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Supporting Information for:

A Coumarin–Hemicyanine Hybrid as a Ratiometric Fluorescent Sensor of Microenvironment Proticity

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Materials and Methods

UV–vis and fluorescence measurements. All UV–vis absorbances (A) were measured on a Cary 300-Bio UV-Vis spectrophotometer using 10 mm light path quartz glass cells with a baseline correction. Samples were prepared in methanol or an appropriate solvent mentioned with the concentration of coumarin 466 (**3**) at 5 μM and probe **1** and probe **2** at 5 μM in methanol and probe **2** at 20 μM in isopropanol, water and dioxane. The molar extinction coefficients (ε) were calculated by using Beer-Lambert's law (A = εcl) from five different solutions of known concentration. To obtain good solubility of the probes, the 4 mM stock solutions were prepared in spectroscopic grade DMSO, prior to dilution with other solvents for spectrophotometric measurements wherein the concentration of DMSO was assumed to be negligible.

The relative fluorescence quantum yield (Φ_{fl}) for all chromophores were calculated using UV–vis absorbances (A) in methanol except for fluorescence standards, quinine bisulfite (QBS, Φ_{std} = 0.46) fluorescein (Φ_{std} = 0.91) which are measured in 0.5 M H_2SO_4 and 0.1 M NaOH, respectively. Integrated fluorescence intensities (FI) were measured by exciting at the wavelengths of maximum absorption for each sample and the fluorescence quantum yields were determined by using the following equation. The differences in solvents is accounted by including the ratio of refractive indices (η) as shown in the equation.

$$\Phi_{fl} = \Phi_{std}\left(\frac{\mathrm{A}_{std}}{\mathrm{A}_{probe}}\right)\left(\frac{\mathrm{FI}_{probe}}{\mathrm{FI}_{std}}\right)\left(\frac{\eta^2{}_{probe}}{\eta^2{}_{std}}\right)$$

All fluorescence measurements to study the effect of solvent polarity and viscosity in appropriate solvents and biopolymer binding in 50 mM sodium phosphate (Na^+) buffer, pH = 7.1 were performed on the Edinburgh Instrument Spectrofluorometer FS5 at 1 μM concentration. Both excitation and emission spectra were measured in quartz cells (108.002F-QS) with a path length of 10 mm at 25 °C and both excitation and emission slit widths were kept constant at 3 nm. The fluorescence emissions were measured by exciting at 352 nm, unless otherwise mentioned to observe the dual-fluorescence emission spectrum. For biopolymer binding, up to 2 equivalents of desired biopolymers were directly added to

the dye solution in Na^+ buffer and the fluorescence spectra were immediately recorded after manual mixing of the samples. The fluorescence titrations with BnBtC probe (1 μM) in Na^+ buffer were carried out with systematic addition of desired biopolymer (either PS2.M or BSA) from a stock solution in water until a final concentration of 4 equiv. was reached.

Table S1. Photophysical parameters of **1** and **2** in MeOH.

Probe[a]	$\lambda_{Abs,max}$ (nm)	ε (M^{-1}, cm^{-1})	$\lambda_{Fl,max}$[b] (nm)	Φ_{Fl}	Brightness (B)[c]	B_{Rel}[d]	B_{Probe}[e]
MeBtC (**1**)	341	8950	447, 639	0.0165[f]	147.68	0.0186	1.8925
	545	73950		0.1073[g]	7934.84		
BnBtC (**2**)	351	7150	446, 651	0.0356[f]	254.54	0.0352	
	561	61150		0.1183[g]	7234.05		

[a]Measured in MeOH, [b](λ_{Ex} = 352 nm), [c]Brightness (B = $\varepsilon\Phi_{Fl}$), [d]B_{Rel} = B_{350}/B_{550}, [e]B_{Probe} = $B_{Rel,BnBtC}/B_{Rel,MeBtC}$, [f]obtained by comparative method using quinine bisulfate in 0.5 M H_2SO_4 standard (Φ_{Std} = 0.46, λ_{Ex} = ~350 nm), [g]obtained by comparative method using fluorescein in 0.1 M NaOH standard (Φ_{Std} = 0.91, λ_{Ex} = ~550 nm),

