Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information
for

A Zwitterionic Squaraine Dye with Large Stokes Shift for in vivo and Site-Selective Protein Sensing

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Experimental Methods

Reagents. All chemicals and reagents were used directly as obtained commercially unless otherwise noted. Water used was ultra filter deionized and purchased from Fisher Scientific. BSA (≥98%), lysozyme, trypsin, formaldehyde dehydrogenase, lipase, chymotrypsin and fibrinogen were purchased from SIGMA or from GE Healthcare.

Spectroscopic Measurements. NMR spectra were collected on a Varian 300 Gemini spectrometer. Mass spectrometric data were obtained on a HP1100LC/MSD mass spectrometry. HRMS data were performed on an ESI-TOF MS system(Waters, Milford, MA). UV-Vis spectra were acquired on a Hewlett-Packard 8453 diode-array spectrometer. Fluorescence spectra were obtained on a HORIBA Jobin Yvon NanoLog spectrometer. The quantum yield of fluorescence of the sample was measured using bis(3-ethylbenzothiazol-2-ylidene) squaraine in ethanol (Φ = 0.21) as a standard[S1] and calculated using eq 1:

$$\Phi_{unk} = \Phi_{std} \times \left(\frac{I_{unk}}{I_{std}}\right) \times \left(\frac{A_{std}}{A_{unk}}\right) \times \left(\frac{n_{unk}}{n_{std}}\right)^2 \quad (1)$$

Where Φ_{unk} is the fluorescence quantum yield of the sample, Φ_{std} is the fluorescence quantum yield of the standard, I_{unk} and I_{std} are the integrated emission intensities of the sample and the standard, respectively, A_{unk} and A_{std} are the absorbance of the sample and the standard at the excitation wavelength, respectively, and n_{unk} and n_{std} are the refractive indexes of the corresponding solution.

S1. Das, S.; Thomas, K. G.; Ramanathan, R.; George, M. V.; Kamat, P. V. *J. Phys. Chem.* **1993**, *97*, 13625-13628.

Zebrafish fluorescence labeling experiments: Zebrafish embryos were obtained by breeding in-house adult zebrafish (e.g. pairing of 4 male with 4 female). Both the adult zebrafish and embryos were maintained at 28ºC with a photoperiod of 14 h light and 10 h dark. Injected and uninjected control embryos were from the same breeding and processed side by side (except no BSA injection for the control embryos).

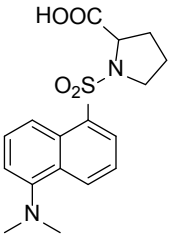
Microinjection procedure was described in detail in the Zebrafish Book.[S2] Briefly, the newly laid eggs were placed in troughs produced in an agarose gel plate. A BSA solution (0.5 mmol/L) was pressure injected (3-4 nL) into either the blastomere(s) or regions of yolk immediately below the blastomeres of the embryo using a Narishige MI300 microinjector. Phenol red was added to the injection solution (1:4 phenol red: BSA solution) as a tracer. After the injections, the embryos were put into 250 ml plastic beakers with 150 ml filtered fish tank water supplemented with PTU (1-phenyl-2-thiourea, 0.003%) to inhibit pigment development, and allowed to develop (in a 28.5ºC water bath) to specific stages (e.g. 2-3 hpf, 72 hpf). To detect BSA distribution, 50 μL of **DNSA-SQ** dissolved in DMSO (0.5 mmol/L) was added to a 20 mL petri dish containing 10 mL of the fish tank water and 10 embryos. After incubating with the dye solution in dark at 26ºC for one hour, the embryos were washed twice in fresh fish tank water, and placed on a glass slide for fluorescent microscopy using an Olympus BX51 microscope equipped with epifluorescence, D.I.C. optics and SPOT digital camera system. The labeling was viewed on an Olympus (BX51) fluorescent microscope by using green light for excitation, with the aid of selective NG filter (575-615 emission).

S2. Westerfield, M. *The zebrafish book. A guide for the laboratory use of zebrafish*; 4th ed., University of Oregon Press, Eugene, **2000**.

