Self-assembly of phospholipid-PEG coating on nanoparticles through dual solvent exchange

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SUPPORTING INFORMATION

S1. Stability of DSPE-mPEG coated IONPs with 6.5 nm iron oxide cores

We coated 6.5 nm iron oxide nanocrystals with DSPE-mPEG (DSPE-mPEG:iron = 4:1). Empty micelles were removed from the IONP colloidal solution by three rounds of ultracentrifugation. After that, the solution was sterilized by passing through a 0.2 μm syringe filter and stored at 4°C. The solution remained optically clear for several months. Dynamic light scattering measurements were performed on the same sample at three time points: 0, 3 and 6 months after coating (Figure S1). There was no notable change in the size distribution. It has been reported that DSPE-mPEG2000 formed micelles are metastable.[1] Prolonged storage of the micelle solution (~ 1 week) at 25°C leads to precipitation. Therefore, DSPE-mPEG2000 coated IONPs were more stable than empty micelles, presumably due to a stronger hydrophobic interaction between the surfactants on the crystal surface and the lipid portion of DSPE-mPEG2000.

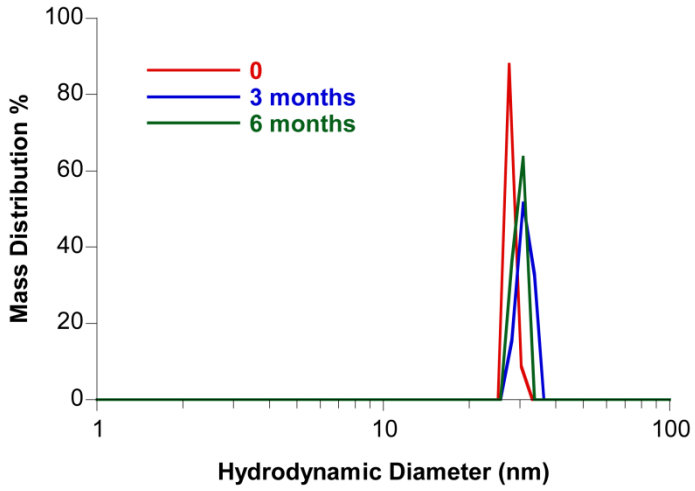

Figure S1. Size distribution of DSPE-mPEG coated IONPs in water. Dynamic light scattering measurements were performed on the sample (IONPs with 6.5 nm iron oxide cores) at three time points after coating.

S2. Absorbance and emission spectra of DSPE-mPEG coated quantum dots

Two CdSe/ZnS quantum dots with nominal emission peaks at 629 nm and 655 nm were coated with DSPE-mPEG (see materials and methods, S4). The absorbance spectra of QD cores in toluene and coated QDs in water were scanned with a UV/Visible spectrophotometer (Ultraspec 2100 pro). The emission spectra were scanned with a microplate reader (Tecan Safire) (Figure S2). The emission spectra were measured with the excitation wave length fixed at 350 nm. There was no significant change in the QD absorbance and emission spectra before and after coating.