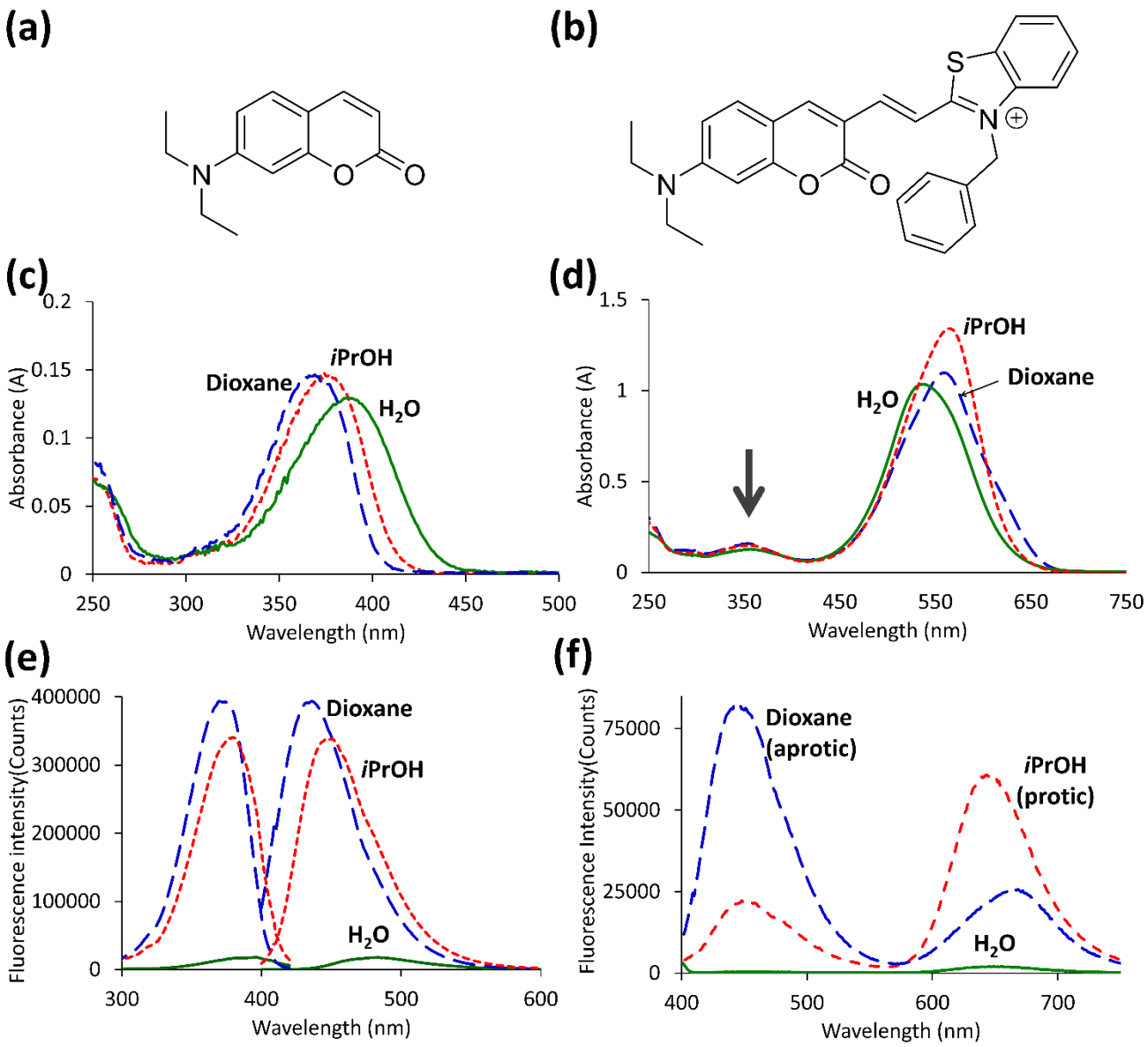

Figure S1. (a) Structure of Comarin-466 dye (**3**). (b) Structure of BnBtC probe (**2**). Absorption spectra of (c) **3** (5 μM) and (d) **2** (20 μM); and fluorescence emission spectra (λ_{Ex} = 352 nm) of (e) **3** (1 μM) and (d) **2** (1 μM) in water (solid green traces), isopropanol (dotted red traces) and 1,4-dioxane (dashed blue traces).