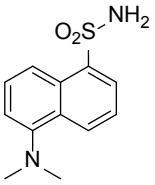
Synthesis of DNSA-SQ: Dansylamide (DNSA) (1.50 g, 6 mmol) was added to a solution of **1a** (0.59 g, 1 mmol) in anhydrous CH_2Cl_2 (30 mL) under N_2 atmosphere, and the mixture was stirred at room temperature for 5-7 days in the presence of catalytic amounts of Et_3N and DMAP [4-(dimethylamino)pyridine]. The mixture was extracted with cold H_2O, and the organic layer was dried over anhydrous Na_2SO_4, solvent was removed in vacuum. The resulting residue was purified on a silica gel column (by using CH_2Cl_2/MeOH as eluent) to give **DNSA-SQ** (0.47 g, 71.1%).^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm) 1.19 (t, 6H, J = 7.2 Hz), 2.83 (s, 6H), 4.19 (q, 4H, J = 7.2 Hz), 6.16 (s, 2H), 7.34-7.24 (m, 3H), 7.32 (dd, 2H, J = 7.2 Hz, J = 7.8 Hz),

7.49 (dd, 2H, J = 7.8 Hz, J = 7.8 Hz), 7.60-7.58 (m, 4H), 7.88 (m, 2H), 8.15 (d, 1H, J = 7.5 Hz), 8.39 (d, 1H, J = 8.4 Hz), 8.62 (d, 1H, J = 9.0 Hz), ^{13}C NMR (DMSO-d_6, 75 MHz) δ (ppm) 166.20, 159.78, 140.40, 130.21, 129.96, 128.61, 128.52, 127.24, 127.03, 126.35, 124.20, 123.27, 122.07, 114.72, 111.54, 45.46, 41.34, 12.20. HRMS (ESI+) found 664.1642 $(M)^+$, calcd for C_{36} H_{32} N_5OS_2, 664.1636.

Structures.



Dansylproline (DP)



Dansylamide (DNSA)

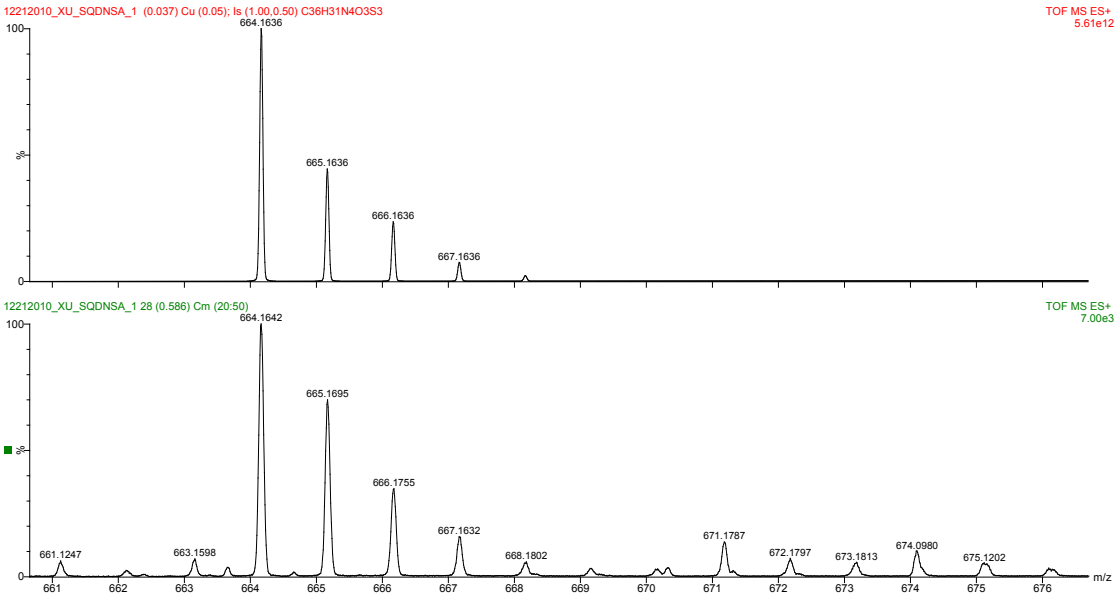


Fig. S1. HRMS spectra of **DNSA-SQ** (upper: theoretical data; bottom: experimental data).

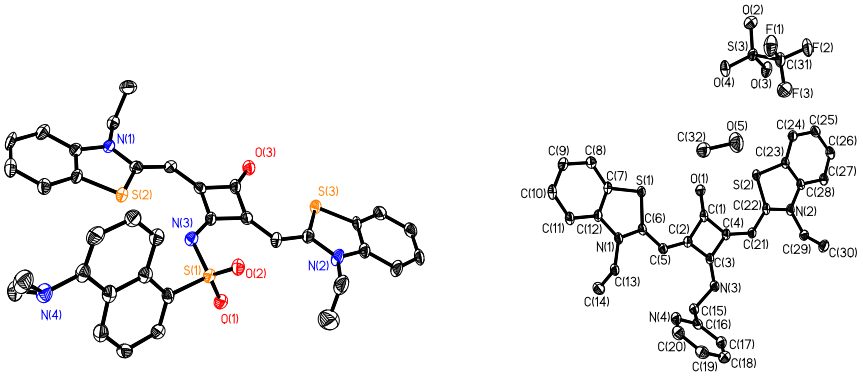


Fig. S2. ORTEP view of the molecular structure of **DNSA-SQ** (left) and **1b** (right) from the front. All hydrogen atoms are omitted for clarity.

