Supporting information

Synthesis of sialic acid conjugates of the clinical near-infrared dye as next-generation theranostics for cancer phototherapy

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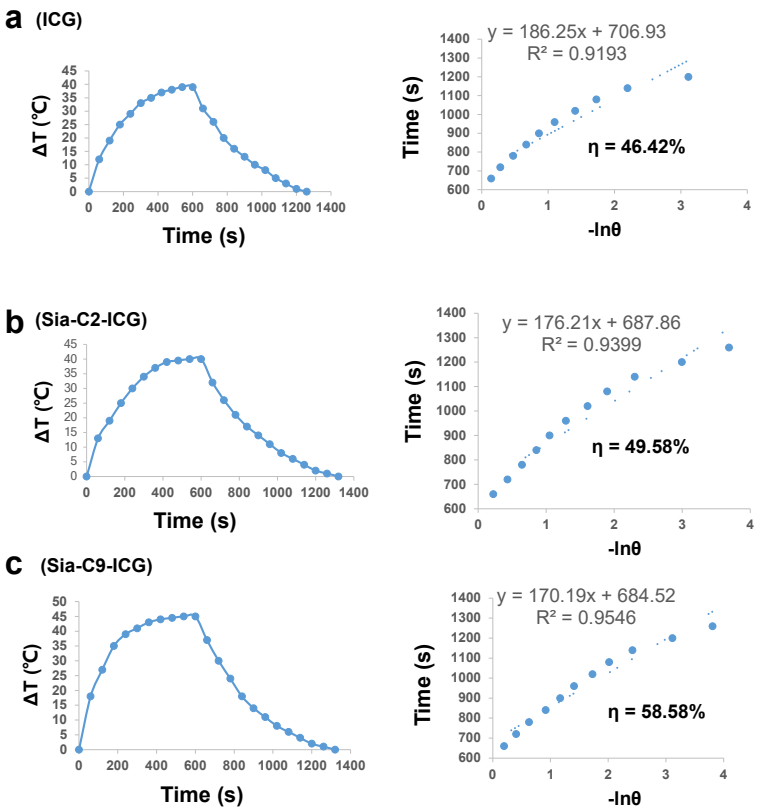


Fig. S1 Photothermal conversion efficiency of ICG and Sia-ICGs. The photothermal effect and time constant for heat transfer of (a) ICG, (b) Sia-C2-ICG, and (c) Sia-C9-ICG with the same amount of ICG (50 ppm) in PBS (pH 7.4) at 808 nm NIR laser for 600 s and then the light was turned off. It is calculated that the photothermal conversion efficiency of ICG, Sia-C2-ICG and Sia-C5-ICG is 46.42%, 49.58% and 58.58%, respectively.

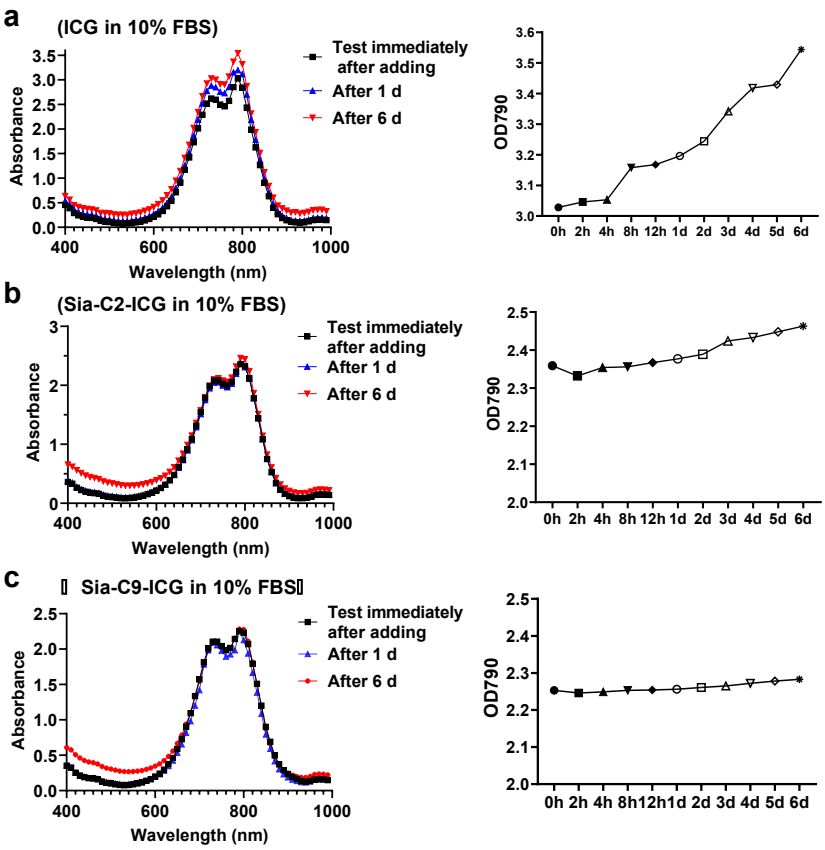


Fig. S2 Absorption spectra of Sia-ICGs and ICG in PBS with 10 % FBS. Absorption spectra of (a) ICG, (b) Sia-C2-ICG, and (c) Sia-C9-ICG in PBS (pH 7.4) containing 10% FBS with the same amount of ICG (40 μg) after storing for different periods of time. This indicates that free ICG can non-specifically bind to serum proteins, while the non-specific binding of Sia-C2-ICG and Sia-C9-ICG to serum proteins is significantly reduced.

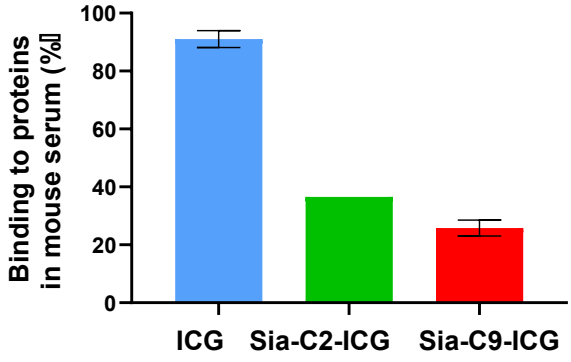


Fig. S3 Determination of the binding rates of ICG, Sia-ICGs to serum proteins. It was found that 91% of ICG is bound to serum proteins, while 37% of Sia-C2-ICG and 26% of Sia-C9-ICG are bound to serum proteins, indicating that Sia-ICGs can significantly reduce non-specific binding to serum proteins.

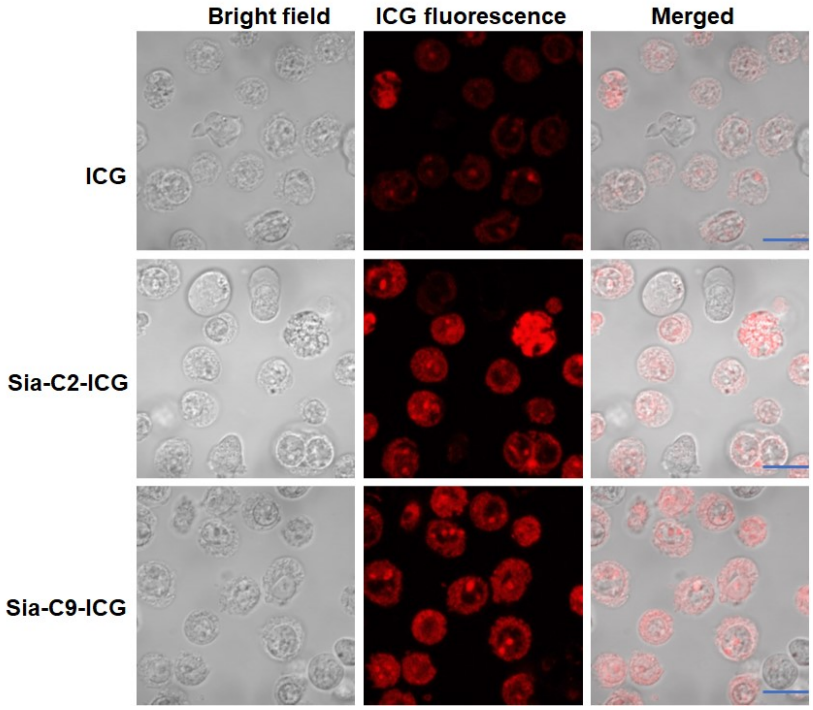


Fig. S4 Uptake of ICG and Sia-ICGs by EL4 cells. The results showed that compared with ICG, the incubation of Sia-C9-ICG and Sia-C2-ICG significantly enhanced the fluorescence signal of EL4 cells, indicating that Sia-coupling can enhance NIR imaging of cancer cells. Scale bars: 20 μm.

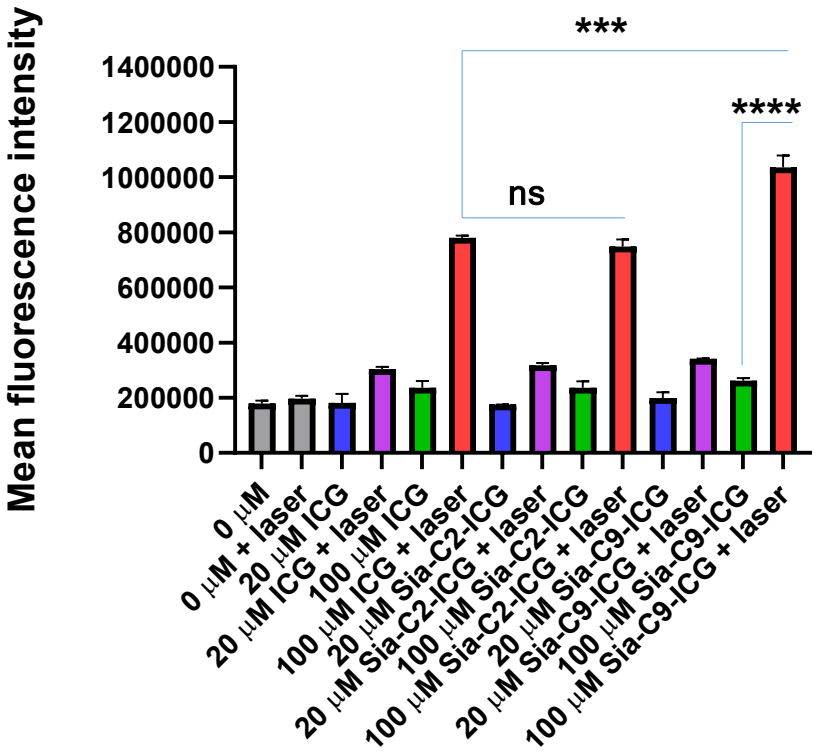


Fig. S5 ROS production in the cell culture environment. For the laser-treated cell groups, the cells were irradiated the 808 nm laser (1 W cm^{-2}) for 3 min in a 37-degree water bath. It was found that Sia-C9-ICG treatment plus laser irradiation led to superior production of ROS in 4T1 cells.

