Supplemental information

A FRET-based toolkit for quantifying lipid incorporation into nanoparticles

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Materials

Reagents

1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DSPG), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), L-α-phosphatidylcholine (HSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)- 2000] (ammonium salt) (DSPE-PEG(2000)), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(Cyanine 5) (DSPE-Cy5), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(Cyanine 3) (DSPE-Cy3), 1-(1Z-octadecenyl)-2-oleoyl-sn-glycero-3-phosphocholine (Plasm PC 18:1), 1-(1Z-octadecenyl)-2-arachidonoyl-sn-glycero-3-phosphocholine (Plasm PC 20:4), 1-(1Z-octadecenyl)-2-oleoyl-sn-glycero-3-phosphoethanolamine (Plasm PE 18:1), 1-(1Z-octadecenyl)-2-arachidonoyl-sn-glycero-3-phosphoethanolamine (Plasm PE 20:4), and cholesterol were purchased from Avanti Polar Lipids. Sulfo-cyanine3 NHS ester and sulfocy-cyanine5 NHS ester were purchased from Lumiprobe. Deuterated dimethyl sulfoxide (DMSO-d6) and deuterated ethanol (EtOD) were purchased from Cambridge Isotope Laboratories.

Dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA) was purchased from MedChemExpress. 1,2-dimyristoyl-*rac*-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG-2000) was purchased from Cayman Chemical Company. Cholesterol and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) were purchased from Avanti Polar Lipids. Absolute ethanol (200 proof), molecular-biology grade and UltraPure DNAse/RNAse-free Distilled Water were purchased from Fisher Scientific. siRNA (Silencer Select pre-designed siRNA) negative control no. 1 (siScramble) was purchased from Life Technologies. HPLC grade acetonitrile, HPLC grade methanol, HPLC grade isopropyl alcohol, and HPLC grade DMSO were purchased from Sigma. Nunc F96 MicroWell Black polystyrene plates, and 3 M sodium acetate solution, pH 5.2, RNAse free, were purchased from Fisher Scientific. Triton X-100 and 1M HEPES, pH 9.0, and pH-indicator strips pH 5.0 - 10.0 were purchased from Sigma. 1x Tris-EDTA solution, pH 8.0 (IDTE) was purchased from Integrated DNA Technologies. 1cc 28Gx1/2” insulin syringes were purchased from Amazon.

1.5 mL Eppendorf tubes were purchased from Genesee Scientific. Borosilicate glass scintillation vials and 50/5/2 mL DNA/Protein loBind Eppendorf tubes were purchased from VWR. were purchased from VWR. Spectra/Por dialysis membrane tubing (3.5 kDa MWCO) were purchased from Repligen. Polystyrene semi-micro cuvettes for the Malvern Zetasizer were purchased from Sarstedt and DTS1070 folded capillary cells were purchased from Fisher Scientific. Carbon coated mesh 400 copper grids were purchased from Electron Microscopy Sciences.

Methods

Dye-Labeled Liposome Synthesis

Dye-labeled liposomes were formed via the thin-film hydration method and freeze-thaw cycles (refs). Cholesterol and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), L-α-phosphatidylcholine (HSPC), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)- 2000] (ammonium salt) (DSPE-PEG(2000)), 1-(1Z-octadecenyl)-2-oleoyl-sn-glycero-3-phosphocholine (Plasm PC 18:1), 1-(1Z-octadecenyl)-2-arachidonoyl-sn-glycero-3-phosphocholine (Plasm PC 20:4), 1-(1Z-octadecenyl)-2-oleoyl-sn-glycero-3-phosphoethanolamine (Plasm PE 18:1), 1-(1Z-octadecenyl)-2-arachidonoyl-sn-glycero-3-phosphoethanolamine (Plasm PE 20:4) were dissolved in chloroform. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) and 1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DSPG) were dissolved in a 65:35:8 mixture of chloroform, methanol and deionized water (milli-Q).

