# Supporting Information

## Electrostatically Mediated Liposome Fusion and Lipid Exchange with a Nanoparticle Supported Bilayer for Control of Surface Charge, Drug Containment, and Delivery

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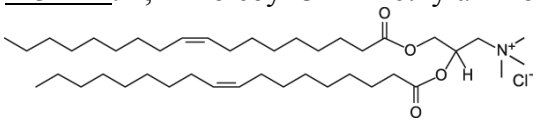
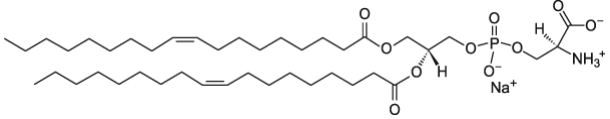
**Materials**: All phospholipids were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Hoechst 33342, FM 1-43FX membrane probe, ProLong Gold mounting agent, mammalian cell viability assay kit, Alexa fluor 647 succinimidyl ester, and fluorescein isothiocyanate (FITC) were purchased from Invitrogen (Carlsbad, CA). Doxorubicin, all the silanes, CTAB (Cetyl trimethylammonium bromide), ammonium molybdate, 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. All fluorescence data were obtained on a Horiba Jobin Yvon Fluoromax-4 fluorometer; and all light scattering and zeta potential data were collected on a Zetasizer Nano dynamic light scattering instrument (Malvern). All epifluorescence images were acquired on a Nikon Eclipse Ti inverted fluorescence microscope with a 40X objective and a Coolsnap HQ2 CCD camera (Roper Scientific). Confocal fluorescence images for mesoporous silica microparticles (~15 μm) were acquired on a Zeiss LSM 510-META system mounted on an inverted (Axiovert 100) microscope with a 63X oil immersion objective. A Bio-Rad Radiance 2100 confocal fluorescence microscope system was used for imaging cells. All TEM images were acquired on a JEOL 2010 200 kV high resolution transmission electron microscope.

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



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



**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. CTAB was selected as the structure directing template. In a typical synthesis of anionic silica nanoparticles, 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 cationic mesoporous silica nanoparticles, 9.36g TEOS and 1.33g 3-[2-(2-aminoethylamino)ethylamino]propyltrimethoxysilane (AEPTMS) were used and the pH of the system was adjusted to 2.0 by concentrated HCl. Other procedures were the same.

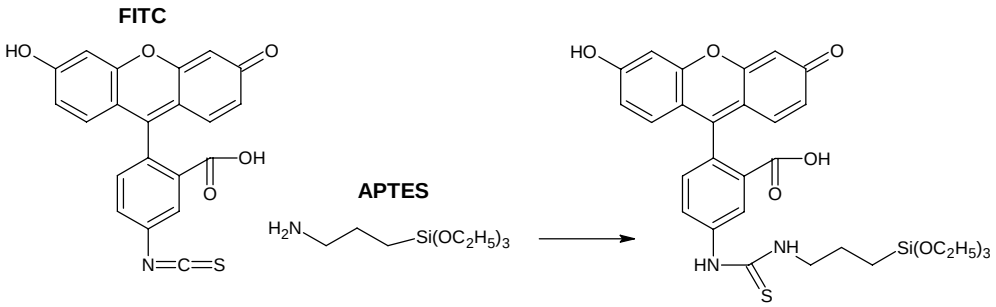



**Figure S1**. Reaction of FITC with APTES to form silane covalently labeled with fluorescein.

To prepare FITC-labeled cationic silica nanoparticles, 18 mg FITC and 10 μL 3-aminopropyltriethoxysilane (APTES) were reacted in 1 mL 200 proof ethanol in dark for four hrs. The reaction equation is presented below. HCl was then added to adjust the pH to 2.0 and the whole solution was added to the water/ethanol/HCl/CTAB/TEOS/AEPTMS mixture described previously to make FITC-labeled particles. To remove the CTAB template, the particles (typically about 500 mg) were dispersed in 100 mL 200 proof ethanol with 1% HCl and refluxed at 70 °C overnight. The particles were collected by centrifugation and washed twice in ethanol and dried in a vacuum oven at room temperature. To prepare Alexa fluor 647-labeled cationic silica nanoparticles, 1 mg amine reactive Alexa fluor 647 succinimidyl ester was used to react with APTES and the remaining procedures were the same.