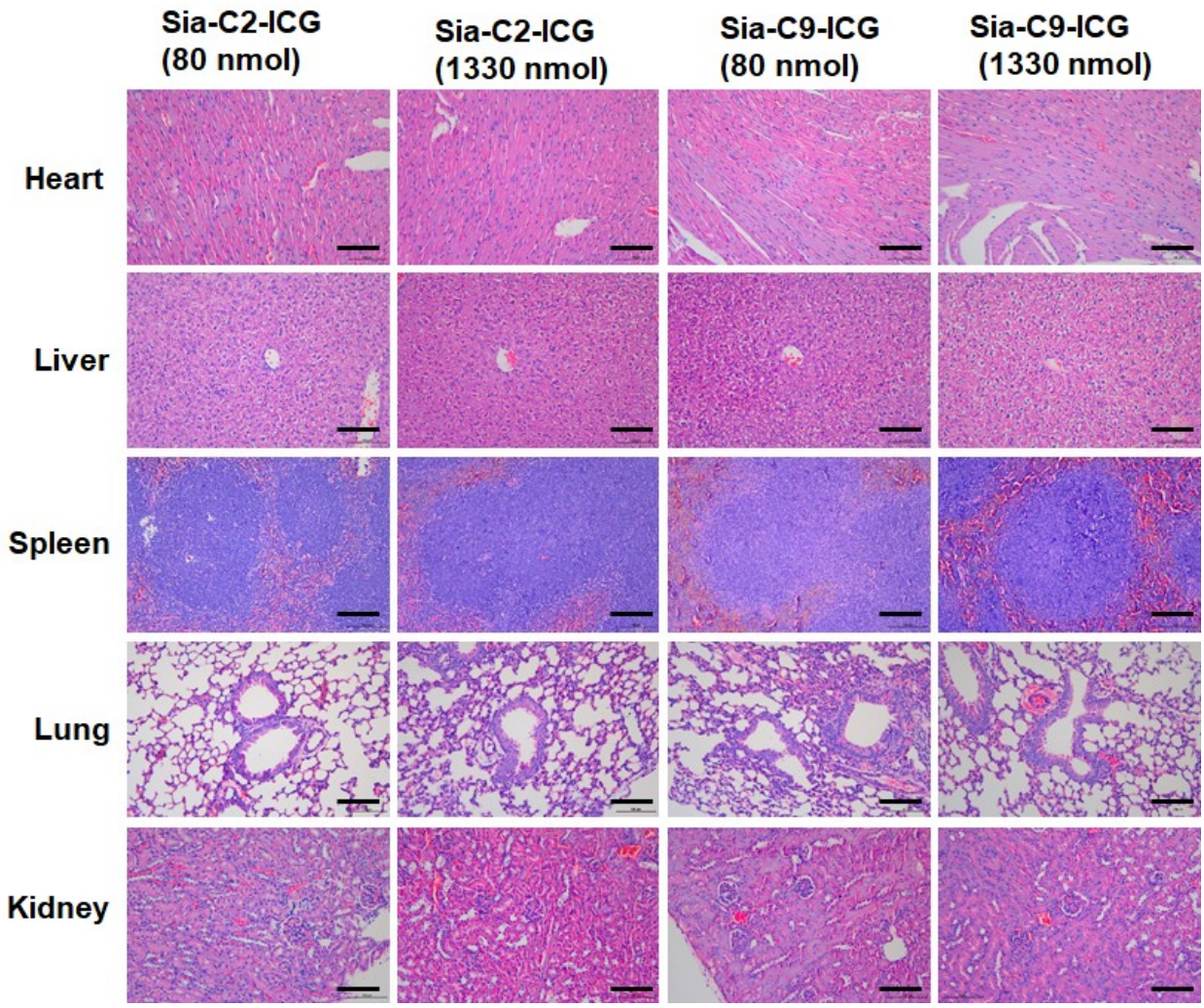


Fig. S6 *In vivo* toxicity of Sia-ICGs by histological analysis. The organs of mice were harvested, sectioned, and stained with hematoxylin/eosin. No lesions were observed from these slides. Scale bars: 100 μm.

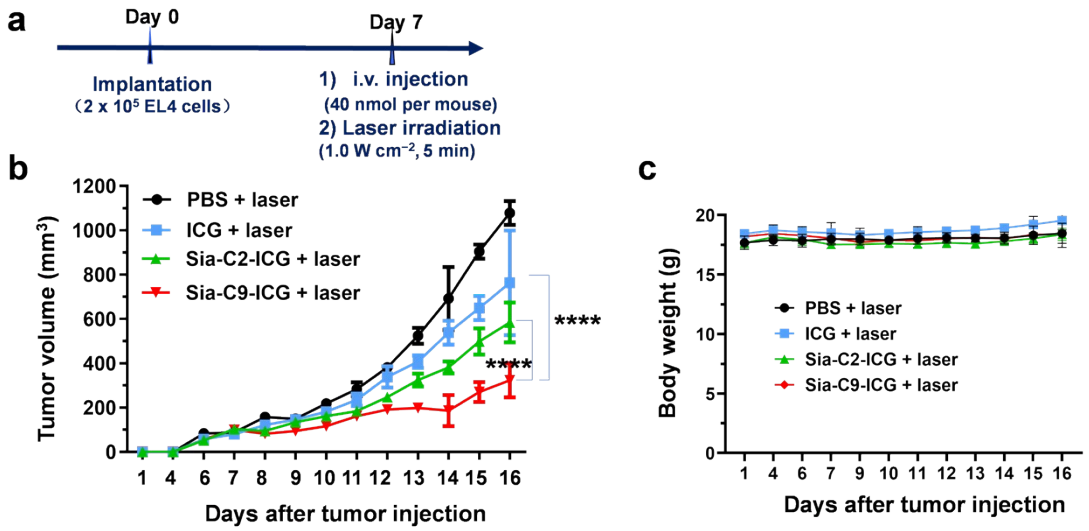


Fig. S7 EL4 tumor challenge. (a) C57BL/6 female mice were inoculated with EL4 cells (2×10^5) by subcutaneous injection on day 0 and were given PBS or Sia-ICGs intravenously on day 7 (40 nmol per mouse). 2 h after the injection of Sia-ICGs or PBS, the mice were irradiated with the 808 nm laser (1.0 W cm^{-2}, 5 min). (b) Growth curves of EL4 tumors in tumor-bearing mice. (c) Body weight of tumor-bearing mice. $n = 6$ mice for each group. The p value was analyzed by GraphPad Prism with a two-way ANOVA test. **** $p < 0.0001$.

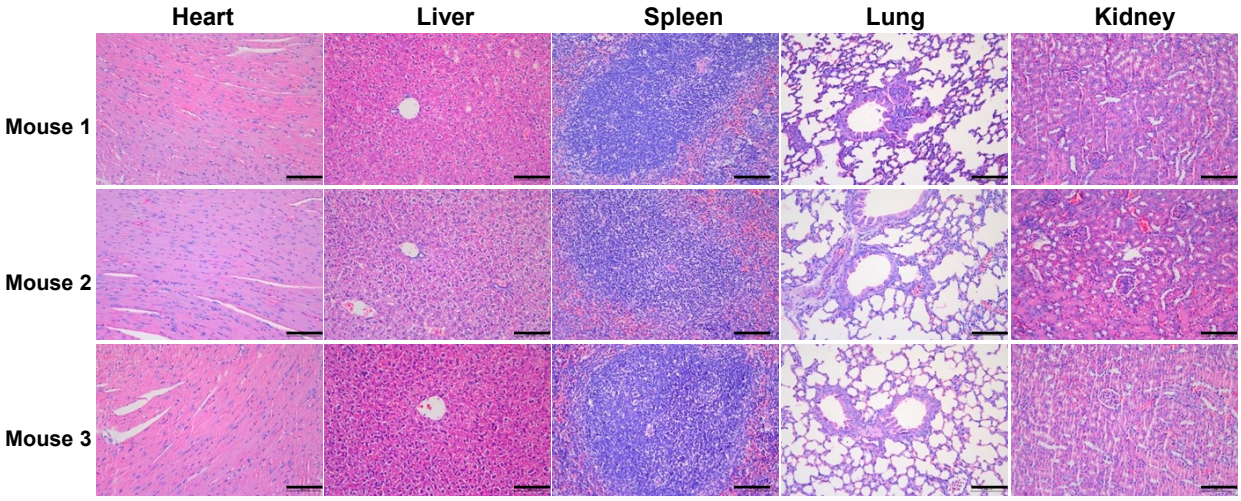


Fig. S8 Histological analysis of mouse organs treated with Sia-C9-ICG plus laser irradiation. The mouse organs are from the 4T1 tumor challenge study mentioned in **Fig. 9**. The mouse organs were harvested, sectioned and stained with hematoxylin/eosin. No lesions were observed from these slides. Scale bars: 100 μm.

