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Electronic Supplementary Information for

Rapid and high-efficiency discrimination of different sialic acid species using dipeptide-based fluorescent sensors

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Part S1. Experimental section

Chemicals. All peptides that contain Asp-Pro-Ala-Asp (DPAD) sequence and six dipeptides (Pro-Asp (PD), Asp-Ala (DA), Pro-Ala (PA), Ala-Asp (AD), Asp-Pro (DP) and Asp-Asp (DD)) were purchased from China–Peptides Corp. (Shanghai) with high purity (>98%). Fluorescein isothiocyanate (FITC, 99%), trishydroxymethyl-aminomethane (Tris), and various saccharides, such as *D*-glucose, *D*-galactose, *N*-Ac-glucose, *N*-Ac-galactose, fucose, mannose, ribose, lactose, maltose, sucrose and tetramaltose with high purity (>98%), were purchased from Alfa Aesar (Germany). The six model sialic acid (SAs), *N*-Acetylneuraminic acid (SA-1), 2-Keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid (SA-2), *N*-Acetylneuraminic acid methyl ester (SA-3), *N*-Acetyl-2,3-didehydro-2-deoxyneuraminic acid (SA-4), *N*-Glycolyl-neuraminic acid (SA-5) and 2,4,7,8,9-Penta-*O*-acetyl-*N*-acetyl-neuraminic acid methyl ester (SA-6), were provided by Tokyo Chemical Industry (TCI, Japan) with high purity (>97%). Triethylamine (Et_3N), hydrochloric acid (HCl), acetonitrile (CH_3CN) and *N, N*'-dimethylformamide (DMF) were used as received from Sigma-Aldrich (Germany), all these regents were chromatographic pure. Milli-Q ultrapure water was used in all needed experiments.

Instruments. Hydrogen and carbon nuclear magnetic resonance (^1H and ^{13}C NMR) experiment were carried out on a Varian Inova 500-MHz spectrometer. Fourier translation infrared (FT-IR) spectra were recorded on a Bruker Optics Vertex 80V FT-IR spectrometer. Elemental analysis was performed on a Vario EL cube (Elementar, Germany). Isothermal titration calorimetry (ITC) experiment was performed on a MicroCal iTC200 system. Fluorescence spectra were recorded on a Perkin-Elmer LS55 fluorescence spectrophotometer. Mass spectra were obtained with an electrospray ionization-quadrupole time-of-flight mass spectrometer (ESI-Q-TOF MS) (Waters, Manchester, UK).

Synthesis of fluorescein-labelled (FL) peptides. FL peptides were synthetized through one-step reaction according to the literature.[1] The synthesis of FL-Pro-Asp (FL-PD) was taken as an example and illustrated in Scheme S1. Et_3N (0.100 g, 1.0

mmol) was added to a solution of PD (0.460 g, 2 mmol) in 30 mL dry DMF, the mixture was stirred for 10 min, then FITC (0.778 g, 2 mmol) was added dropwise to this mixture at ambient temperature, and continued to be stirred for 24 hours. After rotary evaporation of solvent, the crude product was purified by reversed-phase liquid chromatography on C18 modified silica gel with an elution of CH_3CN/H_2O (3:1, v/v), to obtain the pure product as yellow powder (1.015 g, yield: 82.2%). The other FL peptides were synthesized and purified through the similar experiments. The characterization data for FL-PD is listed as follows: ^{1}H NMR (500 MHz, d_6-DMSO): δ (ppm): 3.60 (s, 1H, CH_2C), 3.65–3.78 (m, 2H, NCH_2), 3.85 (s, 1H, CH_2C), 4.54 (ddd, J_1 = 28.5 Hz, J_2 = 13.8 Hz, J_3 = 6.6 Hz, 1H, NC*H*), 4.87, 4.96 (dd, J_1 = 7.5, J_2 = 7.9, 1H, COC*H*), 6.55–6.62 (m, 2H, fluorescein-Ph-*H*), 6.64 (d, *J* = 8.6 Hz, 2H, fluorescein-Ph-*H*), 6.69 (s, 2H, fluorescein-Ph-*H*), 7.18 (d, *J* = 8.2 Hz, 1H, fluorescein-Ph-*H*), 7.87 (d, *J* = 8.2 Hz, 1H, fluorescein-Ph-*H*), 8.07 (d, *J* = 24.5 Hz, 1H, fluorescein-Ph-*H*), 8.22 (dd, J_1 = 24.3 Hz, J_2 = 8.1 Hz, 1H, CON*H*), 9.29 (d, *J* = 17.6 Hz, 1H, CSN*H*), 10.12 (s, 2H, fluorescein-O*H*), 12.50 (s, 2H, COO*H*); ^{13}C NMR (500 MHz, d_6-DMSO): δ (ppm): 24.8, 30.4, 36.4, 49.0, 50.1, 65.7, 102.9, 110.5, 113.2, 120.8, 123.8, 126.7, 129.7, 132.6, 142.7, 147.9, 152.2, 159.9, 169.1, 172.1, 178.6; Infrared (IR) spectroscopy: 3302, 3086, 2879, 1583, 1536, 1456, 1385, 1268, 1172, 848, 719 cm^{-1}; ESI-MS: m/z calcd. for $C_{30}H_{25}N_3O_{10}S$: 619.13; found: 620.1260 (M+H). Elemental analysis calcd. (%) for $C_{30}H_{25}N_3O_{10}S$: C, 58.16; H, 4.07; N, 6.78; S, 5.17. Found: C, 58.10; H, 4.16; N, 6.59; S, 5.32.

