Supporting Information

Active Loading Liposomal Cyanine 5.5 Derivatives with Deep Self-quenching Property and Its Applications in Deep Tissue Imaging

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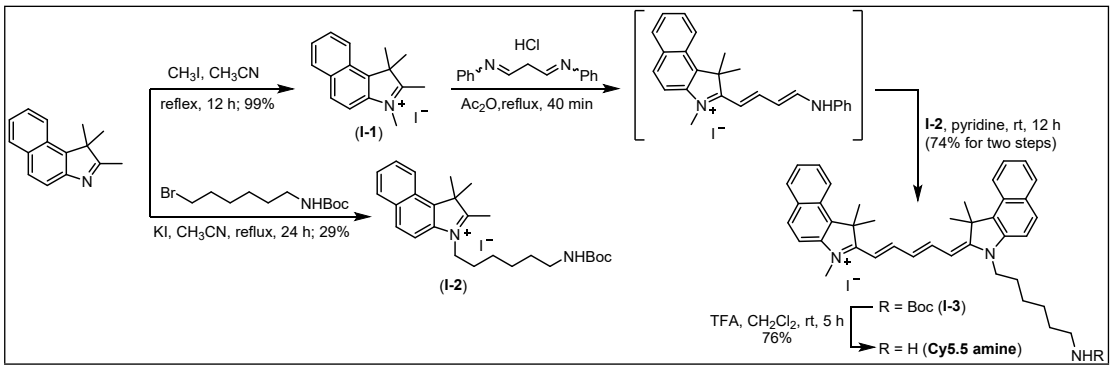
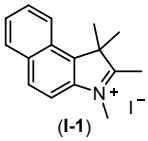
Experimental Section

Synthesis of Cy5.5 amine:



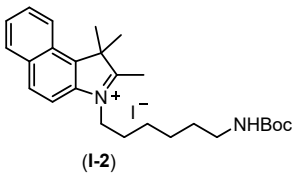
1,2,3,3-Tetramethylbenz[*e*]indolium iodide (I-1)



(**I-1**)

1,1,2-Trimethylbenz[*e*]indole (3 g, 14.3 mmol) was dissolved in acetonitrile (15 mL), and then iodomethane (1.86 mL, 30 mmol) was added. The reaction mixture was heated to reflux for 12 h, and then cooled to room temperature. The precipitate was collected by filtration, washed with diethyl ether, and dried in vacuo. The desired product (**I-1**) was obtained as a pale olive solid (4.98 g, 99% yield). mp 248-249 °C; TLC (5% methanol in CH_2Cl_2) R_f = 0.25; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.35 (d, J = 8.5 Hz, 1H), 8.28 (d, J = 8.5 Hz, 1H), 8.20 (d, J = 8.1 Hz, 1H), 8.09 (d, J = 8.5 Hz, 1H), 7.77 (td, J = 7.0, 1.0 Hz, 1H), 7.70 (td, J = 7.0, 1.0 Hz, 1H), 4.08 (s, 3H), 2.86 (s, 3H), 1.74 (s, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 195.8, 139.4, 136.4, 132.9, 130.4, 129.7, 128.3, 127.0, 123.3, 113.1, 55.2, 35.0, 21.2, 13.9; HRMS (ESI) m/z: $[M-I]^+$ Calcd for $C_{16}H_{18}N$ 224.1434; Found 224.1438.

1-(6-*N*-((*tert*-Butoxycarbonyl)amino)hexyl)-2,3,3-trimethyl benz[*e*]indolium iodide (I-2)

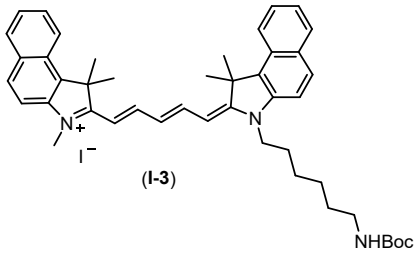


(**I-2**)

Potassium iodide (KI, 3.98 g, 24 mmol) was suspended in acetonitrile (20 mL), and then *tert*-butyl 6-bromohexylcarbamate (3.39 g, 12 mmol) was added. The reaction mixture was stirred at 50 °C for 10 min, and then 1,1,2-trimethylbenz[*e*]indole (3.14 g, 15 mmol) was added. The resulting mixture was heated to reflux for 24 h, and then cooled to room temperature. The reaction mixture was diluted with CH_2Cl_2 (100 mL)

and washed with water. The organic layer was separated and dried over $MgSO_4$, filtered and concentrated. The residue was dissolved in a small volume of CH_2Cl_2 and precipitated with diethyl ether. The precipitate was filtered off, washed with diethyl ether and dried in vacuo. The desired product (**I-2**) was obtained as a pale-blue solid (1.86 g, 29% yield). mp 51-52 °C; TLC (5% methanol in CH_2Cl_2) R_f = 0.25; ^{1}H NMR (500 MHz, DMSO-d_6)δ 8.36 (d, J = 8.4 Hz, 1H), 8.26 (d, J = 9.0 Hz, 1H), 8.20 (d, J = 9.0 Hz, 1H), 8.13 (d, J = 9.0 Hz, 1H), 7.77 (td, J = 7.0, 1.0 Hz, 1H), 7.71 (td, J = 7.0, 1.0 Hz, 1 H), 6.74 (t, J = 5.5 Hz, 1H), 4.56 (t, J = 7.6 Hz, 2H), 2.93 (s, 3H), 2.90-2.86 (m, 2H), 1.90-1.83 (m, 2H), 1.75 (s, 6H), 1.46-1.40 (m, 2H), 1.38-1.35 (m, 2H), 1.31 (s, 9H), 1.30-1.29 (m, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 196.2, 155.5, 138.4, 136.9, 133.0, 130.6, 129.6, 128.3, 127.2 (2 x), 123.4, 113.2, 77.2, 55.4, 47.8, 29.1, 28.2, 27.4, 25.8, 25.4, 21.5, 13.8; HRMS (ESI) m/z: $[M-I]^+$ Calcd for $C_{26}H_{37}N_2O_2$ 409.2850; Found 409.2855.