Materials

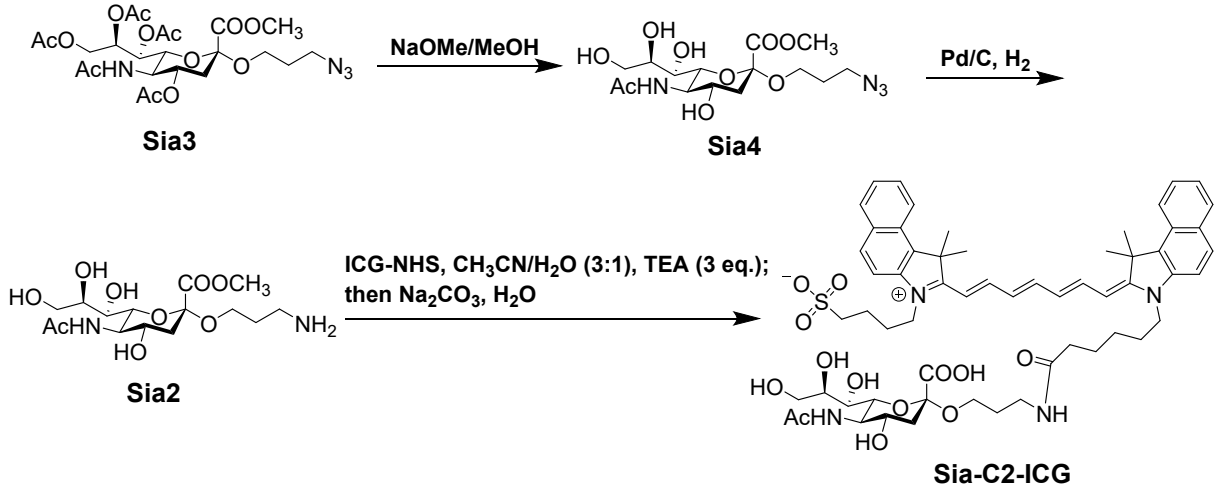
All chemicals were reagent grade and were used as received from the manufacturer unless otherwise noted. ICG-NHS (CAS number: 1622335-40-3) were purchased from Confluore Biotechnology. Gel filtration chromatography was performed using a column (100 cm × 2.5 cm) packed with BioGel P-2 Fine resins (Bio-Rad, Hercules, CA). NMR spectra were calibrated using solvent signals (^{1}H: δ 4.79 for D_2O). EL4 and 4T1 cells from the American Type Culture Collection (ATCC) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate. The cells were cultured at 37 ℃ in a 5% CO_2/air incubator. 6−8 weeks old C57BL/6 and BALB/c female mice from Shandong University Laboratory Animal Center were used for all studies. All animal experiments were conducted by the guidelines of the Animal Care and Use Committee of Shandong University.

General methods for the synthesis

Synthesis of Sia-C9-ICG. 12 mg of 9-amino-Sia methyl ester **Sia1**[1] (0.037 mmol) was dissolved in 300 μl of ultrapure water and added to 1 mL of acetonitrile containing ICG-NHS (20 mg, 0.024 mmol) and 10 μL trimethylamine (TEA). The reaction was stirred at room temperature (rt) for 6 h. Next, acetonitrile was removed by rotary evaporation. 2 mL of Na_2CO_3 (10 mg, 0.097 mmol) in an aqueous solution was added to the flask. The reaction was stirred at rt and monitored by TLC (EtOAc/MeOH/H_2O/HOAc, 12:3:2:1) until the reaction was complete. The product was purified by flash column chromatography and BioGel P-2 gel column chromatography (eluted with water) to obtain Sia-C9-ICG in 91% yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.25 (t, J = 7.4 Hz, 2H), 8.05 (q, J = 8.0 Hz, 4H), 7.98 (d, J = 13.1 Hz, 2H), 7.77 (d, J = 8.9 Hz, 2H), 7.71 (d, J = 9.4 Hz, 1H), 7.64 (q, J = 7.0 Hz, 2H), 7.49 (q, J = 7.8 Hz, 2H), 6.70 – 6.55 (m, 2H), 6.53 (d, J = 14.3 Hz, 1H), 6.41 (d, J = 13.5 Hz, 1H), 4.92 (s, 1H), 4.83 (s, 1H), 4.42 – 4.11 (m, 4H), 3.88 – 3.68 (m, 1H), 3.61 (s, 2H), 3.17 – 2.95 (m, 2H), 2.84 – 2.60 (m, 1H), 2.55 (s, 1H), 2.12 (s, 2H), 1.91 (s, 12H), 1.87 (s, 3H), 1.81 – 1.76 (m, 2H), 1.74 – 1.71 (m, 3H), 1.56 (q, J = 7.4 Hz, 2H), 1.41 (t, J = 7.7 Hz, 2H), 1.31 – 1.27 (m, 1H), 1.23 (s, 2H). ^{13}C NMR (150 MHz, DMSO-d6) δ 173.24, 172.85, 172.17, 171.73, 167.43, 150.89, 150.24, 140.31, 140.25, 133.66, 133.33, 132.44, 132.06, 131.69, 131.56, 130.73, 130.33, 129.11, 128.44, 128.05, 127.99, 125.94, 125.15, 124.60, 122.63, 112.89,

112.18, 111.96, 71.38, 70.79, 70.21, 68.57, 67.82, 66.90, 63.50, 55.38, 53.63, 51.11, 50.89, 50.72, 44.12, 43.87, 38.13, 36.30, 30.22, 29.46, 28.79, 27.48, 27.24, 27.19, 26.27, 25.54, 23.11, 22.88. HRMS (ESI) m/z calcd for $C_{56}H_{68}N_4O_{12}S$ $[M+H]^+$ 1021.4627, found 1021.4536.

Synthesis of Sia-C2-ICG



Scheme S1. Synthesis of Sia-C2-ICG.

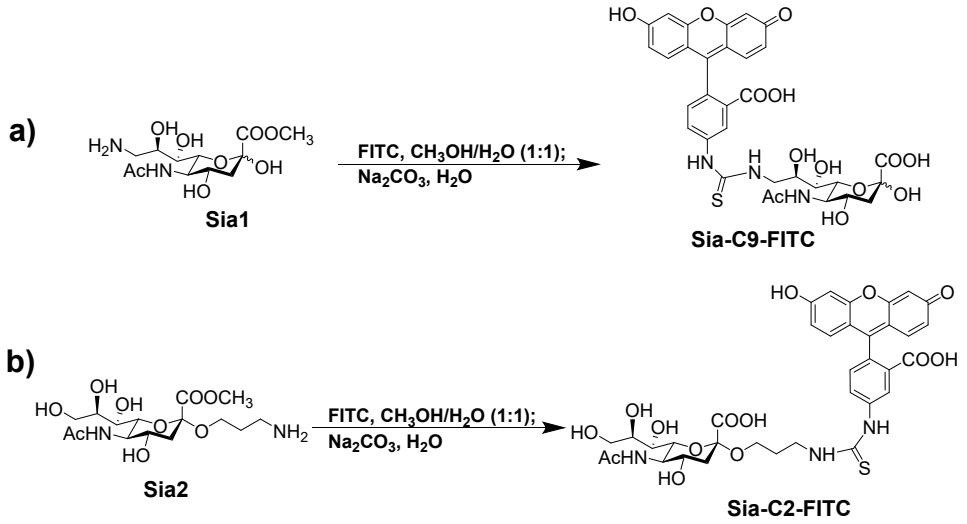
Synthesis of Sia4. 2.44 g of *O*-Acetyl sialic azide **Sia3**[2] (4.25 mmol) was dissolved in methanol (8 mL). NaOMe (0.6 mL, 5.4 M in methanol) was added to the solution. After stirring for 1 h at rt, the reaction solution was neutralized by DOEWX resin and filtered. Then the filtrate was concentrated and purified by flash column chromatography (DCM/MeOH, 15:1) affording **Sia4** as white solid (1.36 g, yield: 74%). ^{1}H NMR (600 MHz, D_2O) δ 3.86 – 3.84 (m, 1H), 3.83 (s, 3H), 3.83 – 3.78 (m, 4H), 3.72 – 3.67 (m, 1H), 3.60 (dd, *J* = 12.1, 6.3 Hz, 1H), 3.54 – 3.49 (m, 2H), 3.37 (t, *J* = 6.5 Hz, 2H), 2.67 (dd, *J* = 12.8, 4.6 Hz, 1H), 1.99 (s, 3H), 1.82 – 1.75 (m, 3H). ^{13}C NMR (150 MHz, D_2O) δ 174.88, 170.04, 98.97, 72.76, 70.49, 68.12, 67.07, 62.97, 61.69, 53.25, 51.62, 47.72, 39.06, 28.07, 21.95.

Synthesis of Sia 2. Sia4 (200.03 mg, 0.49 mmol) was dissolved in 4 mL of methanol. Pd/C (20 mg) and acetic acid (200 μL) were added to the flask. The reaction mixture was stirred under H_2 atmosphere until the **Sia4** was completely consumed. The reaction mixture was then filtered to remove the Pd/C. The resulting filtrate was concentrated and purified by flash column

chromatography (DCM/MeOH, 2:1) to afford **Sia2** (140 mg, 75 %). ^{1}H NMR (600 MHz, D_2O) δ 3.84 – 3.76 (m, 4 H), 3.74 – 3.68 (m, 1 H), 3.66 – 3.61 (m, 1 H), 3.58 (dd, *J* = 12.0, 6.5 Hz, 1H), 3.56 – 3.50 (m, 2H), 3.30 (s, 3H), 3.07 (t, *J* = 6.7 Hz, 2 H), 2.66 (dd, *J* = 12.9, 4.7 Hz, 1 H), 1.98 (s, 3H), 1.93 – 1.86 (m, 2H), 1.65 – 1.61 (m, 1H). ^{13}C NMR (150 MHz, D_2O) δ 175.00, 173.65, 100.47, 72.84, 71.55, 67.98, 62.51, 62.30, 51.78, 48.73, 40.17, 37.86, 26.57, 21.94.