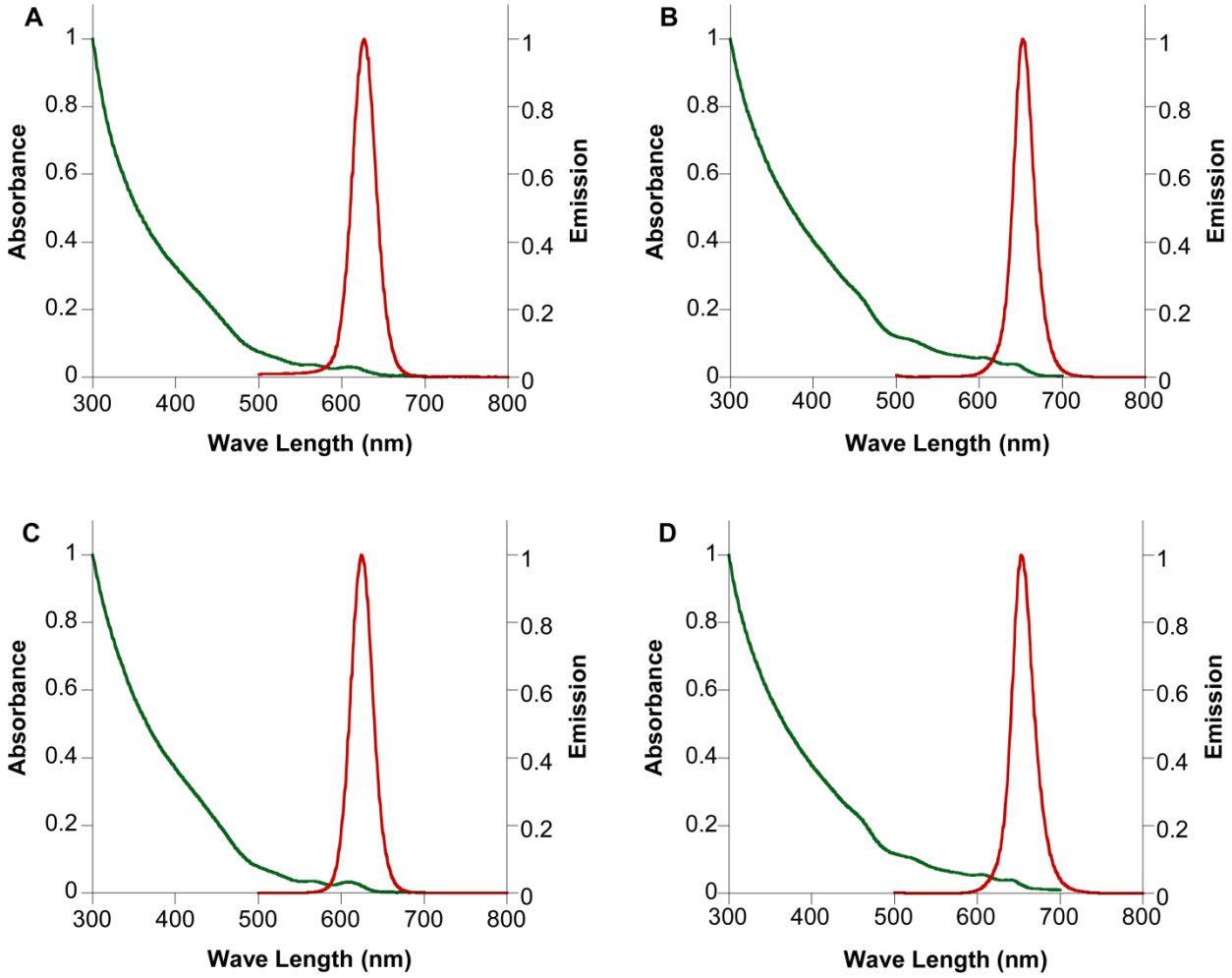

Figure S2. Absorbance and emission spectra of DSPE-mPEG coated quantum dots. (A) QD 629 core in toluene. (B) QD 655 core in toluene. (C) Coated QD 629 in water. (D) Coated QD 655 in water. All curves were normalized with the peak values.

S3. T_2 relaxivity of DSPE-mPEG coated IONPs with different coating densities

We have shown that T_2 relaxivity of superparamagnetic iron oxide nanoparticles decreases when the thickness of PEG coating increases.[2] Here we examined the effect of coating density on T_2 relaxation induced by IONPs. IONPs were synthesized with 6.5 nm iron oxide nanocrystals and DSPE-mPEG using film hydration or solvent exchange method. After large aggregates and empty micelles were removed from the IONPs, all samples were diluted to 20 μg Fe/ml with deionized water. The T_2 measurements were performed with a 0.47 T Bruker Minispec Analyzer MQ20 at 40°C using Hahn spin echo method. The T_2 of IONPs coated with solvent exchange method increased from 7.75 ms to 51.4 ms when the DSPE-mPEG to iron ratio increased from 1:1 to 16:1 (Figure S3). With film hydration method, T_2 of IONPs was not sensitive to the changes in loading ratio. We supplemented DSPE-mPEG to an IONP sample coated with solvent exchange method (1:1 loading ratio) so that the total DSPE-mPEG to iron ratio reached 16:1 in the solution. We found that T_2 of this sample was 7.94 ms, similar to the original sample. Therefore, the change in T_2 was induced by the variation in the coating density of IONPs but not by the free DSPE-mPEG in the solution. The trend in T_2 correlated well with the coating density measurement (Figure 3B in main text). The results confirmed DSPE-mPEG density measurement in Figure 3B.