Synthetic Materials and Description. Synthesis of MeBtC probe (**1**) was performed using reported procedure[S1,S2] and the purity of the final product **1** was confirmed by comparing it's ^{1}H NMR spectrum to the published reports. Although the synthesis and characterization of the *N*-benzyl BnBtC probe (**2**) has been reported previously (called BOB in previous report),[S3] the spectral and NMR chemical shifts in support of dye structure were not accurate. Specifically, dye **2** does not exhibit an absorption band at 426 nm in MeOH and its ^{13}C NMR spectrum is not limited to 12 peaks with a signal at δ 195.60 ppm, and its spectrum should not contain a signal at 63.03 ppm (See Supporting Information for ref. S3; list of peaks for BOB in experimental section and attached ^{13}C NMR spectrum). Therefore, BnBtC (**2**) was rigorously characterized by ^{1}H, ^{13}C and HSQC NMR that were recorded on 300 or 400 MHz spectrometers in DMSO-d_6. Further details are as described below.

MeBtC (1). ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) 1.18 (t, J = 7 Hz, 6H), 2.29 (s, 3H), 3.55 (q, J = 7 Hz, 4H), 4.23 (s, 3H), 6.70 (d, J = 2 Hz, 1H), 6.89 (dd, J = 9 & 2 Hz, 1H), 7.11 (d, J = 8 Hz, 2H), 7.48 (d, J = 8 Hz, 2H), 7.59 (d, J = 9 Hz, 1H), 7.75 (t, J = 7.5 Hz, 1H), 7.85 (t, J = 7.5 Hz, 1H), 8.00 (d, J = 15.5 Hz, 1H), 8.07 (d, J = 15.5 Hz, 1H), 8.23 (d, J = 8.5 Hz, 1H), 8.38 (d, J = 8.5 Hz, 1H), 8.61 (s, 1H).

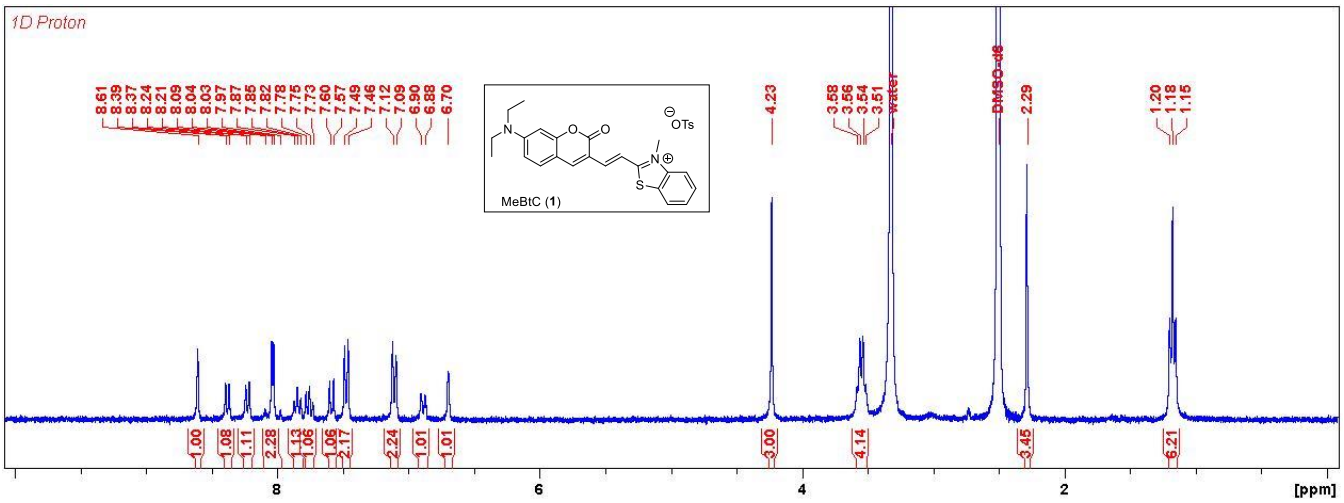

Figure S2. ^{1}H NMR spectra of compound **1**.