2-((1*E*,3*E*,5*E*)-5-(1-(6-((*tert*-butoxycarbonyl)amino)hexyl)-3,3-dimethyl-1,3-dihydro-2*H*-benzo[*f*]indol-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3*H*-benzo[*f*]indolium iodide (I-3)

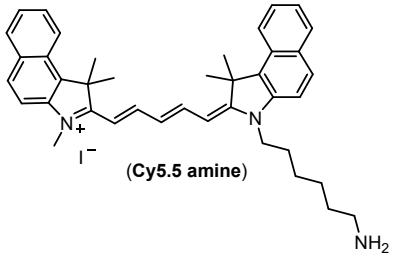


(**I-3**)

1,2,3,3-Tetramethylbenz[*e*]indolium iodide (351 mg, 1 mmol) and malonaldehyde bis(phenylimine) monohydrochloride (311 mg, 1.2 mmol) in acetic anhydride (5 mL) were heated to reflux under nitrogen atmosphere. After 40 min, the reaction was cooled to room temperature and a solution of compound **I-2** (536 mg, 1 mmol) in anhydrous pyridine (4.3 mL) was added. The reaction mixture was stirred at room temperature for 20 h, and then precipitated with diethyl ether. The precipitate was filtered off, washed with diethyl ether and dried in vacuo. The crude product was purified by column chromatography on silica gel (EtOAc to 5% methanol in EtOAc) to afford the desired product (**I-3**) as a dark-purple solid (586 mg, 74% yield). mp 176-177 °C; TLC (10% methanol in EtOAc) R_f = 0.28; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.39 (t, J = 13.0 Hz, 2H), 8.13 (dd, J = 13.0, 8.6 Hz, 2H), 7.88 (d, J = 8.6 Hz, 4H), 7.56 (t, J = 7.8 Hz, 2H), 7.41 (td, J = 8.3, 2.8 Hz, 2H), 7.36 (d, J = 8.8 Hz, 1H), 7.32 (d, J = 8.8 Hz, 1H), 6.80 (t, J = 12.3 Hz, 1H), 6.26 (dd, J = 13.4, 6.6 Hz, 2H), 4.62 (br s, 1H), 4.12 (t, J = 6.7 Hz, 2H), 3.78 (s, 3H), 3.09-3.08 (m, 2H), 2.06 (s, 6H), 2.02 (s, 6H), 1.84-1.81 (m, 2H), 1.49-1.43 (m, 6H), 1.38 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.6, 174.3, 156.0, 152.9, 152.8,

139.9, 139.1, 134.2, 133.6, 131.6, 130.4, 129.8 (x 2), 128.1, 127.6 (x 2), 126.2, 124.9, 124.8, 122.3, 122.2, 110.3 (2 x), 103.3, 103.1, 78.9, 51.3, 51.1, 44.4, 40.2, 32.7, 29.7, 28.3, 27.7, 27.5, 26.4, 26.3; HRMS (ESI) *m*/*z*: $[M\text{-}I]^+$ Calcd for $C_{45}H_{54}N_3O_2$ 668.4211; Found 668.4217.

2-((1*E*,3*E*,5*E*)-5-(1-(6-aminohexyl)-3,3-dimethyl-1,3-dihydro-2*H*-benzo[*f*]indol-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3*H*-benzo[*f*]indolium iodide (Cy5.5 amine)



To a solution of compound **I-3** (35 mg, 44 μmol) in anhydrous CH_2Cl_2 (2 mL) was added trifluoroacetic acid (TFA, 2 mL). The reaction mixture was stirred at room temperature for 5 h, and then was concentrated. The residue was solidified and washed with diethyl ether to afford the desired product as a dark-purple solid (as a TFA salt, 27 mg, 76% yield). mp 154-155 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.06 (d, *J* = 8.4 Hz, 2H), 7.94 (t, *J* = 12.1 Hz, 2H), 7.87-7.84 (m, 4H), 7.60 (br s, 2H), 7.57-7.53 (m, 2H), 7.39 (dd, *J* = 15.0, 7.5 Hz, 2H), 7.29 (dd, *J* = 8.6, 4.3 Hz, 2H), 6.55 (t, *J* = 12.2 Hz, 1H), 6.11 (dd, *J* = 12.8, 8.9 Hz, 2H), 4.0 (br s, 2H), 3.60 (s, 3H), 3.0 (br s, 2H), 1.94 (s, 6H), 1.93 (s, 6H), 1.77-1.69 (m, 4H), 1.42-1.32 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.4, 174.1, 151.9, 151.8, 139.8, 139.1, 133.7, 133.4, 131.8, 130.7, 130.6, 130.0, 128.0, 127.7, 125.4, 125.0 (2 x), 122.0, 110.3, 110.2, 103.0, 102.8, 51.0, 50.9, 44.0, 39.9, 31.3, 27.5, 27.4, 27.1, 26.8, 25.9, 25.5; HRMS (ESI) *m*/*z*: $[M\text{-}I]^+$ Calcd for $C_{40}H_{46}N_3$ 568.3686; Found 568.3688.