Lipid mixtures of the following molar compositions were prepared in scintillation vials in addition to adding equal molar percentage of DSPE-SulfoCy3 and DSPE-SulfoCy5 ranging from 0.5 mol% to 3 mol% by replacing the amount of DSPE/DSPE-PEG:

a) Lipo-A: 56 mol% HSPC, 38 mol% Cholesterol, and 6 mol% DSPE-PEG(2000)
b) Lipo-B: 31 mol% DSPC, 31 mol% DSPG, 31 mol% Cholesterol, 6 mol% DSPE
c) Pre-inserted Lipo: 50 mol% DSPC, 39 mol% Cholesterol, 5 mol% POPG, 6 mol% DSPE-PEG(2000)
d) Plasm-Lipo: 31 mol% 18:1 (20:4) PlasmPE(PC), 31 mol% DSPG, 31 mol% Cholesterol, 6 mol% DSPE

The lipid mixture was evaporated using a rotary evaporator for 30 minutes at < 30 mbar in a 40 °C water bath. The resulting film was hydrated using milli-Q water heated above the transition temperature of the lipids (T_m) or at 70 °C. Hydration was followed by 6 freeze-thaw cycles consisting of 1 minute of vortexing and 1 minute agitation in an ice bath each. Resulting liposomes were sonicated at 65 °C for 10 minutes. Lipid film generation and hydration were similarly applied to all liposomal formulations.

Lipid-Dye Conjugation

DSPE and Plasmalogen PE lipids were conjugated to SulfoCy3 and SulfoCy5 NHS ester. Liposomes comprising of 100% PE lipid were formed via the thin-film hydration method and freeze-thaw cycles described above).

The Liposomes were labeled with SulfoCy3 or SulfoCy5 following standard protocols[1]. Briefly, 50 mM of HEPES, pH 9.0, were added to liposome solution and pH was verified with pH strips. 2.5 molar equivalents, relative to PE lipid, of SulfoCy3 or SulfoCy5 were added dissolved in DMSO and left stirred overnight. The labeled liposome solution was then transferred to a 3.5

kDa MWCO dialysis membrane and dialyzed against water. To ensure total purification from free dye, the dialysate was changed every 6 hours for a period of 3-4 days in addition to measuring the fluorescence decrease in the dialysate in a plate reader until negligible fluorescence was observed. After dialysis, the labeled liposomes were frozen using liquid nitrogen and lyophilized for 1 day. The final solution was then resuspended with a mixture of 65:30:5 of chloroform, methanol, and milli-Q water.

Lipid-Dye Conjugate Characterization

HPLC-UV-Vis characterization was carried out on an Agilent 1260 Infinity II system with a G711B quaternary pump, G7129A autosampler, G7116A MCT, and a G715A DAD WR. A sample of the final lipid-dye and unconjugated lipid solution was dissolved in DMSO and a Poroshell 120 EC-C18 column (4.6 x 100 mm, 2.7 μm) was utilized to determine the absorbance spectra of the lipid-dye conjugate sample using a gradient of 50%-99% water + 0.1% TFA (mobile phase A) to ACN/IPA (90/10 v/v) over 10 minutes with a flow rate of 1.0 mL/min. The lipid-dye conjugates eluted around 6.0 – 6.5 min.

The molar percentages of the lipid PE-dye conjugate and unconjugated lipid PE were determined via ^{1}H NMR. Samples were dissolved in a 1:1 mixture of DMSO-d6 and EtOD and measured in a 500 MHz Bruker Avance III HD with SampleXpress.

Post-Insertion of Micelles in Liposomes

Prefabricated liposomes were synthesized using the methodology described above with a molar composition of 56:39:5 DSPC, Cholesterol, and POPG. DSPE-PEG and DSPE-SulfoCy micelles were formed as described in previous post-insertion protocols using respective molar ratios to compare to pre-incorporated PEG liposomes[2,3]. Micelles were incubated with the pre-formed liposomes at respective molar ratios under sonication at 70°C for 30 minutes.

DLS Characterization of Nanoparticles

Dynamic light scattering (Malvern Zetasizer Pro, λ = 633 nm) was used to measure liposome and lipid nanoparticles (LNPs) hydrodynamic diameter and polydispersity (PDI). Zeta potentials for the particles were also obtained with the Zetasizer Pro using laser doppler electrophoresis. Particles were diluted in milliQ water in polystyrene, semi-micro cuvettes for size measurements and DTS1070 folded capillary cuvettes for zeta measurements.

TEM Characterization of Nanoparticles

Images of Liposomes and LNPs were obtained using a FEI Tecnai T12 TEM. Carbon coated mesh 400 copper grids were glow-discharged and 10 μL of LNPs or liposomes at a concentration of 0.25 mg/mL were deposited on the grid. The grid was blotted after 1 minute to remove excess sample. 10 μL of 2% uranyl acetate (UA) solution was deposited on the grid and immediately blotted. After 30 seconds the UA staining process was repeated, and the grid was left to dry for 10 minutes. The grid was mounted on a single tilt sample holder. The microscope was operated

at 120 kV with a magnification of 10,000-40,000x for assessing particle morphology and size distribution. All bright-field images were recorded on a Gatan MSC794 CCD Camera. Particle sizes were analyzed using ImageJ by manually measuring the diameters of clearly resolved vesicles from representative TEM fields (around 50 particles per sample) collected across at least three non-overlapping regions.