For the aerosol assisted self-assembly process[1] used to prepare pure and amine- or fluorophore-modified mesoporous silica nanoparticles, the residence time in the heating zone of the tubular aerosol reactor is only several seconds. During this short time the heat transfer to the particles is insufficient to pyrolyze/oxidize any added organic groups, and the amine and fluorophore moieties are fully retained in the product nanoparticles.

**Characterization of mesoporous silica nanoparticles**: The full characterization of anionic silica nanoparticles has been reported in a previous publication and will not be repeated here.[3] The nitrogen sorption isotherm of the cationic silica particles shown in Figure S2A 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 2.25 nm with a narrow distribution using the Barrett–Joyner–Halenda (BJH) model (Figure S2B). The Brunauer–Emmett–Teller (BET) surface area is 709$m^2$/g. A TEM image of the cationic mesoporous silica nanoparticles are shown in Figure S3A and mesopores structures can be clearly observed. The size distribution was measured by dynamic light scattering in water at a concentration of 0.5 mg/mL at 25 °C, and the distribution histogram is presented in Figure S3B. Majority of the particles were in the size range between 50 to 300 nm, and a fraction of larger particles

were also present. Fusion with DOPS liposome to form protocells did not change the size distribution significantly (Figure S3C), suggesting the colloidal stability of the system was still maintained. Since the particle size distribution is relatively wide, dynamic light scattering cannot provide the resolution of several nanometers needed to resolve the lipid layer, and the presence of lipid bilayer was therefore established by TEM in the paper. It needs to be pointed out here that mixing oppositely charged liposomes and protocells does not result in precipitation. With an excess of liposomes, protocells can be easily dispersed in buffer.

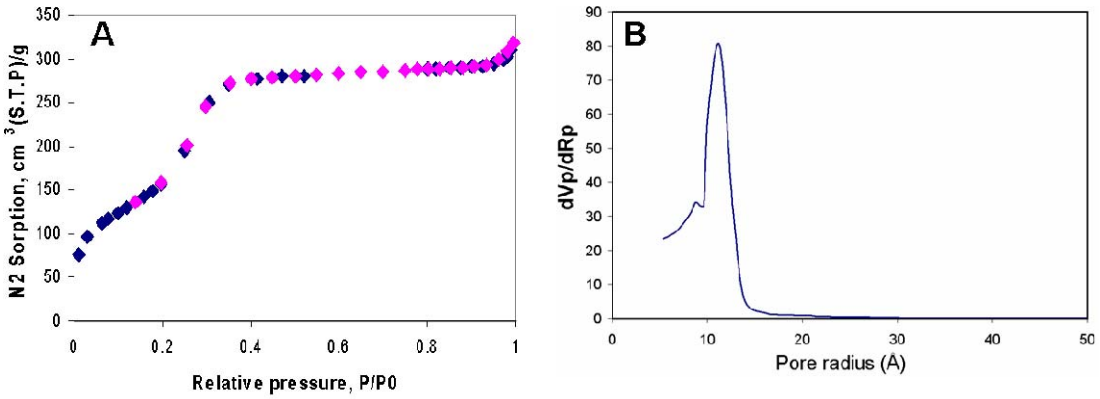



**Figure S2.** (A) The nitrogen sorption isotherm of the cationic mesoporous silica nanoparticles. Blue dots = silica adsorption; red dots: silica desorption. (B) The determination of mesoporous silica nanoparticle pore size with the Barrett–Joyner–Halenda (BJH) model.

