Far-red Fluorescent Liposomes for Folate Receptor-targeted Bioimaging

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1. Synthetic procedures
1.1. Synthesis of SQR23
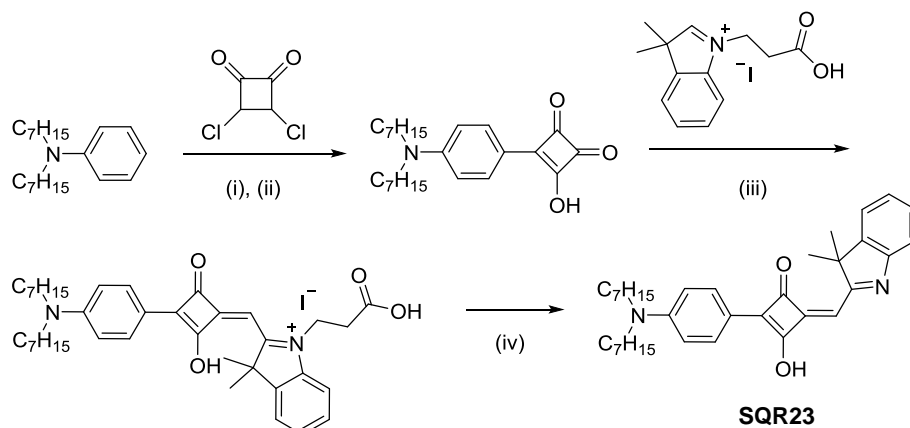
Reagents and conditions: (i) toluene, reflux, 6 h; (ii) 5N HCl, AcOH/H2O (1:1), reflux, 2 h; (iii) n-BuOH/toluene (4:1), reflux, 3 h; (v) NatOBu, THF, rt, 3h.

Figure S1. Synthesis of SQR23.

The synthesis of squaraine far-red dye, SQR23, was outlined in Figure S1. The design contains 3 fragments – aniline, squaric core, and indolium. The sequence in preparing the semi-squaraine intermediate is crucial in order to successfully form the asymmetrical squaraine dyes. The precursors *N,N*-diheptylaniline **1** and squaryl chloride **2** were prepared based on previously reported procedures.[S1, S2] The coupling of **1** and **2** under refluxing toluene gave the chloride intermediate, which was then hydrolysed in a one-pot fashion to obtain squaric acid **3**. Indolium salt **4**, was synthesized using previously published procedures.[S3] Reacting **3** and **4** together in a 1:1 mixture of refluxing *n*-butanol/toluene yielded the butyl ester squaraine dye **5**. This is because the expected carboxylic group of the squaraine dyes reacted with the *n*-butanol solvent to form the butyl ester derivatives instead. We have originally designed the squaraine to contain the carboxylic group as this will serve as an important design for further functionalization and application. However, when **5** was subjected to base hydrolysis using Na^{t}OBu in THF at room temperature, SQR23 was obtained exclusively with 68% yield, instead of the butyl ester deprotected **5**.

