Supplementary materials

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Topology of Surface Ligands on Liposomes: Characterization Based on the Terms, Incorporation Ratio, Surface Anchor Density, and Reaction Yield

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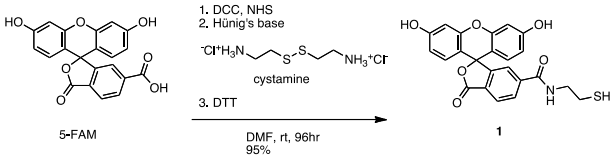
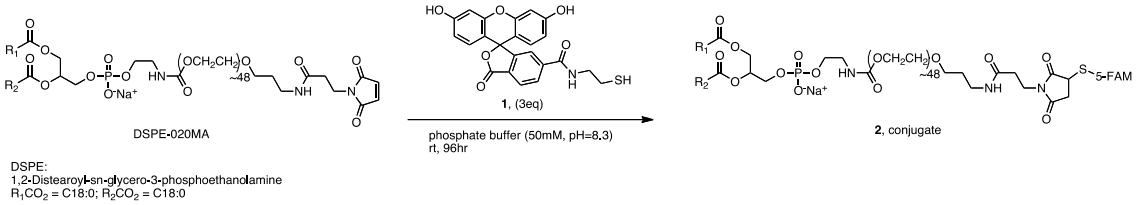
(a)



(b)



DSPE:
1,2-Distearoyl-sn-glycero-3-phosphoethanolamine
R_1CO_2 = C18:0; R_2CO_2 = C18:0

Chart S1. The synthetic route for preparing thiolated 5-FAM and a 5-FAM-PEG_{2000}-DSPE conjugate lipid.

(a) 5-FAM carboxylic acid **1** and cystamine were conjugated by dehydrate reagent, *N,N'*-dicyclohexylcarbodiimide (DCC), mediated peptide coupling reaction. Following the reductive cleavage by dithiothreitol (DTT), thiolated 5-FAM **1** was gotten in yield 82%. All steps were in one reaction pot under DMF, rt for 96 hr. (b) maleimide-PEG_{2000}-DSPE (DSPE-020MA) and **1** performed thio-maleimide Michael addition to get 5-FAM-PEG_{2000}-DSPE conjugate **2** under phosphate buffer, pH = 8.3, rt, for 96 hr.