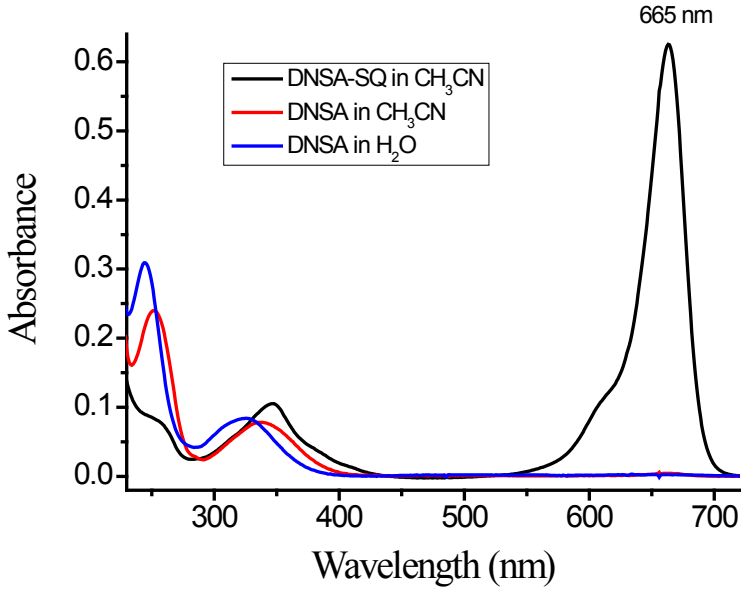


Fig. S3. Absorption of **DNSA-SQ** (5 μM) **and DNSA** (5 μM).

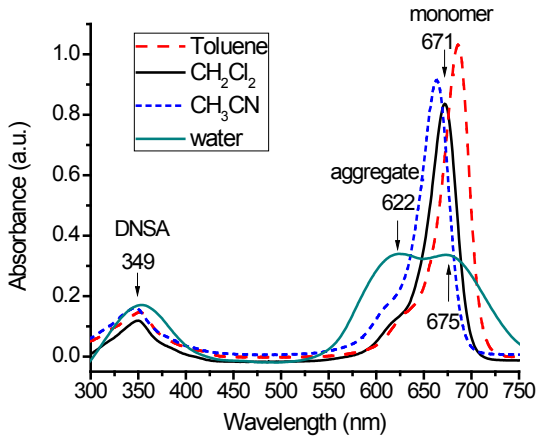


Fig. S4. Absorption of **DNSA-SQ** (5 μM) in different solvents. The absorption spectrum in water has been multiplied by 4 for clarity.

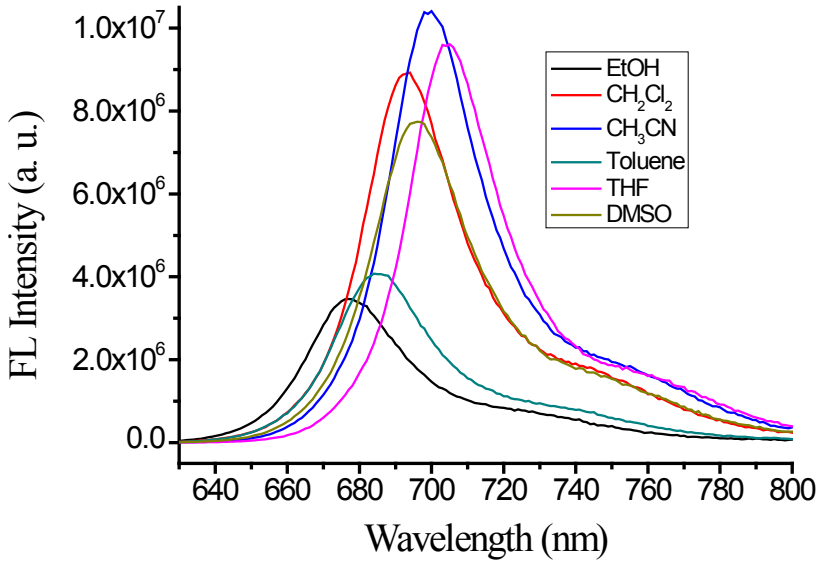


Fig. S5. Fluorescence spectra of **DNSA-SQ** (5 μM) in different solvents.