Particle Size and Zeta Potential of Cy5.5-loaded Liposomes.

The particle size and ζ-potential characterizations of all Cy5.5-loaded liposomes were analyzed using the dynamic light scattering (DLS). The liposome suspension was diluted 100 times with HEPES buffer (pH 7.4) before being measured for particle size and ζ-potential on a Zetasizer Ultra (Malvern Panalytical) at 25 °C. Disposable polystyrene cuvettes were employed to assess particle size, while a folded capillary cell (DTS 1070) was utilized to measure zeta potential.

Cryo-EM Imaging of Cy5.5-loaded Liposomes.

For the cryo-EM imaging of Cy5.5-loaded liposomes, sample concentrations were adjusted to ~0.7 mM of total lipid and imaged by the FEI Tecnai G2 F20 TWIN TEM (FEI, Hillsboro). Approximately 4 μL of liposome sample was dropped onto a 200-mesh carbon-coated grid (HC200-Cu, Electron Microscopy Sciences) that had been glow-discharged in an Ar/O_2 atmosphere for 15 seconds, paper-blotted for 3 seconds in a 100% humidified chamber at 4 °C, and then plunge-frozen into liquid ethane using a Vitrobot system (FEI, Hillsboro) to generate vitreous ice. The frozen grids were then kept in liquid nitrogen until imaged. In bright-field mode, EM imaging was conducted at 200 kV. Images were captured at a magnification of 50,000 with a high-resolution 4k × 4k CCD camera (Glatan, Pleasanton) at a defocus value of ~1.8 μm under low-dose conditions (25-30 e/Å^2). All experiments were carried out at the Academia Sinica Cryo-EM Facility (Taipei, Taiwan).

Stability of Cy5.5-Loaded Liposomes in Various Media.

The stability of Cy5.5-loaded liposomes was evaluated by dye release over time. The release of encapsulated Cy5.5 dyes from liposomes was detected using EnSpire Multimode Plate Readers (PerkinElmer) at an excitation wavelength of 685 nm and fluorescence emission wave-length of 707 nm. Twenty microliters of size exclusion-purified Cy5.5-loaded liposomes were incubated with 180 μL of HEPES buffer solution/fetal bovine se-rum/DMEM/RPMI at 37 °C for 7 days. The percentage of Cy5.5 dye released was calculated as $(I_T-I_0)/(I_{max}-I_0) \times 100\%$, where I_T represents the fluorescence at time point T, I_0 represents the fluorescence at the start of the incubation time, and I_{max} represents the maximal fluorescence after 5 μL of 30% Triton X-100 was added.