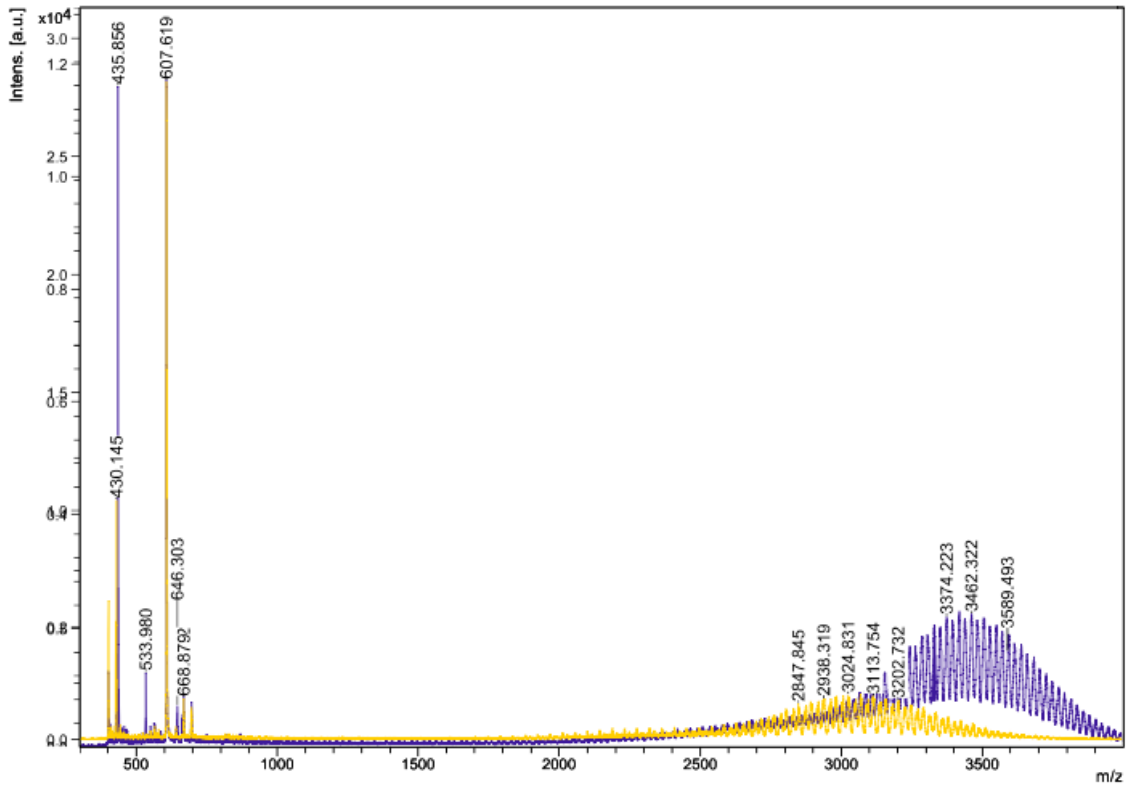


Figure S1. MALDI-TOF mass spectroscopy of the 5-FAM-PEG_{2000}-DSPE conjugate lipid.

Two mass spectroscopies (MS) were aligned. Yellow peaks indicate anchor lipid, maleimide-PEG_{2000}-DSPE, calculated for $C_{148}H_{286}N_3O_{61}P$ 3112.9107, found 3112.754. Blue peaks indicate the product, 5-FAM-PEG_{2000}-DSPE conjugate **2**, calculated for $C_{167}H_{295}N_4O_{65}PS$ 3460.9393, found 3461.322. The left blue peak indicates 5-FAM-SH **1**, calculated for $C_{23}H_{17}NO_6$ 435.0777, found 434.856. The shift from yellow to blue peaks ensures the product was synthesized. 2,5-dihydroxy benzoic acid (DHB) is used as matrix to mix with sample in molar ratio 3000 to 1.

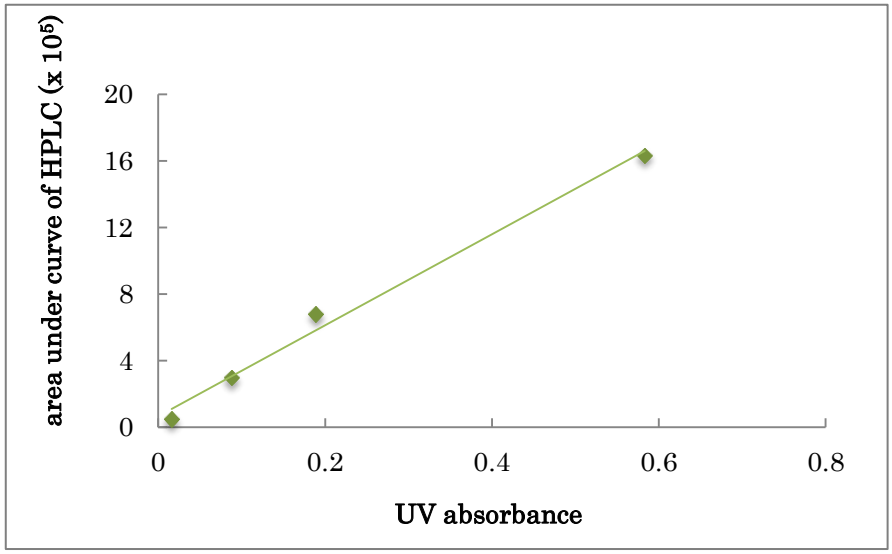


Figure S2. HPLC versus UV calibration curve for the 5-FAM-PEG_{2000}-DSPE conjugate lipid.

Linearity of the area under the curve (AUC) of HPLC versus UV absorbance for 5-FAM-PEG_{2000}-DSPE conjugate lipid **2** dissolved in HEPES-NaOH buffer (pH = 7.4 10 mM) (λ_{ex} = 495 nm) (y = 27.355x + 0.6577, R^2 = 0.99027). The linearity range of UV absorbance covers the analysis samples of liposomal solution for HPLC analysis.

Table S1. Physical data for the liposomes in Chart 1.

