

Fast tumor imaging using pH-responsive aggregation of cyanine dyes with rapid clearance

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1. Experimental Section

1.1 Materials and methods

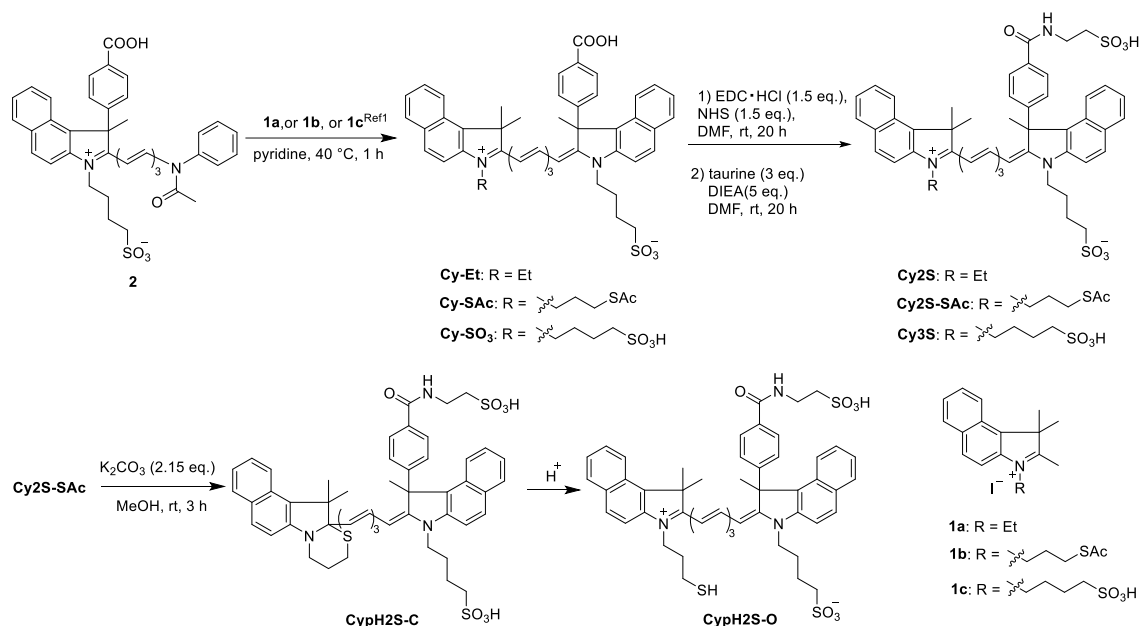
Dichloromethane (CH_2Cl_2), pyridine, dimethylsulfoxide (DMSO), hydrogen peroxide (H_2O_2), methanol (MeOH), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl), sodium hypochlorite solution (NaClO), acetonitrile (MeCN) and Dulbecco's modified Eagle's medium (DMEM) with/without phenol red were purchased from FUJIFILM Wako Pure Chemical Inc. (Japan). Triton X-100, potassium carbonate (K_2CO_3), *N*-hydroxysuccinimide (NHS), *N,N*-dimethylformamide (DMF), *N,N*-diisopropylethylamine (DIEA), acetone, cysteine (Cys), glutathione (GSH), glutamic acid (Glu), glycine (Gly), lysine (Lys), bovine serum albumin F-V (BSA), and chloroform (CHCl_3) were purchased from Wako Pure Chemical Inc. (Japan). Taurine was purchased from Tokyo Chemical Industry Co. Ltd. (Japan). Anhydrous solvents were distilled over CaH_2 under N_2 atmosphere. Silica gel (SiO_2 , 230–400 mesh) for column chromatography was purchased from Silicycle (Canada). Wakogel 50C18 (octadecyl, 38–63 μm) for reversed-phase column chromatography was purchased from FUJIFILM Wako Pure Chemical Inc. (Japan). Saline solution for in vivo experiments was purchased from Otsuka Pharmaceutical Factory, Inc. Japan. Isoflurane inhalation solution used as anesthesia agent was purchased from Pfizer Inc. (Japan). Endothelial cell growth medium was purchased from PromoCell (Germany). Carbon Film C20-C10 (based on Cu100P, #10-1023) purchased from Okenshoji Co., Ltd. (Japan) was used for TEM measurements.

Buffered aqueous solutions (pH 2.0–2.2) were prepared by dissolving hydrochloric acid (HCl) and potassium chloride (KCl) in water (MilliQ). Buffered aqueous solutions (pH 3.1–6.9) were prepared by dissolving citric acid and sodium dihydrogenphosphate (NaH_2PO_4) in water (MilliQ). Buffered aqueous solutions (pH 7.2–8.7) were prepared by dissolving NaH_2PO_4 and disodium hydrogenphosphate (Na_2HPO_4) in water (MilliQ). Buffered aqueous solutions (pH 9.0–11.0) were prepared by dissolving sodium bicarbonate (NaHCO_3) and sodium carbonate (Na_2CO_3) in water (MilliQ). All buffered aqueous solutions were prepared in a concentration of 0.1 M. The solutions were stored in refrigerator and used within one week.

HPLC analytical experiment was performed to purify the cyanine dyes by instrument equipped with HPLC column oven, UV detector (GL-7450, GL Sciences Inc., Japan) and HPLC pump (GL-7410, GL Sciences Inc., Japan). High resolution mass spectra were measured by EXACTICVE (ESI, Thermo Fisher Scientific Inc., USA).

Images and fluorescence intensities from IVIS-lumina were analyzed using Living Image 2.50-Igor Pro 4.09 software (PerkinElmer Inc., USA).

1.2 Synthesis of dyes



Scheme S1. Synthetic procedure of probe **CypH2S** and control dyes **Cy2S** and **Cy3S**.

Cy2S-SAc: ¹H NMR (400 MHz, CD₃OD, 25 °C) δ 1.95 (s, 3H), 1.97 (s, 3H), 2.08–2.15 (m, 6H), 2.22 (s, 3H), 2.31 (s, 3H), 2.94–3.01 (m, 4H), 3.05 (t, J = 6.4 Hz, 2H), 3.76 (t, J = 6.4 Hz, 2H), 4.27 (m, 4H), 7.21–7.30 (m, 2H), 7.47–7.67 (m, 6H), 7.84–7.88 (m, 2H), 7.97–8.02 (m, 3H), 8.24 (d, J = 8.8 Hz, 1H). ¹³C-NMR (400 MHz, CD₃OD, 25 °C) δ 29.08, 30.99, 31.67, 32.05, 32.17, 33.51, 34.58, 39.92, 45.53, 45.93, 51.11, 51.64, 52.23, 53.99, 56.75, 92.98, 94.70, 102.69, 105.09, 108.23, 108.97, 110.68, 111.21, 111.90, 112.18, 112.81, 112.97, 113.22, 113.52, 113.65, 114.68, 115.01, 115.58, 115.84, 116.52, 116.98, 117.46, 118.04, 118.64, 119.97, 122.72, 123.65, 127.93, 131.03, 132.75, 135.74, 145.50, 147.89, 150.96, 167.69. HRMS (ESI) calcd for C₅₂H₅₄N₃O₈S₃ ([M–H⁺]): 944.3079, found: 944.3073.