FRET Characterization

Labeled liposomes or LNPs containing single-labeled SulfoCy3 and SulfoCy5 particle controls, FRET particle (containing both SulfoCy3 and SulfoCy5 in the same particle), and a negative control containing a mixture of both single-labeled controls were pipetted into a Nunc F96 MicroWell Black polystyrene plate. Fluorescence of the particles was quantified using a BioTek Synergy Hybrid H1 plate reader using three channels: Cy3, Cy5, and FRET channels. The Cy3 and Cy5 channels consisted of 530/560 nm (Ex/Em) and 635/665 nm (Ex/Em), respectively. The FRET channel consisted of 530/665 (Ex/Em). FRET efficiency was determined by measuring the fluorescence of SulfoCy3 in the single-labeled SulfoCy3 control particle relative to the FRET particle using

$$FRET_{Eff} = \frac{F_{Cy3} - F_{FRET}}{F_{Cy3}}$$

where F_{cy3} and F_{Fret} are the fluorescence of the particles containing only SulfoCy3 and the FRET formulation containing both SulfoCy3 and SulfoCy5 (mixed single-color control), respectively, measured in the Cy3 channel (Ex = 530 nm, Em = 560 nm).[4] To compare incorporation across formulations, FRET efficiencies were normalized between two reference states, which were the mixed single-color control ($FRET_{0\%}$), and a fully labeled Doxil-like control (Lipo-A) ($FRET_{100\%}$), using the equation

$$\%Lipid\ Incorporation = \frac{FRET_{Eff} - FRET_{0\%}}{FRET_{100\%} - FRET_{0\%}}$$

Dye-Labeled LNP Synthesis

Cholesterol and DSPC were dried from chloroform stock solutions using a rotary evaporator and resuspended in 100% ethanol. Dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA) and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG-2000) were dissolved in 100% ethanol. Lipid mixtures consisting of required molar concentrations were prepared in 4 mL scintillation vials.

Silencer Select pre-designed scrambled siRNA was dissolved in 35 mM of sodium acetate, pH 4.0 at 2.5 μM. 4 volumes parts of the aqueous phase were added to a 4 mL scintillation vial and stirred gently at room temperature. 1 volume part of the organic phase was injected rapidly, and solution was removed from stirring for 5 minutes. Solution was restirred and 5 volume parts of

RNAse free water were added to the solution. The solution was then removed from stirring. Ethanol was allowed to evaporate overnight.

Nanoparticle Tracking Analysis

Labeled liposomes and LNP solutions were diluted in Mili-Q water and injected into a Particle Metrix Zetaview to analyze size distribution and fluorescence colocalization. A Cy3 channel using the 520 nm laser and the 550 filter and a Cy5 channel using the 610 nm laser and the 640 nm filter were used to determine the size distribution using the respective filters. The fluorescence colocalization script consisted of both channels recording data sequentially to determine fluorophore colocalization.

FRET Liposome Model

The number of lipids in the inner and outer layers using the following equations:

$$A_{\text{outer}} = 4\pi \left(\frac{D}{2}\right)^2 \quad (1)$$

$$A_{\text{inner}} = 4\pi \left(\frac{D}{2} - d\right)^2 \quad (2)$$

$$N_{\text{lipid\_outer}} = \frac{A_{\text{outer}}}{A_{\text{lipid}}} \quad (3)$$

$$N_{\text{lipid\_inner}} = \frac{A_{\text{inner}}}{A_{\text{lipid}}} \quad (4)$$

where D and d represent the diameter and membrane thickness of the liposome, respectively. A_{inner}, A_{outer}, A_{lipid} represent the inner and outer membrane and lipid head surface area of the liposome, respectively. N_{lipid_inner} and N_{lipid_outer} represent the total number of lipids on the inner and outer surfaces. The membrane thickness (d) and the surface area of the lipid head (A_{lipid}) for 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) were determined from the literature[5–7]. The fraction of lipid in the outer membrane (f_{outer}) was determined using

$$f_{\text{outer}} = \frac{N_{\text{lipid\_outer}}}{N_{\text{lipid\_outer}} + N_{\text{lipid\_inner}}} \quad (5)$$

.