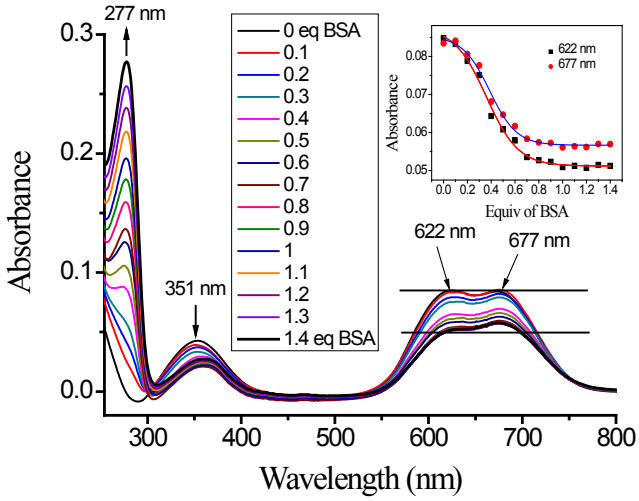


Fig. S6. Absorbance of **DNSA-SQ** (5 μM) in aqueous solution with different concentration of BSA.

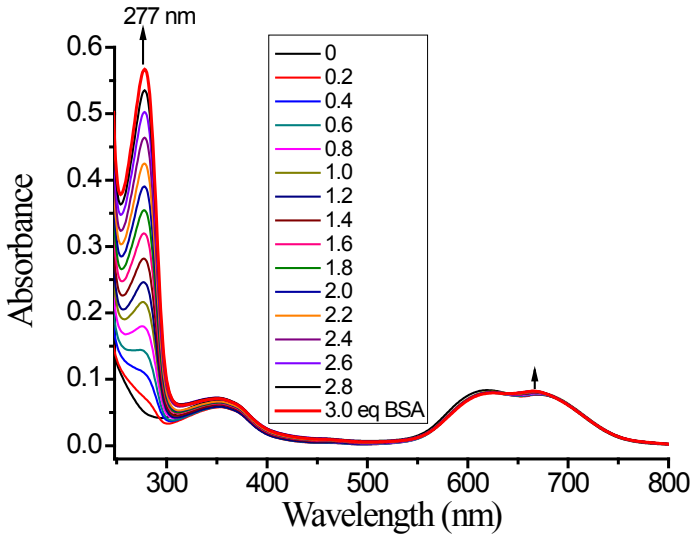


Fig. S7. Absorbance of **DNSA-SQ** (5 μM) in phosphate buffer solution (10 mM, pH7.2) with different concentration of BSA.

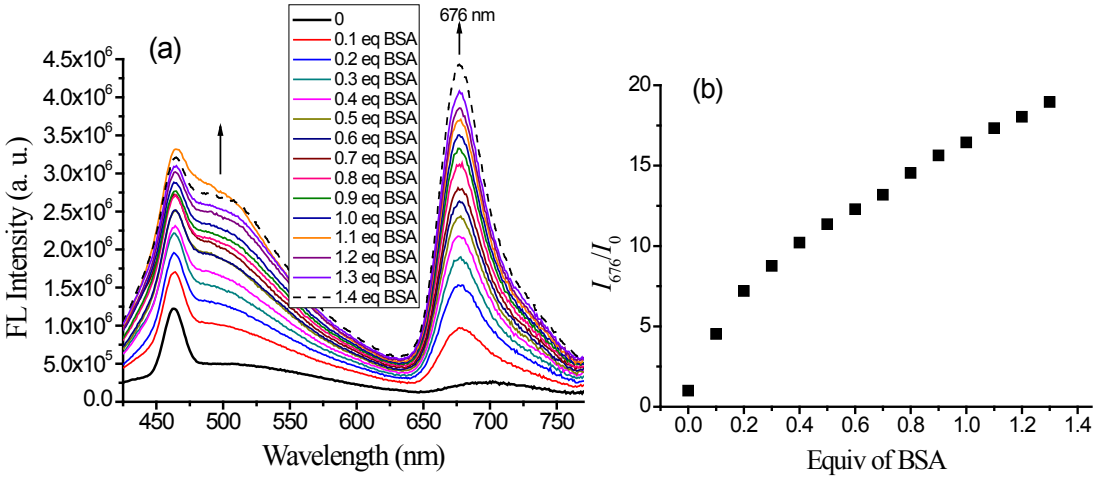


Fig. S8. Fluorescence of **DNSA-SQ** (5 μM) in phosphate buffer solution (10 mM, pH7.2) with different concentration of BSA (a) and relative fluorescence intensity (I_{676}/I_0) of **DNSA-SQ** (5 μM) at 676 nm (b) in phosphate buffer solution (10 mM, pH7.2) to different concentration of BSA. (excitation at 400 nm)

Note: the broad peak at ~500 nm in Fig. S8a is attributed to emission from DNSA segment.