Cy2S: (73%, green solid) Purity: > 96%, ¹H NMR (400 MHz, CD₃OD, 25 °C) δ 1.40 (t, J = 7.4 Hz, 3H), 1.92 (s, 3H), 1.94 (s, 3H), 2.01–2.07 (m, 4H), 2.20 (s, 3H), 2.94 (t, J = 6.4 Hz, 2H), 3.05 (t, J = 6.4 Hz, 2H), 3.75 (t, J = 6.4 Hz, 2H), 4.28–4.89 (m, 4H), 7.15–7.18 (m, 1H), 7.25–7.27 (m, 1H), 7.48–7.67 (m, 6H), 7.83–7.85 (m, 2H), 7.96–8.03 (m, 3H), 8.24, (d, J = 8.6 Hz, 1H). ¹³C-NMR (400 MHz,

CD₃OD, 25 °C) δ 13.08, 23.64, 27.24, 27.40, 30.77, 37.23, 40.69, 44.52, 51.14, 51.90, 52.79, 58.03, 102.56, 102.61, 111.89, 112.08, 122.55, 123.58, 125.46, 126.49, 128.37, 128.46, 128.88, 129.18, 129.36, 130.65, 131.09, 131.90, 132.06, 132.38, 132.80, 133.72, 134.69, 136.19, 140.29, 142.07, 147.62, 150.04, 150.09, 153.89, 156.61, 169.33, 171.21, 174.98, 176.36. HRMS (ESI) calcd for C₄₉H₅₀N₃O₇S₂ (M–H⁺) 856.3086, found 856.3096.

Cy3S: (75%, green solid) Purity: > 97%, ¹H NMR (400 MHz, CD₃OD, 25 °C) δ 1.94–1.99 (m, 6H), 1.99–2.01 (m, 2H), 2.17 (s, 3H), 2.89–2.95 (m, 4H), 3.07 (t, *J* = 6.8 Hz, 2H), 3.33 (s, 6H), 3.77 (t, *J* = 6.8 Hz, 2H), 4.23–4.28 (m, 4H), 7.17–7.27 (m, 2H), 7.48–7.68 (m, 6H), 7.84–7.86 (m, 2H), 7.96–8.02 (m, 3H), 8.24 (d, *J* = 8.8 Hz, 1H). ¹³C-NMR (400 MHz, CD₃OD, 25 °C) δ 23.48, 23.68, 26.06, 27.34, 27.53, 27.71, 30.41, 37.22, 44.53, 45.40, 51.18, 51.69, 51.94, 52.81, 58.04, 79.47, 111.65, 111.90, 112.46, 119.33, 122.56, 123.56, 125.40, 126.44, 128.02, 128.27, 128.38, 128.68, 128.70, 128.82, 129.07, 129.16, 129.32, 129.42, 130.59, 130.65, 131.08, 131.90, 132.02, 132.81, 133.74, 134.76, 136.04, 140.75, 142.12, 142.14, 144.32, 169.34, 171.16. HRMS (ESI) calcd for C₅₁H₅₄N₃O₁₀S₃ (M–H⁺) 964.2958, found 964.2977.

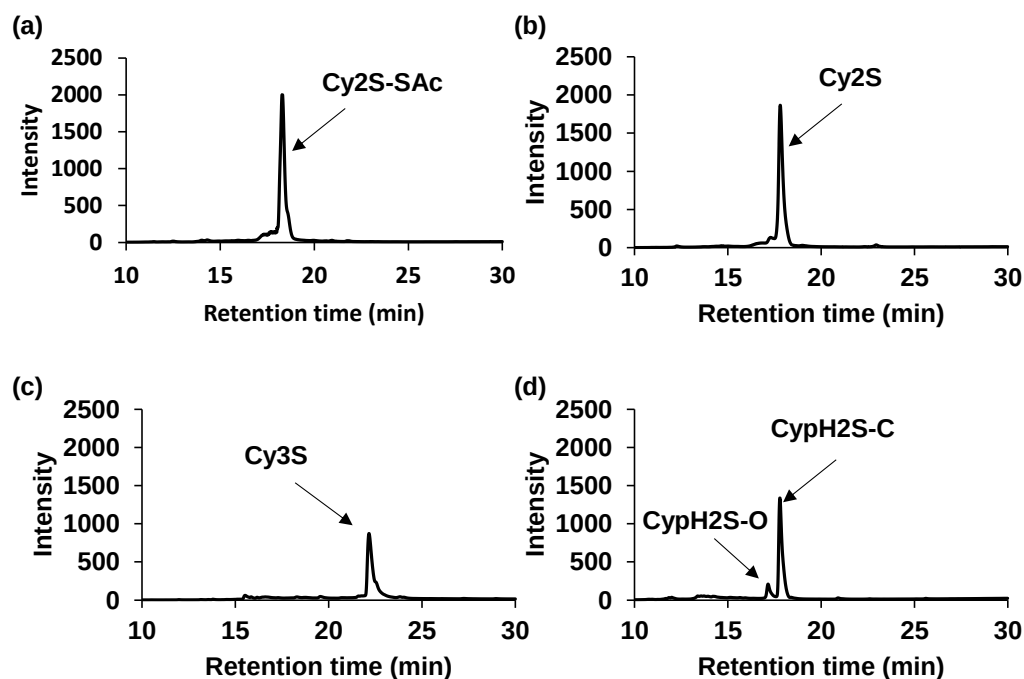


Figure S1. UV–vis trace of HPLC chromatogram of (a) **Cy2S-SAc**, (b) **Cy2S**, (c) **Cy3S**, and (c) **CypH2S**. The retention time for **Cy2S-SAc**, **Cy2S**, and **Cy3S** was assigned as 18.2 min, 17.8 min, and 22.2 min, respectively. The probe **CypH2S** is in an equilibrium between **CypH2S-C** having a closed-ring structure and **CypH2S-O** having an open-ring structure.

1.3 Photophysical properties of cyanine dye derivatives

Table S1. Photophysical properties of dyes.

dyes	molar extinction coefficient ϵ (L mol ⁻¹ ·cm ⁻¹) ^a	fluorescence quantum yield Φ ^b
Cy2S-SAc	2.76×10^5	0.1348
CypH2S	—	0.0940
Cy2S	1.2×10^5	0.1228
Cy3S	1.6×10^5	0.1260

^aDetermined at 780 nm in EtOH. ^bDetermined in EtOH, indocyanine green (ICG, $\Phi = 0.132$ (EtOH)) was used as a standard.¹ Due to low absorbance intensity of CypH2S in EtOH, its molar extinction coefficient was not determined.