Synthesis of Sia-C2-ICG. 0.036 mmol of **Sia2** was dissolved in 500 μL ultrapure water and added to 1.5 mL of acetonitrile containing ICG-NHS (20 mg, 0.024 mmol) and 10 μL TEA. The mixture was stirred at rt until ICG-NHS disappeared completely. Removal of the solvent by evaporation affords the crude Sia(CO_2CH_3)-C2-ICG. To hydrolyze its methyl ester (CO_2CH_3), 10 mg of Na_2CO_3 (0.097 mmol) in an aqueous solution (2 mL) was added to the flask containing Sia(CO_2CH_3)-C2-ICG. The reaction mixture was stirred at rt and monitored by TLC (EtOAc/MeOH/H_2O/HOAc, 12:3:2:1) until the methyl ester of Sia(CO_2CH_3)-C2-ICG was hydrolyzed completely. Afterward, the product was purified by flash column chromatography and BioGel P-2 gel column chromatography (eluted with water) to afford Sia-C2-ICG in 94% yield. ^{1}H NMR (600 MHz, DMSO-d6) δ 8.29 – 8.21 (m, 2H), 8.05 (t, *J* = 8.4 Hz, 3H), 8.02 – 7.88 (m, 2H), 7.82 – 7.70 (m, 3H), 7.64 (d, *J* = 8.5 Hz, 2H), 7.49 (q, *J* = 6.6 Hz, 2H), 6.70 – 6.52 (m, 2H), 6.48 (s, 1H), 6.42 (d, *J* = 13.5 Hz, 1H), 5.06 (s, 1H), 4.78 (s, 1H), 4.62 (s, 1H), 4.41 (s, 1H), 4.22 (d, *J* = 17.6 Hz, 4H), 3.71 (s, 3H), 3.61 (d, *J* = 11.8 Hz, 4H), 3.49 (d, *J* = 13.5 Hz, 4H), 3.25 – 3.15 (m, 2H), 3.05 – 3.00 (m, 1H), 2.69 (d, *J* = 11.0 Hz, 1H), 2.08 – 2.00 (m, 2H), 1.91 (s, 12H), 1.86 – 1.76 (m, 4H), 1.73 (d, *J* = 11.9 Hz, 2H), 1.58 – 1.49 (m, 3H), 1.38 (t, *J* = 7.9 Hz, 2H), 1.31 – 1.22 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.27, 171.91, 171.52, 170.50, 170.15, 149.80, 145.19, 139.74, 132.90, 132.84, 132.04, 131.10, 131.03, 130.17, 129.78, 127.48, 125.71, 125.32, 124.86, 124.48, 124.02, 122.68, 122.08, 116.77, 111.60, 111.12, 106.18, 103.81, 102.36, 100.08, 95.29, 94.82, 72.52, 71.24, 70.37, 69.50, 68.99, 67.22, 65.27, 63.45, 63.32, 60.75, 59.34, 53.03, 52.19, 50.63, 50.30, 43.54, 42.03, 35.96, 35.06, 29.39, 27.01, 26.69, 26.27, 25.56, 24.86, 22.44, 22.35. HRMS (ESI) m/z calcd for $C_{59}H_{74}N_4O_{13}S$ $[M+H]^+$ 1079.5046, found 1079.4961.

Synthesis of Sia-C9-FITC and Sia-C2-FITC



Scheme S2. Synthesis of Sia-C9-FITC and Sia-C2-FITC.

Synthesis of Sia-C9-FITC. To 8 mL of CH_3OH/H_2O (1:1) containing 100 mg of FITC (0.26 mmol) was added **Sia1** (125.7 mg, 0.39 mmol) and Na_2CO_3 (109.17 mg, 1.03 mmol). After stirring at rt for 24 h, the product was purified by flash column chromatography (DCM/MeOH, 2:1) and BioGel P-2 gel column chromatography (eluted with water) to afford Sia-C9-FITC with a yield of 68%. ^{1}H NMR (600 MHz, D_2O) δ 7.67 (s, 1H), 7.51 (d, J = 8.1 Hz, 1H), 7.30 – 7.09 (m, 3H), 6.62 (dd, J = 9.2, 2.3 Hz, 2H), 6.57 (d, J = 2.3 Hz, 2H), 4.07 (qd, J = 7.0, 3.1 Hz, 1H), 4.03 – 3.94 (m, 3H), 3.91 (t, J = 10.2 Hz, 1H), 3.71 – 3.64 (m, 1H), 3.54 – 3.47 (m, 1H), 2.19 (dd, J = 13.1, 4.8 Hz, 1H), 2.02 (s, 3H), 1.80 (t, J = 12.3 Hz, 1H). ^{13}C NMR (150 MHz, D_2O) δ 179.96, 179.47, 177.97, 176.66, 174.61, 173.80, 158.19, 140.94, 137.30, 131.44, 130.94, 128.91, 125.67, 124.58, 122.00, 113.44, 103.35, 96.36, 70.00, 69.74, 68.82, 68.38, 67.19, 62.36, 52.19, 47.99, 40.99, 39.29, 22.05, 19.93. HRMS (ESI) m/z calcd for $C_{32}H_{31}N_3O_{13}S$ $[M+H]^+$ 698.1650, found 698.1580.

Synthesis of Sia-C2-FITC. To 8 mL of CH_3OH/H_2O (1:1) containing 100 mg of FITC (0.26 mmol) was added **Sia2** (148.35 mg, 0.39 mmol) and Na_2CO_3 (109.17 mg, 1.03 mmol). After stirring at rt for 24 h, the product was purified by flash column chromatography (DCM/MeOH, 2:1) and BioGel P-2 gel column chromatography (eluted with water) to afford Sia-C2-FITC in 58% yield. ^{1}H NMR (600 MHz, D_2O) δ 7.84 – 7.68 (m, 1H), 7.61 (s, 1H), 7.35 – 7.21 (m, 3H),

6.72 (t, *J* = 2.4 Hz, 1H), 6.70 (t, *J* = 2.5 Hz, 1H), 6.67 – 6.60 (m, 2H), 3.93 – 3.86 (m, 3H), 3.83 – 3.78 (m, 2H), 3.71 (dd, *J* = 10.4, 1.9 Hz, 2H), 3.63 – 3.57 (m, 3H), 2.76 – 2.69 (m, 1H), 2.04 (s, 1H), 2.02 (s, 3H), 1.97 – 1.91 (m, 2H), 1.67 (td, *J* = 12.2, 5.0 Hz, 1H). ^{13}C NMR (150 MHz, D_2O) δ 179.40, 174.98, 174.72, 173.66, 173.55, 173.32, 157.62, 144.15, 131.66, 130.72, 128.77, 126.53, 124.31, 120.90, 114.84, 103.13, 101.64, 100.56, 72.55, 71.64, 70.06, 68.15, 68.06, 66.56, 63.37, 62.45, 61.45, 51.89, 42.21, 40.37, 38.68, 37.58, 28.42, 27.89, 22.01. HRMS (ESI) m/z calcd for $C_{35}H_{37}N_3O_{14}S$ $[M+H]^+$ 756.2069, found 756.2017.

Stability evaluation of ICG and Sia-ICGs

ICG, Sia-C2-ICG, and Sia-C9-ICG containing the same amount of ICG (40 μg) were dispersed in 1 mL of phosphate-buffered saline (PBS, pH 7.4) at rt for 0−5 days. Their UV-vis absorption spectra on days 0, 1, 2 and 5 were collected, respectively. Their photographs were taken after storing ICG or Sia-ICG in PBS for 1 day.

***In vitro* photothermal performances**

An 808 nm fiber-coupled NIR laser (MW-GX-808/1−5000 mW, Changchun Laser Technology Co., Ltd. Jilin, China) was utilized for this study. To PBS was added ICG (0−50 ppm), Sia-C2-ICG (internal ICG concentrations of 0−50 ppm) or Sia-C9-ICG (internal ICG concentrations of 0−50 ppm). The solution was irradiated with the 808 nm laser (1 W cm^{-2}) for 10 min, and the temperature of the solution was recorded over irradiation time. To test their photostabilities, free ICG, Sia-C9-ICG and Sia-C2-ICG (internal ICG concentrations of 50 ppm) in PBS (pH 7.4) were irradiated for three lasers on/off cycles under irradiation with the 808 nm laser (1 W cm^{-2}). The temperature of the solution was recorded over irradiation time. The photothermal conversion efficiency (η) of ICG, Sia-C2-ICG and Sia-C9-ICG was calculated using the following equation:[3]

$$\eta = \frac{hS\Delta T_{max} - Q_{Dis}}{I\left(1 - 10^{-A808}\right)}$$

Binding of Sia-ICGs to serum proteins

To PBS (pH 7.4) containing 10% fetal bovine serum (FBS) was added ICG, Sia-C2-ICG and Sia-C9-ICG, respectively. At the indicated time, their UV-vis absorption spectra were collected. The results indicated that the non-specific binding of Sia-C2-ICG and Sia-C9-ICG to serum proteins was significantly reduced (see **Fig. S2**). To confirm this, the mouse serum was mixed with ICG, Sia-C9-ICG and Sia-C2-ICG, respectively. After incubating for 24 h, the free ICG or Sia-ICGs without binding to serum proteins were separated from the serum proteins by ultrafiltration, and the amounts of ICG and Sia-ICGs bound with serum proteins were determined (see **Fig. S3**).

Hemolysis test of ICG and Sia-ICGs

Fresh mouse blood was collected. After centrifugation at 1000 rpm (4 ℃) for 5 min, the red blood cells (RBCs) obtained were washed three times with PBS and resuspended in PBS to a concentration of 1×10^8 cells mL^{-1}. To each well of a 96-well plate were added the resulting RBCs (200 μL, 2×10^7 cells per well) containing ICG (20 μM, 100 μM), Sia-C2-ICG (20 μM, 100 μM) and Sia-C9-ICG (20 μM, 100 μM), respectively. After incubating for 1 h, the cells were centrifuged at 12000 rpm for 5 min. The absorbance of the resulting supernatant was measured at 541 nm using a microplate reader. All samples were tested in triplicate.