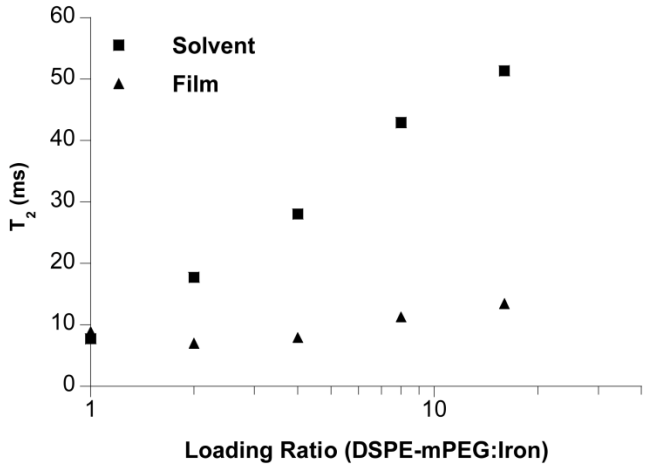

Figure S3. T_2 measurements of DSPE-mPEG coated IONPs coated with solvent exchange or film hydration methods.

S4. Materials and Methods

Materials. Hydrochloric acid, perchloric acid, hydroxylamine HCl, sodium hydroxide, ammonium acetate, fluorescamine and ferrozine were purchased from Sigma-Aldrich. Mouse IgG and horse anti-mouse IgG antibody were purchased from Vector Laboratories. 2-Mercaptoethylamine•HCl was purchased from Thermo Scientific. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium salt), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[carboxyl(polyethylene glycol)-2000] (ammonium salt) and 1,2-distearoyl-*sn*-

glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (ammonium salt) were purchased from Avanti Polar Lipids. For simplicity, the four molecules are referred to as DSPE-mPEG, DSPE-PEG-NH_2, DSPE-PEG-COOH and DSPE-PEG-maleimide, respectively. DSPE-PEG is used for all four molecules when no functional group is specified. Goat anti-human folate receptor-1 antibody and control goat IgG were purchased from R & D systems. HeLa and MDA-MB-435s cells were purchased from ATCC and cultured following the instructions provided by the company.

The iron oxide cores were synthesized according to a published procedure.[3] The iron oxide cores were stabilized by oleic acid and oleylamine in toluene. Mean diameters of the iron oxide nanocrystals, which were 6.5 nm and 17.1 nm respectively, were measured from TEM images with software ImagePro Plus. Triangular CdSe/ZnS quantum dots with trioctylphosphine oxide (TOPO) coating were provided by Dr. Andrew Smith and Dr. Shumin Nie at Emory University. The quantum dots were dispersed in toluene at a concentration of ~15 μM. Rod shaped CdSe/ZnS quantum dots with TOPO coating were purchased from Invitrogen. The quantum dots were dispersed in decane at 1 μM particle concentration. Figure S4 shows the TEM images of uncoated nanocrystals dispersed in toluene.