1.4 pH-Dependent spectral changes of CypH1S and CypH2S

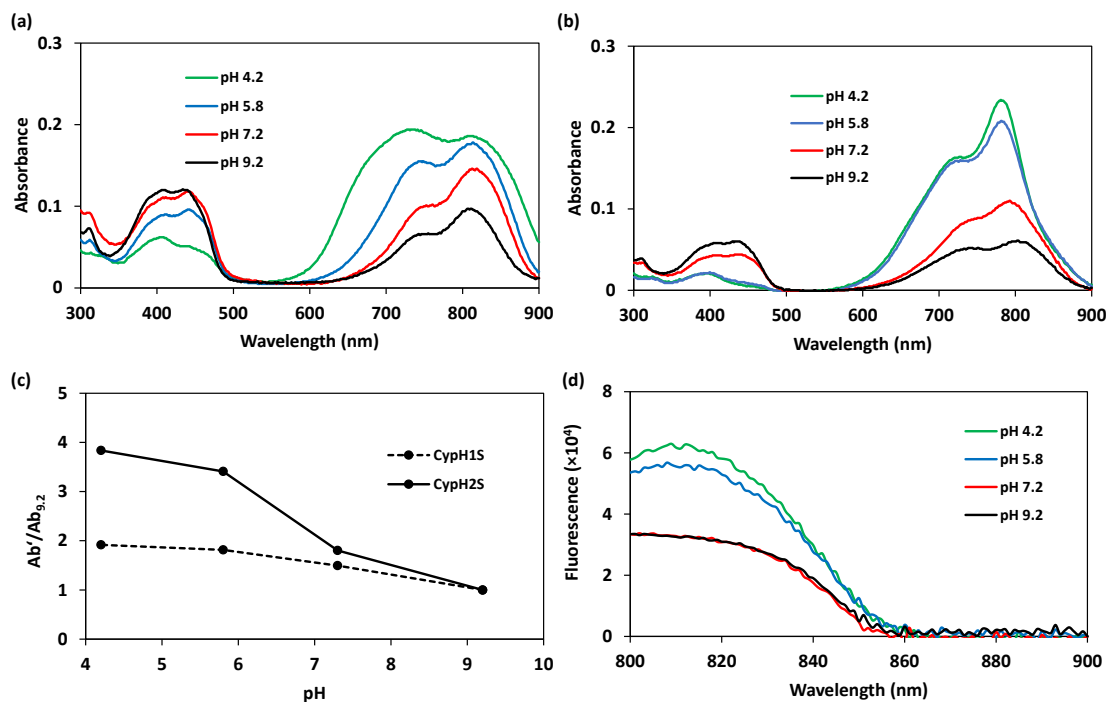


Figure S2. pH-Dependent UV-vis absorption spectra of (a) **CypH1S** and (b) **CypH2S** (5 μ M) in buffered aqueous solutions (pH= 9.2, 7.2, 5.8, 4.2). (c) $Ab'/Ab_{9.2}$ ratio of **CypH1S** and **CypH2S**. Ab' : maximum absorption intensities around 800 nm at different pH values; $Ab_{9.2}$: maximum absorption intensity around 800 nm at pH 9.2. The peak around 800 nm was assigned to non-aggregated dye form. (d) pH-Dependent fluorescence spectra of **CypH2S** (5 μ M) in buffered aqueous solutions.

1.5 Dynamic light scattering (DLS) measurements of probes

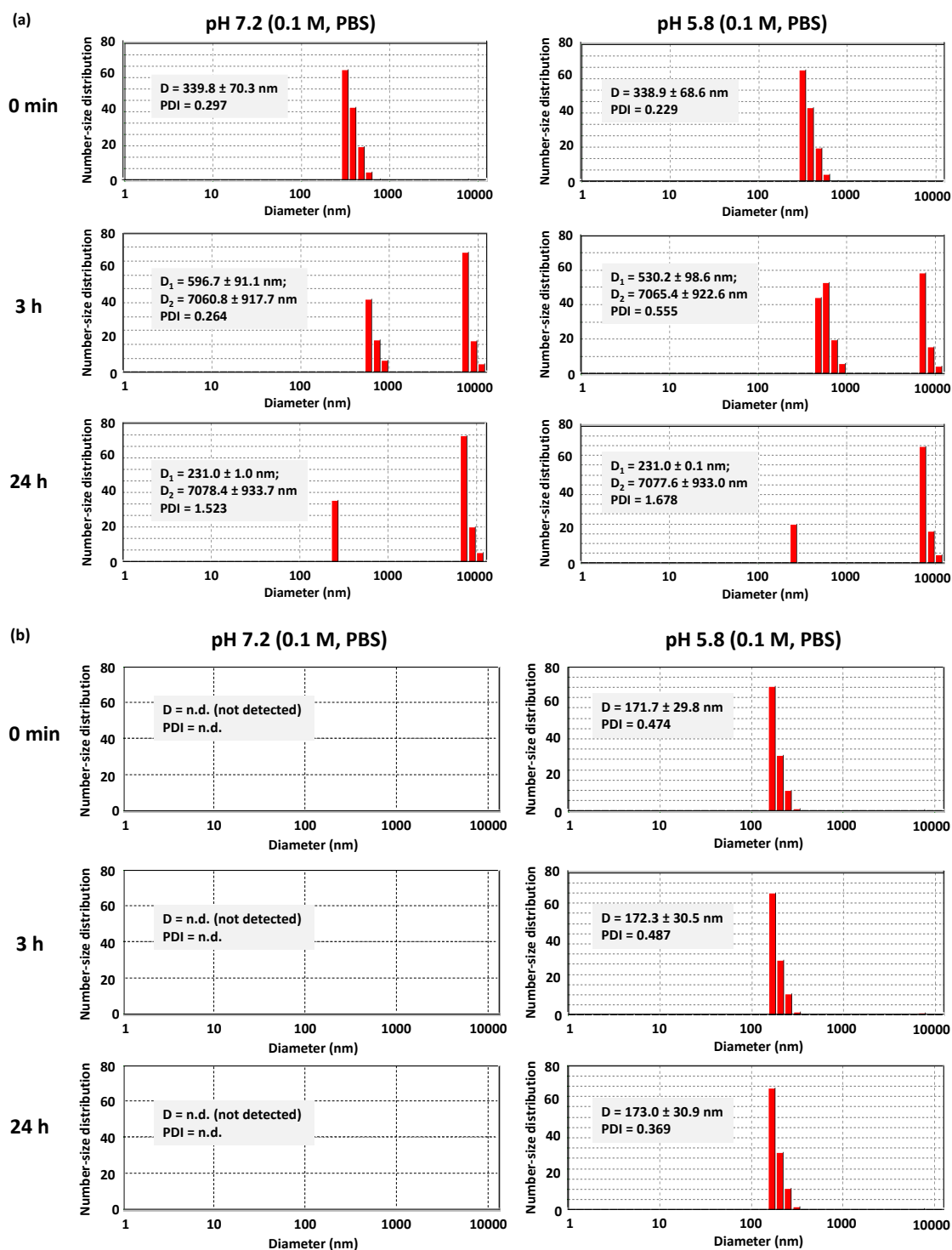


Figure S3. Time-dependent size-distribution of self-assemblies of (a) CypH1S and (b) CypH2S (100 μ M) in buffered solution (pH 7.2 and pH 5.8).