Förster showed that FRET efficiency depends on the inverse distance to the sixth power between donor and acceptor via:

$$FRET_{\text{eff}} = \frac{1}{1 + \left(\frac{\langle r \rangle}{R_0}\right)^6} \quad (6)$$

Where <r> is the random distance between the donor and acceptor and R_o is the Förster radius[8]. Next, to determine the FRET efficiency as a function of the concentration of DSPE-Cy3 and

DSPE-Cy5, the random distance between DSPE-Cy3 and DSPE-Cy5 using a nearest neighbor analysis of random distributions on a sphere was modeled. Scott and Tout use a Poisson-like distribution to describe the nearest neighbor points in a sphere

$$P_1(r) = 2\pi n r \mathrm{e}^{-\pi n r^2} \tag{7}$$

Where n is the number density in a plane[9]. The mean nearest neighbor can be derived from this via:

$$\langle r \rangle = \int_0^\infty r P_1(r) dr = \frac{1}{2\sqrt{n}} \tag{8}$$

This gives an elegant solution to determine the distance between Poisson-like distributed points in a spherical plane. The number density of the DSPE-SulfoCy conjugate was estimated using

$$n = \frac{N_{\text{DSPE-sulfocy}}}{A_{\text{total}}} \tag{9}$$

Where $N_{DSPE\text{-}sulfocy}$ represents the total number of DSPE-SulfoCy molecules in the liposome. $N_{DSPE\text{-}sulfocy}$ can be estimated using the total number of lipid molecules and the mole percentage of the DSPE-SulfoCy in the formulation.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism. Multiple comparisons were performed using Kruskal-Wallis test with post hoc Dunn's multiple comparisons test and one-way ANOVA with Tukey's multiple comparison test.