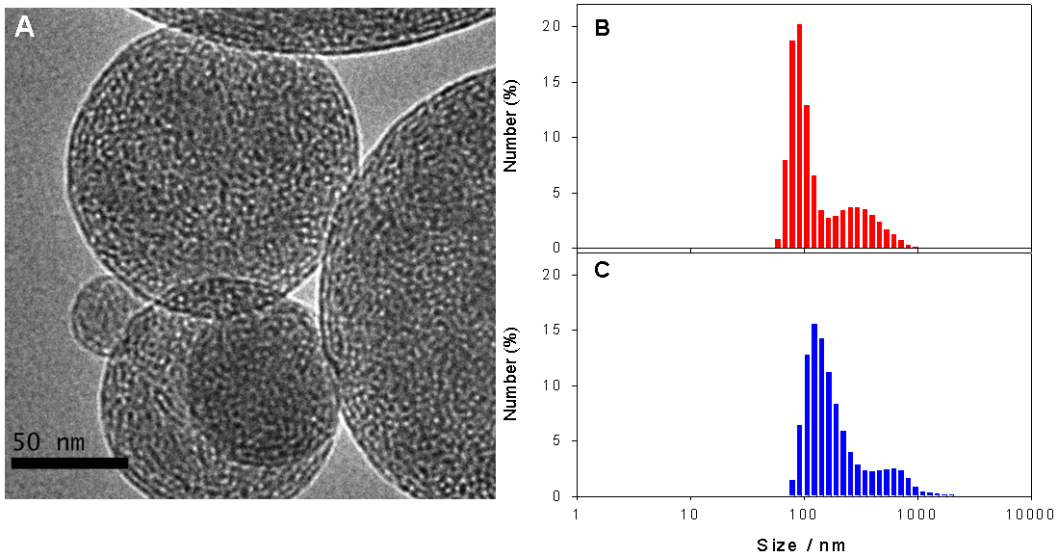



**Figure S3**. (A) TEM image of cationic mesoporous silica nanoparticles. Size distribution of cationic mesoporous silica nanoparticles (B) and after fusion with DOPS liposome (C) studied by dynamic light scattering.

**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. Some lipids were mixed with a small fraction (2 %) of Texas Red-labeled DHPE or 2% 1-Palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-Glycero-3-Phosphocholine. 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 buffer A (10 mM MOPS, pH 7.0, 60 mM NaCl) 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 following standard protocols. 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.

**Preparation of calcein loaded supported lipid bilayers and subsequent liposome mixing**: For calcein delivery, the CTAB extracted cationic silica nanoparticles were dispersed in water (25 mg/mL) containing 1 mM calcein and the calcein molecules were completely adsorbed by the silica particles (<0.1% free calcein left in the supernatant). The loading of calcein was calculated to be 2.5% by weight relative to silica. If saturating concentration of calcein was used, the loading can reach 24.2%. 10 μL of such solution was transferred to a microcentrifuge tube, and 30 μL of 2.5 mg/mL DOPS liposome was added and the two solutions were mixed by manual pipetting. The mixture was allowed to sit at room temperature for at least 45 min and agitated by pipetting occasionally. Finally, the mixture was centrifuged at 4000 rpm for 2 min. The supernatant was removed and the mixture was washed by buffer A and centrifuged again. After removing the supernatant, the mixture was dispersed in 20 μL of buffer A, and silica nanoparticle supported DOPS bilayers were prepared. To mix with additional free DOTAP liposomes, the supported DOPS bilayers were mixed with 30 μL of 2.5 mg/mL DOTAP liposomes and the same incubation, centrifugation, and washing processes were performed. Similar processes were used for additional mixing steps of liposomes. All the particles and supported bilayers were finally dispersed in 50 μL of buffer A before adding to cells (5 mg/mL when calculating silica particles).