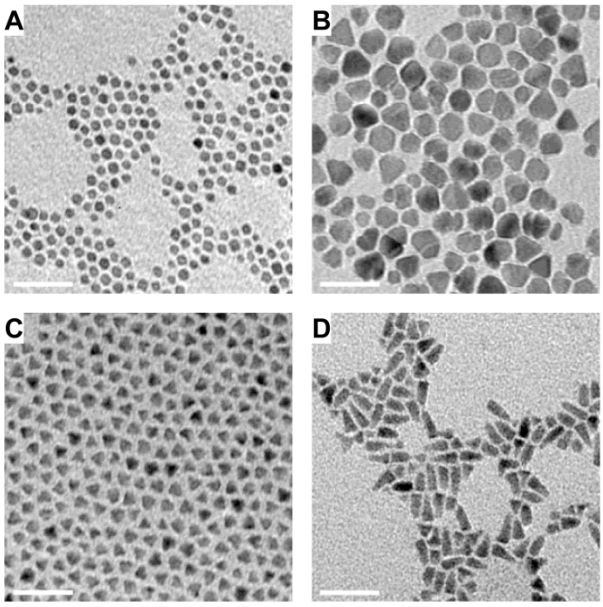

Figure S4. TEM images of nanocrystals dispersed in toluene. (A) 6.5 nm iron oxide nanocrystals. (B) 17 nm iron oxide nanocrystals. (C) QD Type 1: Triangular quantum dots. (D) QD Type 2: Rod-like quantum dots. Scale bar = 40 nm.

Nanoparticle coating with dual solvent exchange method. The iron oxide nanocrystals were initially dispersed in toluene at 5 mg Fe/ml. To coat 1 mg Fe iron oxide nanocrystals, 0.2 ml nanocrystal colloidal solution was mixed with DSPE-PEGs in 0.8 ml chloroform in a round bottom flask. The concentration of DSPE-PEGs in chloroform was determined based on the weight ratio between DSPE-PEG and iron (ranging from 1:1 to 32:1). 4 ml of DMSO was added slowly to the mixture. The mixture was incubated on a shaker at room temperture for 30 minutes. Then chloroform and toluene were removed completely by vaporization under vaccum. After that, deionized water was added to the colloidal solution in DMSO to reach a total volume of 20 ml. DMSO was completely substituted with

deionized water by three rounds of centrifugation in centrifugal filter tubes (Vivaspin 20, 100 KDa cutoff size). The triangular quantum dots were coated using the same procedure.

The quantum dots purchased from Invitrogen were initially dispersed in decane. Decane needs to be removed before addition of DMSO because the two solvents are not miscible. To avoid aggregation, DSPE-PEG chloroform solution was added to the quantum dot. After the solvents were evaporated under a beam of dry nitrogen gas, the powder was placed under vacuum overnight to ensure complete removal of decane. Then the powder was dispersed in chloroform and the solvent exchange procedure was performed as that of iron oxide nanocrystals. The molar concentration of purchased quantum dots was 15 times lower than that of 6.5 nm iron oxide nanocrystals. It is difficult to maintain the same molar ratio during coating. Instead, we kept the concentration of DSPE-PEG the same as that of 2:1 coating ratio for 6.5 nm iron oxide nanocrystals. The coating efficiency of quantum dots varied significantly among different batches of quantum dots we purchased. This might be due to the different amount of free surfactant and stability of quantum dots in the dispersion. Our experiment and published studies showed that attempts to remove free surfactants led to decreased stability of quantum dots in chloroform.[4] Therefore, the quantum dots were coated without purification. Aggregated quantum dots were removed after coating by centrifugation at 14,000 g for 5 minutes.

Nanoparticle Coating with film hydration method. The nanocrystals were mixed with DSPE-PEG in a round bottom flask as aforementioned. Then chloroform and toluene were completely removed with a rotary evaporator (Buchi Rotavapor R-205) followed by incubation in a vacuum chamber overnight. To compare nanoparticle coating under the same conditions, 20 ml deionized water was added to the flask. The flask was sonicated for 3 hours to obtain DSPE-PEG coated nanoparticles.

Transmission electron microscopy. Negative staining was used to visualize the polymer coating of nanoparticles dispersed in water. Before TEM, empty micelles were removed from the samples by three rounds of ultracentrifugation. Coated nanoparticles were negatively stained on glow discharged copper grids. 6.5 nm iron oxide nanoparticles were stained with phosphotungstic acid. The other three nanoparticles were stained with methylamine tungstic acid. TEM images were recorded with a Hitachi H-7500 Transmission Electron Microscope connected to a CCD camera. The negative staining and TEM procedures were conducted by Robert P. Apkarian Integrated Electron Microscopy Core at Emory University.