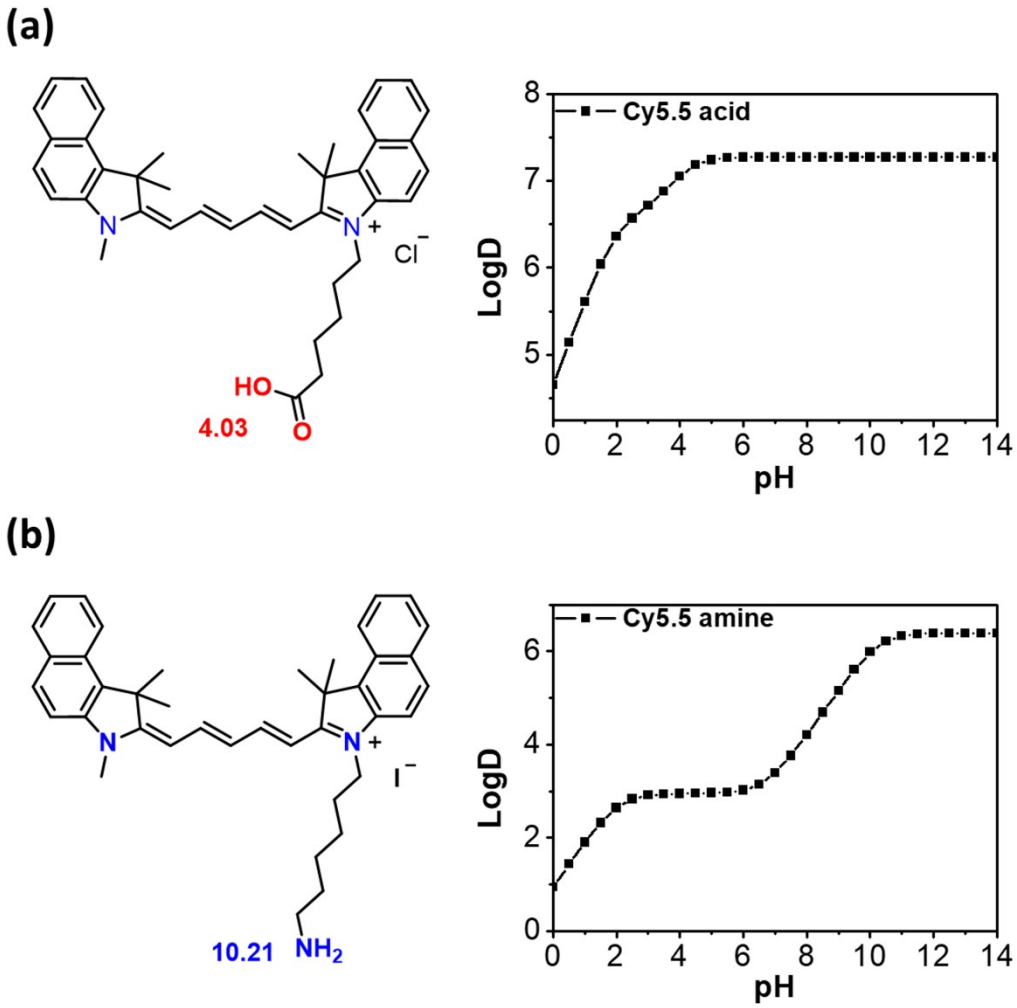


Figure S1. The structure, pKa of ionizable heteroatoms, and logD profile for (a) Cy5.5 acid and (b) Cy5.5 amine were calculated by ChemAxon software.

Table SI-1. Particle size and ζ-potential analysis of Cy5.5-liposomes with the candidate trapping agents at a D/L ratio of 0.02.

Liposome composition		Particle Size (nm)	PDI	ζ-Potential (mV)
Gradient-free liposome	Blank	116.4±0.4	0.027±0.011	-0.42±0.49
	Cy5.5 acid	117.9±0.5	0.023±0.017	-0.95±1.36
	Cy5.5 amine	118.4±0.7	0.033±0.019	-1.23±0.55
Ca(OAc)2-liposome	Blank	122.3±0.7	0.047±0.016	-0.94±0.61
	Cy5.5 acid	113.1±1.3	0.018±0.015	-4.47±0.85
	Cy5.5 amine	116.2±0.9	0.023±0.020	-1.93±0.65
La(OAc)3-liposome	Blank	110.0±1.5	0.020±0.015	-1.65±0.79
	Cy5.5 acid	118.1±1.9	0.057±0.020	-1.73±0.71
	Cy5.5 amine	109.7±0.9	0.041±0.008	-3.06±0.32
NH4(SO4)2-liposome	Blank	139.8±1.7	0.038±0.030	-1.47±0.55
	Cy5.5 acid	140.6±0.5	0.032±0.018	-2.02±0.98
	Cy5.5 amine	140.1±1.0	0.053±0.023	-1.59±0.50
ASO-liposome	Blank	115.6±0.5	0.02±0.017	-0.94±0.26
	Cy5.5 acid	115.2±0.5	0.04±0.024	-0.73±0.74
	Cy5.5 amine	111.7±0.8	0.06±0.014	-1.57±0.61

Table SI-2. Particle size and ζ-potential analyses of ASO-Cy5.5-liposomes at different D/L ratios.

Liposome composition	D/L ratio	Particle Size (nm)	PDI	ζ-Potential (mV)
ASO-Liposome	Blank	119.5±1.2	0.039±0.025	-2.50±1.29
ASO- Cy5.5 acid-liposome	0.02	119.7±0.5	0.026±0.012	-1.44±0.59
	0.05	117.4±0.7	0.046±0.009	-3.89±1.93
	0.075	116.0±0.2	0.038±0.029	-0.11±0.14
	0.10	113.7±0.4	0.057±0.019	-1.76±0.68
	0.20	109.6±0.4	0.058±0.006	-0.01±0.02
ASO-Cy5.5 amine-liposome	0.02	116.7±1.2	0.049±0.011	0.04±0.21
	0.05	114.0±0.2	0.067±0.008	-2.87±0.57
	0.075	116.9±0.2	0.078±0.026	-3.11±0.51
	0.10	125.1±0.9	0.070±0.010	-3.10±1.00
	0.20	218.4±4.0	0.227±0.009	-1.99±0.25