The characterization data for FL-DP is listed as follows: ^{1}H NMR (500 MHz, d_6-DMSO): δ (ppm): 1.79–2.00 (m, 2H, CCH_2), 2.41 (dd, J_1 = 16.4, J_2 = 7.6, 1H, CCH_2), 2.59–2.66 (m, 1H, CCH_2), 3.49 (s, 1H, NCH_2), 3.53–3.70 (m, 1H, NCH_2), 4.22 (dd, J_1 = 8.7 Hz, J_2 = 3.9 Hz, 1H, COC*H*), 4.88 (dd, J_1 = 14.1, J_2 = 7.6, 1H, NC*H*), 6.55–6.60 (m, 2H, fluorescein-Ph-*H*), 6.61 (s, 1H, fluorescein-Ph-*H*), 6.62 (s, 1H, fluorescein-Ph-*H*), 6.68 (d, *J* = 9.6 Hz, 2H, fluorescein-Ph-*H*), 7.16 (d, *J* = 9.9 Hz, 1H, fluorescein-Ph-*H*), 7.76 (d, *J* = 7.6 Hz, 1H, fluorescein-Ph-*H*), 8.17 (s, 1H, CSN*H*), 8.24 (d, *J* = 8.1 Hz, 1H, fluorescein-Ph-*H*), 8.28 (s, 1H, CSN*H*), 9.99 (s, 2H, fluorescein-O*H*); ^{13}C NMR (500 MHz, d_6-DMSO): δ (ppm): 25.0, 29.1, 36.4, 44.2,

47.4, 58.8, 102.5, 110.7, 113.0, 116.7, 124.4, 129.5, 142.7, 147.6, 152.5, 159.9, 169.1, 171.8, 173.4; Infrared (IR) spectroscopy: 3302, 3073, 2867, 1709, 1598, 1538, 1454, 1271, 1177, 1132, 915, 721 cm^{-1}; ESI-MS: m/z calcd. for $C_{30}H_{25}N_3O_{10}S$: 619.13; found: 620.1420 (M+H). Elemental analysis calcd. (%) for $C_{30}H_{25}N_3O_{10}S$: C, 58.16; H, 4.07; N, 6.78; S, 5.17. Found: C, 58.21; H, 4.12; N, 6.64; S, 5.24.

The characterization data for FL-DA is listed as follows: ^1H NMR (500 MHz, d_6-DMSO): δ (ppm): 1.27 (d, J = 8.2 Hz, 3H, CCH_3), 2.53–2.59 (m, 1H, CCH_2), 2.72–2.81 (m, 1H, CCH_2), 4.15–4.24 (m, 1H, COC*H*), 4.56–4.66 (m, 1H, NC*H*), 6.55–6.63 (m, 4H, fluorescein-Ph-*H*), 6.69 (d, J = 2.1 Hz, 2H, fluorescein-Ph-*H*), 7.17 (d, J = 8.2 Hz, 1H, fluorescein-Ph-*H*), 7.71 (s, 1H, CSN*H*), 7.76 (d, J = 2.3 Hz, 1H, fluorescein-Ph-*H*), 8.06–8.10 (m, 1H, CON*H*), 8.22 (s, 1H, fluorescein-Ph-*H*), 10.02 (s, 2H, fluorescein-O*H*), 10.16 (s, 2H, COO*H*), 10.33 (s, 1H, CSN*H*); ^{13}C NMR (500 MHz, d_6-DMSO): δ (ppm): 17.5 32.1, 35.7, 36.5, 42.3, 48.1, 49.4, 102.9, 110.2, 113.2, 124.8, 129.4, 152.2, 159.9, 169.5, 171.1, 172.1; 174.4 Infrared (IR) spectroscopy: 3302, 3061, 2933, 1714, 1639, 1533, 1455, 1231, 1141, 914, 834, 721 cm^{-1}; ESI-MS: m/z calcd. for $C_{28}H_{23}N_3O_{10}S$: 593.11; found: 594.1212 (M+H). Elemental analysis calcd. (%) for $C_{28}H_{23}N_3O_{10}S$: C, 56.66; H, 3.91; N, 7.08; S, 5.40. Found: C, 56.60; H, 3.98; N, 7.12; S, 5.35.

The characterization data for FL-DD is listed as follows: ^1H NMR (500 MHz, d_6-DMSO): δ (ppm): 2.09–2.16 (m, 2H, CCH_2), 2.57–2.72 (m, 2H, CCH_2), 4.53 (dd, J_1 = 14.0 Hz, J_2 = 6.0 Hz, 1H, COC*H*), 4.62 (dd, J_1 = 13.5, J_2 = 8.2, 1H, COC*H*), 6.55–6.65 (m, 4H, fluorescein-Ph-*H*), 6.69 (d, J = 2.1 Hz, 2H, fluorescein-Ph-*H*), 7.17 (d, J = 8.2 Hz, 1H, fluorescein-Ph-*H*), 7.77 (d, J = 2.2 Hz, 1H, fluorescein-Ph-*H*), 8.01 (d, J = 8.1 Hz, 1H, CSN*H*), 8.12 (d, J = 8.0 Hz, 1H, CON*H*), 8.23 (s, 1H, fluorescein-Ph-*H*), 10.08 (s, 2H, fluorescein-O*H*); ^{13}C NMR (500 MHz, d_6-DMSO): δ (ppm): 35.2, 36.5, 43.7, 48.5, 49.4, 102.9, 110.2, 113.2, 124.4, 127.0, 129.4, 142.3, 147.6, 152.2, 158.9, 160.2, 169.1, 169.1, 171.1, 172.1; Infrared (IR) spectroscopy: 3302, 3058, 2934, 1666, 1636, 1591, 1537, 1387, 1177, 967, 797, 764 cm^{-1}; ESI-MS: m/z calcd. for $C_{29}H_{23}N_3O_{12}S$: 637.10; found: 636.1158 (M−H). Elemental analysis calcd. (%) for $C_{29}H_{23}N_3O_{12}S$: C, 54.63; H, 3.64; N, 6.59; S, 5.03. Found: C, 54.50; H, 3.59; N,

6.63; S, 5.11.