Supplemental Figures

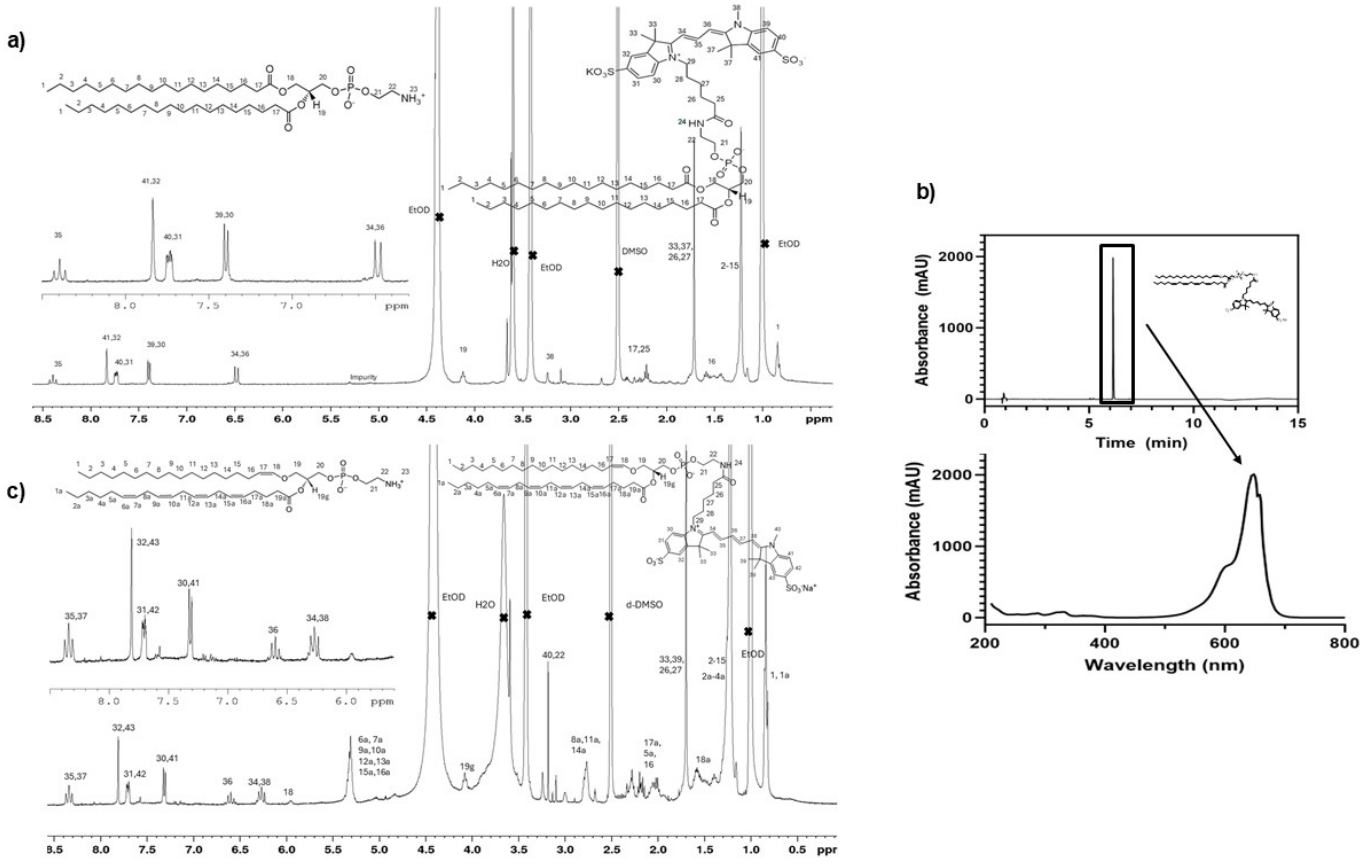

Figure S1. (a) ^{1}H NMR characterization of DSPE-SulfoCy3 in 1:1 DMSO-d6 and EtOD. (b) UV Vis Characterization using HPLC to characterize the absorbance of the purified DSPE-SulfoCy5 conjugate, showing maximum absorbance peak at expected wavelength. (c) ^{1}H NMR characterization of Plasm 20:4 PE-SulfoCy5 in 1:1 DMSO-d6 and EtOD.