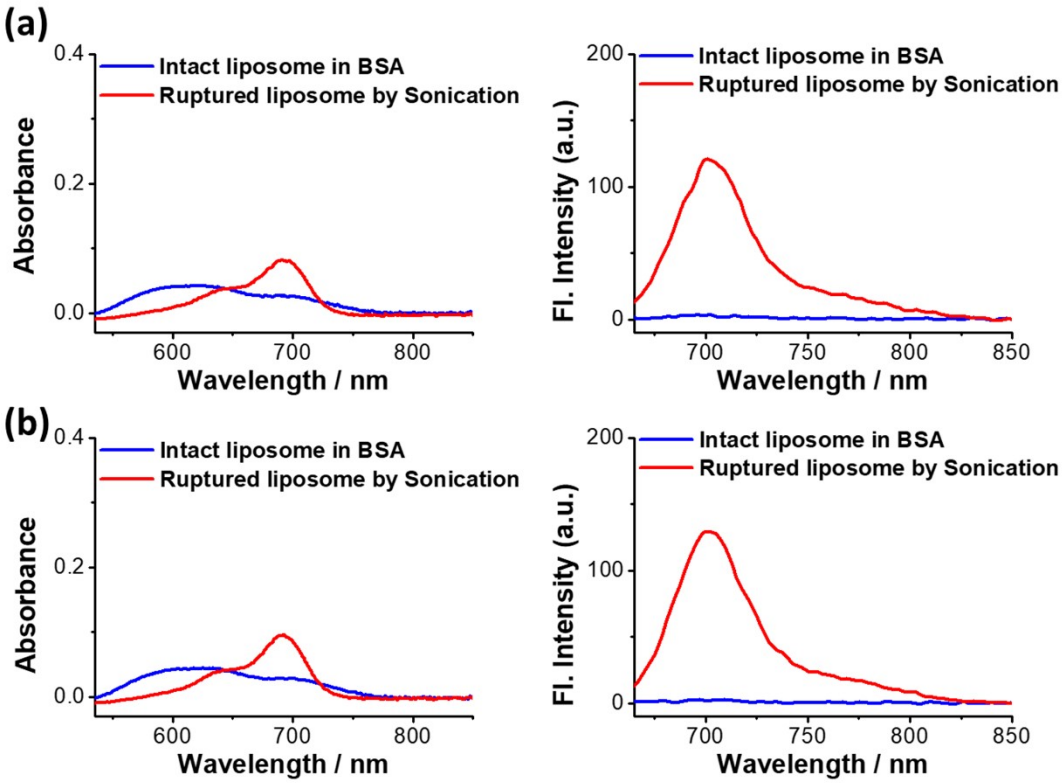


Figure S2. Absorption and fluorescence spectrum of the Cy5.5-liposomes before and after rupture by sonication. BSA was added to the buffer to bind and rigidify the structure of the released Cy5.5 dyes for fluorescence enhancement purposes. (a) Cy5.5 acid-loaded liposomes at a D/L ratio of 0.02 (b) Cy5.5 amine-loaded liposomes at a D/L ratio of 0.02. This result shows that Triton X-100 does not significantly affect fluorescence.