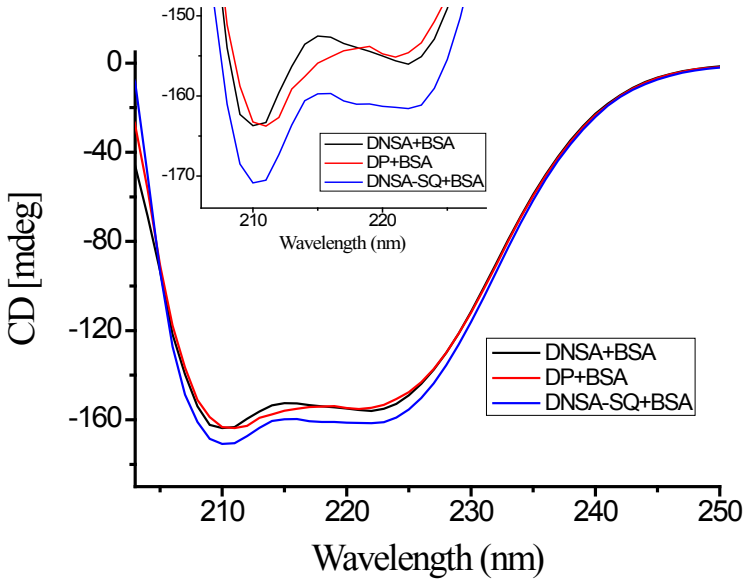


Fig. S9.Circular dichroism of **DNSA-SQ** (2.5 μM) in the presence of 1 equiv of BSA. The concentration for DNSA or DP is 30 μM.

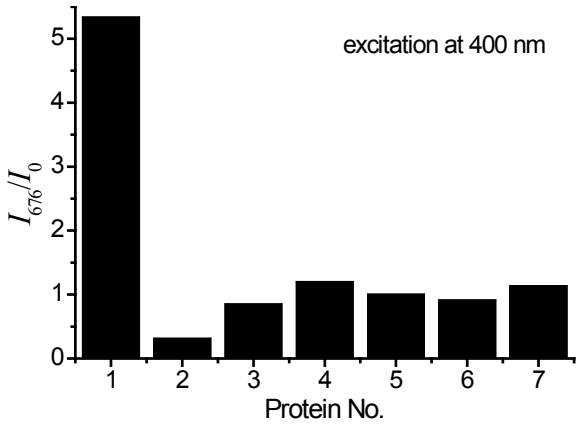


Fig. S10. Relative fluorescence intensity (I_{676}/I_0) of **DNSA-SQ** (5 μM) at 676 nm in phosphate buffer solution (10 mM, pH7.2) to different protein/enzyme (30 μg/mL) (Excitation at 400 nm). 1: BSA; 2: Lysozyme; 3: Trypsin; 4: Formaldehyde dehydrogenase; 5: Lipase; 6: Chymotrypsin; 7: Fibrinogen.

Note: For BSA of molecular weight 60,000, the concentration 30 μg/mL is corresponding to 0.5 μM. This is about 0.1 equivalent of DNSA-SQ.

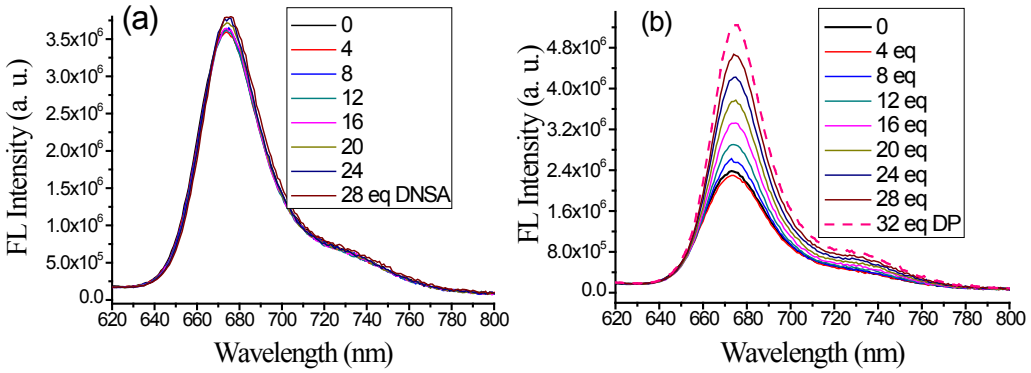


Fig. S11. Fluorescence of **DNSA-SQ** (5 μM) in phosphate buffer solution (10 mM, pH7.2) with different concentration of (a) DNSA and (b) DP in the presence of 3 eq BSA.