**Doxorubicin loading**: Doxorubicin was dissolved at 5 mg/mL in 1:1 water:DMSO solution. In a typical experiment, anionic silica nanoparticles were dispersed in PBS at 25 mg/mL. 50 μL of such silica nanoparticles were diluted with 200 μL of PBS and mixed with 25 μL of the doxorubicin solution for 5 min at room temperature (doxorubicin in excess and the final loading was determined to be 4%). The mixture was centrifuged at 5000 rpm for 3 min. After removal of the supernatant, the precipitants were dispersed in 50 μL of buffer A. 10 μL aliquots were taken out and were either mixed with buffer A or with liposomes. In the current study, three liposome mixing steps were used (DOTAP/DOPS/DOTAP). Finally, each sample was dispersed in 50 μL of buffer A.

**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 medium was changed every two to three days and the cells were passaged by trypsinization. The cells were kept in an incubator at 37 $^{o}$C with 5% $CO_2$ and 95% humidity.

**Cellular uptake of drug loaded silica nanoparticles or protocells:** To study cellular uptake, 5 μL of particles or protocells prepared above containing calcein or doxorubicin (silica amount equals to 25 μg) were mixed with 1 mL of serum free F-12K media. CHO cells were cultured in a 12-well plate and grown to ~70% confluence. The old media was removed and new media containing nanoparticles or protocells were added. The cells were incubated at 37 °C for 4 hrs, washed with PBS and media, and analyzed by fluorescence microscopy or flow cytometry.

**Flow cytometry**: After removal of the media and washed twice with PBS, the above nanoparticle incubated CHO cells were treated with 500 μL of trypsin/EDTA solution (0.25% trypsin, 0.53mM EDTA in Hanks' Balanced Salt Solution (HBSS), modified without calcium chloride or magnesium chloride) at room temperature for ~5 min. The detached cells were then transferred to 500 μL serum containing F-12K media for flow cytometry studies. The flow cytometry measurement was performed on a FACScalibur instrument. The excitation laser was at 488 nm and the green emission of calcein and red emission of doxorubicin were monitored.

**Epifluorescence microscopy**: After the 4 hr incubation with nanoparticles or protocells, 1 μL of 5 mg/mL Hoechst 33342 dye was added to the media and the cells were incubated for another 10 min at 37 °C. The media was removed and the cells were washed twice with PBS and dispersed in 500 μL of fresh F-12K serum free media. The cells were taken to an inverted Nikon Eclipse fluorescence microscope for imaging. A 40x objective was used and fluorescence images with blue (Hoechst 33342), green (for calcein) or red filter cubes (for doxorubicin) were acquired. Transmission images were also taken.

**Confocal fluorescence microscopy for observing silica microparticle supported bilayers**: A Zeiss LSM 510-META confocal system mounted on an inverted (Axiovert 100) microscope was used. Anionic mesoporous silica microparticles prepared by the aerosol-assisted self-assembly method)[4] were first fused with Texas Red-labeled DOTAP and then mixed with NBD-labeled DOPS. Argon 488 nm line was used for imaging the NBD fluorescence; green HeNe (543 nm) was used for imaging Texas Red. In a typical experiment, 3 μL of dilute protocells were spotted on a glass slide and sealed with a cover slip by super glue. The images were collected with a 63X oil immersion objective.

**Uptake of cationic mesoporous silica nanoparticles with covalently labeled FITC**. As described in the paper, there was no observable calcein delivery when calcein was loaded in cationic mesoporous silica nanoparticles. To understand whether the failure of calcein delivery was due to inhibited nanoparticle uptake or other reasons, cationic mesoporous silica nanoparticles containing covalently labeled FITC (25 μg) was incubated with CHO cells in serum free F-12K media at 37 °C for 4 hrs. After washing away free particles the cells were imaged under the inverted fluorescence microscope. Representative images are presented in Figure 2D in the paper, which clearly showed that a significant number of silica nanoparticles were taken by the cells, and the failure of calcein delivery should be due to the displacement of calcein by other molecules/ions into the media.