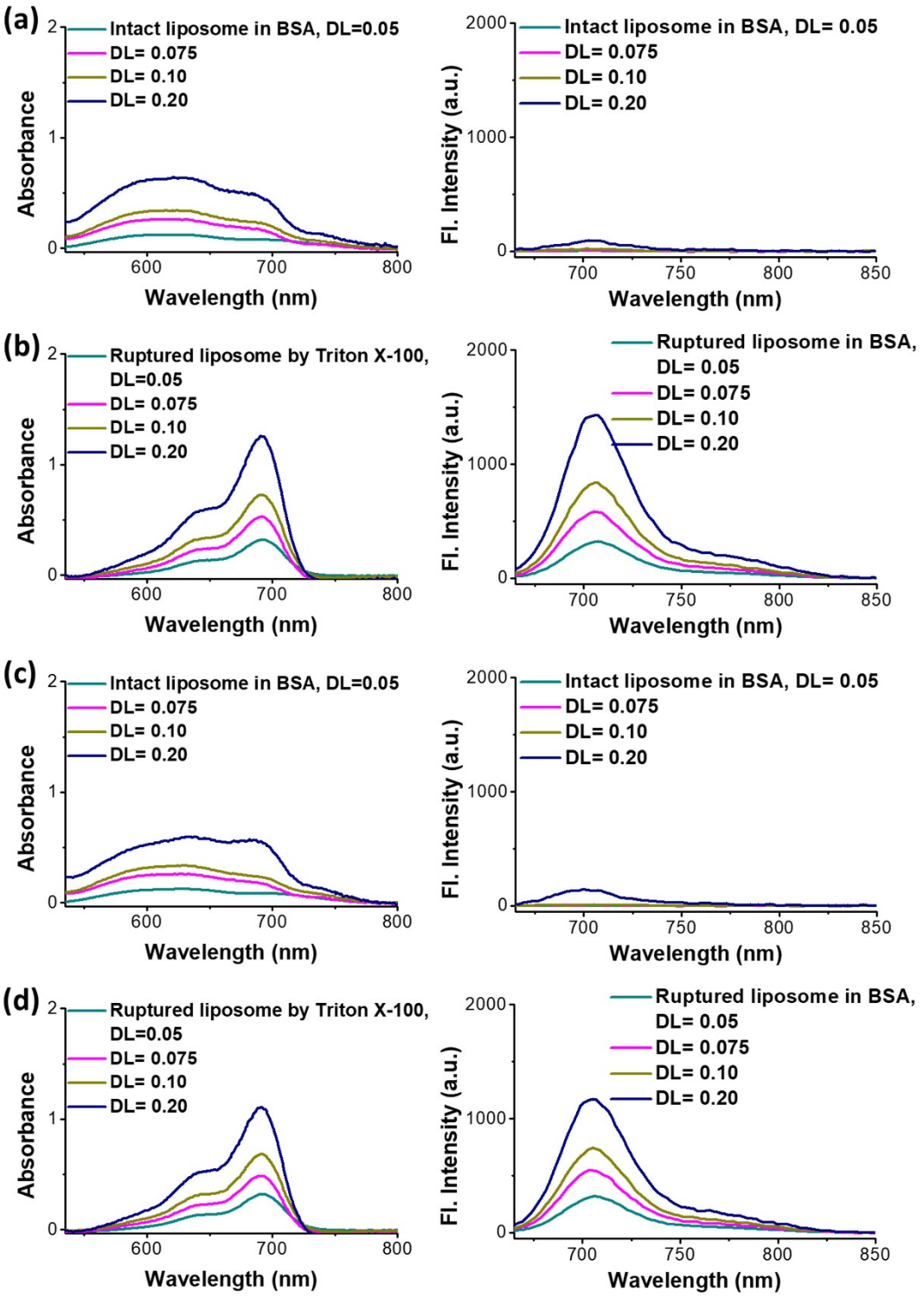


Figure S3. Absorption and fluorescence spectrum of the Cy5.5-liposomes before and after rupture by Triton X-100. (a) Cy5.5 acid-loaded liposomes at different D/L ratios before rupture. (b) Cy5.5 acid-loaded liposomes at different D/L ratios after rupture. (c) Cy5.5 amine-loaded liposomes at different D/L ratios before rupture. (d) Cy5.5 amine-loaded liposomes at different D/L ratios after rupture.

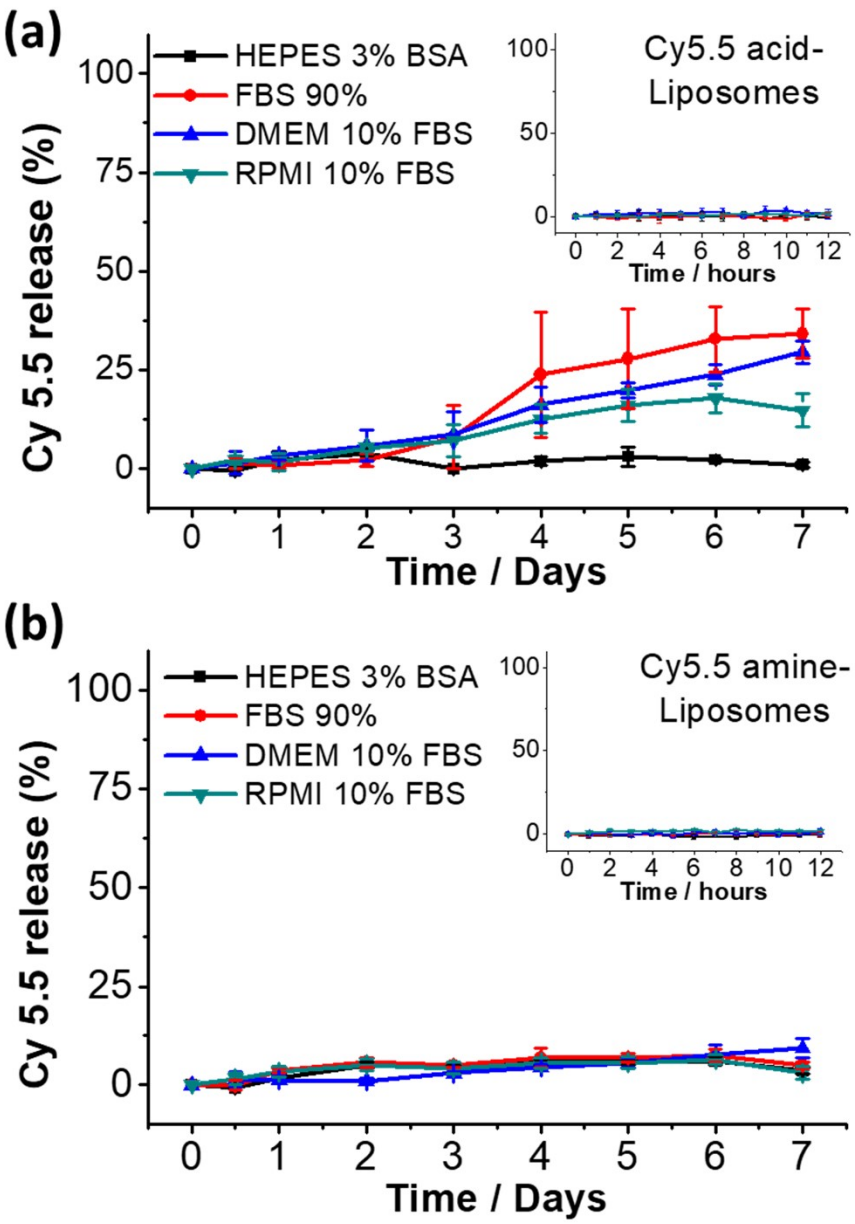


Figure S4. Stability test of Cy5.5-liposomes in various media at 37 °C for 7 days. (a) Cy5.5 acid-loaded liposomes and (b) Cy5.5 amine-loaded liposomes. All liposomes were at a D/L ratio of 0.02. Inset figures are stability test of Cy5.5-liposomes in the early stage (within 12 hours).