The characterization data for FL-PA is listed as follows: ^{1}H NMR (500 MHz, d_6-DMSO): δ (ppm): 1.20–1.45 (m, 3H, CHC*H*$_3$), 1.45–1.63 (m, 2H, CH$_2$C*H*$_2$) 1.68–1.92 (m, 2H, CHC*H*$_2$), 3.32–3.78 (m, 2H, NC*H*$_2$), 4.12–4.29 (m, 1H, COC*H*), 4.34 (ddd, J_1 = 31.9 Hz, J_2 = 16.9 Hz, J_3 = 6.5 Hz, 1H, NHC*H*), 6.57 (dd, J_1 = 8.7 Hz, J_2 = 1.8 Hz, 2H, fluorescein-Ph-*H*), 6.61 (d, J = 8.6 Hz, 2H, fluorescein-Ph-*H*), 6.69 (s, 2H, fluorescein-Ph-*H*), 7.18 (d, J = 8.3 Hz, 1H, fluorescein-Ph-*H*), 7.75 (d, J = 6.8 Hz, 1H, fluorescein-Ph-*H*), 8.10 (d, J = 7.3 Hz, 1H, fluorescein-Ph-*H*), 8.15 (s, 1H, CON*H*), 8.27 (s, 1H, CSN*H*), 9.97 (s, 2H, fluorescein-O*H*); ^{13}C NMR (500 MHz, d_6-DMSO): δ (ppm): 17.8, 24.8, 26.7, 44.6, 48.3, 59.1, 102.6, 110.5, 113.2, 116.7, 124.7, 127.4, 129.4, 141.9, 152.5, 158.9, 159.1, 160.1, 169.1, 170.8, 172.4, 174.8; Infrared (IR) spectroscopy: 3302, 3071, 2943, 1590, 1538, 1453, 1385, 1271, 963, 849, 764 cm^{-1}; ESI-MS: m/z calcd. for $C_{29}H_{25}N_3O_8S$: 575.14; found: 576.2060 (M+H). Elemental analysis calcd. (%) for $C_{29}H_{25}N_3O_8S$: C, 60.51; H, 4.38; N, 7.30; S, 5.57. Found: C, 60.45; H, 4.26; N, 7.39; S, 5.49.

The characterization data for FL-AD is listed as follows: ^{1}H NMR (500 MHz, d_6-DMSO): δ (ppm): 1.19 (d, J = 7.1 Hz, 3H, CC*H*$_3$), 2.09–2.21 (m, 2H, CC*H*$_2$), 4.24–4.39 (m, 1H, COC*H*), 4.53 (dd, J_1 = 13.9 Hz, J_2 = 6.4 Hz, 1H, NC*H*), 6.58 (dd, J_1 = 8.7 Hz, J_2 = 1.9 Hz, 2H, fluorescein-Ph-*H*), 6.61 (s, J = 8.6 Hz, 2H, fluorescein-Ph-*H*), 6.69 (d, J = 1.9 Hz, 2H, fluorescein-Ph-*H*), 7.16 (d, J = 8.3 Hz, 1H, fluorescein-Ph-*H*), 7.76 (d, J = 6.4 Hz, 1H, fluorescein-Ph-*H*), 8.11 (d, J = 7.7 Hz, 1H, fluorescein-Ph-*H*), 8.15 (s, 1H, CON*H*), 8.28 (s, 1H, CSN*H*), 10.00 (s, 2H, fluorescein-O*H*); ^{13}C NMR (500 MHz, d_6-DMSO): δ (ppm): 15.8, 25.1, 26.5, 35.4, 40.7, 44.0, 48.6, 49.3, 102.9, 110.2, 113.2, 124.4, 127.0, 129.4, 142.0, 147.5, 152.2, 158.9, 160.2, 169.1, 172.1, 172.8; Infrared (IR) spectroscopy: 3302, 3061, 2934, 1590, 1454, 1269, 1175, 1118, 915, 796, 720 cm^{-1}; ESI-MS: m/z calcd. for $C_{28}H_{23}N_3O_{10}S$: 593.11; found: 594.1560 (M+H). Elemental analysis calcd. (%) for $C_{28}H_{23}N_3O_{10}S$: C, 56.66; H, 3.91; N, 7.08; S, 5.40. Found: C, 56.50; H, 3.96; N, 6.99; S, 5.38.