**Zeta-potential measurement**: A similar process was employed to prepare supported lipid bilayers for zeta-potential measurement (with cationic mesoporous silica nanoparticle core). The difference is that no calcein was used when prepare the cationic silica nanoparticle suspension, and other procedures were the same. In a typical experiment, cationic silica nanoparticles were dispersed in 25 mg/mL with a

volume of 84 μL. 10 μL was taken out for measuring the zeta-potential of cationic silica nanoparticle cores. For the remaining 74 μL, 148 μL 2.5 mg/mL DOPS liposome was added to prepare supported DOPS bilayers (protocells). The mixture was allowed to sit at room temperature for 1 hr and protocells and free DOPS liposomes were separated by centrifugation. The zeta-potential of both the supernatant liposome and the protocells were measured. In the subsequent liposome addition steps, the volume of free liposomes was always kept at twice that of the protocells, and the silica concentration in the protocells was kept at 25 mg/mL. For the titration experiment, 10 μL of 25 mg/mL cationic silica nanoparticles coated with the DOPS liposomes were mixed with varying concentrations of free DOTAP liposomes, and the mixture was centrifuged and washed, the resulting supported bilayer zeta-potential was measured. The particles and protocells were dispersed in 0.6 mL of buffer A (10 mM MOPS, pH 7.0, 60 mM NaCl) and loaded into a disposable cuvette for zeta-potential measurement. The measurements were performed on a Zetasizer Nano dynamic light scattering instrument (Malvern) at 25 °C.

**Steady-state fluorescence measurement**: 25 μL of the above prepared silica particles (5 mg/mL) and supported lipid layers were dispersed into 1 mL of F-12k media at room temperature for 20 min. The media was centrifuged at 8000 rpm for 10 min and 50 μL of the supernatant was mixed with 450 μL of buffer A for fluorescence measurement. Calcein was excited at 480 nm and doxorubicin was excited at 467 nm.

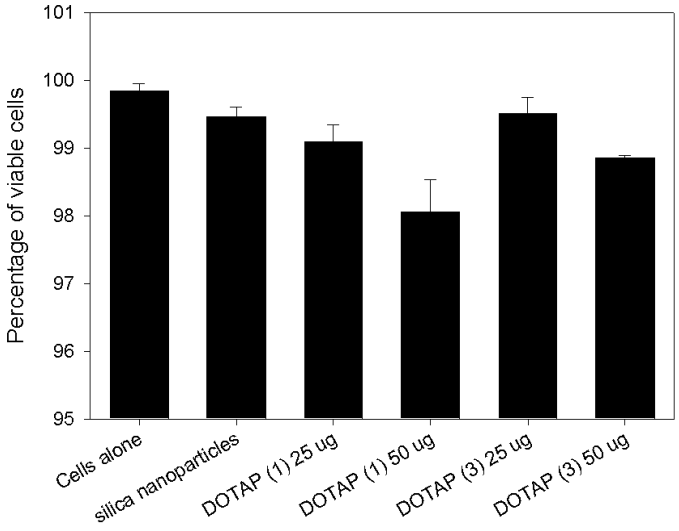



**Figure S4**: Cell viability assay after treating cells with various silica nanoparticles and silica nanoparticle supported lipid bilayers.

**Cell viability assays**: CHO cells were cultured in 12 well plates to ~70% confluence. After exchanged to fresh serum free media, anionic mesoporous silica nanoparticles and protocells with DOTAP or DOTAP/DOPS/DOTAP (25 or 50 μg) were added to the cells. After the cells were incubated with particles for 4 hrs at 37 $^{o}$C, the old media containing particles were removed and the cells were allowed to sit in fresh media for another 3 hrs. Cell viability was assayed with calcein AM and ethidium homodimer (kit purchased from Invitrogen) following the vendor's instruction. Briefly, to 12 mL PBS buffer, 10 μL of 2 mM ethidium homodimer and 2.5 μL of 4 mM were added and mixed. 500 μL of this