Characterization of SQR23. ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.11 (d, *J* 9.0 Hz, 2H), 7.36 – 7.27 (m, 3H), 7.23 – 7.16 (m, 1H), 6.67 (d, *J* 9.2 Hz, 2H), 5.83 (s, 1H), 3.41 – 3.30 (m, 4H), 1.68 (br s, 1H), 1.66 – 1.58 (m, 4H), 1.49 (s, 6H), 1.37 – 1.24 (m, 16H), 0.90 (t, *J* 6.9 Hz, 6H); ^{13}C NMR (101 MHz, CDCl$_3$) δ 187.1, 185.3, 181.6, 181.1, 169.1, 151.0, 141.4, 139.8, 130.7, 128.6, 125.0, 122.5, 118.6, 113.0, 111.7, 89.6, 77.3, 77.0, 76.7, 51.1, 49.9, 31.8, 29.1, 27.4, 27.0, 25.4, 22.6, 14.1; MS (ESI) m/z (M+H) 527.
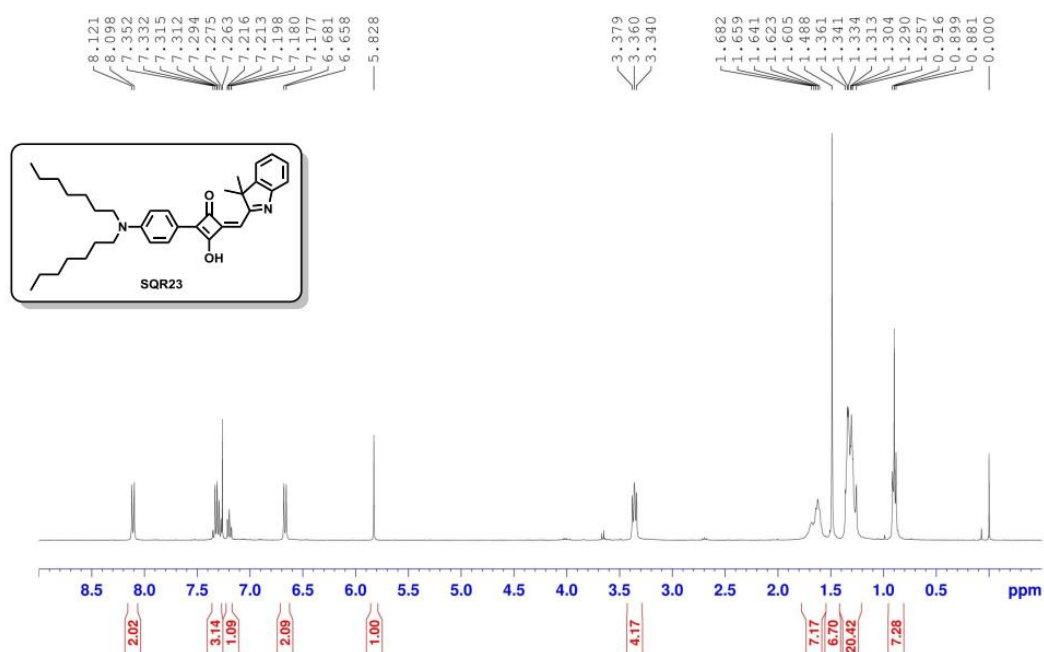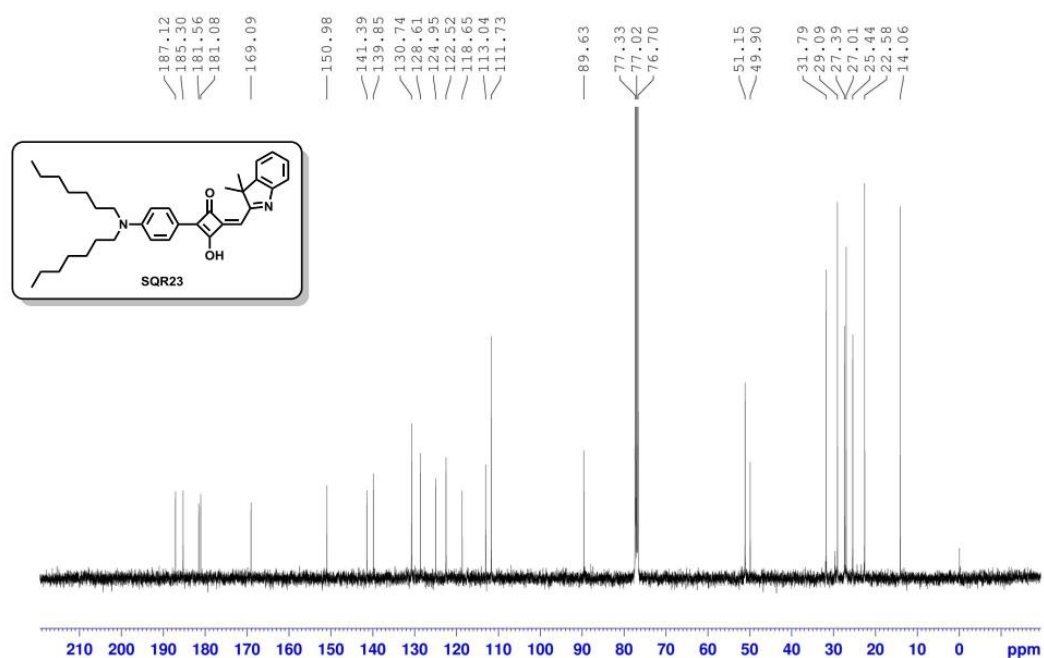
Figure S2. ^{1}H NMR of SQR23

Figure S3. ^{13}C NMR of SQR23
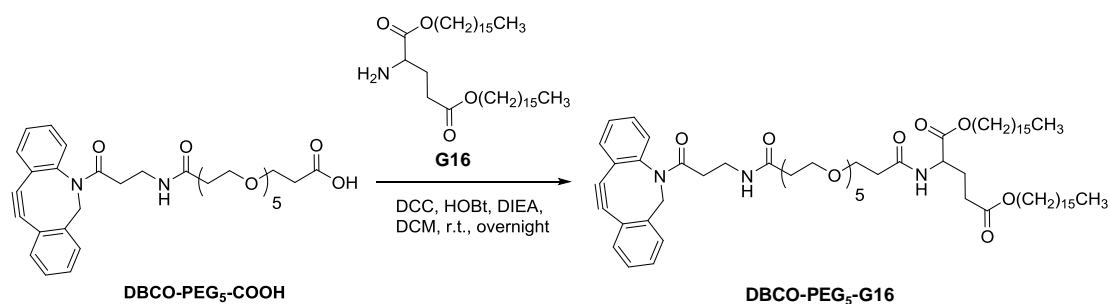
1.2. Synthesis of DBCO-PEG$_5$-G16