Cellular uptake of Sia-ICGs

EL4 cells were cultured in 96-well plates at a density of 5×10^5 cells per well. The plates were placed in an incubator (37 ℃, 5% CO_2) for 12 h. The culture medium was replaced with the fresh medium containing 100 μM of ICG, Sia-C2-ICG and Sia-C9-ICG, respectively. After incubating for 24 h, the cells were washed 3 times with sterile PBS and then imaged using a confocal fluorescence microscope (see **Fig. S4**).

Cytotoxicity of Sia-ICGs

EL4 cells were cultured in 96-well plates at a density of 1.5×10^4 cells per well. After culturing for 12 h, the cells were incubated with ICG, Sia-C2-ICG and Sia-C9-ICG (0.1, 1, 6.25, 12.5, 25, 50, 100 μM), respectively. After incubating for 24 h, 10 μL MTT (3-[4,5-dimethylthiazol-

2-yl]-2,5-diphenyltetrazolium bromide) (5 mg mL^{-1}) was added to each well and incubated for 4 h. Then, the medium was removed, and 100 μL of dimethyl sulfoxide (DMSO) was added. The absorbance was measured at 570 nm using a microplate reader. All samples were tested in triplicate.

In vitro photothermal killing of tumor cells

Cells (EL4 and 4T1) were seeded into a 96-well culture plate at a density of 5000 cells per well and placed in an incubator (5% CO_2) at 37 ℃ for 12 h. The cells were supplemented with ICG (0−20 ppm), Sia-C2-ICG (internal ICG concentrations of 0−20 ppm), and Sia-C9-ICG (internal ICG concentrations of 0−20 ppm) for 24 h and irradiated with the 808 nm laser (1 W cm^{-2}) for 10 min then re-cultured for 6 h. Then 10 μL MTT (5 mg mL^{-1}) was added to each well and incubated for 4 h. Afterward, 100 μL of DMSO was added. The absorbance was measured at 570 nm using a microplate reader. All samples were tested in triplicate.

Reactive oxygen species detection

The cells (EL4 and 4T1) were seeded into a 24-well culture plate at a density of 2×10^6 cells per well. To the cells, was added ICG (20 μM and 100 μM), Sia-C2-ICG (20 μM and 100 μM) and Sia-C9-ICG (20 μM and 100 μM), respectively. After culturing 12 h, the cells were collected and washed for 3 times with PBS. Then, the solution of 2'-7'dichlorofluorescin diacetate (DCFH-DA) (1mL) was added. After culturing at 37 ℃ for 20 min, the cells were washed 3 times in serum-free 1640 medium and then suspended with 1640 medium. The cells were irradiated the 808 nm laser (1 W cm^{-2}) for 3 min on ice or at 37 ℃ for the laser-treated cell groups. The fluorescence intensities of cells were determined by flow cytometry. All samples were tested in triplicate.

NIR fluorescence imaging of tumors by Sia-ICGs

C57BL/6 mice were xenografted by subcutaneous injections of EL4 cells. When the tumor volume reached approximately 100 mm^3, free ICG (40 nmol per mouse), Sia-C2-ICG (40 nmol per mouse) and Sia-C9-ICG (40 nmol per mouse) were respectively injected into tumor-bearing mice via the tail vein (n = 3 mice per group). At 0, 2, 5, 9 and 24 h following injection, the mice

were analyzed for whole-body NIR fluorescence imaging. To perform *in vivo* organ analysis, C57BL/6 mice bearing EL4 tumors were intravenously injected with ICG (80 nmol per mouse), Sia-C2-ICG (80 nmol per mouse) and Sia-C9-ICG (80 nmol per mouse) *via* tail vein, respectively (n = 6 mice per group). At 5 h or 24 h after injection, 3 mice in each group were sacrificed by anesthesia. Their tumors and representative organs were dissected and analyzed for *ex vivo* fluorescence imaging.

For *in vivo* NIR imaging of 4T1 tumors, BALB/c mice bearing 4T1 tumors were intravenously injected with ICG (80 nmol per mouse), Sia-C2-ICG (80 nmol per mouse) and Sia-C9-ICG (80 nmol per mouse), *via* tail vein (n = 6 mice per group). At 5 h or 24 h post-injection, 3 mice in each group were sacrificed by anesthesia. The tumors and representative organs were dissected and analyzed for *ex vivo* fluorescence imaging.

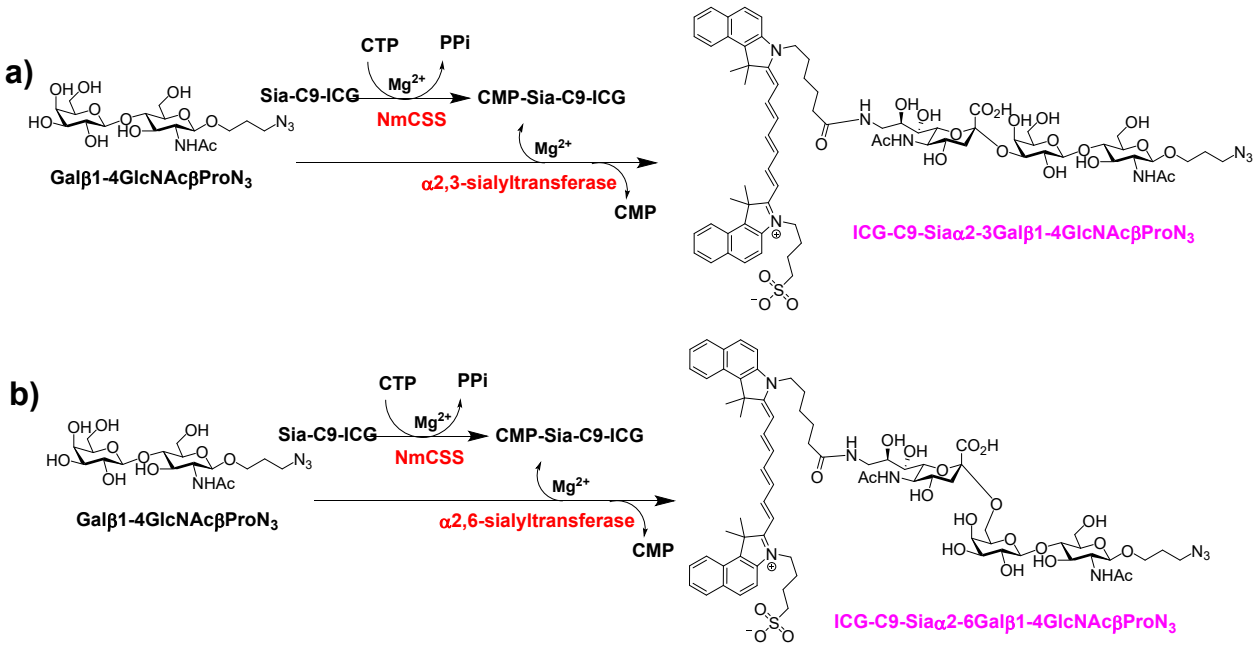
Cellular uptake of Sia-FITCs

4T1 cells were seeded in 96-well plates at a density of 5×10^5 cells per well. The plates were placed in an incubator (37 ℃, 5% CO_2) overnight. The culture medium was replaced with the fresh medium containing FITC (0−100 μM), Sia-C2-FITC (0−100 μM), and Sia-C9-FITC (0−100 μM), respectively. The cells were incubated for 24 h. Next, the cells were digested into a single cell suspension, washed with sterile PBS, and then analyzed by flow cytometry.

Biodistribution of Sia-FITCs in mice

BALB/c mice bearing 4T1 tumors were intravenously injected with FITC (80 nmol per mouse), Sia-C2-FITC (80 nmol per mouse) and Sia-C9-FITC (80 nmol per mouse), respectively, *via* tail vein (n = 3 mice per group). At 5 h post-injection, the tumors and representative organs were dissected and imaged for *ex vivo* fluorescence analysis.

Recognition of Sia-C9-ICG by CMP-Sia synthetase and sialyltransferase



Scheme S3 (a) Enzymatic reaction of Sia-C9-ICG with Galβ1-4GlcNAcβProN_3 by CMP-Sia synthetase (NmCSS) and α2,3-sialyltransferase (PmST1) in the presence of CTP. (b) Enzymatic reaction of Sia-C9-ICG with Galβ1-4GlcNAcβProN_3 by CMP-Sia synthetase (NmCSS) and α2,6-sialyltransferase (PdST) in the presence of CTP.

Recognition of Sia-C9-ICG by α2,3-sialyltransferase in the presence of NmCSS. α2,3-sialyltransferase 5 μL of Galβ1-4GlcNAcβProN_3 (100 mM) and 5 μL of Sia-C9-ICG (120 mM, 1.2 eq.) were dissolved in 0.1 M Tris-HCl (pH 8.5) containing 4 mM of $MgCl_2$ (the final reaction volume was 50 μL). To the solution was added 5 μL of cytidine-5'-triphosphate (CTP, 150 mM, 1.5 eq.). The pH of the mixture was maintained at pH 8.5. Next, 10 μL of *Pasteurella multocida* α2,3-sialyltransferase (PmST1) (4 mg mL^{-1}) and 10 μL of *Neisseria meningitidis* CMP-Sia synthetase (NmCSS) (4 mg mL^{-1}) were added to the reaction mixture. The reaction was kept at 37 °C for 18 h. The crude product ICG-C9-Siaα2-3Galβ1-4GlcNAcβProN_3 was characterized by mass spectrometry. HRMS of the product (ESI) m/z calcd for $C_{73}H_{96}N_8O_{22}S$ $[M+H+Na]^{2+}$ 746.3162, found 746.3092.