working solution was added to each well. After 20 min at room temperature, the cells were washed with PBS and observed with the inverted fluorescence microscope with a 4X objective. In each field of view, there were ~7000 cells. The dead cells (red dots) were counted from three random regions in three independent samples. As plotted in Figure S4, with the dosage used in the experiments (25 μg), cells have ~99% viability. Cells incubated with DOTAP/DOPS/DOTAP protocells (DOTAP (3)) have slightly higher viability compared to those incubated with pure DOTAP protocells (DOTAP (1)). Since DOTAP (3) protocells have lower zeta-potential and contain less positive charges, this experiment suggests that by tuning the surface charge, the toxicity of protocells can be minimized.

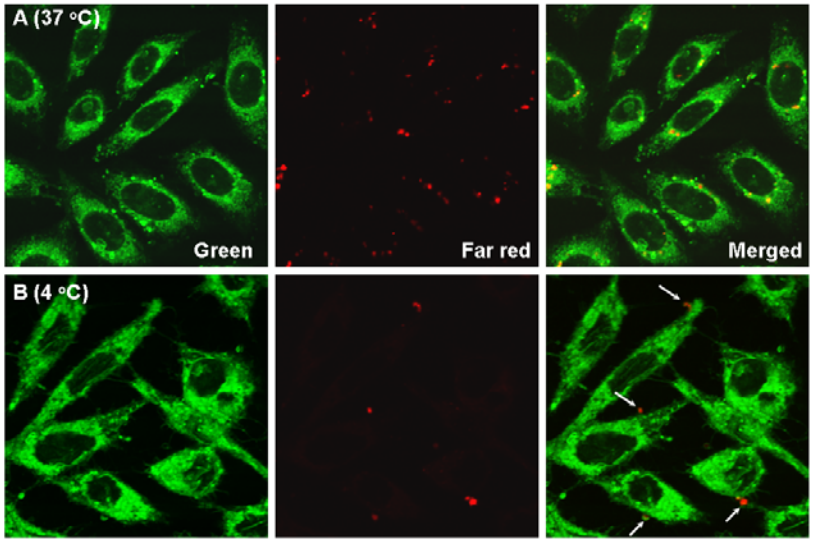



**Figure S5**: Confocal fluorescence microscopy study of temperature-dependent uptake of protocells (cationic silica with DOPS/DOTAP liposomes). The silica nanoparticles were covalently attached with Alexa fluor 647. At 4 $^{o}$C, most red dots (highlighted by white arrows) are close to cell surface instead of inside cells, suggesting internalization of protocells require energy-dependent mechanisms.

**Temperature dependent protocell uptake**: To confirm that protocell uptake by cells is through energy-dependent mechanisms, confocal fluorescence microscopy was used to observe cells incubated with protocells made by Alexa fluor 647-labeled cationic silica nanoparticles mixed with DOPS/DOTAP liposomes. The cells were grown on glass coverslips and were incubated with the protocells either at 4 $^{o}$C or 37 $^{o}$C for 4 hrs. Free protocells were washed away by three washes (five minutes each with shaking) with PBS at 4 $^{o}$C. The cells were stained by FM 1-43FX membrane probe at 4 $^{o}$C following the vendor's protocols, fixed with 3.6% paraformaldehyde at 4 $^{o}$C for 20 min and then at room temperature for 10 min, and mounted with ProLong Gold mounting agent. The samples were observed on a Bio-Rad confocal fluorescence microscopy system. Argon 488 nm line was used for imaging FITC and calcein and Red Diode (633 nm) –was used for imaging Alexa fluor 647-labeled silica nanoparticles. All images were collected with a 40X oil immersion objective. As can be observed from Figure S5, at 37 $^{o}$C, most red dots (Alexa fluor 647) were close to the cell nucleus; while for samples incubated at 4 $^{o}$C, the red colored dots were close to cell surface. This experiment confirmed that protocell uptake is through energy dependent mechanisms, such as endocytosis.