Figure S4. Synthesis of DBCO-PEG$_5$-G16

    1, 5-dihexadecyl-L-glutamate (G16) was synthesized according to the previously reported procedures.[S4] 0.01 mmol (1 equiv.) DBCO-PEG$_5$-COOH, 0.02 mmol (2 equiv.) G16, 0.015 mmol (1.5 equiv.) DCC, 0.02 mmol (2 equiv.) HOBt and 0.02 mmol (2 equiv.) DIEA were dissolved in 5 mL DCM. The mixture was kept stirring at room temperature for overnight and then concentrated with rotary evaporator. The mixture was then purified with preparation TLC with eluent as DCM: MeOH= 15: 1. The pure product was obtained with 50% yield.

Characterization of DBCO-PEG$_5$-G16. ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.67 (1 H, d, *J* 7.4), 7.45 – 7.27 (6 H, m), 6.98 (1 H, d, *J* 7.9), 6.56 (1 H, br), 5.13 (1 H, d, *J* 13.9), 4.60 (1 H, td, *J* 7.9, 5.3), 4.07 (4 H, dt, *J* 25.1, 6.8), 3.80 – 3.44 (21 H, m), 3.39 – 3.20 (2 H, m), 2.54 – 2.10 (9 H, m), 1.96 (2 H, m), 1.70 – 1.52 (4 H, m), 1.27 (52 H, s), 0.87 (6 H, t, *J* 6.8). ^{13}C NMR (101 MHz, CDCl$_3$) δ 172.8 , 172.0, 171.4, 171.0, 151.1, 148.1, 132.1, 129.1, 128.6, 128.3, 128.2, 127.8, 127.2, 125.6, 123.1, 122.6, 114.8, 107.8, 70.6, 70.5, 70.4, 70.2, 67.2, 67.1, 65.7, 64.9, 55.5, 51.5, 36.9, 36.8, 35.2, 34.8, 31.9, 30.3, 29.7, 29.6, 29.5, 29.3, 29.2, 28.6, 28.5, 27.6, 25.9, 25.8, 22.7, 14.1; MS (ESI) m/z (M+H) 1174.5.
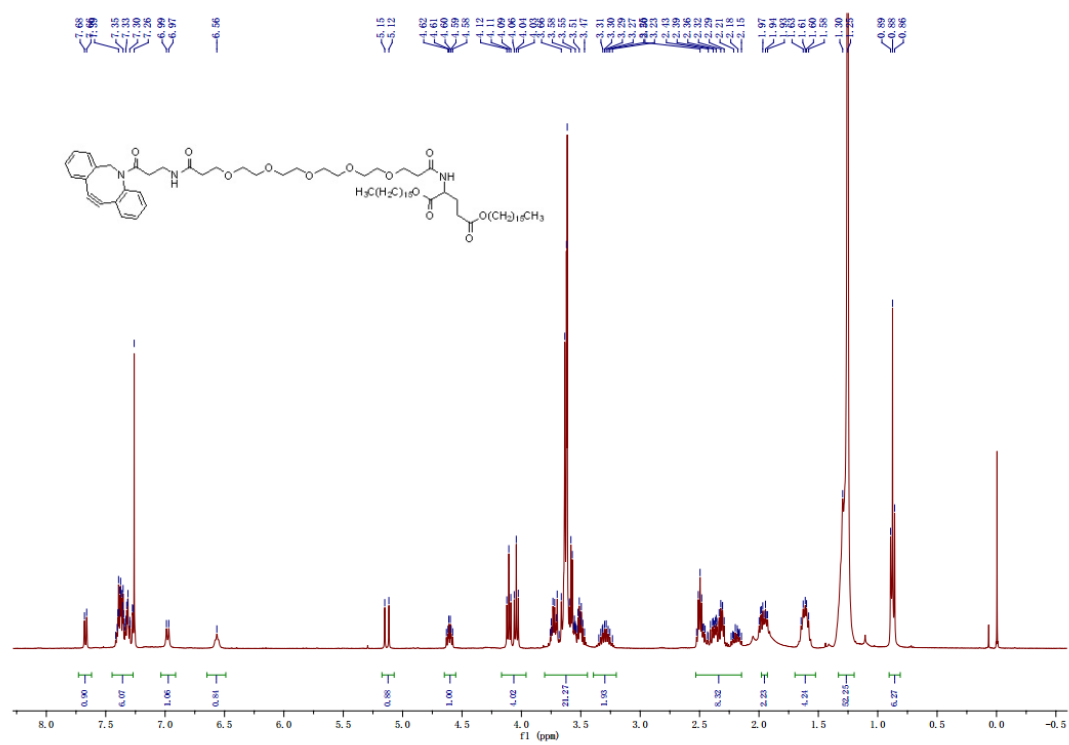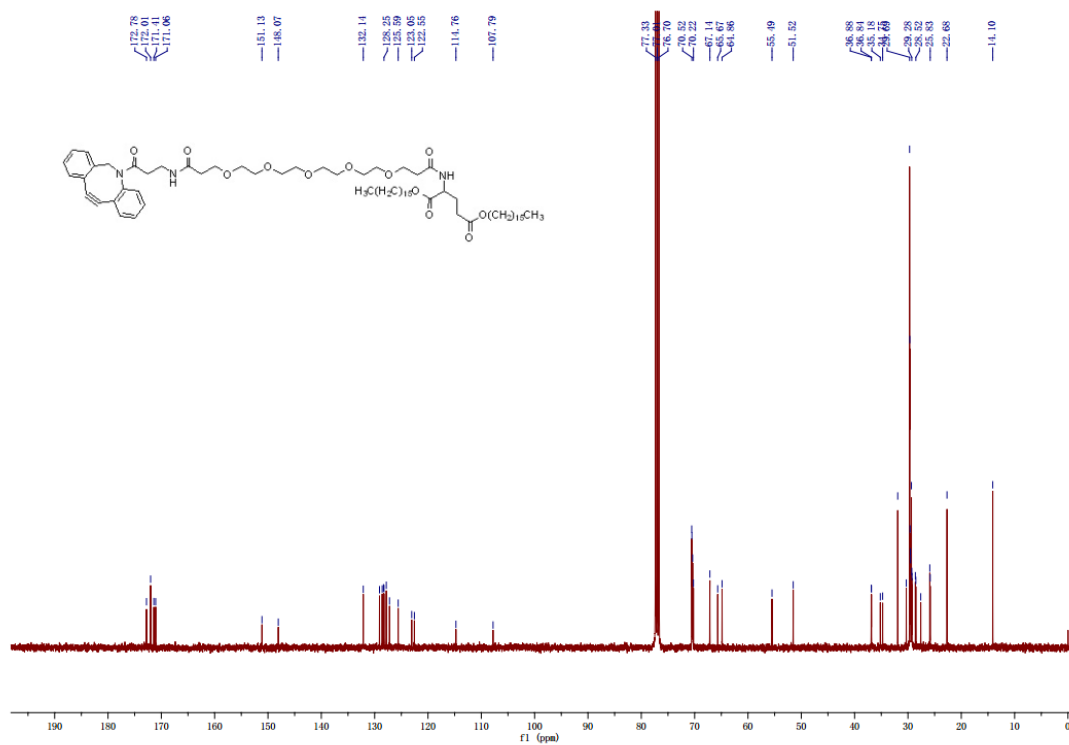
Figure S5. ^{1}H NMR of DBCO-PEG$_5$-G16