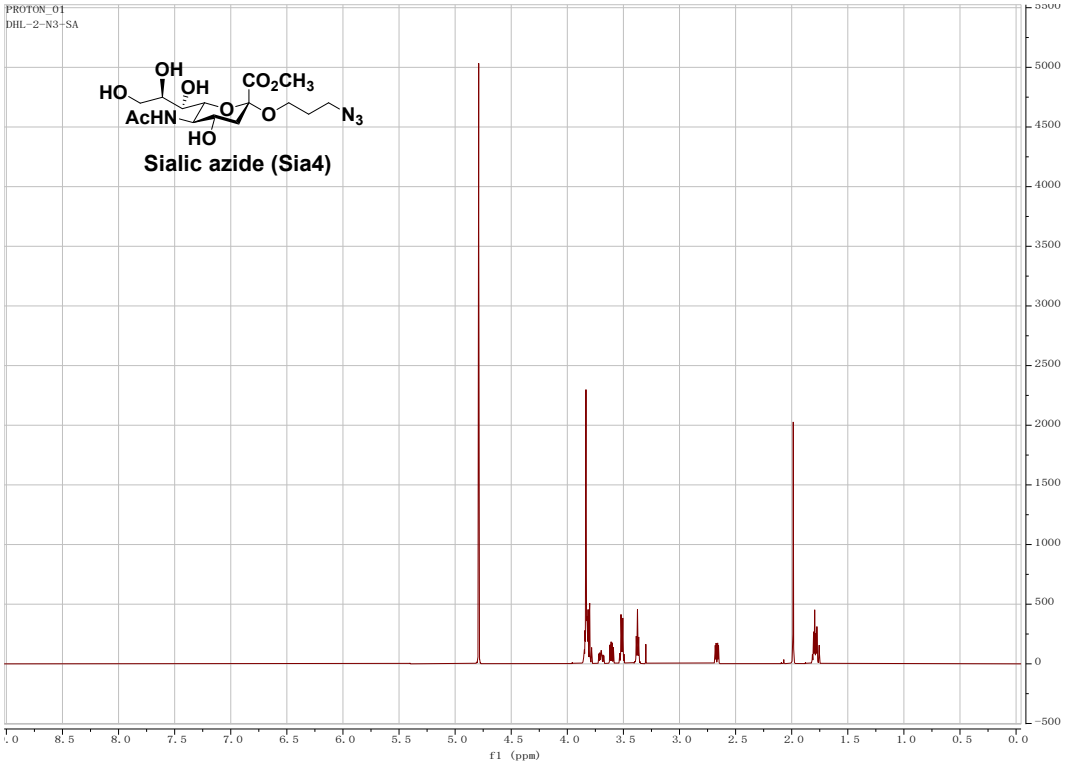
Recognition of Sia-C9-ICG by α2,6-sialyltransferase in the presence of NmCSS. 5 μL of Galβ1-4GlcNAcβProN_3 (100 mM) and 5 μL of Sia-C9-ICG (120 mM, 1.2 eq.) were dissolved in 0.1 M Tris-HCl (pH 8.5) containing 4 mM of $MgCl_2$ (the final reaction volume was 50 μL). To the solution was added 5 μL of cytidine-5'-triphosphate (CTP, 150 mM, 1.5 eq.). The pH of the mixture was maintained at pH 8.5. Next, 10 μL of *Photobacterium damsela* α2,6-sialyltransferase (PdST, from Sigma) (1 U mL^{-1}) and 10 μL of *Neisseria meningitidis* CMP-Sia synthetase (NmCSS) (4 mg mL^{-1}) were added to the reaction mixture. The reaction was kept at 37 ℃ for 6 h. The crude product ICG-C9-Siaα2-6Galβ1-4GlcNAcβProN_3 was characterized by mass spectrometry. HRMS of the product (ESI) m/z calcd for $C_{73}H_{96}N_8O_{22}S$ $[M+2H]^{2+}$ 735.3253, found 735.3130; $[M+H+Na]^{2+}$ 746.3162, found 746.3036; $[M+2Na]^{2+}$ 757.3072, found 757.2961.

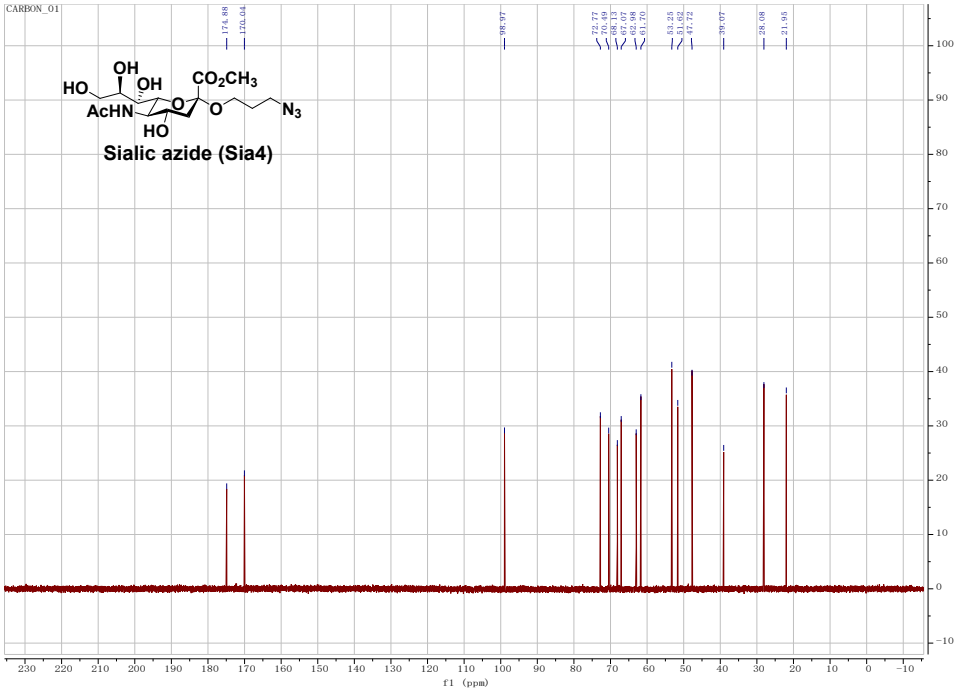
***In vivo* tumor challenge studies**

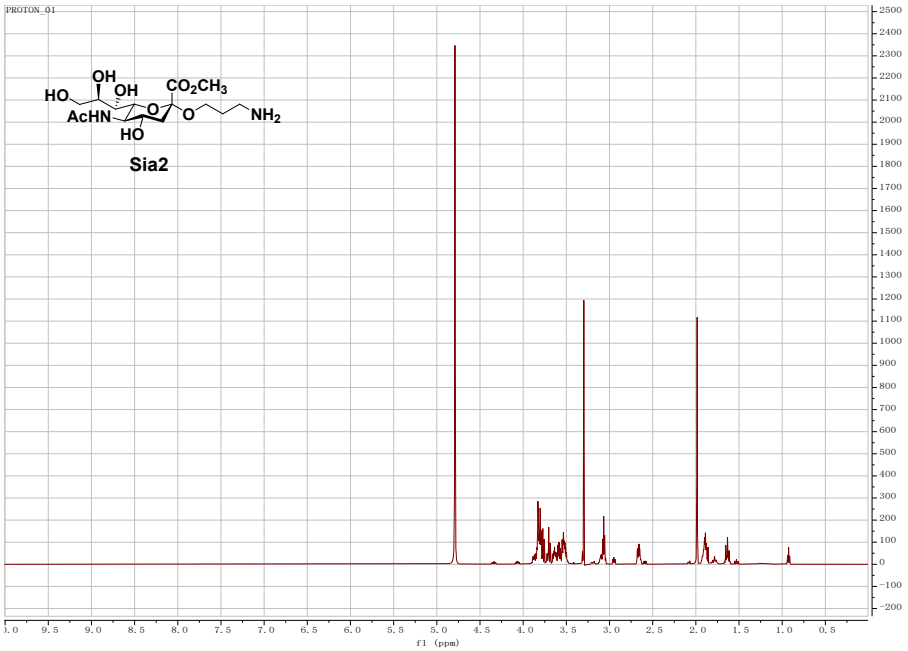
For EL4 tumor challenge study, C57BL/6 female mice were injected subcutaneously with EL4 cells (2×10^5) on day 0, and intravenously injected with PBS, ICG (40 nmol per mouse), Sia-C2-ICG (40 nmol per mouse), and Sia-C9-ICG (40 nmol per mouse), respectively, on day 7. The mice were irradiated with the 808 nm laser (1.0 W cm^{-2}, 5 min) at 2 h following injection of Sia-ICGs or PBS in mice. n = 6 mice per group. Tumor volume was calculated with the formula: Volume (mm^3) = 1/2 (length × width × height) [4].

For 4T1 tumor challenge study, 4T1 cells (2×10^5) were injected subcutaneously into BALB/c mice. The tumor-bearing mice were randomly divided into eight groups (n = 6 mice per group). On days 10 and 16, the mice were intravenously given two injections with PBS, ICG (40 nmol per mouse), Sia-C2-ICG (40 nmol per mouse), and Sia-C9-ICG (40 nmol per mouse), respectively. For the laser-treated groups, the mice were irradiated with the 808 nm laser (1.0 W cm^{-2}, 10 min) 2 h after the injection of Sia-ICGs, ICG or PBS. The growth curves of 4T1 tumors in mice and the body weights of tumor-bearing mice were recorded. In order to test the *in vivo* toxicity of Sia-C9-ICG for phototherapy, 3 mice in the group treated with Sia-C9-ICG plus laser irradiation, were sacrificed after 24 days. Their organs were dissected, fixed in paraformaldehyde, and analyzed by hematoxylin and eosin (H&E) staining.

