures were centrifuged and the Texas Red absorbance from the supernatant and the silica nanoparticles was measured. As can be observed from Figure S5, positively charged DOTAP and neutral DOPC liposomes almost quantitatively associated with silica nanoparticles when <20 μg of liposome was used for 0.5 mg silica particles, suggesting high binding affinity. Further addition of liposomes did not increase association, possibly due to the saturation of the silica surface. Negatively charged DOPS did not associate with silica, which could be attributed to the electrostatic repulsion between them at neutral pH.

Colloidal stability of the silica/lipid mixture as a function of lipid concentration: We found that depending on the relative amount of liposome added, silica particles first aggregated at low lipid concentrations to form large aggregates, which disappeared upon adding more liposomes. We

characterized this process by dynamic light scattering as shown in the inset of Figure S5. For both DOTAP and DOPC, there is a significant increase in the average size of particles at low liposome contents. Similar observations were also reported for polystyrene beads, where aggregation was attributed to liposome mediated nanoparticle assembly at low lipid concentrations.[4] Therefore, to form supported bilayers with good colloidal stability, excess amount of liposomes (50 μg liposome per 0.5 mg silica) were used.

Additional References:

1. Lu, Y. F.; Fan, H. Y.; Stump, A.; Ward, T. L.; Rieker, T.; Brinker, C. J., *Nature* **1999,** 398, 223-226.
2. Jiang, X. M.; Brinker, C. J., *J. Am. Chem. Soc.* **2006,** 128, 4512-4513.
3. Davis, R. W.; Flores, A.; Barrick, T. A.; Cox, J. M.; Brozik, S. M.; Lopez, G. P.; Brozik, J. A., *Langmuir* **2007,** 23, 3864-3872.
4. Troutier, A. L.; Delair, T.; Pichot, C.; Ladaviere, C., *Langmuir* **2005,** 21, 1305-1313.