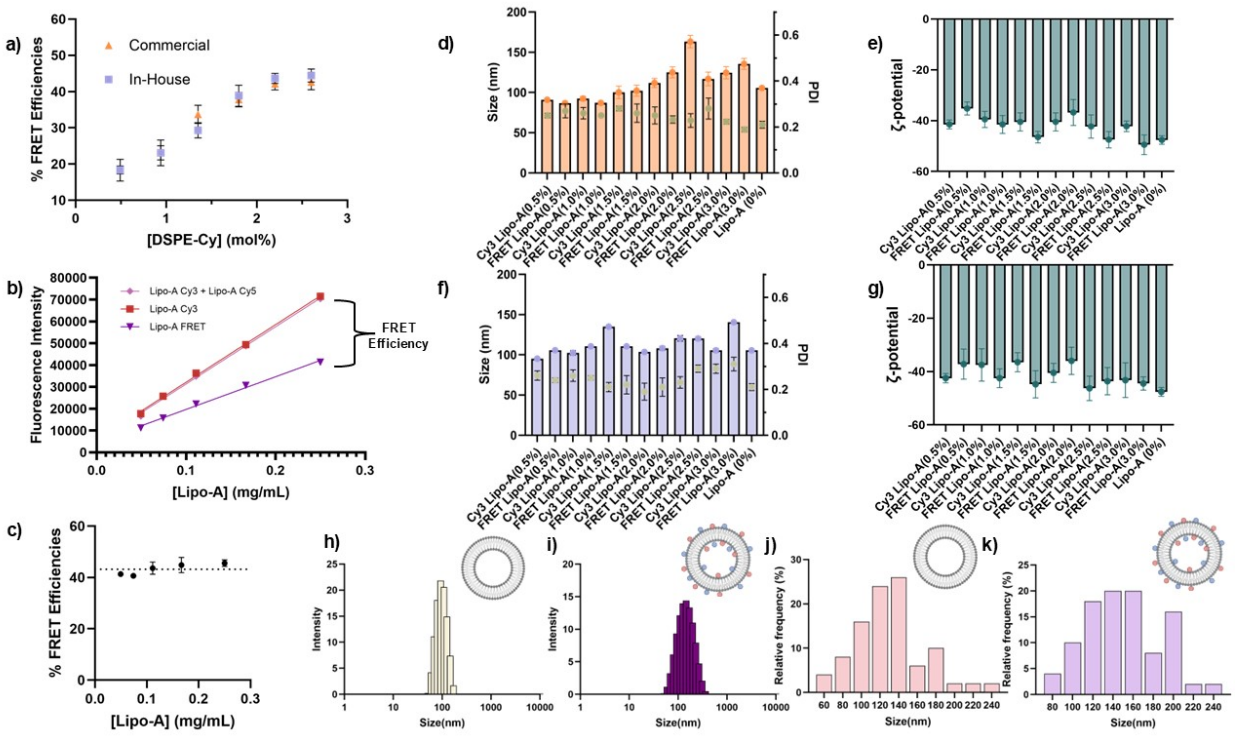

Figure S2. (a) % FRET efficiencies of Lipo-A formulation using commercially available non-sulfonated cyanine dyes and "in-house" synthesized sulfonated cyanine dyes, respectively. (b) Fluorescence Intensity for the formulations Lipo-A Cy3, Lipo-A FRET, and the negative control, Lipo-A Cy3 and Lipo-A Cy5 in the same solution. (c) The FRET efficiency of the Lipo-A formulation at various concentrations. (d-e) DLS characterization of Lipo-A using commercially available lipid-dye conjugates. (f-g) DLS characterization of Lipo-A using "in-house" synthesized lipid-dye conjugates. Data is presented as mean ± standard deviation of three technical replicates. (h) DLS size distribution of unlabeled Lipo-A. (i) DLS size distribution of FRET labeled Lipo-A (3% DSPE-SulfoCy). (j) Size distribution histogram generated from TEM representative fields for unlabeled Lipo-A (n=50). (k) Size distribution histogram generated from TEM representative fields for FRET-labeled Lipo-A (n=50). Data is presented as mean ± standard deviation of three technical replicates.

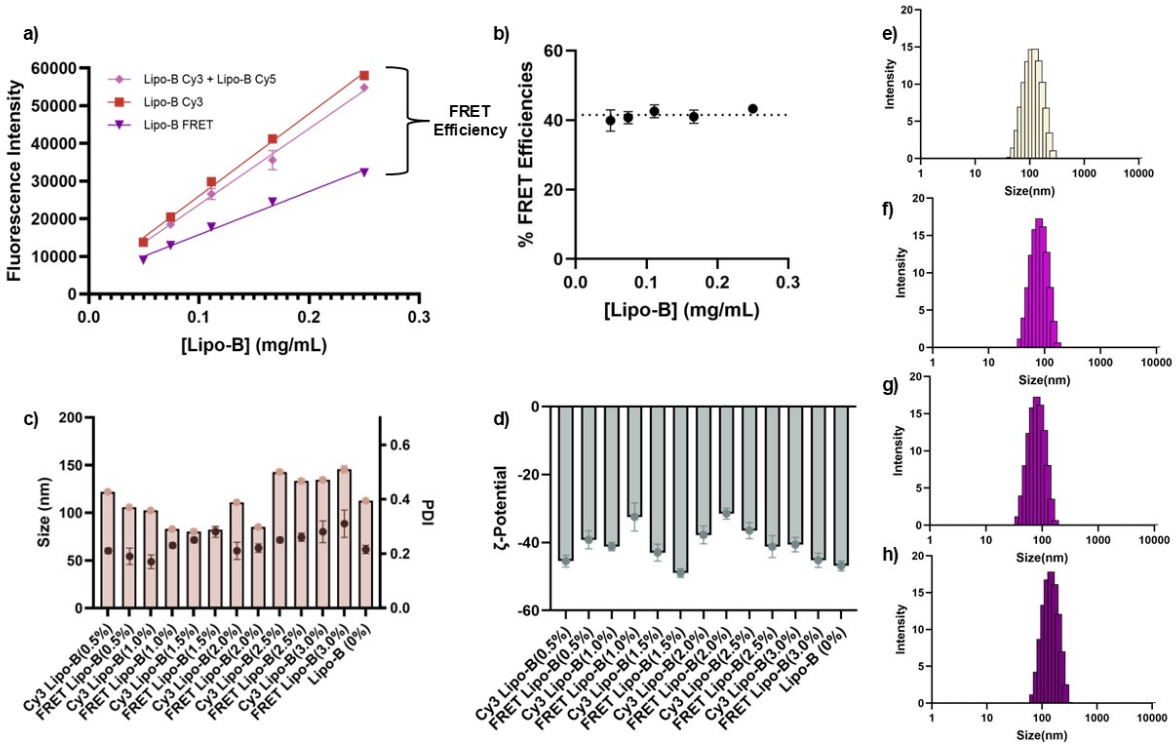

Figure S3. (a) Fluorescence intensity of the Lipo-B formulation as a function of liposome concentration. (b) FRET efficiencies of the Lipo-B at different liposome concentrations. (c-d) DLS characterization of the Lipo-B formulations, including size, polydisperisity index, and ζ potential. Data is presented as mean ± standard deviation of three technical replicates. (e) DLS size distribution of unlabeled Lipo-B liposomes. (f) DLS size distribution of labeled Lipo-B liposomes with 1 mol% DSPE-Sulfocy. (g) DLS size distribution of labeled Lipo-B liposomes with 2 mol% DSPE-Sulfocy. (h) DLS size distribution of labeled Lipo-B liposomes with 3 mol% DSPE-Sulfocy. Data is presented as mean ± standard deviation of three technical replicates.