Product Characterization Spectra



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NH2
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Sia2
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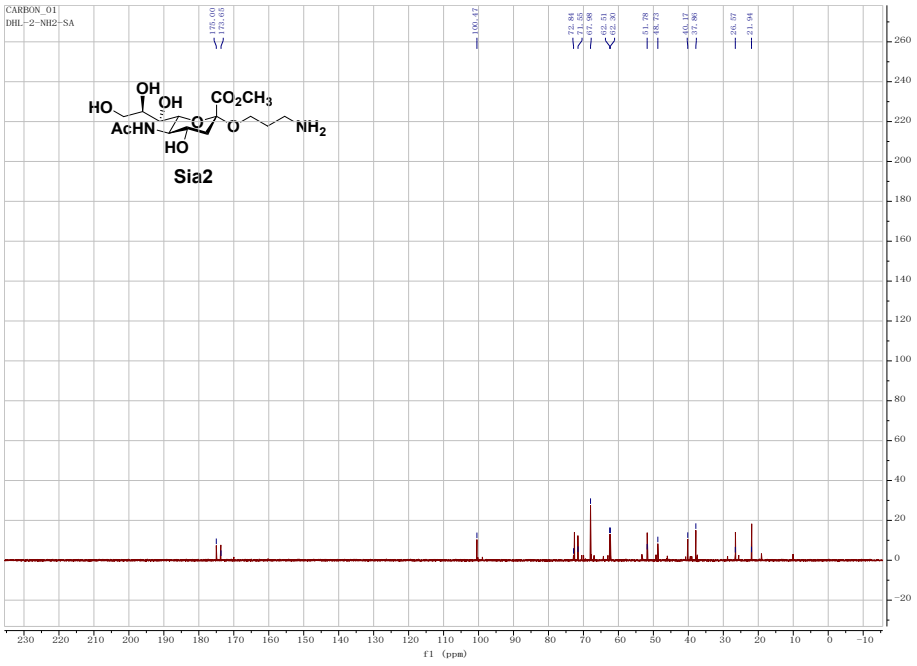
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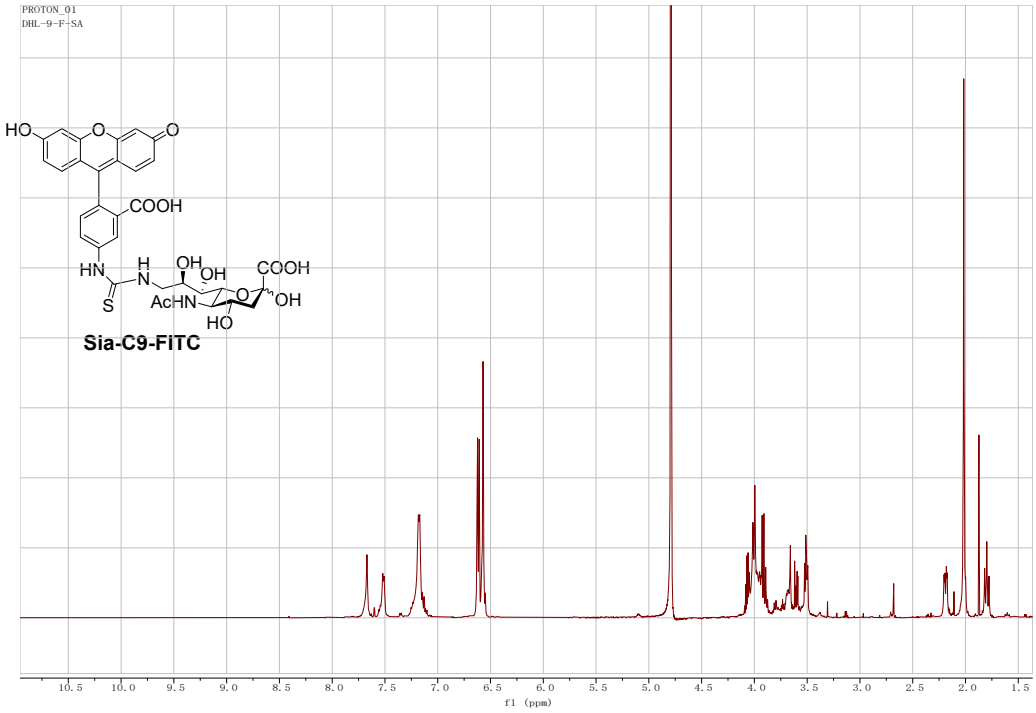
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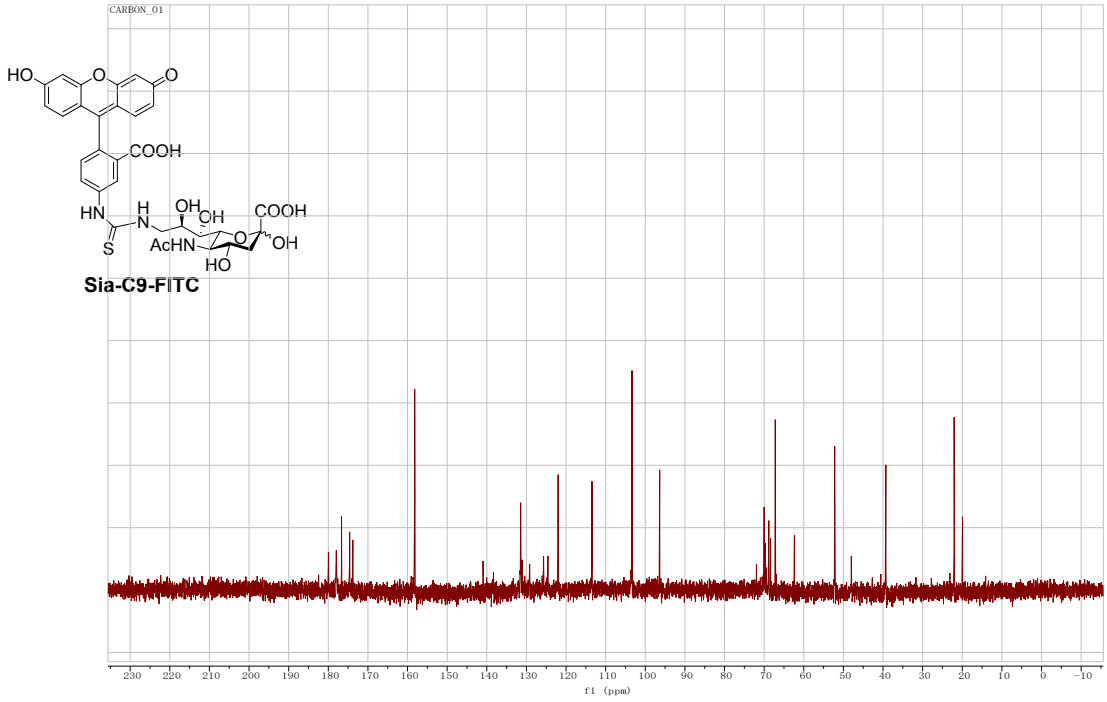
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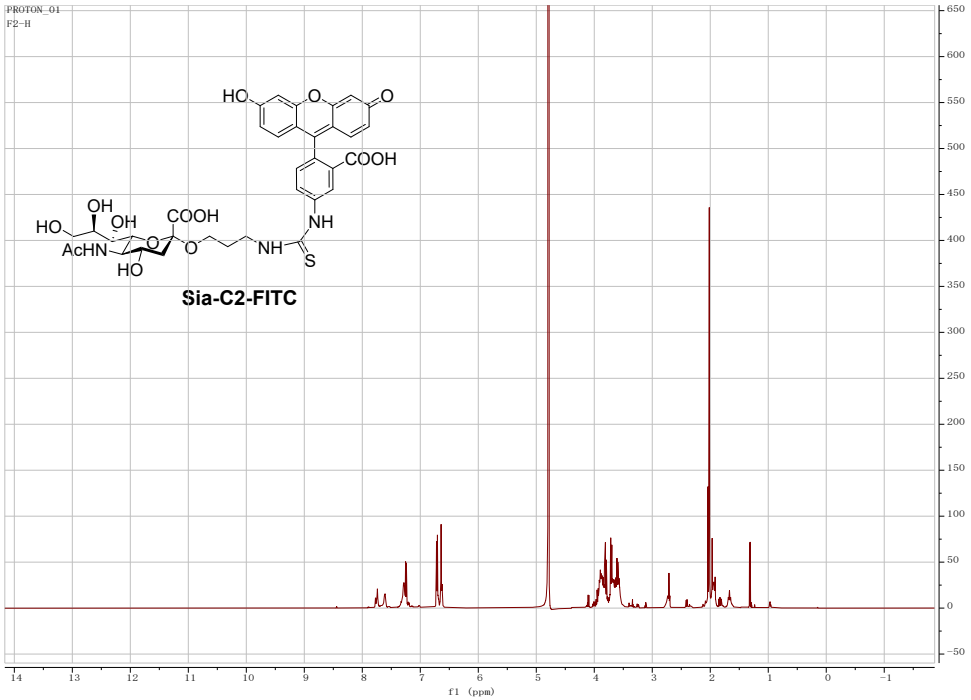
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550
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450
400
350
300
250
200
150
100