Fluorescent experiments. Host FL peptide probe was prepared as stock solution in Tris-HCl buffer solution (1.0 mM) for 6.0×10^{-4} mol·L^{-1}. Guest SA was prepared to

0.0175 and 0.175 mol·L^{-1} of stock solution in H_2O. The work solutions were prepared by adding different volumes of guest solution to a series of test tubes, and then same amount of stock solution of host FL peptide was added into each test tube, followed by dilution to 3.00 mL by Tris-HCl buffer solution. After being shaken for 1 min, the work solutions were measured immediately at 25 °C. Every experiment was repeated three times to ensure reproducibility.[2]

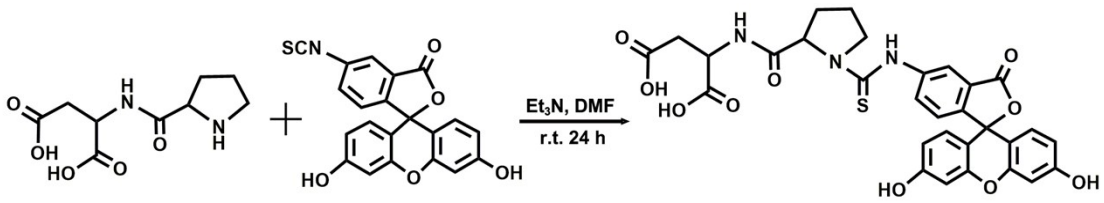
^{1}H NMR experiment. ^{1}H NMR experiments were undertaken to investigate the binding details between FL-PD and SA-1 in d_6-DMSO.[3] The chemical shifts of protons of FL-PD (2 mmol·L^{-1}), SA-1 (2 mmol·L^{-1}), and an equimolar mixture of them were recorded and analyzed. Similar method was used to study the complexation between FL-PD and SA-3 in d_6-DMSO. To avoid the interference of D_2O with strong suppression effect on hydrogen bonding, d_6-DMSO was chosen as the solvent because both FL-PD and SA derivatives were well soluble in it.

FT-IR experiment (Bio-ATR Mode).[4] The infrared spectra were recorded on a Bruker Vertex 80v FT-IR spectrometer in Bio-ATR cell II accessory (the accessory is based on dual crystal technology: the top crystal is made of silicon, and the second crystal has a hemispherical design and is made of zincselenide (ZnSe)). The Bio-ATR II unit is factory-prealigned; hence, no alignment is required. All samples were dissolved in 20 μL d_6-DMSO. For each sample, the concentration (FL-PD: 0.2 mmol·L^{-1}, SA-1: 0.2 mmol·L^{-1}) and total volume (20 μL) were strictly controlled. For each measurement, the equipment remained in standby mode for 15 min to ensure the equilibrium of temperature (20 °C) prior to the test, and all the spectra of samples were obtained by 1200 scans subtracting the d_6-DMSO background at a 4 cm^{-1} resolution. Before each measurement, the ATR crystal was cleaned with distilled water and ethanol and dried sufficiently under nitrogen gas flow. Similar method was used to study the complexation between FL-PD and SA-3 in d_6-DMSO.

ITC experiment.[5] The binding enthalpies, entropies and associated constant were determined at T = (298.15 ± 0.01) K and atmospheric pressure p = (101.3 ± 5.0) kPa, and calculated by software of MicroCal Analysis Launcher. The sample cell was loaded with 200 μL FL-PD aqueous solution (0.1 mmol·L^{-1}) while the reference cell

was loaded with 200 μL of pure water. The 40 μL syringe was filled with a SA-1 aqueous solution (5 mmol·L^{-1}). A run of ITC consists of 18 times successive injections of 2 μL titrant solution with 5 s duration each, and an interval of 2 min between two injections. The apparent heat effect per injection, which corresponds to the change in molality of titrated solution in the sample cell, was determined by automatic peak integration of thermal power vs time curve. The thermal effects from the friction in the process of injection were considered to be negligible according to the literature.[6] Similar method was used to study the complexation between FL-PD and SA-3 in H_2O.

Part S2. Supplementary figures



Scheme S1 Synthesis of fluorescein-labelled (FL) dipeptide sensors (here FL-PD was taken as an example).