1.6 pH-dependent images of CypH1S and CypH2S

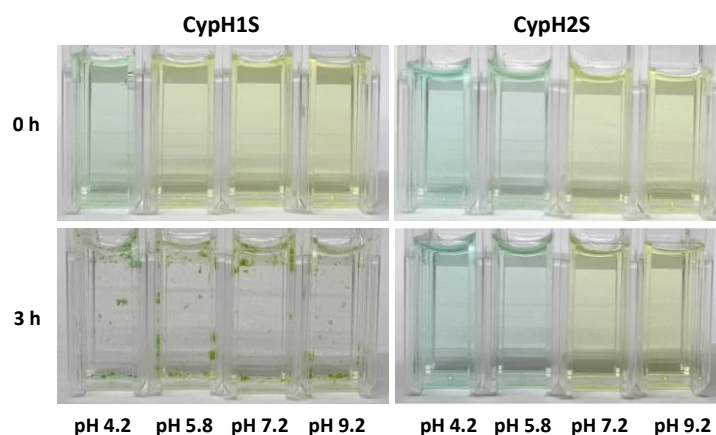


Figure S4. Presentative images of CypH1S and CypH2S (20 μM) in PBS with different pH values after just preparation or stored for 3 h.

1.7 Stability of CypH2S in aqueous solutions with different pH values

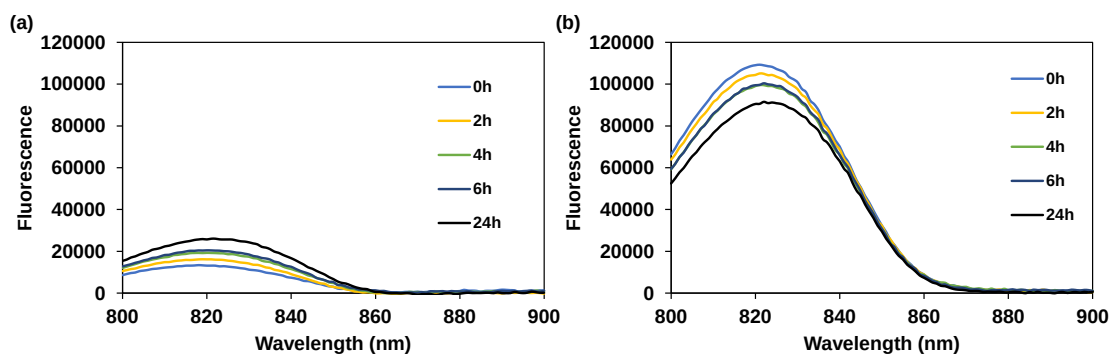


Figure S5. Time-dependent fluorescence spectra of CypH2S (5 μM) in buffered solutions with Triton X-100 (2.0×10^{-3} M) at (a) pH 7.2 and (b) pH 5.8.

1.8 Concentration-dependent fluorescence of dyes

Concentration-dependent fluorescence spectra of Cy1S-SAc, and Cy2S-SAc was evaluated. The fluorescence loss of dyes with concentration of dyes was determined by determination of fluorescent intensities at 820 nm according to the known method.³ The concentrations for fluorescent quenching of Cy1S-SAc and Cy2S-SAc were assigned at < 0.001 and 2 μM, respectively.

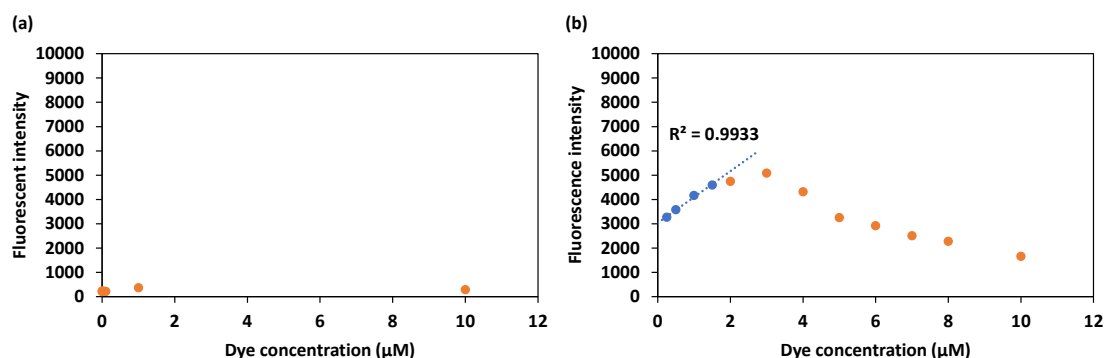


Figure S6. Fluorescence intensity at the maximum emission spectra with different concentrations (0–10 μM) of (a) **Cy1S-SAc** and (b) **Cy2S-SAc**. Dotted lines represent ideal linear relationship between fluorescence intensity and concentration, indicating non-quenching in blue dotted range.

1.9 pH-Responsive aggregation of CypH2S

pH-Activatable size change of **CypH2S** was evaluated in aqueous solutions. Firstly, the PBS buffered solution containing **CypH2S** (100 μM , 3 mL) stood for 24 h before DLS measurement. Then the pH value was adjusted to pH 5.2 by addition of 10% HCl aqueous solution (67 μL). Next, the size-distribution of self-assemblies of **CypH2S** was detected (Figure S7).

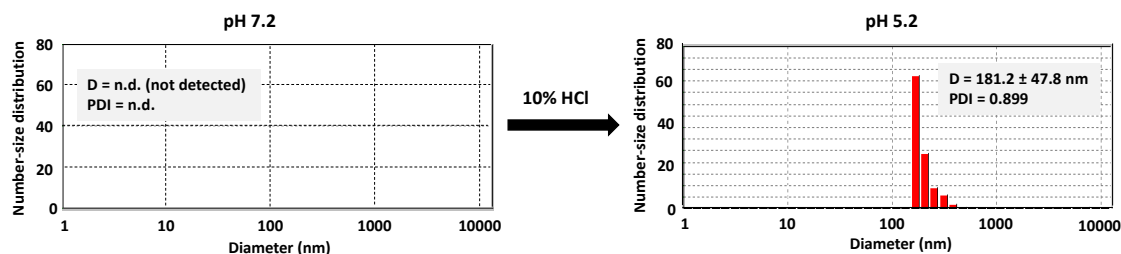


Figure S7. Size-distribution of self-assemblies of **CypH2S** (100 μM) in different pH buffered solution adjusted by 10% HCl aqueous solution.