Liposomes stage	Equivalent of 5-FAM-SH	Size (d.nm)	PDI	ζ(mV)
Before reaction	0	75.7	0.187	-20.1
After reaction	0	75.0	0.183	-14.0
	0.5	75.9	0.163	-10.5
	1	76.9	0.197	-13.8
	10	128.1	0.431	-18.0
After purify	0.5	76.1	0.169	-24.2
	1	76.8	0.183	-26.9
	10	111.6	0.478	-19.6

The physical data, including size, PDI value and zeta potential, ζ (mV) of different stages of liposomes in Chart 1 were measured by Zetasizer. The liposomal surface reaction were performed in four groups, including 0, 0.5, 1, 10 equivalent of 5-FAM-SH as probes.

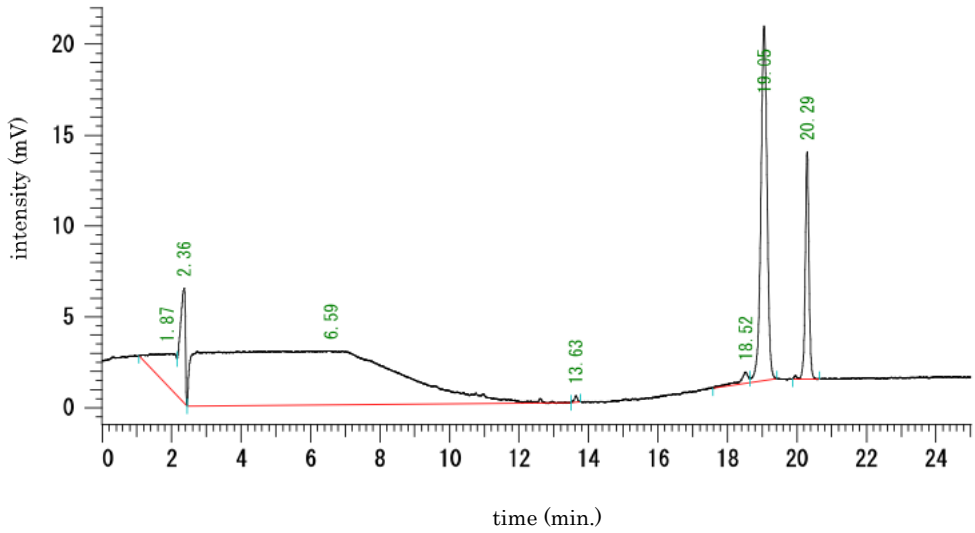


Figure S3. HPLC chromatogram of the 5-FAM-PEG_{2000}-DSPE conjugate lipid on the liposomes.

In the analysis of liposomal samples, the first peak indicates 5-FAM-PEG_{2000}-DSPE in retention time 19.05 min, and the second peak indicates rodamine-DOPE in 20.29 min. The AUC of peaks were calculated to estimate molar amount of each lipid. The samples were analyzed by C4 column, and eluted by triethylammonium acetate (TEAA) buffer (100 mM, pH = 7.4) / acetonitrile 0-90% in 25 min at 40 ^{o}C, λ_{ex} = 495 nm.

Table S2. Physical data for the anchor grafted liposomes in Chart 3.

Liposomes stage	Size (d.nm)	PDI	ζ (mV)
Plain	109.0	0.265	-26.4
After insertion	114.4	0.206	-5.67
After purification of Sephadex® G-25	114.6	0.241	-16.0

The physical data, including size, PDI value and zeta potential, ζ (mV) of the liposomes in different stages in Chart 3 were all measured by Zetasizer.

Table S3. Physical data of anchor grafted liposomes purified in Chart 3.

Liposomes stage	Tube No.	Size (d.nm)	PDI	ζ (mV)
After purification of Sepharose® CL-4B	5	127.7	0.190	-14.2
	6	115.5	0.178	-19.2
	7	105.7	0.214	-19.0
	8	95.7	0.253	-9.95

The physical data of four liposomal collected fractions with size, PDI value and zeta potential, ζ (mV) that purified by Sepharose® CL-4B in Chart 3 were measured by Zetasizer.

Table S4. Physical data for the functionalized liposomes in Chart 4.

Temperature (°C)	Size nm	PDI	ζ (mV)
4	114.3	0.174	-5.72
25	120.8	0.193	-10.4
37	119.0	0.197	-12.5
60	117.9	0.185	-14.7
60**	107.9	0.184	-14.4

The physical data of five liposomal surface reaction groups, including size, PDI value and zetapotential, ζ (mV) that purified by Sephadex® G-25 in Chart 4 were measured by Zetasizer. These reactions were carried out with 1.1 equivalent of 5-FAM-SH at 40 °C to 60 °C in 1 hr.