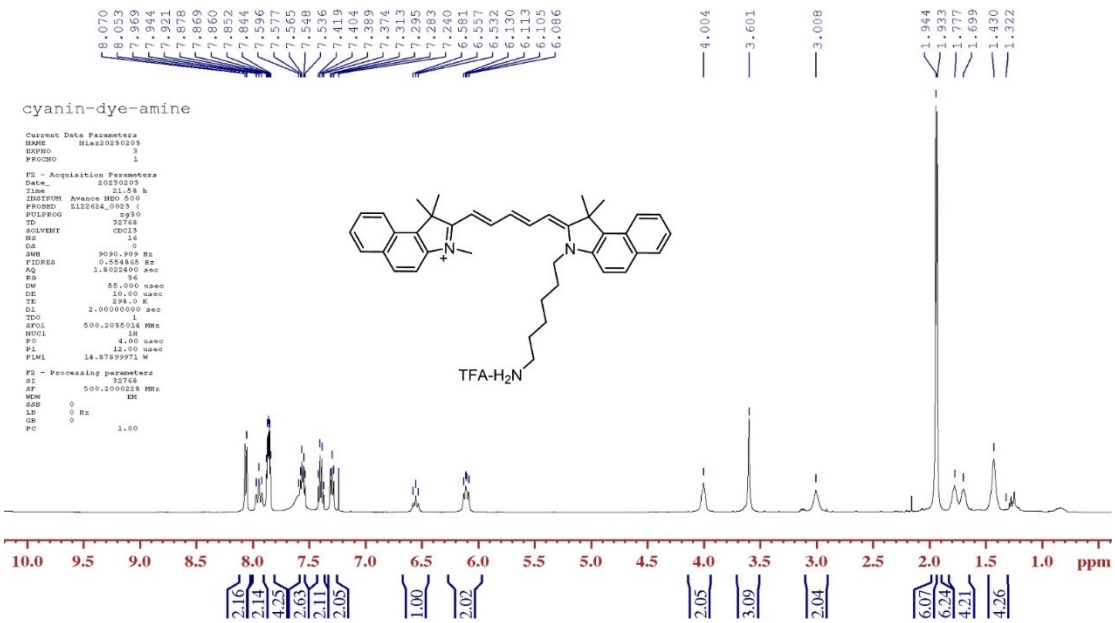


Figure S5. ^{1}H NMR spectrum of compound **Cy5.5 amine** (500 MHz, $CDCl_3$)

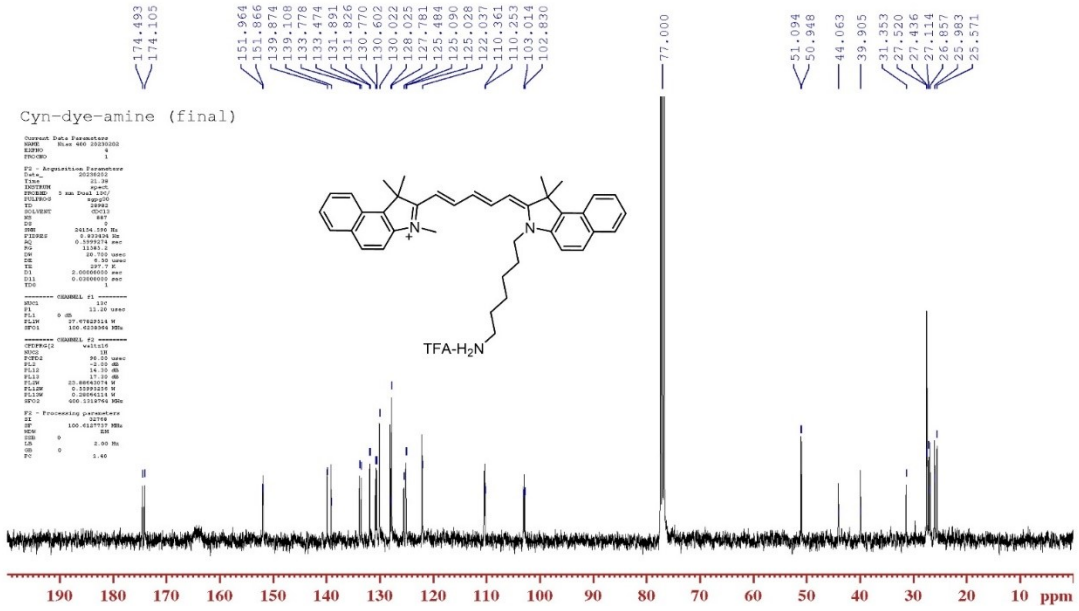


Figure S6. ^{13}C NMR spectrum of compound **Cy5.5 amine** (100 MHz, $CDCl_3$)