Table S1: Photophysical data of **DNSA-SQ**, **1a-1b**, **DNSA** and **DP**.[a]

Compound	Absorption				Emission		
	λ_{abs}[nm][b]	logε_{max}	λ_{abs}[nm][c]	logε_{max}	λ_{em}[nm][d]	FEF[e]	Φ_f[f]
DNSA-SQ	346, 655	4.15	655	5.06	675	15.9	0.12
1a	330	4.51	631	5.25	664	1	0.01
1b	340	4.17	656	5.16	679	-	0.036
DNSA	340	4.11	-	-	526	-	0.04
DP	341	3.63	-	-	549	-	0.11

[a] Measurements recorded in MeOH. [b] The maximal absorption of the component; [c] The maximal absorption of the squaraine component. [d] The maximal emission of the cassette. [e] Fluorescence enhancement relative to the acceptor **1a**. [f] Fluorescence quantum yields were determined using quinine sulfate (Φ_f=0.53 in 0.1 M H_2SO_4) as a standard.[S3] [g] Pseudo-Stokes shifts of the cassette and the Stokes shift of the acceptor **1a-1b**.

(S3. Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Kluwer Academic: New York, 1999; pp 51–55.)

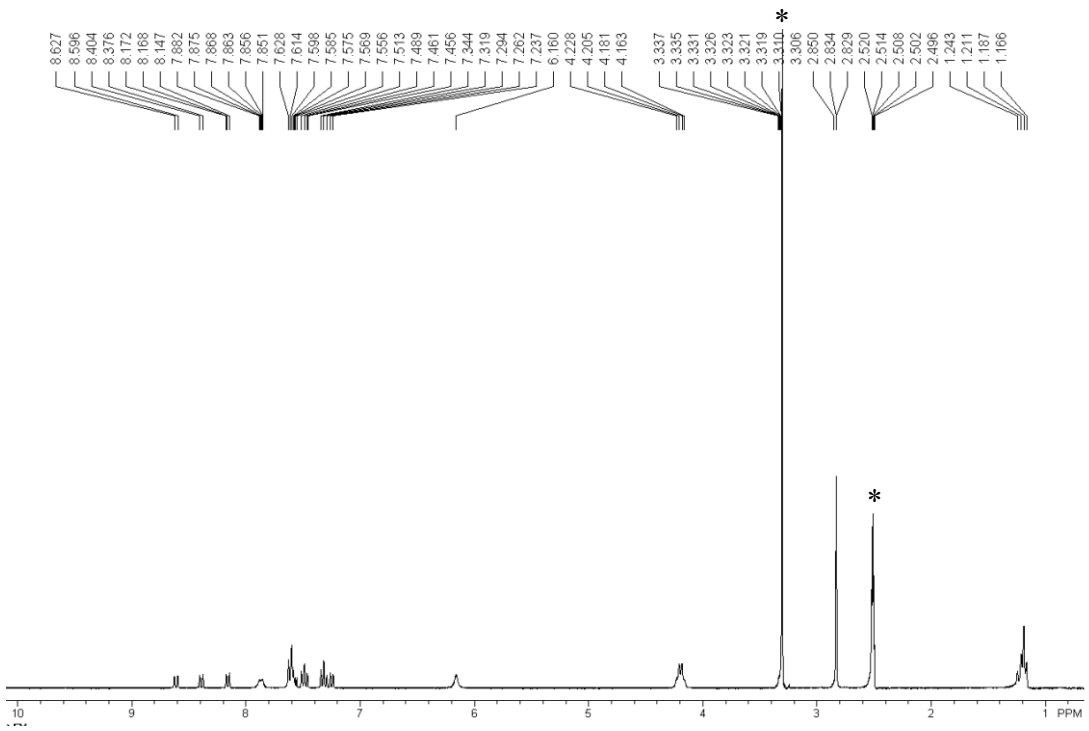


Fig. S15. ^{1}H NMR spectrum of **DNSA-SQ** in DMSO-d_6. The starred signals at 2.51 and 3.3 ppm are attributed to DMSO and water, respectively.