Determination of IONP concentration. The iron content of IONPs was determined using a ferrozine assay. In brief, 50 μl sample was mixed with equal volume of 12 M HCl and incubated at room temperature for 30 minutes. Then, 240 μl of 2 M NaOH, 50 μl of 4 M ammonium acetate and 110 μl of 5% hydroxylamine HCl were added to the solution sequentially. After 30 minutes incubation, the solution was mixed with 0.02% ferrozine solution. Light absorption was read at 562 nm with 810 nm as the reference using a Tecan Safire microplate reader. The average diameter of iron oxide nanocrystals was measured in TEM images using ImageProPlus® software. The average diameters of the two iron oxide nanocrystals used in this study are 6.5 nm and 17.1 nm. The number of iron atoms per iron oxide

nanoparticle was estimated to be 5.78 x 10^3 and 105 x10^3 for 6.5 nm and 17.1 nm nanoparticles respectively based on the average volume of iron oxide nanocrystals with a density of 5.17g/cm^3. It was assumed that the iron oxide nanocrystals were Fe_3O_4. The molar concentration of IONPs was calculated by dividing the mass concentration with the total molecular weight of iron in a particle.

Quantification of DSPE-mPEG molecules on IONPs. An assay was developed to quantify the concentration of DSPE-mPEG in IONP colloidal solution based on the phosphate concentration. Each DSPE-mPEG contains a phosphate group. To release the inorganic form of phosphate from DSPE-mPEG, aliquots of 100 µl IONP were added to glass test tubes. After the solvents were evaporated under vacuum, 500 µl 70% perchloric acid was added to each sample and the mixture was heated at 160°C for 20 minutes. 580 µl of 10 M NaOH was added to neutralize perchloric acid. The concentration of phosphate in the solution was quantified with a Malachite green phosphate detection kit (R&D Systems). This assay was validated with DSPE-mPEG solutions.

Quantification of DSPE-PEG-NH_2 on IONPs. The density of DSPE-PEG-NH_2 on 6.5 nm IONPs was measured using fluorescamine assay. 200 µl IONP colloidal solution (100 µg Fe/ml in water) was mixed with 50 µl fluorescamine solution (3 mg/ml in acetone) in a 96-well quartz plate. After 20 minutes incubation at room temperature, the fluorescence intensity was measured with a microplate reader (λ_{ex} = 395 nm, λ_{em} = 470 nm). The standard solutions was prepared with DSPE-PEG-NH_2 and contained the same amount of 6.5 nm IONPs coated with DSPE-mPEG.

Dynamic light scattering measurements. The hydrodynamic diameters of the nanoparticles were measured with Wyatt DynaPro Nanostar dynamic light scattering instrument. The nanoparticles were dispersed in deionized water unless otherwise specified. Before measurements, air bubbles and dusts were removed from the samples by passing through 0.2 µm syringe filters. All measurements were performed at 25°C. Mass weighted size distribution was reported.

Gel electrophoresis assays. All 6.5 nm IONP samples used in gel electrophoresis were unpurified in order to reflect size and charge distribution of IONPs due to the two coating procedures. Coated rod-like quantum dots were centrifuged at 14,000g for 5 minutes as mentioned above. 6.5 nm IONPs or coated quantum dots were dispersed in deionized water and mixed with glycerol The final solution contained 200 µg Fe/ml IONPs or ~20 nM quantum dots and 10% glycerol. 0.5% agarose gel was prepared with tris-acetate EDTA buffer (pH 8.3). Gel electrophoresis was performed in tris-acetate EDTA buffer (pH 8.3) at 135 V. The running time varied between 30 minutes to 60 minutes. The gel images were recorded with Gel Logic 100 Imaging System equipped with a UV lamp.