Figure S6. ^{13}C NMR of DBCO-PEG$_5$-G16
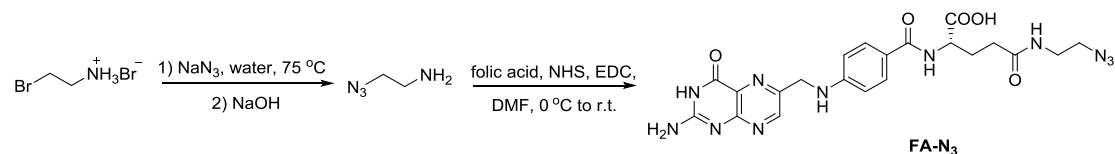
1.3 Synthesis of FA-N₃

Figure S7. Synthesis of FA-N$_3$.

Procedures adapted from published literature (details see S5).

Characterization of FA-N$_3$: ^{1}H NMR (400 MHz, DMSO) δ 8.50 (s, 1H), 8.03 (t, J = 5.4 Hz, 1H), 7.69 (d, J = 8.5 Hz, 2H), 6.76 (s, 2H), 6.58 (d, J = 8.4 Hz, 3H), 4.32 (d, J = 5.0 Hz, 3H), 4.17 – 4.06 (m, 2H), 2.25 – 1.81 (m, 6H); MS (ESI) m/z (M+H) 510.1.
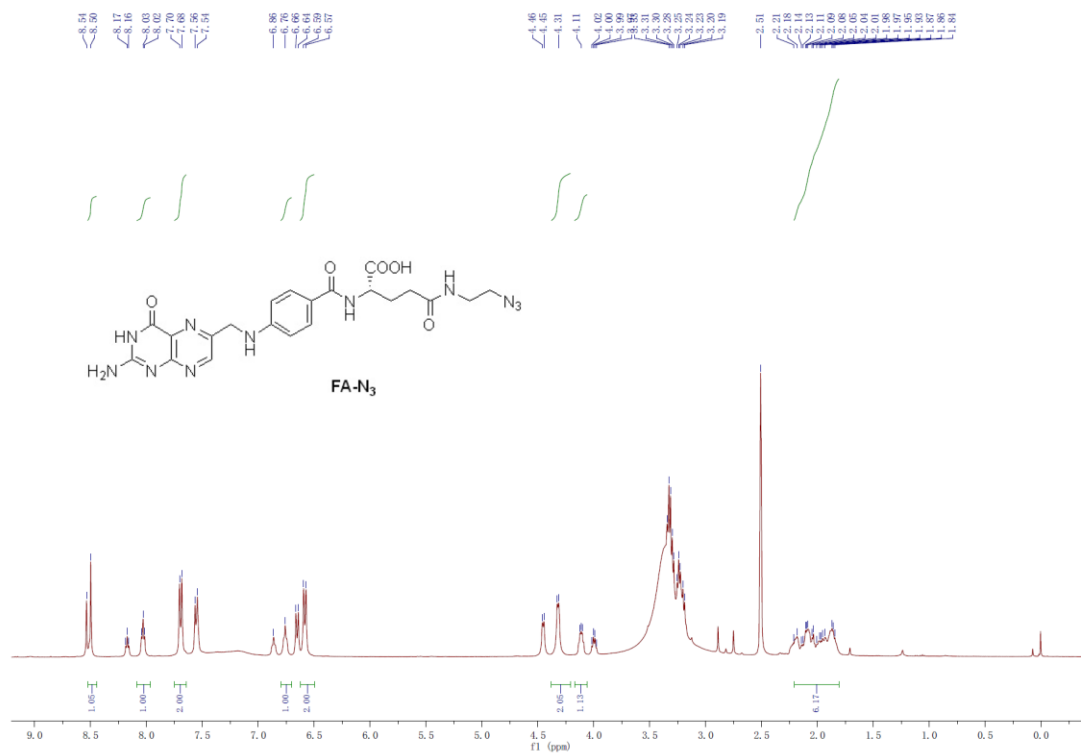

Figure S8. ^{1}H NMR of FA-N$_3$.

2. Spectroscopic properties of SQR23