1.10 DLS measurements of CypH1S, CypH2S, Cy2S or Cy3S in saline

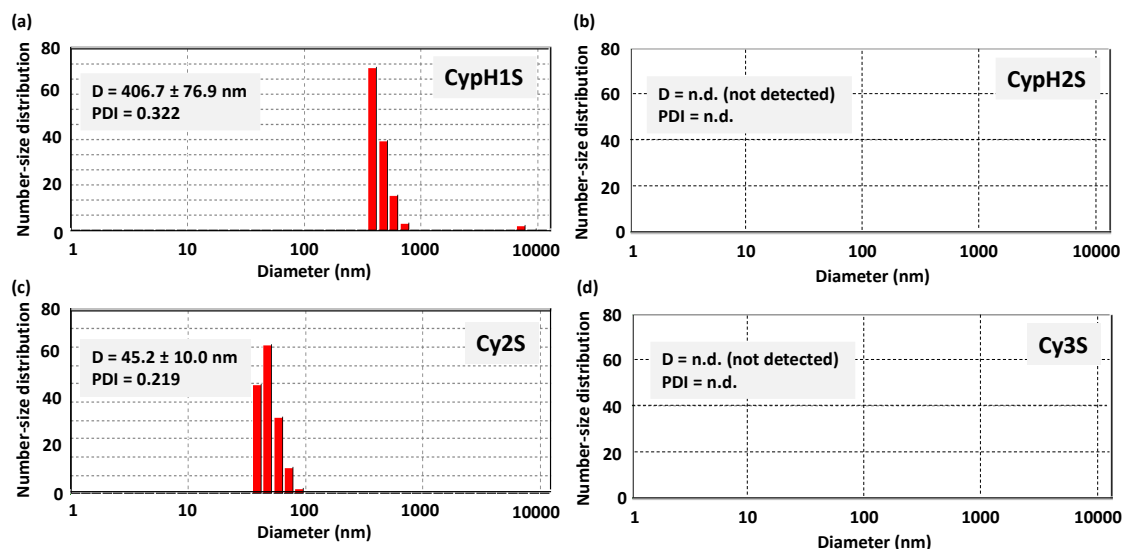


Figure S8. Size-distribution of self-assemblies of (a) CypH1S, (b) CypH2S, (c) Cy2S, and (d) Cy3S (100 μM) in saline (pH 7.2).

2. In vitro living cell experiments

cell culture

Human lung carcinoma cell line, A549, was purchased from American Type Culture Collection (Manassas, VA). Normal cells, Human Umbilical Vein Endothelial Cells (HUVEC), were purchased from Riken cell bank, Japan. A549 cells were cultured in 10% FBS-Dulbecco's modified Eagle's medium (DMEM) containing 1% penicillin-streptomycin. HUVEC cells were cultured in Endothelial cell growth medium. Cells were cultured in well-humidified incubator with 5% CO_2 at 37 $^{\circ}\text{C}$.

MTT assay

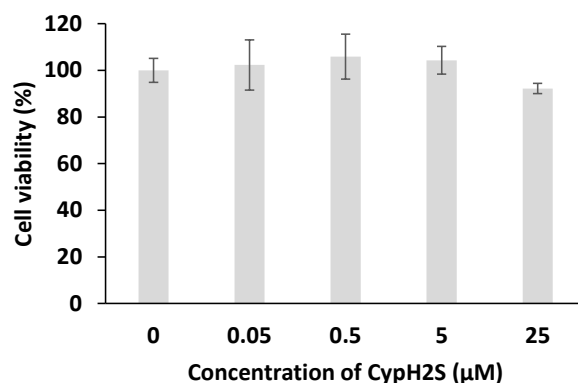


Figure S9. Cytotoxicity of CypH2S in varied concentrations (0, 0.05, 0.5, 5, 25 μM) in living

A549 cells for 12 h. Error bar indicates s.d., $n = 6$.

3. In vivo experiments

3.1 Optical imaging of tumor-bearing mice

Fifteen BALB/cSlc-nu/nu nude mice were prepared for in vivo experiments. A549 cells were transplanted into the right leg of each mouse and the tumor-size carefully monitored at the Institute of Laboratory Animals at Graduate School of Medicine of Kyoto University. All the IVIS imaging of mice were operated under a general anesthesia of isoflurane.

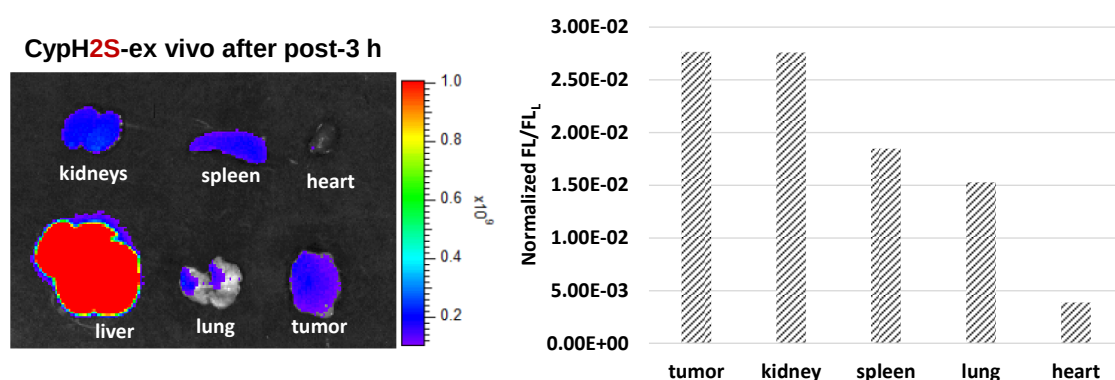


Figure S10. NIRF image of organs and tumor tissue excised from the mouse treated with **CypH2S** (100 μ M in 200 μ L saline) at 3 h post-injection. FL: the fluorescent intensities from organs and tumor; FL_L: the fluorescent intensity from liver.

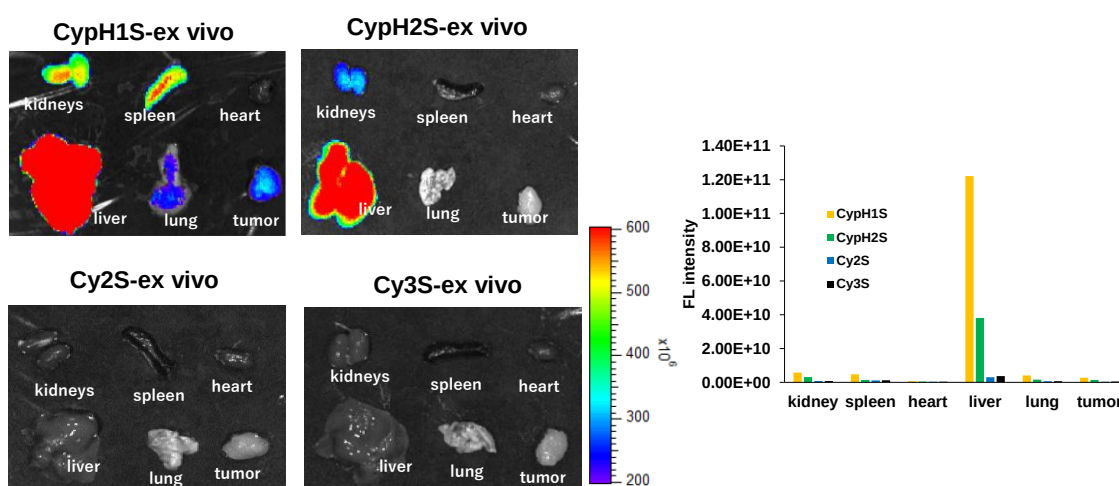


Figure S11. NIRF images and quantification of organs and tumor tissue excised from the mice treated with **CypH1S**, **CypH2S**, **Cy2S**, and **Cy3S** (100 μ M in 200 μ L saline) after 24 h injection.