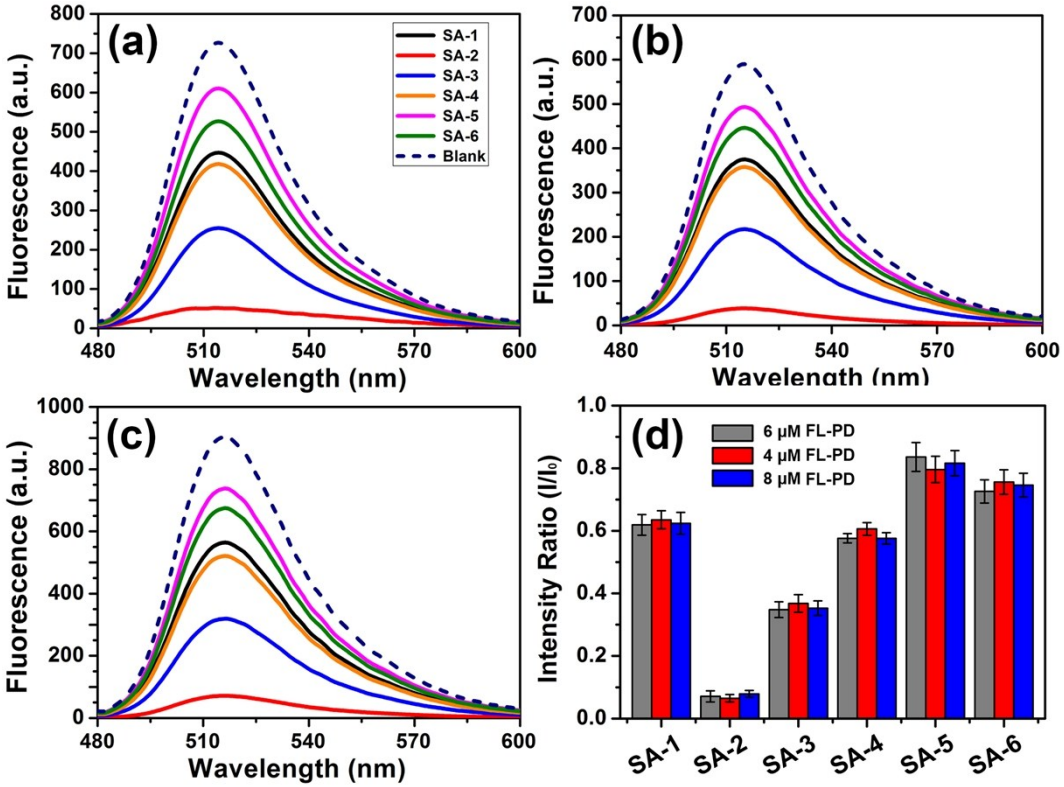


Fig. S1 (a-c) Fluorescence spectra (λex: 470 nm) of FL-DP solutions upon the addition of 20 equivalents of diverse SA derivatives in Tris-buffer solution (1 mM, pH 7.4) at 20 °C; FL-PD concentrations: 6 μM (a), 4 μM (b) and 8 μM (c). (d) Comparative analysis of corresponding fluorescence intensity (λem: 514 nm) changes of FL-DP solutions (concentrations: 6 μM, 4 μM and 8 μM) with the addition of 20 equivalents of diverse SA derivatives in Tris-buffer solution (1 mM, pH 7.4) at 20 °C; all data are shown as mean ± standard error (n = 3).

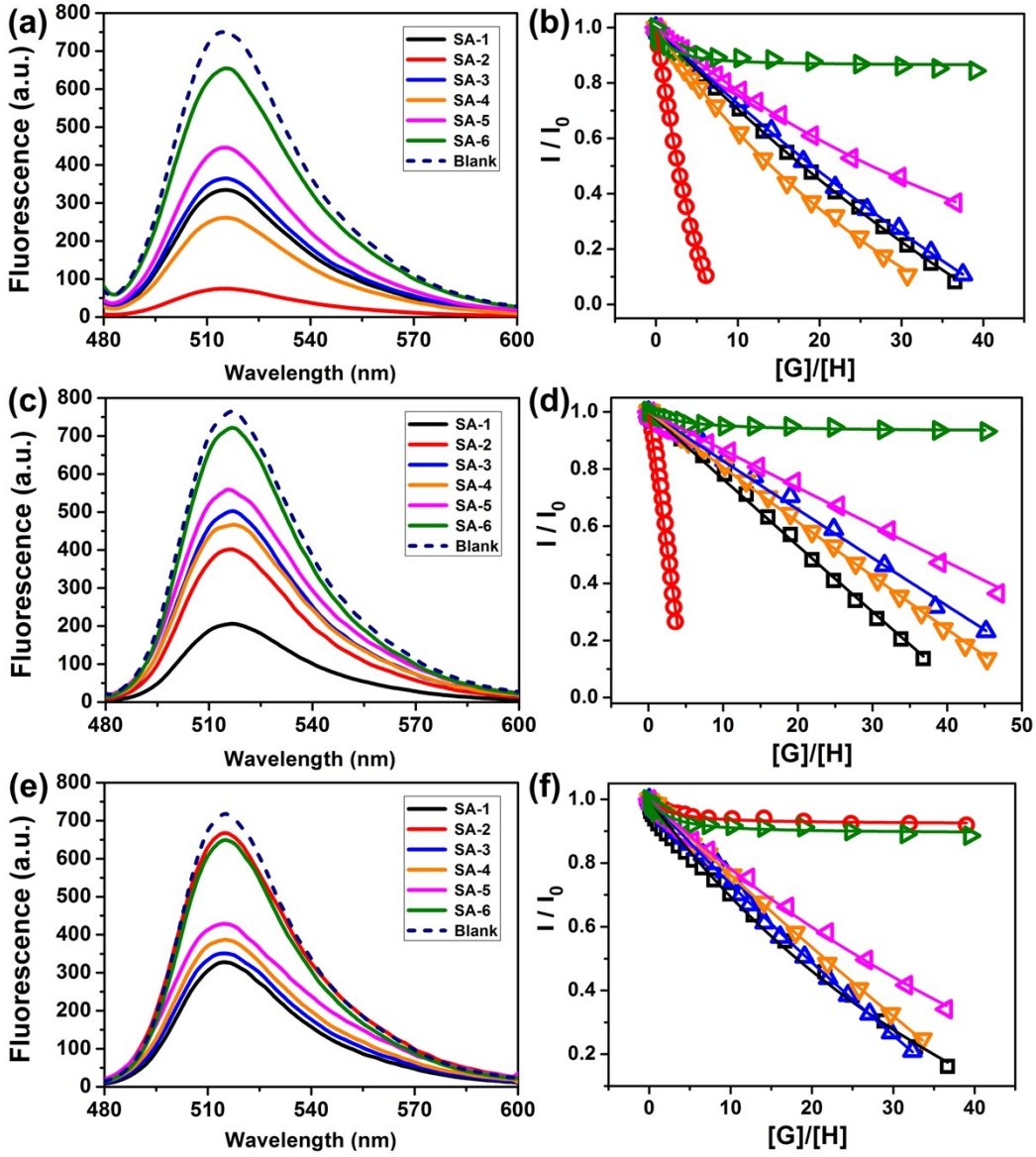


Fig. S2 Fluorescence spectra (λex: 470 nm) of FL-DP (a), FL-DA (c) and FL-DD (e) solutions (6 μM) upon the addition of 20 equivalents of SA derivatives in Tris-buffer solution (1 mM, pH 7.4) at 20 °C, and corresponding dynamic fluorescent intensity (λem: 514 nm) changes of FL-DP (b), FL-DA (d) and FL-DD (f) solutions with the addition of various equivalents of different SA derivatives in Tris-buffer solution (1 mM, pH 7.4) at 20 °C.