**Calcein displacement by the individual components in the media**: The F-12K media is a complex mixture of inorganic and organic molecules. To understand the exact component(s) in the media that are responsible for the displacement of calcein, several negatively charged molecules with high concentrations are made into individual solutions at concentrations similar or higher than those present in the media. Several other chemicals that are not present in the media were also tested (e.g. citrate, MOPS, and acetate). The particles were mixed with the solutions (pH adjusted to 7.0) for 20 min at room temperature and the fluorescence in the supernatant after centrifugation was measured (Figure S6). Interestingly, small anions with multiple charges (e.g. phosphate, sulfate, citrate, pyruvate, carbonate) and high concentrations of chloride are the most effective in displacing calcein; while larger monovalent anions such as aspartate and glutamate are ineffective in the displacement. Calcein itself carries four negative charges at neutral pH and therefore the adsorption should be relatively strong to cationic silica. It needs to be pointed out that in the final mixture, the calcein concentration after complete release is only 10 μM, while the competing anions are at millimolar levels. If at equal concentration, calcein is still strongly adsorbed.

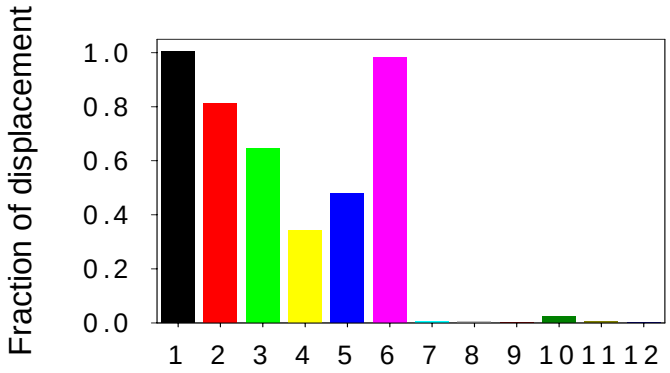



**Figure S6**. Determination of the fraction of calcein displacement from the cationic silica particles (no lipid) by various anions. 1 = 10 mM phosphate, 2 = 20 mM bicarbonate, 3 = 20 mM sulfate, 4 = 20 mM pyruvate, 5 = 150 mM NaCl, 6 = 20 mM citrate, 7 = 20 mM aspartate, 8 = 20 mM glutamate, 9 = 20 mM MOPS, 10 = 50 mM acetate, 11 = 100 uM phenol red, 12 = water.

**TEM of negative stained protocells**. In a typical experiment, CTAB templated anionic mesoporous silica nanoparticles prepared from the aerosol process were dispersed in 0.5X PBS at 25 mg/mL. 10 μL of the nanoparticles were transferred to a small centrifuge tube and 30 μL of 2.5 mg/mL DOTAP liposome was added with manual pipetting for mixing. After allowing the mixture for 1 hr at room temperature, the mixture was centrifuged to remove free liposomes. The resulting protocell was wash once with 0.5X PBS and finally dispersed in 100 μL of the same buffer. 100 μL of 2% osmium tetroxide was added to fix the lipid at room temperature for 20 min, and extra osmium was removed by centrifugation. The samples were again dispersed in 100 μL of 0.5X PBS and 10 μL was taken out and mixed with 100 μL of 1% ammonium molybdate. The mixture was added to a holy carbon coated TEM grid treated with 0.1% bacitracin and the grid was dried by wicking away the liquid by a filter paper. Similar preparation method was used for imaging protocells mixed with multiple liposomes. The TEM image in Figure 1E is obtained from silica nanoparticles mixed with DOTAP and the image from Figure 1F is from silica nanoparticles mixed with DOTAP/DOPS/DOTAP.

**Additional References**: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(4) Rao, G. V. R.; Lopez, G. P.; Bravo, J.; Pham, H.; Datye, A. K.; Xu, H. F.; Ward, T. L., *Adv. Mater.* **2002,** 14, 1301-1304.