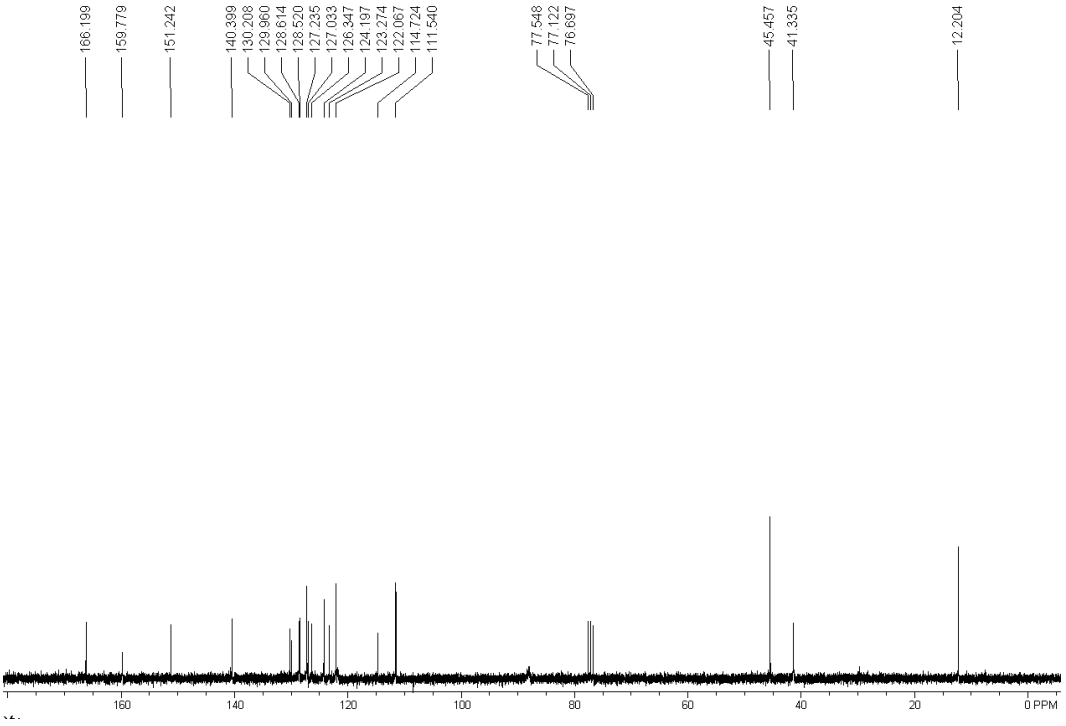


Fig. S16. ^{13}C NMR spectrum of **DNSA-SQ** in DMSO-d_6.

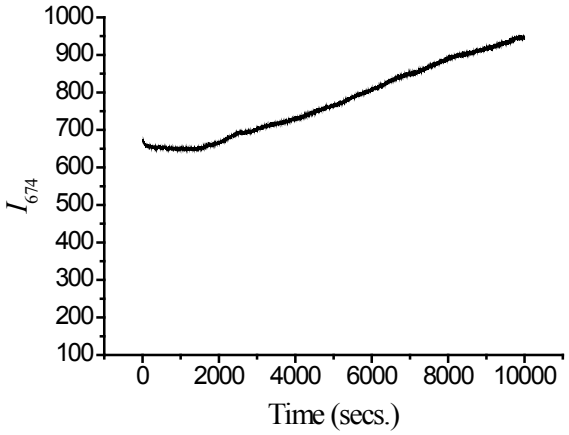


Fig. S17. the fluorescence intensity change of **DNSA-SQ** (5 μM)at 674 nm in phosphate buffer solution (10 mM, pH7.2) with 1 equivalent of BSA with increasing reaction time as indicated (excitation wavelength at 600 nm). Within 30 min, the fluorescence intensity of **DNSA-SQ** at 674 nm remains unchanged. Our titration (Figure 2) was recorded within 3 min after BSA was added.

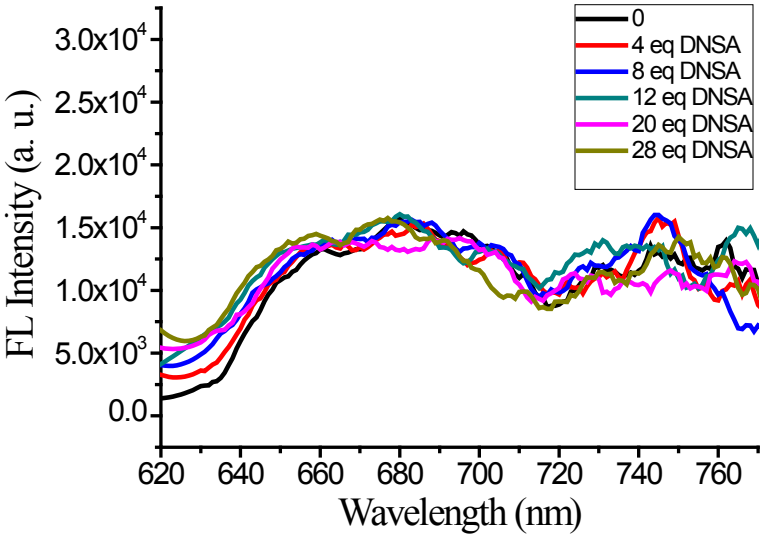


Figure S18. Fluorescence of **DNSA-SQ** (5 μM) in phosphate buffer solution (10 mM, pH7.2) with different concentration of DNSA in the absence of BSA (excitation wavelength at 600 nm).
Note: Although the structure of DNSA is similar to **DNSA-SQ**, their interaction does not change the fluorescence of **DNSA-SQ**.