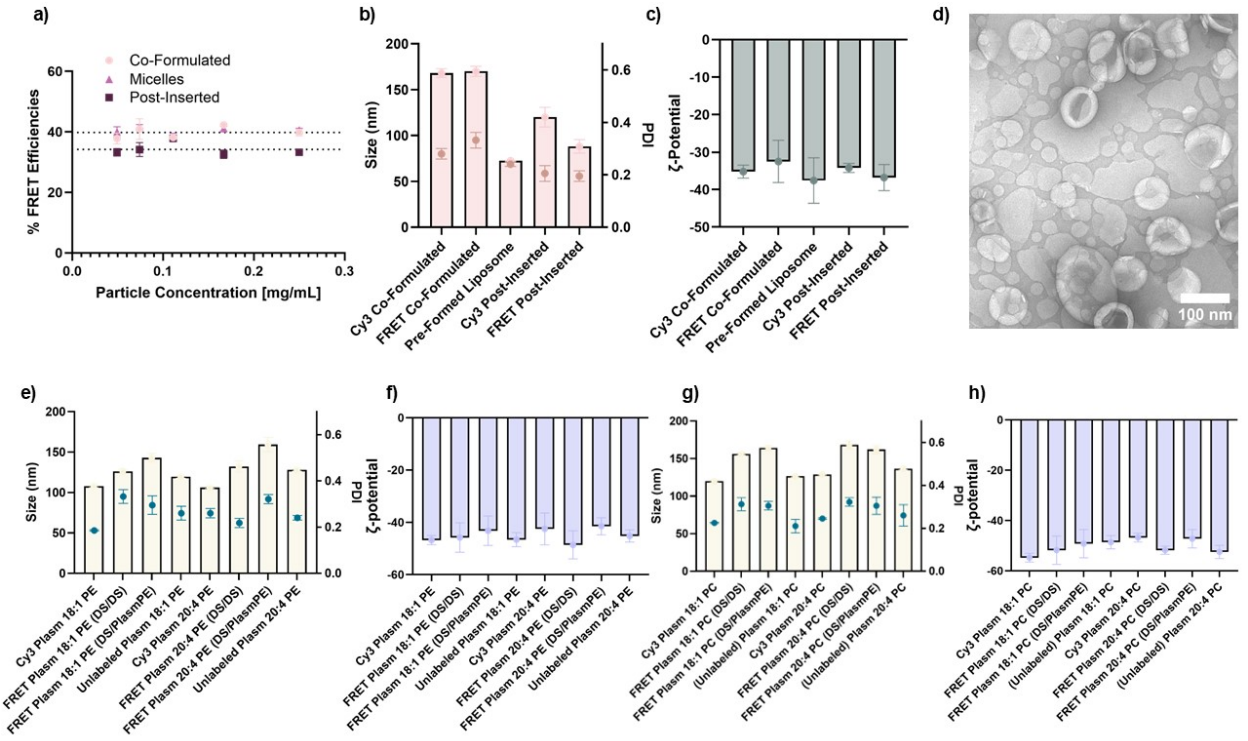

Figure S4. (a) FRET efficiencies of Co-Formulated, PEG and DSPE-SulfoCy micelles, and liposomes with post-inserted micelles. (b-c) DLS characterization of the liposome formulation when the lipid-dye conjugates are co-formulated, the pre-formed liposome formulation without PEG-lipid and lipid-dye conjugate (pre-formed liposome), and the liposome formulation when PEG-lipid and lipid-dye micelles are inserted in the pre-formed liposome (post-inserted). (d) Representative bright-field transmission electron micrographs of FRET-labeled post-inserted liposomes (scale bar = 100 nm). (e-f) DLS characterization of PlasmalogenPE-Lipo system, including size, polydisperisity index, and ζ potential. (g-h) DLS characterization of PlasmPC-Lipo system, including size, polydisperisity index, and ζ potential. Data is presented as mean ± standard deviation of three technical replicates.