To measure the spectroscopic properties, SQR23 solutions in different solvents were prepared (10 μM for absorbance spectrum and 1 μM for fluorescence spectrum). The UV-vis-near infrared spectra were measured using a UV-vis spectrophotometer (Cary 60; Agilent Technologies Inc., Santa Clara, USA). The fluorescence spectra were measured using fluorescence spectrophotometer (Agilent Cary Eclipse; Agilent Technologies Inc., Santa Clara, USA).
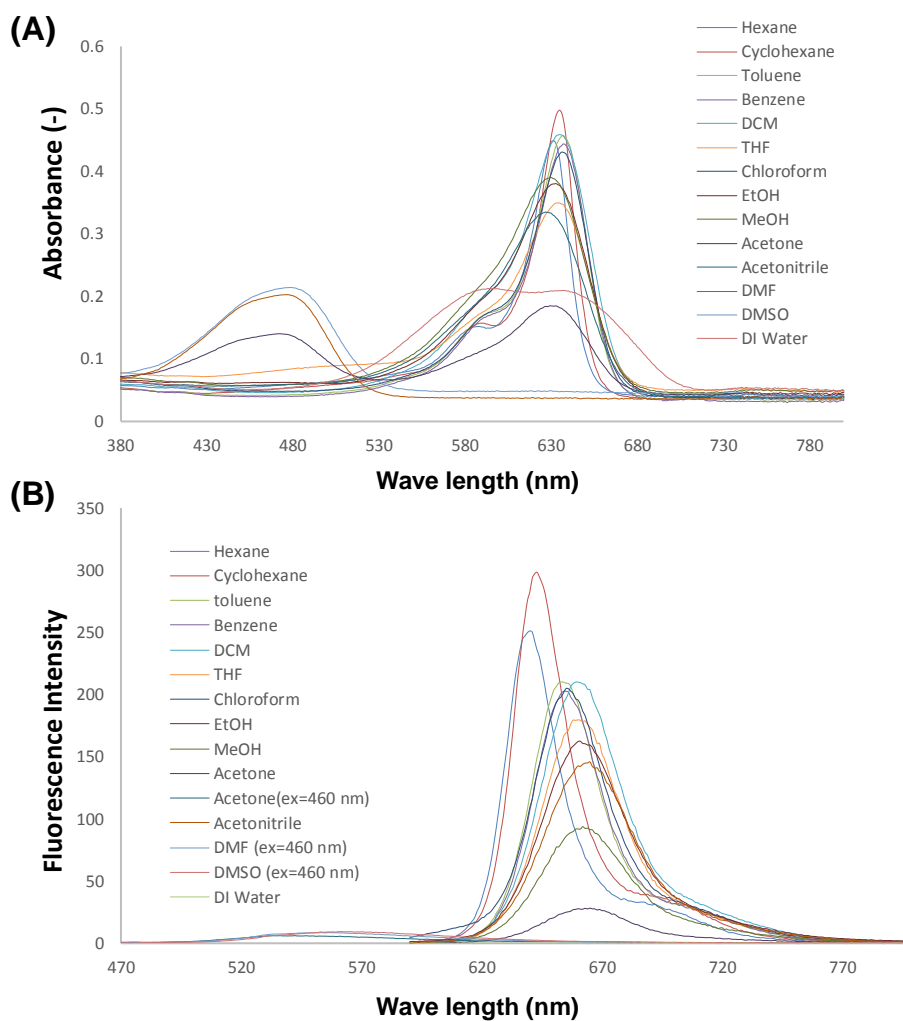
Figure S9. Spectroscopic properties of SQR23. (A) UV-vis-near infrared spectra ([SQR23]=10 μM) and (B) fluorescence emission spectra ([SQR23]=1 μM).