50
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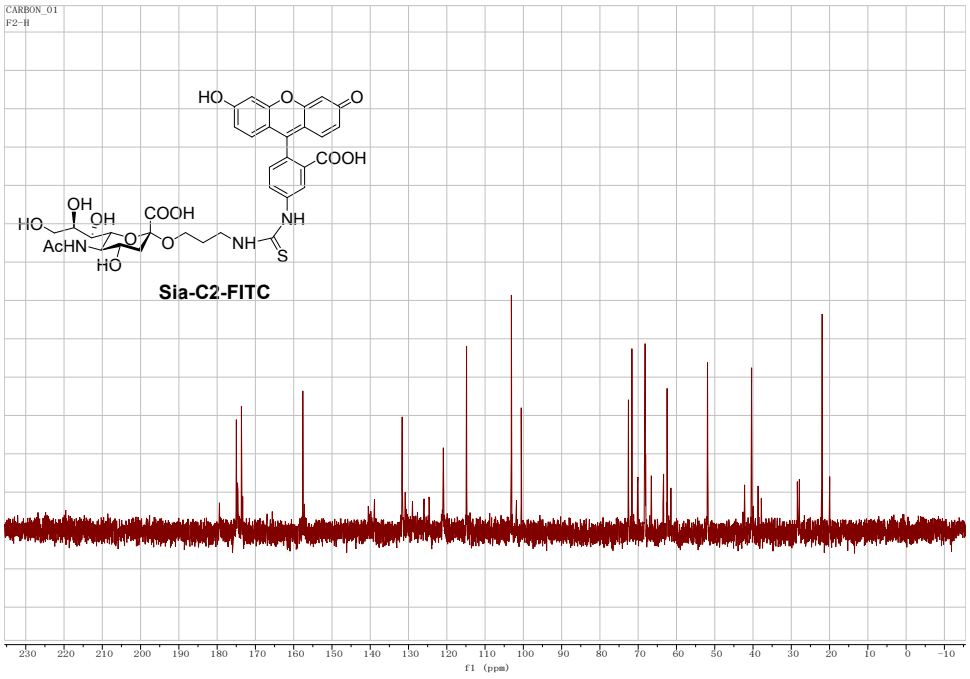
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200
190
180
170
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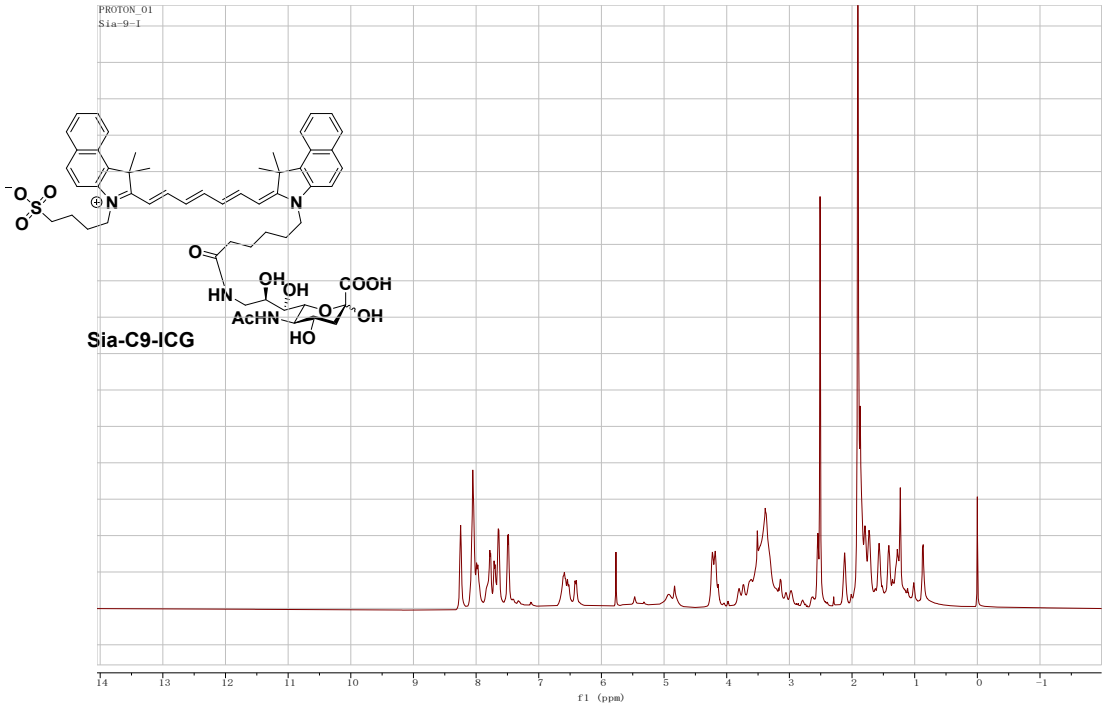
−10
f1 (ppm)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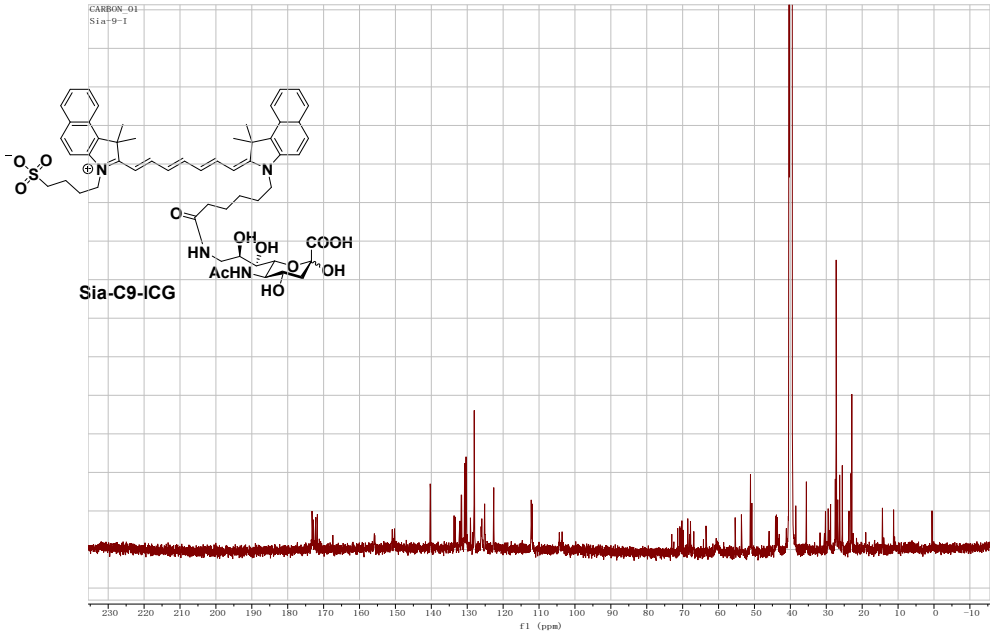
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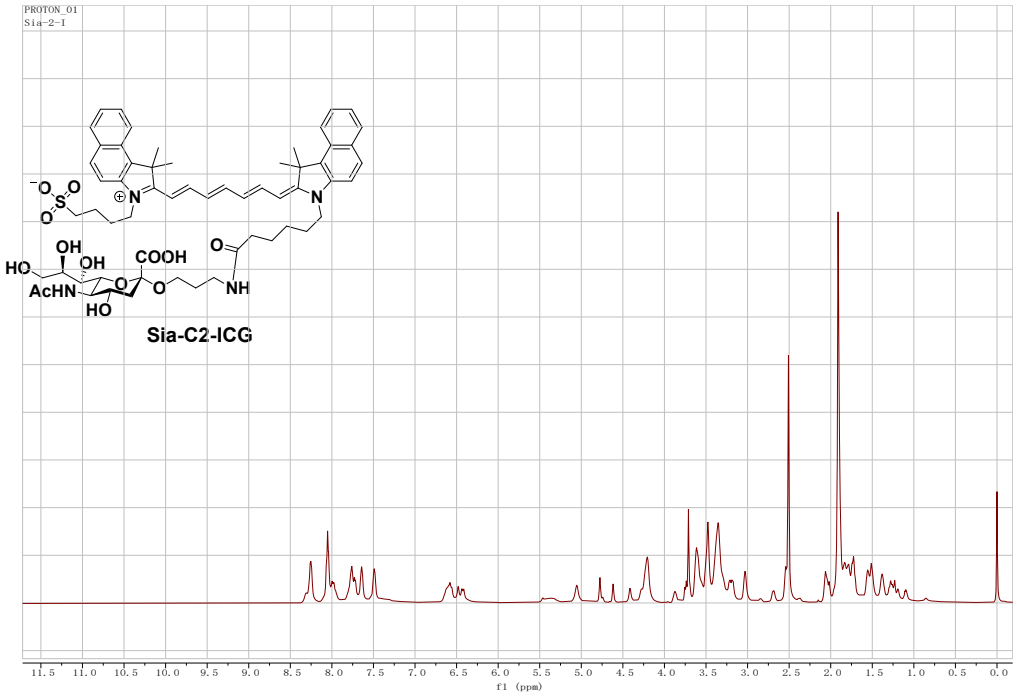
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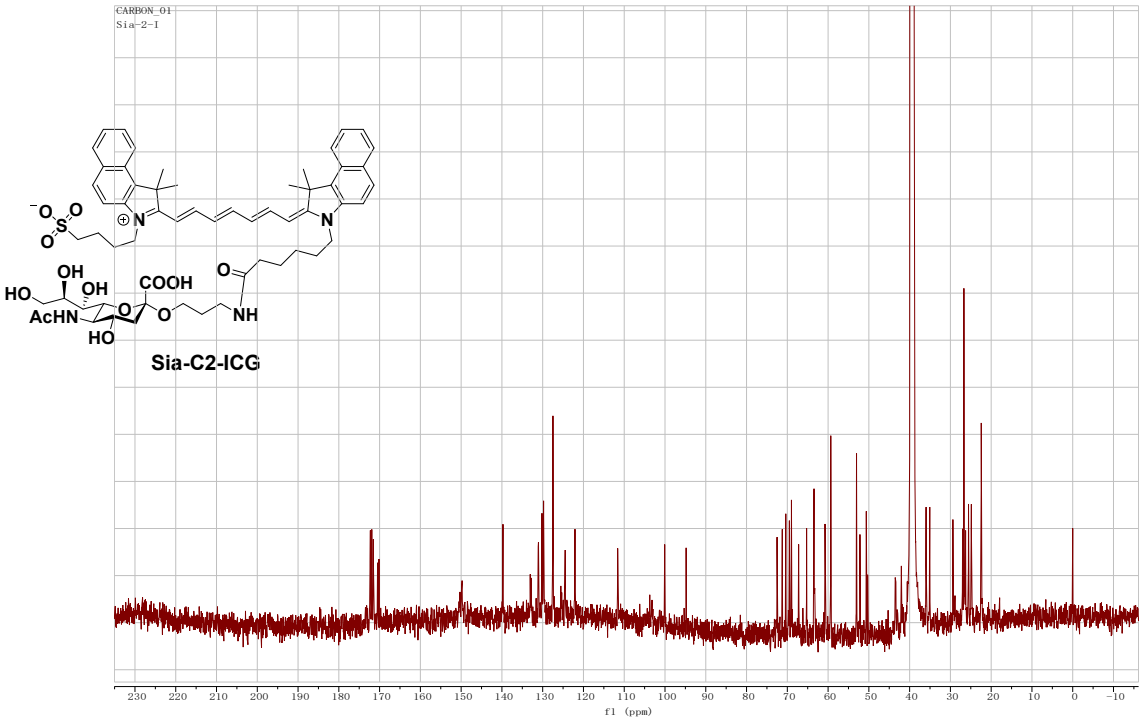
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References

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