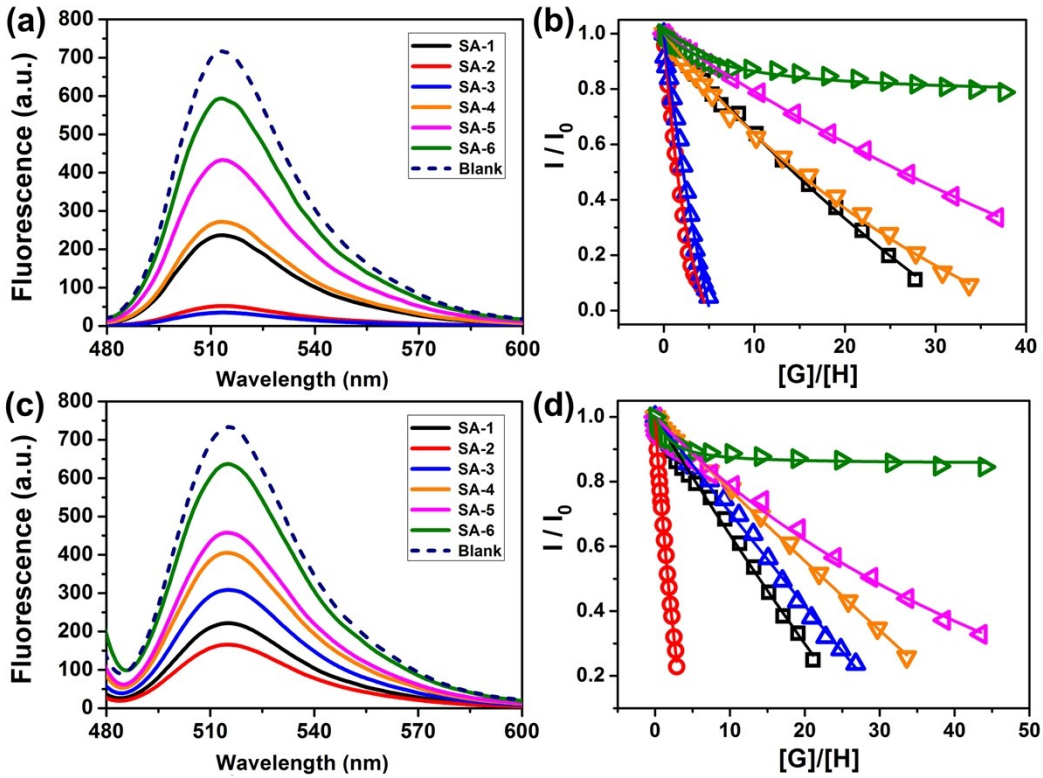


Fig. S3 Fluorescence spectra (λex: 470 nm) of FL-PA (a) and FL-AD (c) solutions (6 μM) upon the addition of 20 equivalents of SA derivatives in Tris-buffer solution (1 mM, pH 7.4) at 20 °C, and corresponding dynamic fluorescent intensity (λem: 514 nm) changes of FL-PA (b) and FL-AD (d) solutions with the addition of various equivalents of different SA derivatives in Tris-buffer solution (1 mM, pH 7.4) at 20 °C.

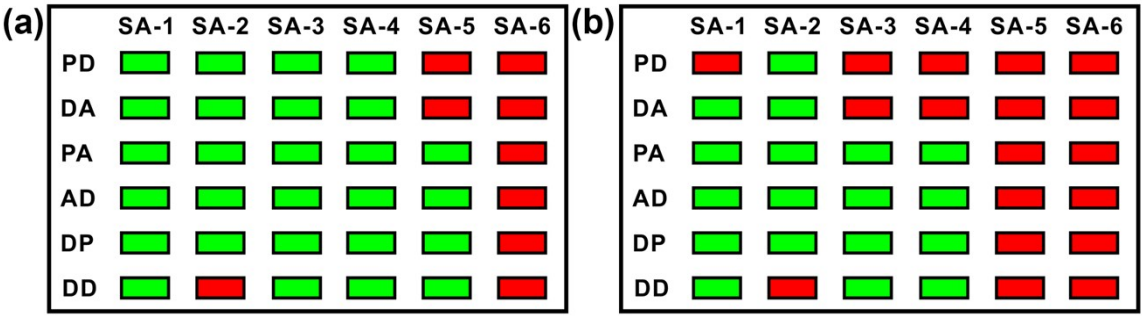


Fig. S4 Corresponding fluorescence response matrix upon the addition of 35 equivalents of six model SAs in Tris-buffer solution (pH 7.4) at °C, when the threshold fluorescence intensity ratio (I/I_0) was set as 0.5 (a) or 0.3 (b). In a, SA-1, SA-3 and SA-4 had the same traffic light pattern, and could not be discriminated. Similarly in b, two pairs of SA species (SA-3 and SA-4; SA-5 and SA-6) could not be discriminated due to the same reason. All the data indicated that neither 0.5 nor 0.3 is the optimal value of threshold fluorescence intensity ratio.