BnBtC (2). ^{1}H NMR (DMSO-d6, 300 MHz): δ (ppm) 1.17 (t, J = 7 Hz, 6H), 3.55 (q, J = 7 Hz, 4H), 6.08 (s, 2H), 6.68 (d, J = 2 Hz, 1H), 6.88 (dd, J = 9 & 2 Hz, 1H), 7.32–7.44 (m, 5H), 7.58 (d, J = 9 Hz, 1H), 7.71–7.85 (m, 2H), 8.10 (d, J = 15 Hz, 1H), 8.18 (d, J = 15 Hz, 1H), 8.21 (d, J = 8 Hz, 1H), 8.43

(d, J = 8 Hz, 1H), 8.60 (s, 1H); ^{13}C NMR (DMSO- d^6, 100 MHz): δ (ppm) 12.9, 45.2, 51.8, 97.0, 109.5, 111.2, 111.5, 112.4, 117.0, 125.0, 128.3, 128.6, 129.1, 129.7, 130.0, 132.5, 134.1, 141.9, 146.1, 149.1, 154.0, 157.8, 160.0, 173.0. HRMS (ESI/Q-TOF) *m/z*: [M]+ = Calcd for C29H27N2O2S+: 467.1788; Found: 467.1794.

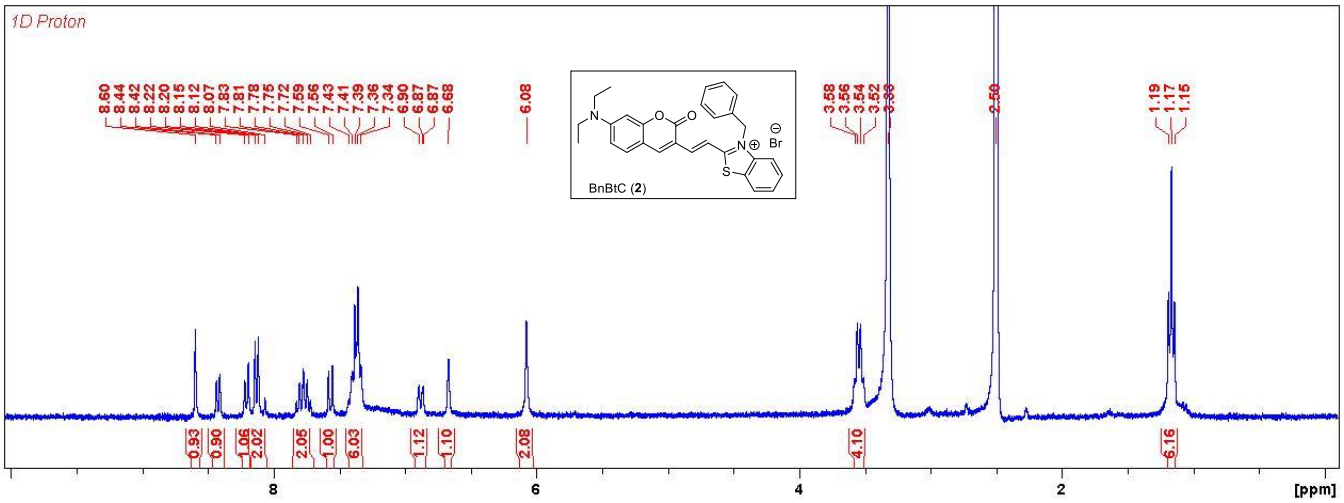

Figure S3. ^{1}H NMR spectra of compound **2**.