3.2 The blood concentration of CypH2S in vivo

Four female BALB/cSlc-nu/nu nude mice in 9-weeks old were purchased from Shimizu Laboratory Supplies Co., Ltd. (Japan) and used for the measurement of the blood concentration of **CypH2S** in vivo. The method for solution preparation and blood harvest was according to the reported procedure.³

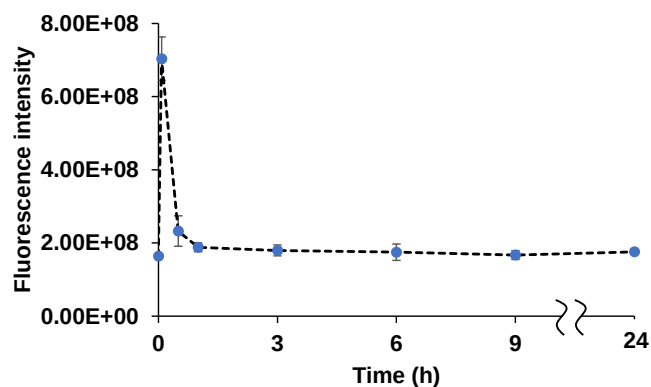
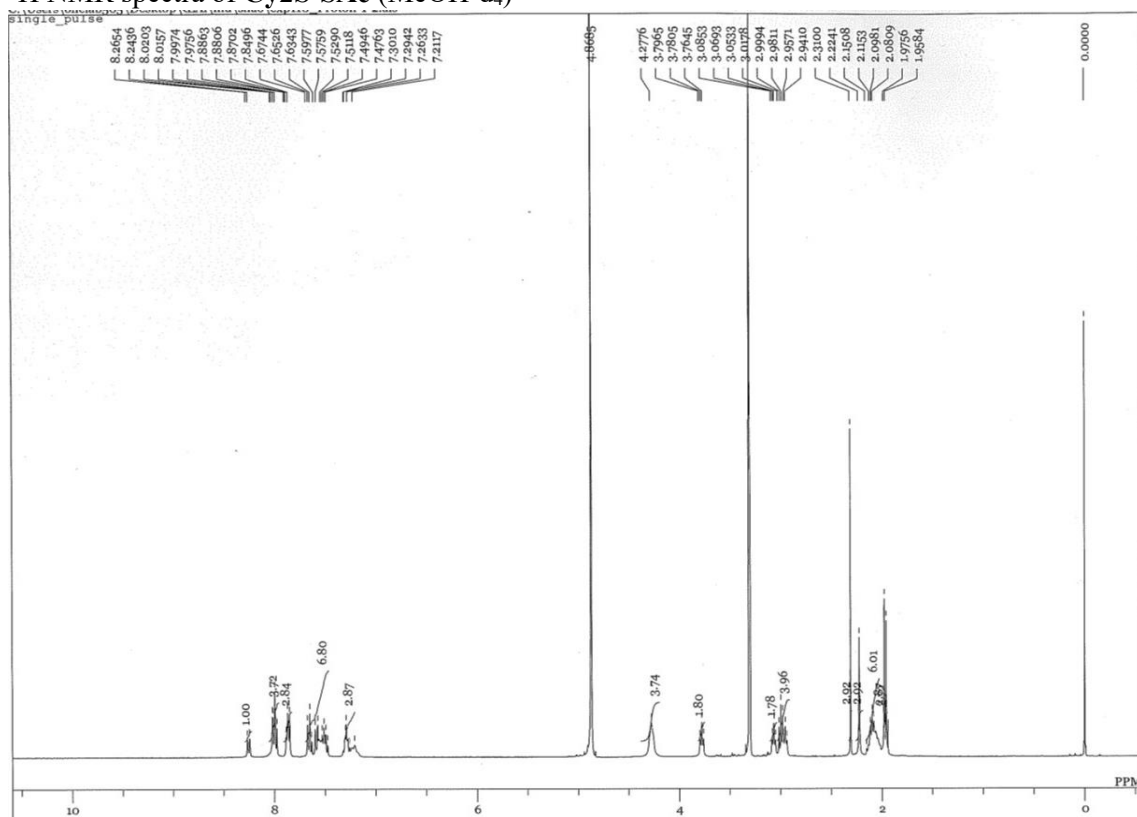


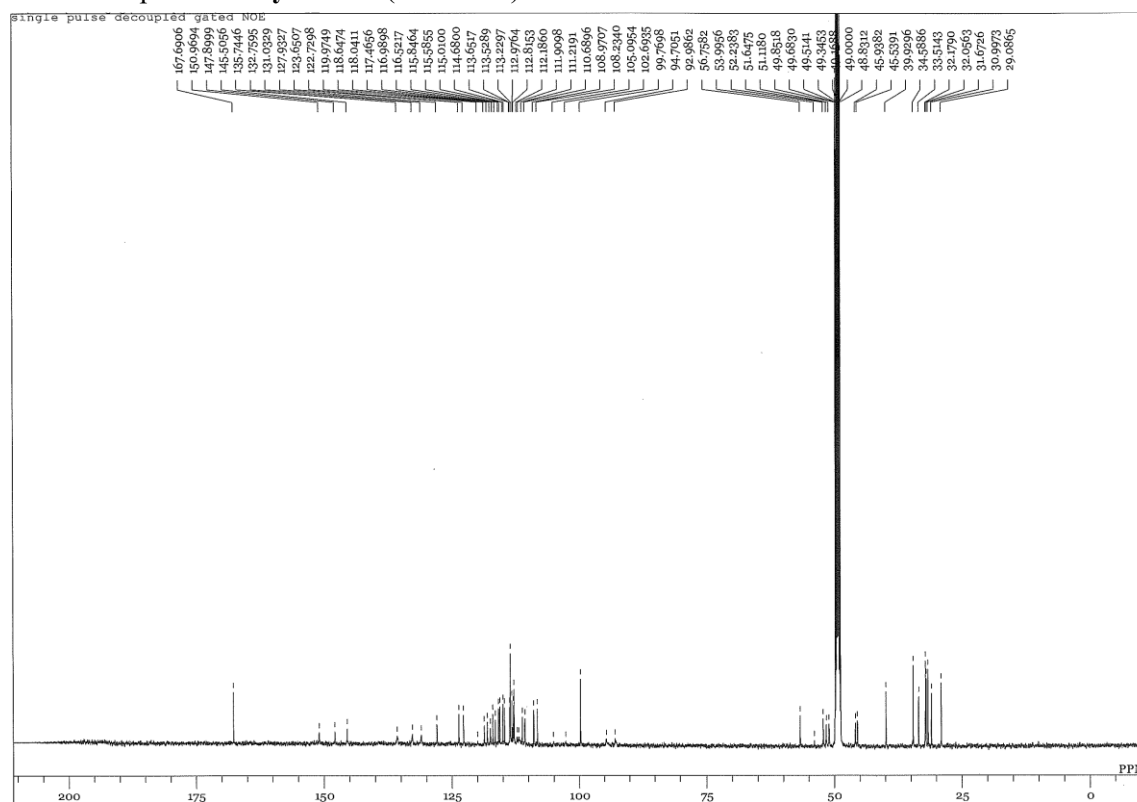
Figure S12. Time-dependent blood concentration of **CypH2S** in live nude mice. Fluorescence intensities were measured of mixed solution with the blood harvested from mouse administrated before-injection, post-5 min, 30 min, 1 h, 3 h, 6 h, 9 h, and 24 h injection of **CypH2S** (100 μ M in 200 μ L saline). Error bar indicates s.d. of independent experiments (n = 4).