Antibody conjugation. Horse anti-mouse IgG antibody was conjugated to 17 nm IONPs by thiol-maleimide reaction. 17 nm IONPs were coated with a mixture of DSPE-mPEG and DSPE-PEG-maleimide. The weight ratio between total DSPE-PEG and iron was 2:1 and the percentage of DSPE-

PEG-maleimide in the mixture was 2%. After transferred to deionized water, wash and centrifugation were always performed at 4°C to minimize hydrolysis of maleimide. The disulfide bond in the antibody was reduced with 2-Mercaptoethylamine•HCl. After 2-Mercaptoethylamine•HCl was removed by centrifugation with a Microcon® centrifugal filter tube (MW = 10,000, Millipore), the reduced antibody was mixed with the IONPs in PBS at 1:1 ratio and incubated at 4°C for 16 hours. Free antibody fragments were removed by ultracentrifugation.

Goat anti-human folate receptor-1 antibody and control goat IgG were conjugated to IONPs using the same protocol. Antibody conjugated IONPs were mixed with a fluorescence molecule, DiI, in water at 4°C for 24 hours. The concentration of IONPs and DiI were 80 μg Fe/ml and 5 μg/ml respectively. In a separate study, we found that DiI molecules could bind to coated IONPs by hydrophobic interactions (data not shown). Unbound DiI molecules were removed from the solutions by ultracentrifugation.

Targeting mouse IgG on ELISA plate. First, a 96-well ELISA plate (R&D Systems) was coated sequentially with mouse IgG and bovine serum albumin. In brief, mouse IgG was dissolved in PBS (0 to 4 μg/ml). 100 μl of mouse IgG solution was added to each well. The plate was sealed and incubated at 4°C for 24 hours and washed with PBS containing 0.05% Tween-20. Then, the plates were incubated with 1% BSA solution in PBS at 4°C for 24 hours. In the coated plate, the amounts of mouse IgG were different but the BSA density was approximately the same on the surface of each well.

In the targeting study, each well was loaded with 100 μl of 17 nm IONPs conjugated with horse anti-mouse IgG antibody. In Figure 6A, the concentrations of the IONPs were fixed at 8.5 nM (particle concentration) but the mouse IgG loading varied from 0 to 4 μg/ml. In Figure 6B, the mouse IgG loading was fixed at 4 μg/ml, while the concentration of IONPs varied from 0 to 8.5 nM. The plate was incubated at 37°C for one hour and washed with PBS containing 0.05% Tween-20. To measure the amount of bound IONPs, the wells were incubated with 50 μl of 6N HCl for 30 minutes at room temperature. Then, 120 μl of 2 N NaOH, 25 μl of ammonium acetate buffer, 25 μl of 5% hydroxylamine HCl and 30 μl of 2% ferrozine were added sequentially. The iron content was determined by light absorption at 562 nm with 810 nm as the reference. All samples were measured in duplicate.

Targeting human folate receptor-1 on culture cells. HeLa and MDA-MB-435 were cultured in T75 cell culture dishes following ATCC instructions. After the cells reached 70% confluent, the cells were detached with trypsin-EDTA and seeded in a 4 well chamber glass. The cells were maintained in the chamber glass for 24 hours to fully attach to the glass surface. After that, the cells were incubated with culture media containing antibody conjugated IONPs (50 μg Fe/ml) for 1 hour at 37°C. At the end of the incubation, cell nuclei were counterstained with Hoechst 33342. Fluorescence images were recorded with a fluorescence microscope connected to a CCD camera. In a separate preparation, cells were detached with trypsin-EDTA after incubation with antibody conjugated IONPs and analyzed with BD LSRII flow cytometer system.

To perform western blots for HeLa and MDA-MB-435S, the cells cultured in a 6-well plate were lysed by RIPA buffer (Santa Cruz). Total protein levels were detected using Coomassie Plus assay kit (pierce) and equal amount of protein samples were used in gel electrophoresis. Gels were transferred on to PVDF and the membrane was probed with goat anti-human folate receptor-1 antibody. Membranes were incubated with horseradish peroxidase conjugated rabbit anti-goat secondary antibody (Santa Cruz) and treated with Vector NovaRED Peroxidase Substrate Kit (Vector Labs).

References

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