Table S1. Spectroscopic properties of SQR23

Entry	Solvent	Polarity Index	λ_{max} (nm)	λ_{em} (nm)	ϕ
1	Hexane	0.1	631	640	0.34
2	Cyclohexane	0.2	636	643	0.42
3	Toluene	2.4	637	654	0.36
4	Benzene	2.7	637	655	0.36
5	DCM	3.1	635	660	0.34
6	THF	4.0	635	660	0.31
7	Chloroform	4.1	636	655	0.39
8	Ethanol	4.3	633	661	0.28
9	Methanol	5.1	630	662	0.14
10	Acetone	5.1	a: 630 b: 472	a: 665 b: 536	a: 0.08 b: 0.02
11	Acetonitrile	5.8	627	665	0.25

12	DMF	6.4	476	556	0.02
13	DMSO	7.2	479	561	0.02
14	DI water	10.2	594 (broad)	none	0

3. Liposome preparation

Components of liposomes were mixed as ratio of DPPC: SA: PEG-DSPE: SQR23: DBCO-PEG$_5$-G16 = 90: 10: 0.3: 0.1: n (molar ratio, n=0.3, 3, 5). The mixture was fully dissolved in t-BuOH and removed the solvent with freeze drier. 12 mg mixed dry powder was hydrated with 4 mL PBS. Then the dispersion was introduced into an extruder and was extruded through membrane filter for 4 times (pore size was 0.22 μm) under N$_2$ gas pressure. At this stage, we obtained unfunctionalized liposomes in the concentration of 3 mg/ mL.
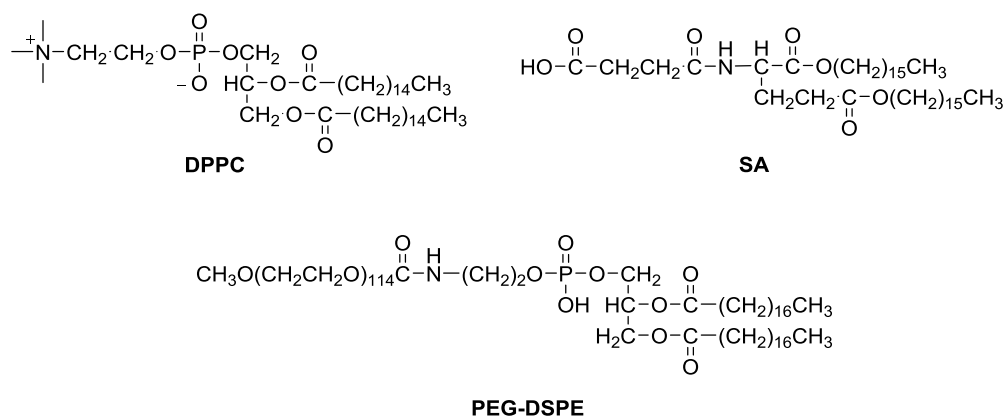