4. NMR spectra

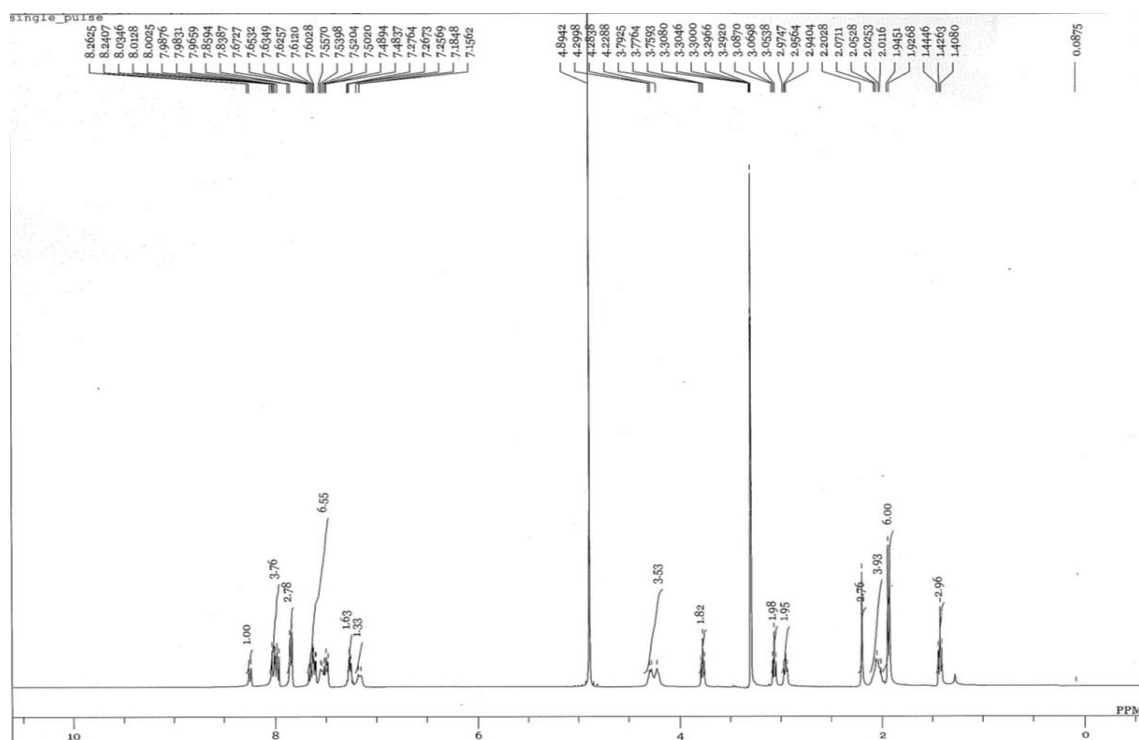
¹H NMR spectra of Cy2S-SAc (MeOH-d₄)



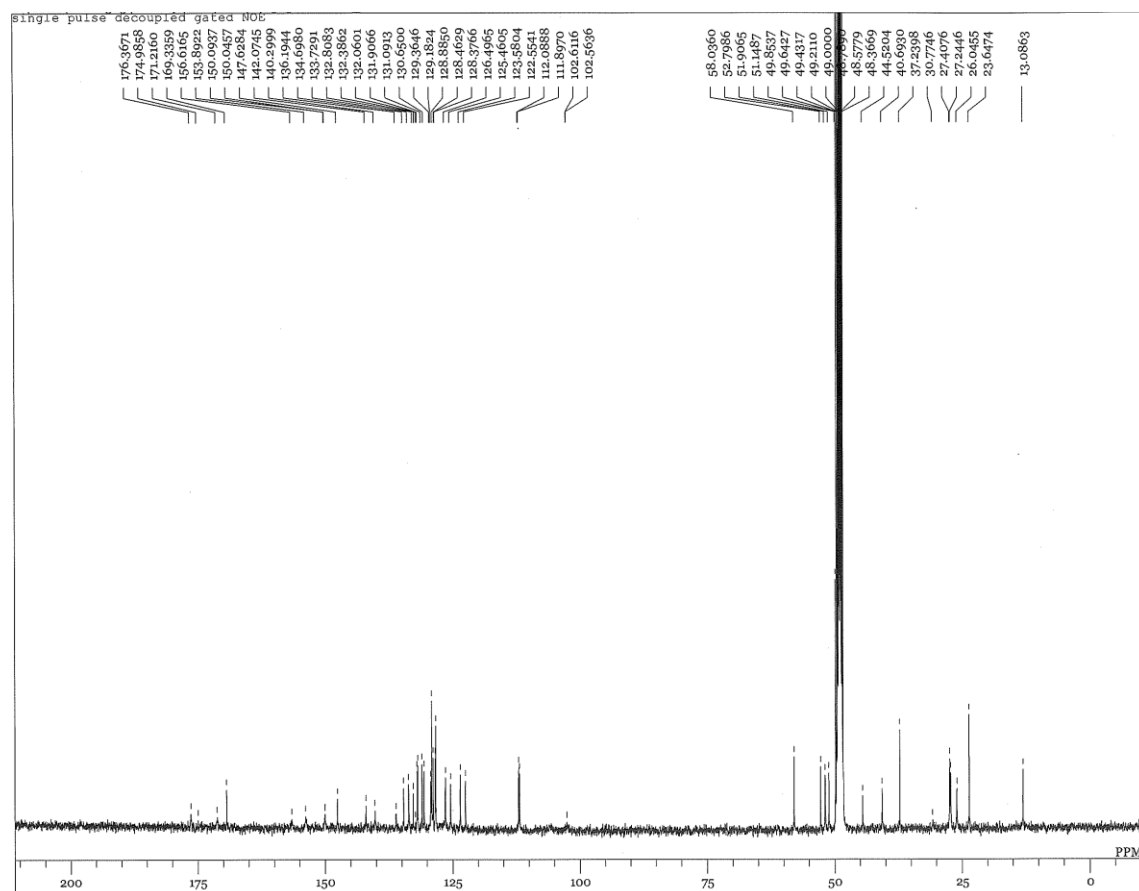
¹³C NMR spectra of Cy2S-SAc (MeOH-d₄)