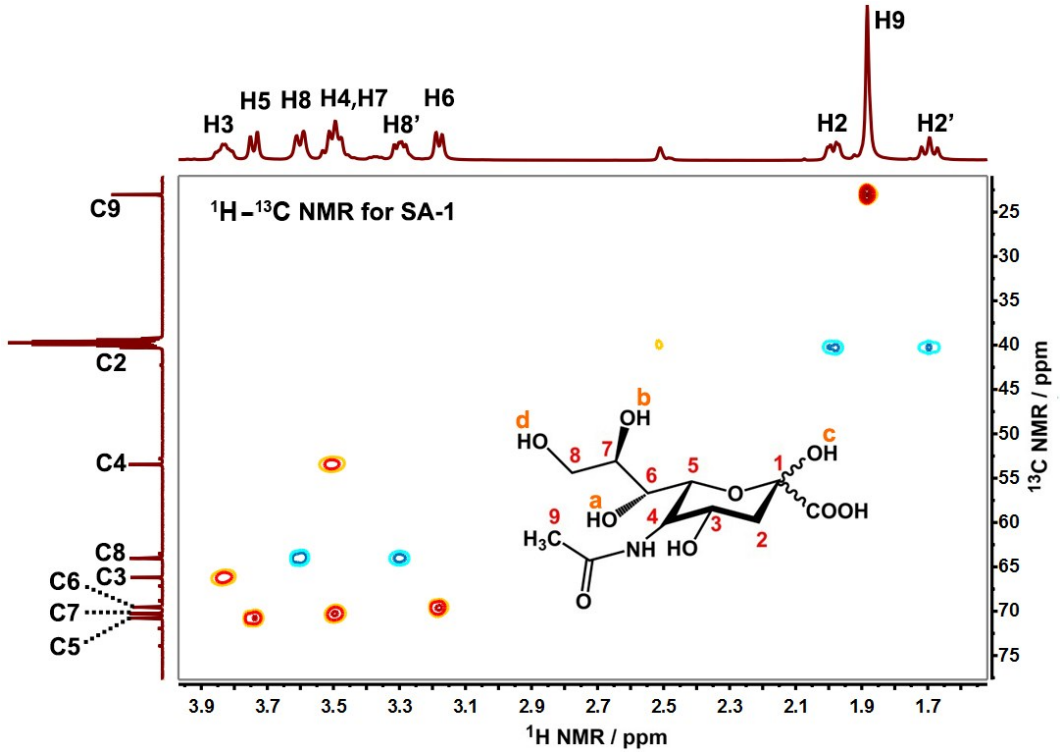


Fig. S5 ^{1}H—^{13}C correlation NMR spectra of Neu5Ac (SA-1) in d_6-DMSO at 20 ºC. This figure clearly indicated the chemical attribution of diverse H and C atoms in SA-1.

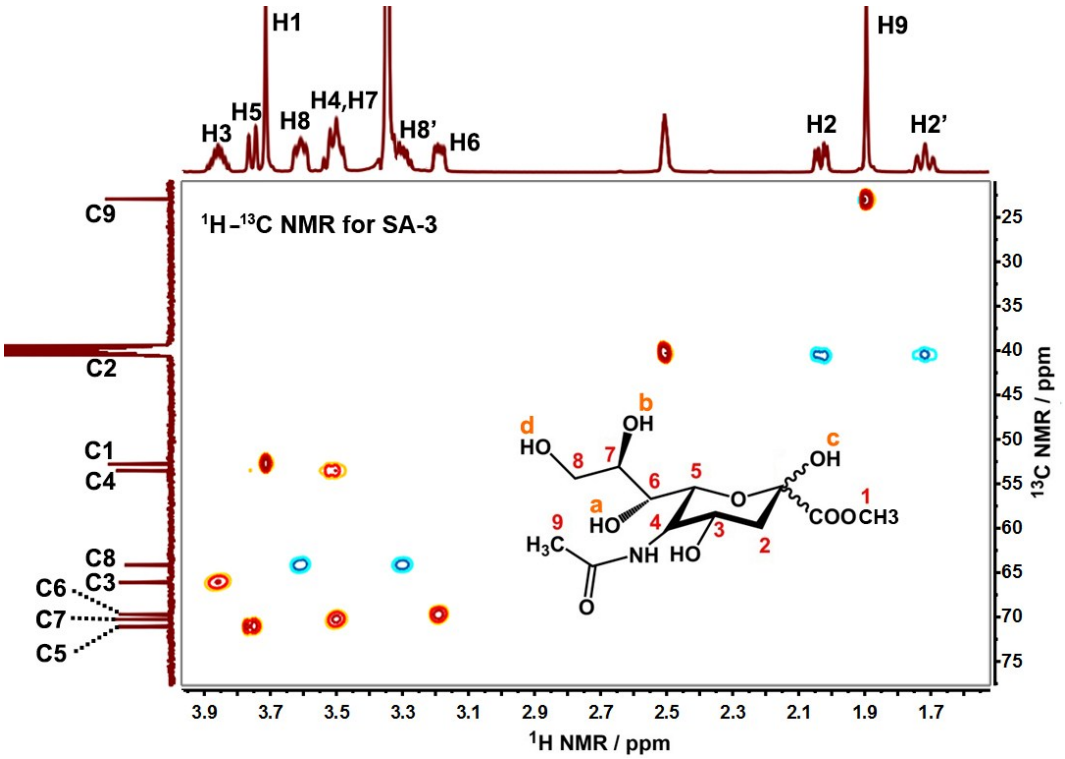


Fig. S6 ^{1}H—^{13}C correlation NMR spectra of *N*-Acetylneuraminic acid methyl ester (SA-3) in d_6-DMSO at 20 ºC. This figure clearly indicated the chemical attribution of diverse H and C atoms in SA-3.