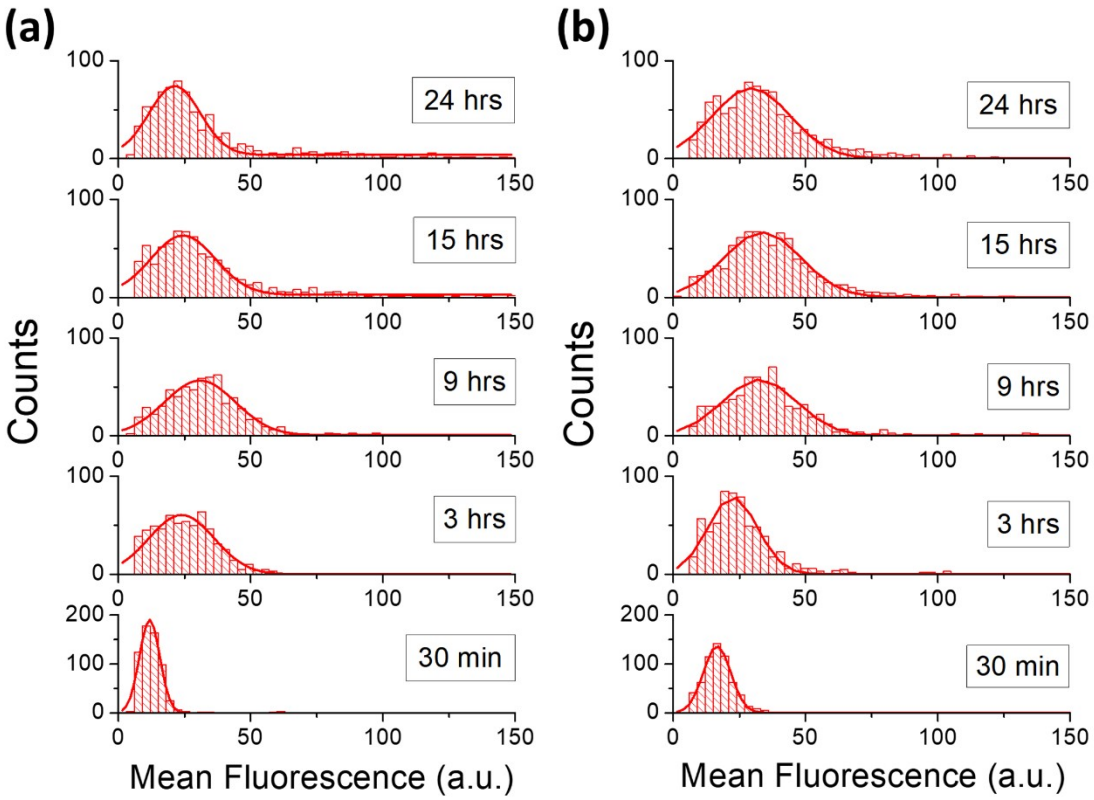


Figure S7. (a) Histogram of A549 treated with Cy5.5 acid-loaded liposomes, (b) Histogram of A549 treated with Cy5.5 amine-loaded liposomes. The histograms are binned with intervals of 3, and the fitting curve was generated using the Gaussian method.

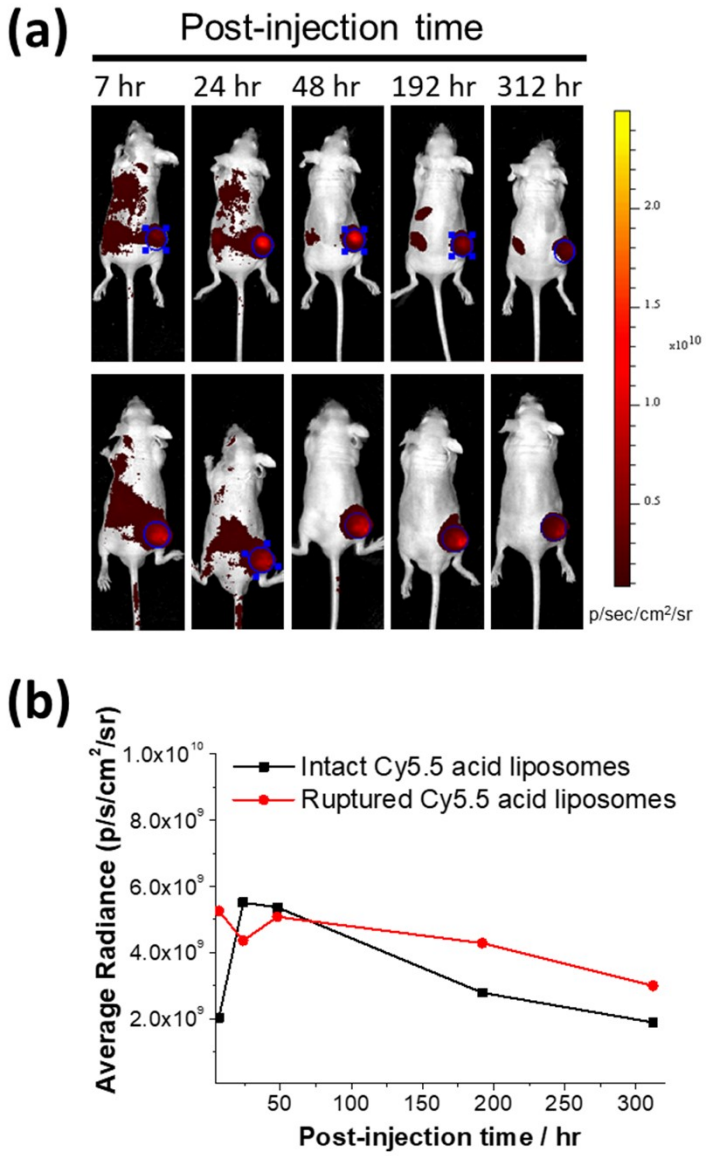


Figure S8. *In vivo* fluorescence imaging. (a) Time course of mouse imaging with IVIS system after intratumor injection of Cy5.5 acid-loaded liposomes into KB tumor-bearing mice. (b) Quantification of fluorescence of IVIS images from regions and time points of interest after intratumor injection of Cy5.5 acid-loaded liposomes. Cy5.5 acid liposomes were directly injected 20 μL liposome (with ~0.26 nmoles Cy5.5 acid) into the intratumor site.