**This sample was treated with excess amount of 5-FAM-SH (3 equivalents).

The definitions of equations and explanations

1. 'Conventional surface anchor density' in the case of the pre-inserted method is calculated as:

$$\text{conventional surface anchor density} = \text{added anchor molar ratio} \times \frac{55}{100} \qquad (1)$$

where 'added anchor molar ratio' is the maleimide-PEG_{2000}-DSPE molar ratio in the lipid film (e.g. the lipids mixture solution), and ' $\frac{55}{100}$ ' indicates the ratio of the anchors on the outer face of the lipid membranes. The density is not experimentally examined but theoretically calculated.

2. 'Surface anchor density' clarifies the real density of the capped liposome (pre-inserted liposome) determined by experiment:

$$\text{surface anchor density} = \frac{\text{capped anchor}}{\text{lipids mixture}} \qquad (2)$$

where 'capped anchor' is the fluorescent-labeled anchor, which represents the molar amount of 5-FAM-PEG_{2000}-DSPE, and 'lipids mixture' is the molar amount of lipid film (e.g. the lipids mixture solution). Here, the probe is 5-FAM-SH.

3. 'Conventional surface anchor density' in the case of the post-inserted method is calculated as:

$$\text{conventional surface anchor density} = \text{added anchor molar ratio} \qquad (3)$$

where 'added anchor molar ratio' is the maleimide-PEG_{2000}-DSPE molar ratio in the anchor insertion solution (post-insertion process). It is assumed that the anchors do not form the micelles.

4. In the case of the post-inserted method, considering the micelles existence, 'surface anchor density' corrects the assumption that the micelles are removing:

$$\text{surface anchor density} = \frac{\text{anchor} - \text{micelle}}{\text{lipids mixture} + \text{anchor} - \text{micelle}} \qquad (4)$$

where 'anchor' is the molar amount of the added maleimide-PEG_{2000}-DSPE in the anchor insertion solution (post-insertion process), 'micelle' is the molar amount of the maleimide-PEG_{2000}-DSPE, which is incorporated into the micelles in the same solution, and 'lipids mixture' is the molar amount of lipid film (e.g. the lipids mixture solution).

This equation expresses an idea that the lipids amount, which is incorporated into the micelles, should be subtracted in the density calculation.

5. In this manuscript, the micelles were removed by size exclusion chromatography, so 'surface anchor density' in the case of the post-inserted method is experimentally determined and calculated as follows:

$$\text{surface anchor density} = \frac{\text{surface capped anchor}}{\text{lipids mixture} + \text{surface capped anchor}} \qquad (5)$$

where 'surface capped anchor' is the fluorescent-labeled anchor, which presents the molar amount of 5-FAM-PEG_{2000}-DSPE on the capped liposome surface, and 'lipids mixture' is the molar amount of lipid film (e.g. the lipids mixture solution). The density is an experimental result, and here, the probe is 5-FAM-SH.

6. 'Reaction yield' refers to the efficiency for conjugation for the surface reaction on the conjugated liposome:

$$\text{reaction yield} = \frac{\text{surface 5-FAM conj.}}{\text{surface anchor}} \qquad (6)$$

where 'surface 5-FAM conj.' is the conjugate, which presents the molar amount of 5-FAM-PEG_{2000}-DSPE on the conjugated liposomal surface after ligand conjugation, and 'surface anchor' refers to the molar amount of maleimide-PEG_{2000}-DSPE on the anchor grafted liposome before ligand conjugation.

Here, 'surface anchor' is equal to 'surface capped anchor', and can be calculated by the 'surface anchor density' × ('lipids mixture' + 'surface capped anchor'), deriving from equation (5). Here, the ligand is 5-FAM-SH.

7. ‘Incorporation ratio’ is defined as:

$$= \frac{\text{surface 5–FAM conj.}}{\text{lipids mixture + surface anchor}}$$

Then, our equation is derived as follows:

$$= \text{surface anchor density} \times \text{reaction yield} \qquad (7)$$

where ‘surface anchor density’ is from equation (5), and ‘reaction yield’ is from equation (6).