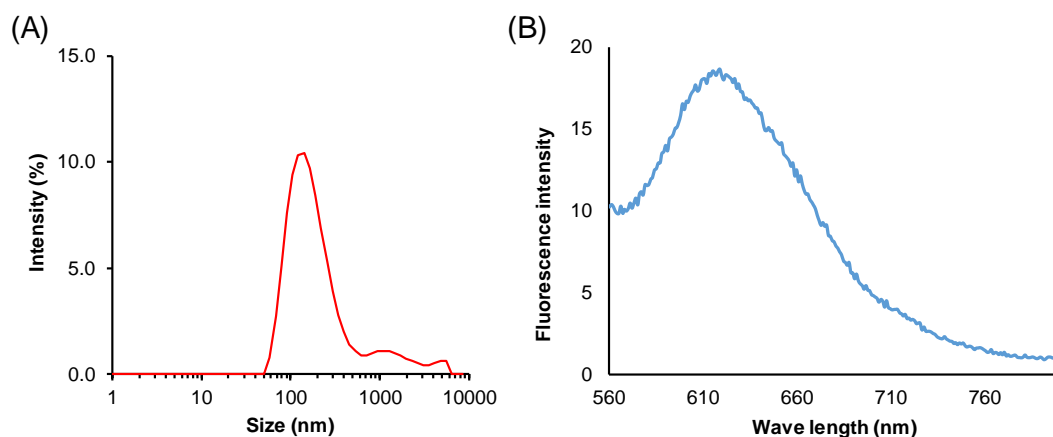**Figure S10.** Chemical structures of DPPC, SA, and PEG-DSPE

Figure S11. Characterization of SQR23-loading liposomes. (A) Size distribution of liposomes measured by dynamic light scattering. (B) Fluorescence emission spectra (λ_{ex}=500 nm).

Unfunctionalized liposome suspension (200 μL) was diluted with PBS to 800 μL. (For 5 mol% sample with doubled concentration, diluted to 400 μL). 0.5 equiv. of

FA-N$_3$ (to DBCO-PEG$_5$-G16) was added to the reaction and the reaction mixture was kept stirring at room temperature for overnight in the dark. The responding liposome suspension was further diluted with PBS to certain concentrations (0.09 mg/ mL, 0.19 mg/ mL and 0.38 mg/ mL). Without further storage, the FA tethered liposomes were used in following bio experiments.

4. Cell culture

SKOV-3 (ATCC #HTB-77) ovarian adenocarcinoma cells were cultured at 37°C, 5% CO$_2$ in folic acid-free RPMI-1640 medium (Gibco, Thermo Fisher Scientific) supplemented with 100 units/mL Penicillin, 100µg/mL Streptomycin and 10% heat inactivated fetal bovine serum (Life Technologies) until confluency. Culture passages 5 to 20 were used for experiments. Upon confluency, culture media was removed from the flask. Cells were washed with sterile 1× phosphate-buffered saline (PBS) (Capricorn Scientific) and treated with 0.05% trypsin-EDTA (Life Technologies, Singapore). Dislodged cells were washed in complete culture media via centrifugation at 300 × g for 5 minutes before assessment of cell viability by Trypan blue staining (Sigma-Aldrich, Singapore). Only cells with viability ≥95% were used for experiments. Cells were either seeded into black 96-well microplates (Greiner Bio-One) or 8-well chamber slides (Nunc, Thermo Fisher Scientific) for subsequent experiments. Seeded microplates and chamber slides were maintained at culture conditions overnight to attain confluency before use for experiments.

5. Fluorescence-based quantitative measurement of folic acid-medicated liposome cellular uptake

SKOV-3 cells were seeded into black 96-well microplates prior to experiments. Upon reaching cell confluency, cell culture medium was removed and cells in each well were washed with PBS 3 times. Liposome suspension (200 µL) was added to the washed cell monolayers in the wells. The microplate was incubated at culture conditions for 2.5 hours for cellular uptake. Liposome suspensions were then removed and the treated cell monolayers were washed 3 times with PBS to remove unbound liposomes. Fluorescence intensity measurements were taken from the cell monolayers in the respective wells of the microplate using a Tecan Infinite m200 microplate reader (Tecan).

6. Confocal microscopy experiment

SKOV-3 cancer cells were seeded into black 8-well chamber slides prior to confocal microscopy imaging. Upon reaching cell confluency, cell culture media was removed and cells in each well were washed with PBS 3 times.

6.1. Visualization of folate receptor-α expression on SKOV-3 cells cultured in folic acid-free cell culture media

PBS-washed confluent cell monolayers in chamber slides were incubated with monoclonal mouse anti-human folate receptor-α IgG1 primary antibody (Thermo Fisher Scientific) at 10µg/mL for 1 hour and then washed twice with wash buffer of