X-ray Structure Determination Details

Crystals of **DNSA-SQ** and **1b** were coated in paraffin oil and mounted on a CryoLoop™ and placed on the goniometer head under a stream of nitrogen cooled to 100K. The data was collected on a Bruker APEX CCD diffractometer with graphite-monochromated Mo K_α radiation (λ = 0.71073 Å). The unit cell was determined by using reflections from three different orientations. The data was integrated using SAINT.[1] An empirical absorption correction and other corrections were applied to the data using multi-scan SADABS. Structure solution, refinement, and modeling were accomplished by using the Bruker SHELXTL package.[1,2] The structure was determined by full-matrix least-squares refinement of F^2 and the selection of the appropriate atoms from the generated difference map. Hydrogen atom positions were calculated and $U_{iso}(H)$ values were fixed according to a riding model.

Table S3. Crystal data and structure refinement for **DNSA-SQ**.

Empirical formula	C36 H32 N4 O3 S3	
Formula weight	664.84	
Temperature	100(2) K	
Wavelength	0.71073 Å	
Crystal system	Triclinic	
Space group	P-1	
Unit cell dimensions	a = 9.237(2) Å	α= 66.612(4)°.
	b = 14.840(3) Å	β= 74.734(4)°.
	c = 15.433(4) Å	γ = 81.644(4)°.
Volume	1871.2(7) Å^3	
Z	2	
Density (calculated)	1.180 Mg/m^3	
Absorption coefficient	0.236 mm^{-1}	
F(000)	696	
Crystal size	0.08 x 0.08 x 0.04 mm^3	
Theta range for data collection	1.48 to 28.37°.	
Index ranges	-11<=h<=11, -19<=k<=19, -20<=l<=20	
Reflections collected	16467	
Independent reflections	8551 [R(int) = 0.0527]	
Completeness to theta = 28.37°	91.2 %	
Absorption correction	Semi-empirical from equivalents	

[1] Bruker (1997). SMART (Version 5.625), SAINT (Version 6.22) and SHELXTL (Version 6.10)

[2] Sheldrick, G. M. (1997). SHELX-97. University of Göttingen, Germany

Max. and min. transmission	0.9913 and 0.8250
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	8551 / 0 / 419
Goodness-of-fit on F^2	1.035
Final R indices [I>2sigma(I)]	R1 = 0.0837, wR2 = 0.1790
R indices (all data)	R1 = 0.1233, wR2 = 0.1967
Largest diff. peak and hole	0.594 and -0.324 e.Å^{-3}

Table S4. Crystal data and structure refinement for **1b**.

Identification code	$\mathbf{C_{32}H_{30}F_3N_4O_5S_3}$	
Empirical formula	C32 H30 F3 N4 O5 S3	
Formula weight	703.78	
Temperature	100(2) K	
Wavelength	0.71073 Å	
Crystal system	Triclinic	
Space group	P-1	
Unit cell dimensions	a = 8.3153(11) Å	α=101.112(2)°.
	b = 13.3256(18) Å	β= 96.674(2)°.
	c = 14.721(2) Å	γ = 96.978(2)°.
Volume	1572.3(4) Å^3	
Z	2	
Density (calculated)	1.487 Mg/m^3	
Absorption coefficient	0.302 mm^{-1}	
F(000)	730	
Crystal size	0.40 x 0.26 x 0.04 mm^3	
Theta range for data collection	1.42 to 26.30°.	
Index ranges	-10<=h<=10, -16<=k<=16, -18<=l<=18	
Reflections collected	11989	
Independent reflections	6241 [R(int) = 0.0335]	
Completeness to theta = 26.30°	97.9 %	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	0.9880 and 0.8886	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	6241 / 0 / 428	
Goodness-of-fit on F^2	1.076	

Final R indices [I>2sigma(I)]	R1 = 0.0629, wR2 = 0.1682
R indices (all data)	R1 = 0.0690, wR2 = 0.1740
Largest diff. peak and hole	1.019 and -0.618 e.Å^{-3}