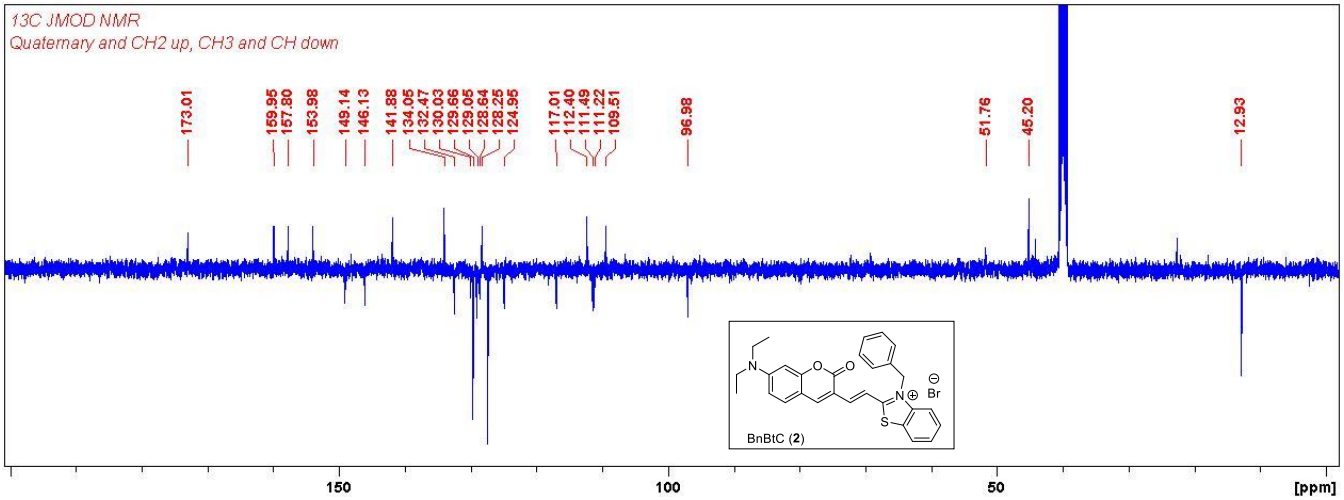

Figure S4. ^{13}C-jmod NMR spectra of compound **2**.

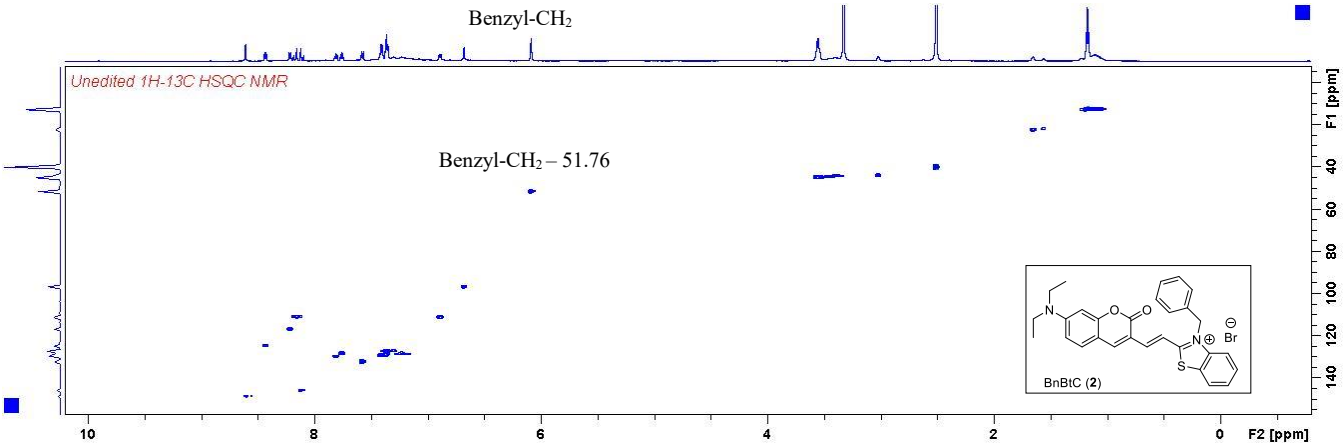

Figure S5. HSQC spectra of compound **2**.