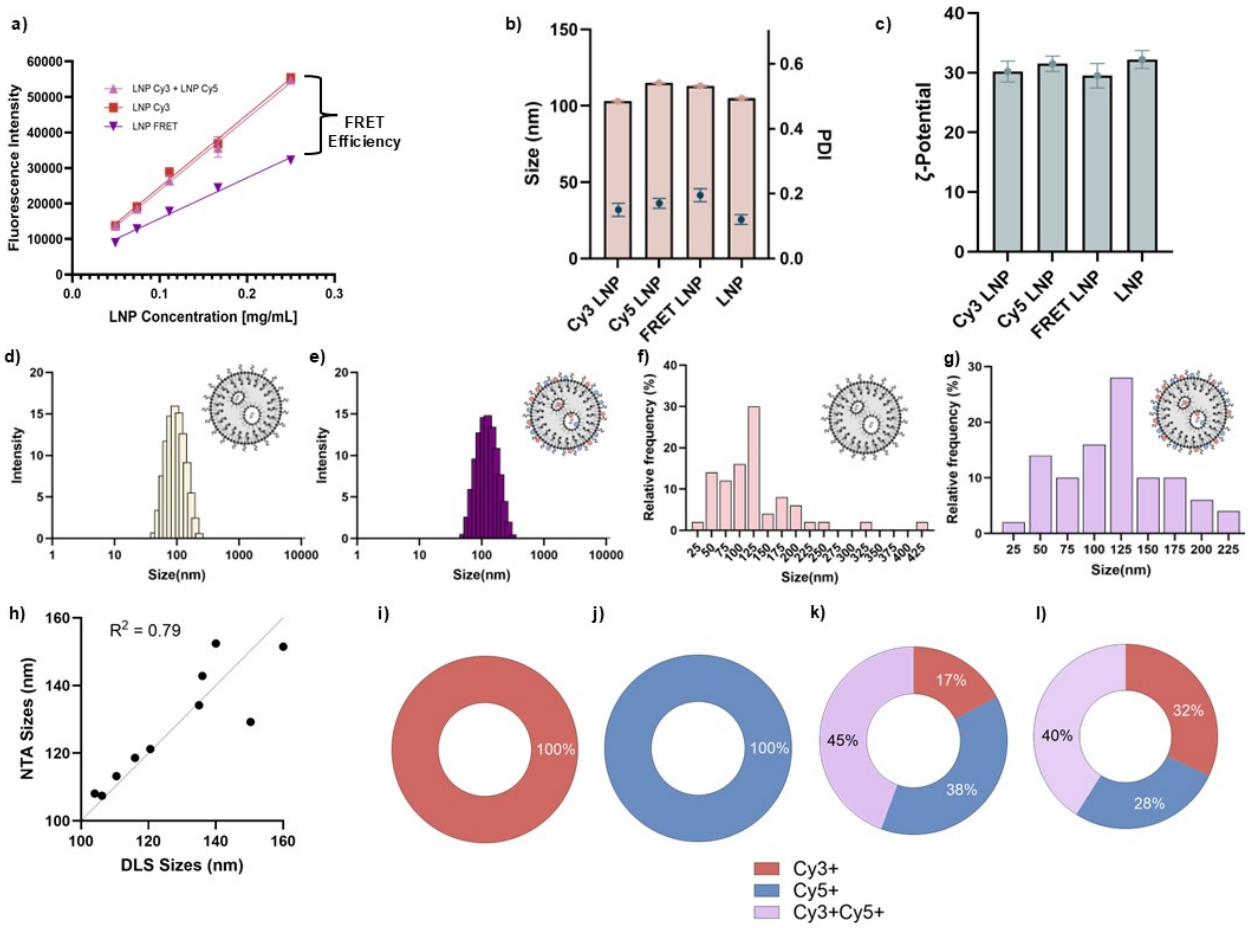

Figure S5. (a) Fluorescence intensity of the LNP formulations at various LNP concentrations. (b-c) DLS characterization of the LNP formulations. (d) DLS size distribution of unlabeled LNP. (e) DLS size distribution of FRET labeled LNP. (f) Size distribution histogram generated from TEM representative fields for unlabeled LNP (n=50). (g) Size distribution histogram generated from TEM representative fields for FRET-labeled LNP (n=50). (h) Relationship between NTA and DLS sizes. NTA sizes were obtained from the Cy3 channel (Ex = 530 nm, Em = 560 nm) and each data point represents a separate nanoparticle batch. (i) NTA analysis of individually labeled SulfoCy3 LNPs. (j) NTA analysis of individually labeled SulfoCy5 LNPs. (k) NTA analysis of the Plasm 20:4 PC FRET formulation using DSPE-SulfoCy3 and Plasm 20:4 PE-SulfoCy5. (l) NTA analysis of Tetraspeck® particles. Data is presented as mean ± standard deviation of three technical replicates.

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