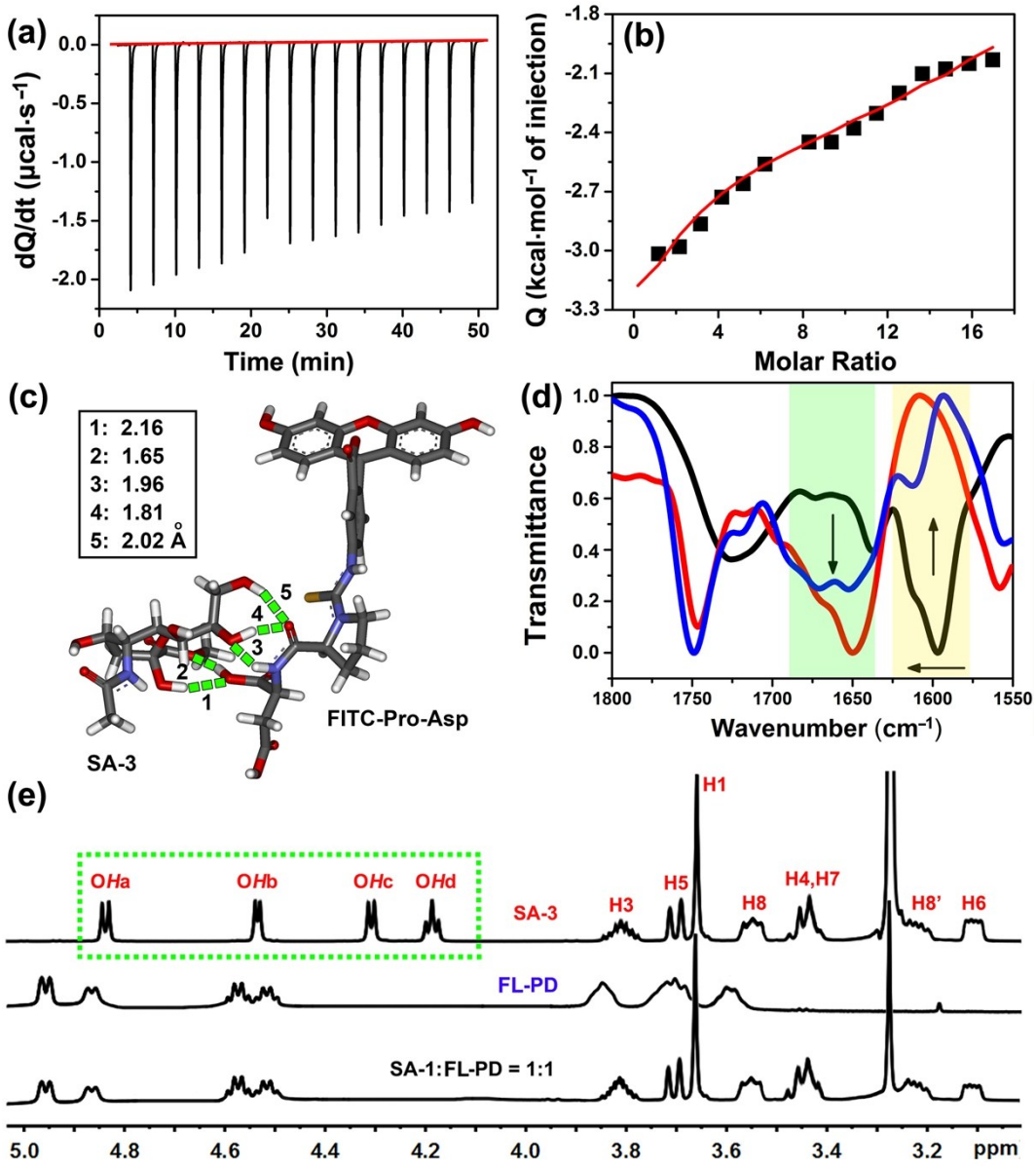


Fig. S7 (a,b) Isothermal calorimetric titration of FL-PD (0.5 mM) with various equivalents of SA-3 (50 mM) in H_2O at 20 °C. (c) Optimized binding model of FL-PD with SA-3, obtained through quantum chemistry calculation (Gaussian, density function theory, at 6-311G level). Hydrogen bonds with different bond lengths are indicated by dashed green lines. (d) Representative Bio-ATR-FTIR spectra of FL-PD (0.2 mM, black), SA-3 (0.2 mM, red), and their equimolar mixture (blue) in d_6-DMSO at 20 °C. The coloured ribbons indicate the characteristic vibration peaks of amide group in PD; green ribbon: amide I band; yellow ribbon: amide II band. (e) Partial ^{1}H NMR spectra of SA-3, FL-PD, and their equimolar mixture in d_6-DMSO at 20 °C. Disappearance of the hydroxyl proton signals (indicated by dashed green box, and their detailed attributions are illustrated in Fig. S6) revealed that hydroxyl groups of SA-3 participated in the binding process.

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