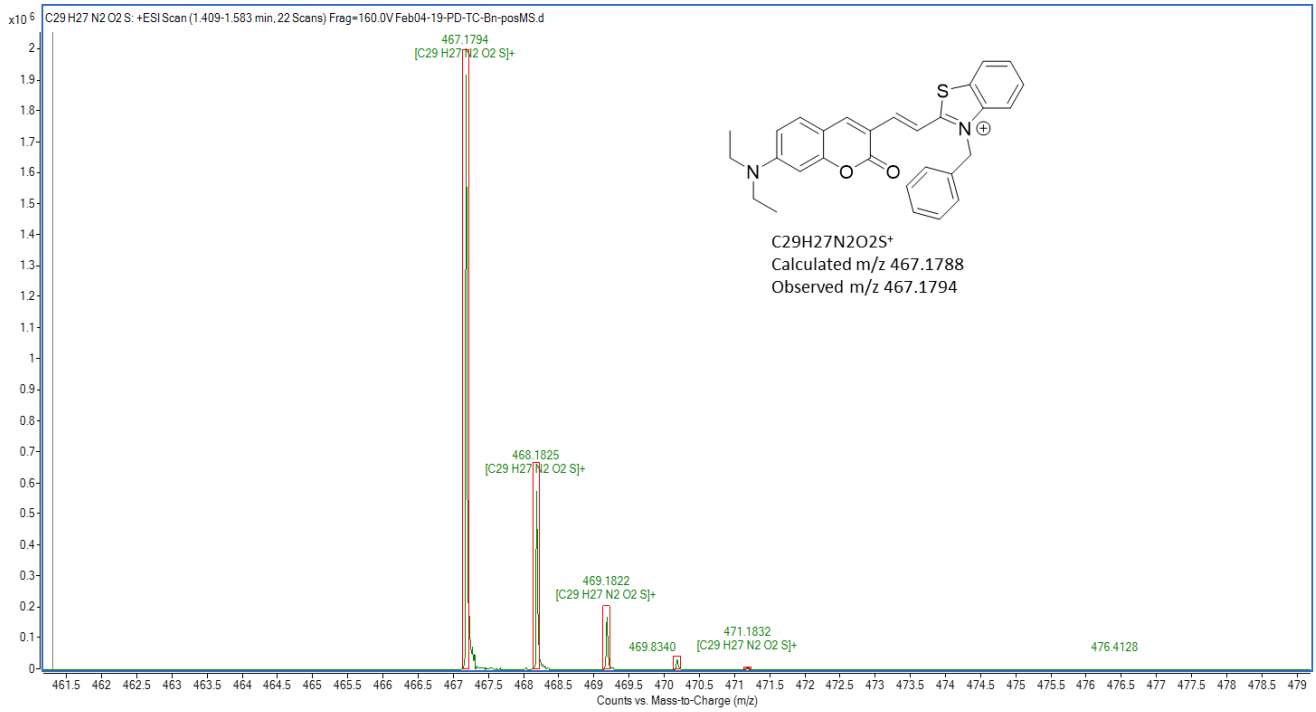

Figure S6. HRMS spectra of compound **2**.

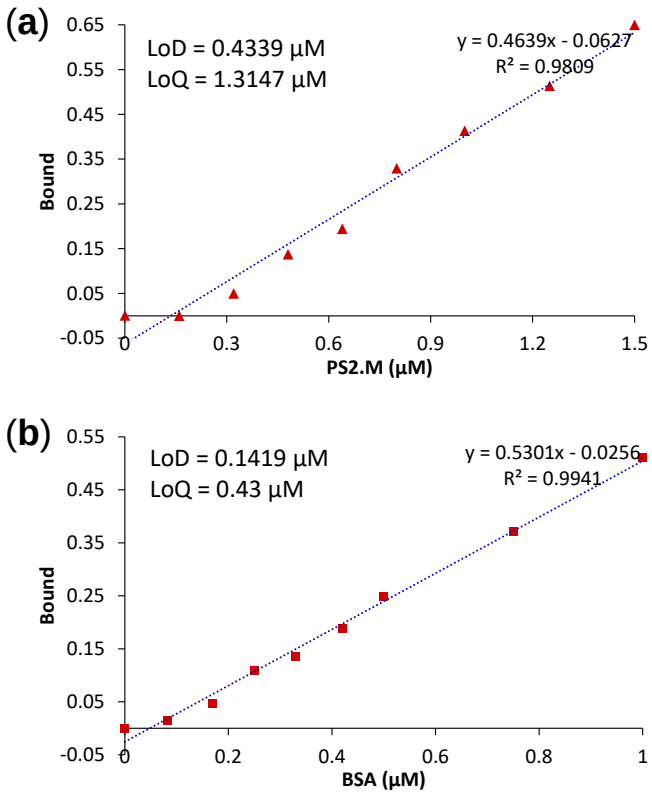

Figure S7. Determination of Limits of Detection (LoD) and quantification (LoQ) for **2** binding to (a) PS2.M and (b) BSA targets.

References

[S1] N. Narayanaswamy, M. Kumar, S. Das, R. Sharma, P. K. Samanta, S. K. Pati, S. K. Dhar, T.. K. Kundu, T. Govindaraju, *Sci. Rep*. **2014**, *4*, 6476.

[S2] J.-W. Yan, Y.-G. Tian, J.-H. Tan, Z.-S. Huang, *Analyst*, **2015**, *140*, 7146-7149.

[S3] N. Jiang, J. Fan, F. Xu, X. Peng, H. Mu, J. Wang, X. Xiong, *Angew. Chem. Int. Ed*. **2015**, *54*, 2510-2514.