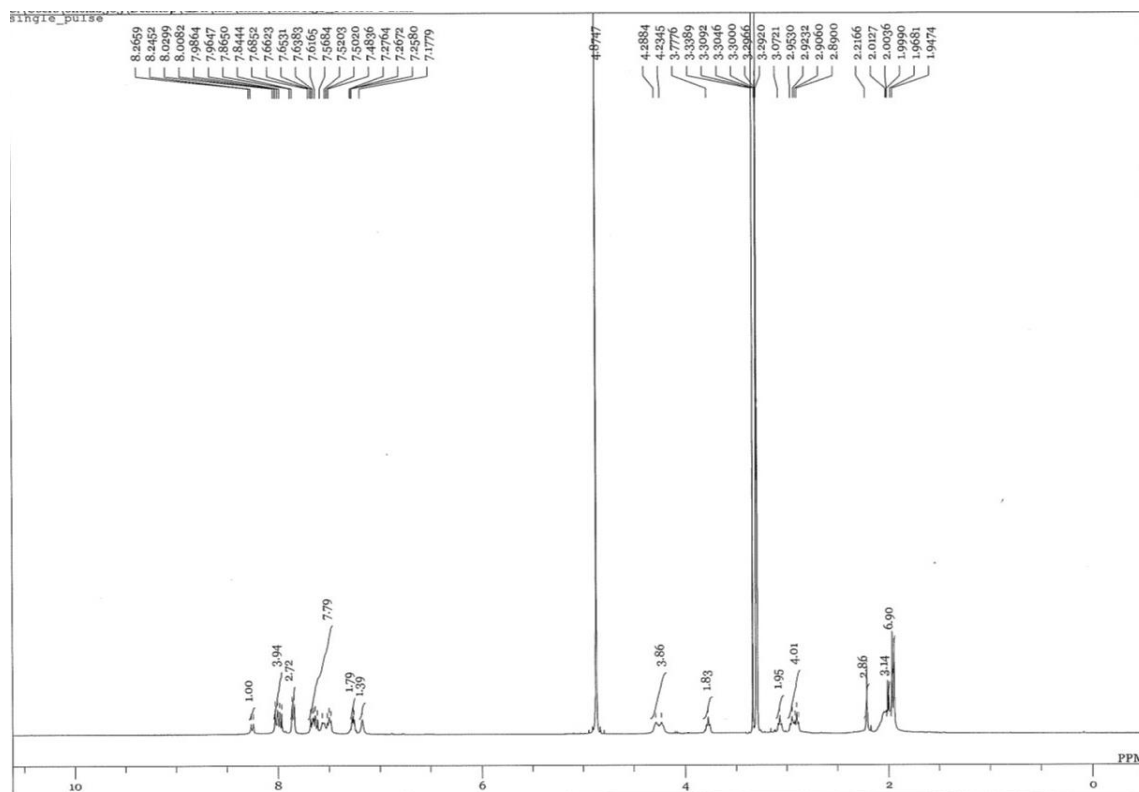
^1H NMR spectra of Cy2S (MeOH- d_4)



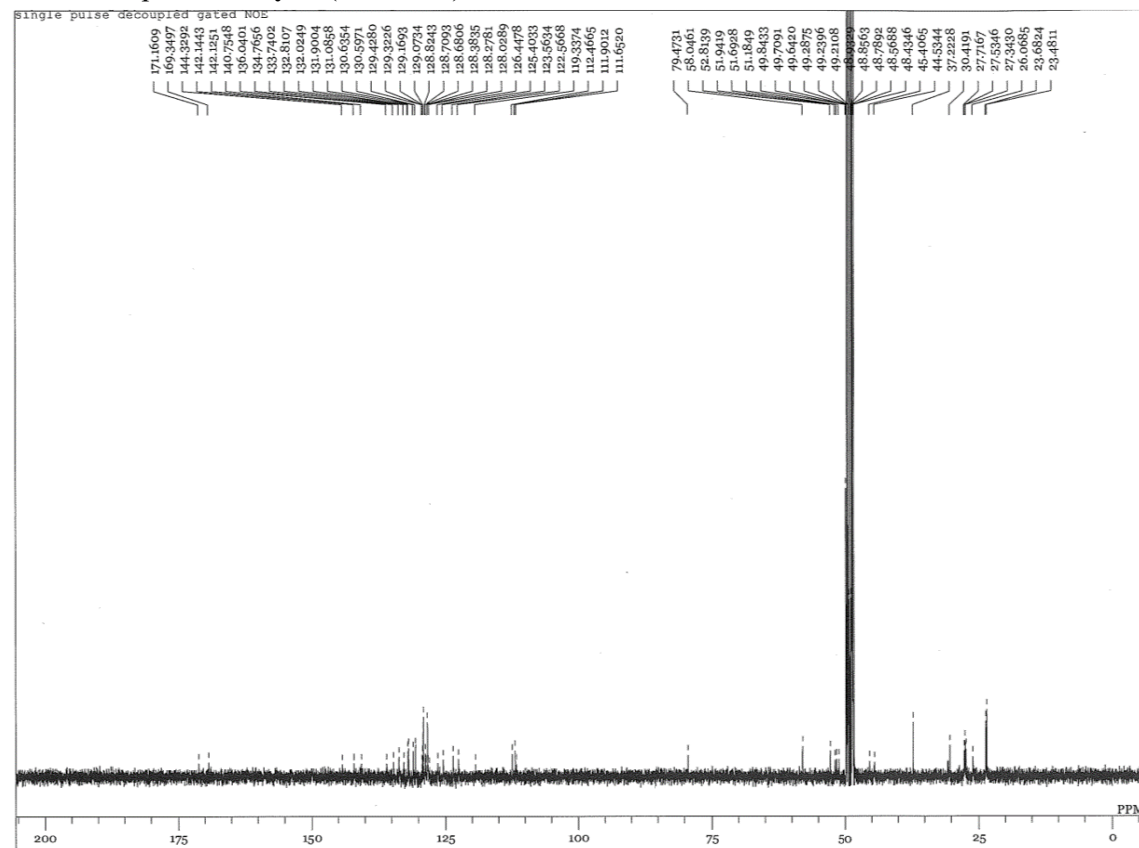
^{13}C NMR spectra of Cy2S (MeOH- d_4)



^1H NMR spectra of Cy3S (MeOH- d_4)



^{13}C NMR spectra of Cy3S (MeOH- d_4)



5. Reference

- [1] K. Rurack, M. Spieles, *Anal. Chem.* 83 (2011) 1232.
- [2] X. Zhang, J. Gao, Y. Tang, J. Yu, S. S. Liew, C. Qiao, G. Liu, H. Fan, Y. Xia, J. Tian, K. Pu, Z. Wang, *Nat. Commun.* 13 (2022) 3513.
- [3] K. Miki, T. Inoue, Y. Kobayashi, K. Nakano, H. Matsuoka, F. Yamachi, T. Yanno, K. Ohe, *Biomacromolecules* 16 (2025) 219–227.