0.1% bovine serum albumin (BSA) in PBS. Cell monolayers labelled with primary antibody were then incubated with polyclonal fluorescein-conjugated goat anti-mouse IgG secondary antibody (Thermo Fisher Scientific) at 10μg/mL for 1 hour and then washed twice with wash buffer. The chamber was removed from the slide and the cell monolayers were covered with glass coverslips and antifade mountant (Thermo Fisher Scientific) before analysis on an Olympus FV1000 confocal microscope (Olympus).
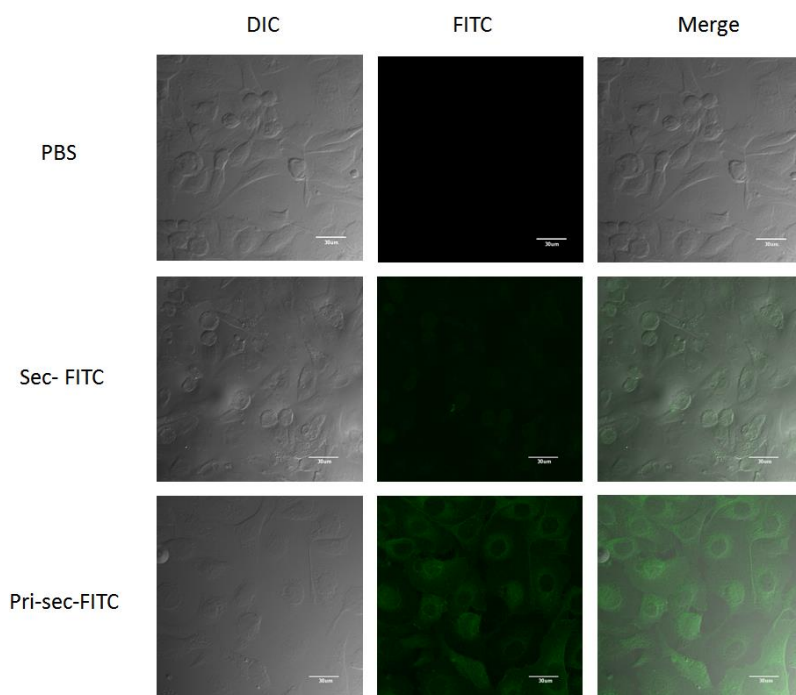

Figure S12. Confocal images of SKOV-3 cells non stained (PBS), stained with polyclonal fluorescein-conjugated goat anti-mouse IgG secondary antibody (Sec-FITC), and stained with folate receptor-α IgG1 primary antibody and then polyclonal fluorescein-conjugated goat anti-mouse IgG secondary antibody (Pri-sec-FITC). The scale bars represent 30 μm.

6.2. Visualization of folic acid-mediated fluorophore-liposome cellular uptake

Liposome suspension or PBS (200 μL) was added to PBS-washed cell monolayers in a chamber slide. The chamber slide was incubated at culture conditions for 2.5 hours for cellular uptake. Liposome suspensions or PBS were then removed by aspiration and the treated cell monolayers were washed 3 times with PBS to remove unbound liposomes. The chamber wall was removed from the slide and the cell monolayers were covered with glass coverslips and antifade mountant (Thermo Fisher Scientific) before analysis on an Olympus FV1000 confocal microscope (Olympus). The fluorescence intensity of ROI on the cells in the confocal images was analyzed using ImageJ software (ImageJ v. 1.51j8, National Institutes of Health, USA).
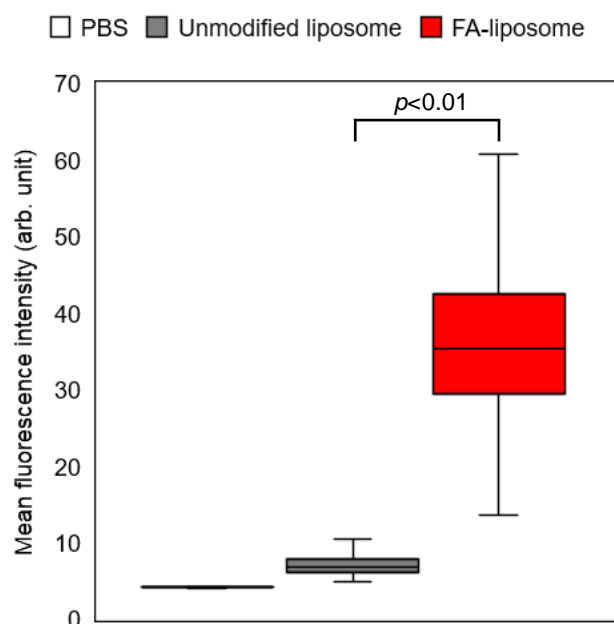
□ PBS ■ Unmodified liposome ■ FA-liposome

Figure S13. Box-whisker plot showing mean fluorescence intensity (MFI) of SKOV-3 ovarian cancer cells treated with PBS (background) (n=36), unmodified liposomes (n=39), and FA-liposomes (n=43). Fluorescence intensity analyses were done from confocal images in Figure 4B. Whiskers indicate 0% and 100% quartiles; the boxes indicate 25% and 75% quartiles; the horizontal lines in the boxes indicate the 50% quartile. Whiskers of FA-liposome box indicates that 100% of FA-liposome-treated SKOV-3 cells had higher MFI than SKOV-3 cells treated with unmodified liposomes. Statistical analysis by Student's *t*-test was conducted on MFI data. MFI of SKOV-3 cells treated with FA-liposomes was found to be significantly higher than that of SKOV-3 cells treated with unmodified liposomes ($p<0.01$).

References

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