Supplementary Information(SI†)

A 'Double Locked' Ratiometric Fluorescent Probe for detection of Cysteine in a viscous system and Its Application in Cancer Cells

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Contents

Synthesis and Structure Characterization:

Synthesis of 4-(6-Hydroxy-naphthalen-2-yl)-benzaldehyde:

To a stirred solution of 6-Bromo-naphthalen-2-ol (500mg, 2.25 mmol), 4 formyl phenylboronic acid (506 mg, 3.37 mmol) and K_2CO_3 (465 mg, 3.37 mmol) in dioxane-water (6:4). De-gassed the reaction mixture for 15 minutes then 130 mg $Pd(PPh_3)_4$ was added. Reaction mixture was stirr for 12 hours at 80^0C. After that, the reaction mixture was filtered and workup with EtOAc and water. The organic layer was separeted and dried over anhydrous Na_2SO_4. The solvent was removed using a rotary evaporator. The crude product was purified by column chromatography (silica gel/ethyl acetate: petroleum ether 5:95) to afford (355 mg, 63%) as a white solid.

Synthesis of 2-Benzothiazol-2-yl-3-[4-(6-hydroxy-naphthalen-2-yl)-phenyl]-acrylonitrile:

To a stirred solution of 4-(6-Hydroxy-naphthalen-2-yl)-benzaldehyde (350 mg, 1.41 mmol) and Benzothiazol-2-yl-acetonitrile (250 mg, 1.42 mmol) were taken into anhydrous EtOH. Catalytic amount of piperidine was added to the solution. The reaction mixture was heated at 80°C for 3 hours. After that, the precipitate was filtered and washed with cold EtOH to get yellow solid (350 mg, 61%).

Synthesis of Probe (ABN):

To a stir solution of 2-Benzothiazol-2-yl-3-[4-(6-hydroxy-naphthalen-2-yl)-phenyl]-acrylonitrile (100 mg, 0.24 mmol) in dry DCM. 0.2 ml Triethylamine was added dropwise at cold condition. Then 0.2 ml Acryloyl chloride was added then stirr the reaction mixture for 5 h at room temperature. Check the TLC and workup with DCM-H_2O. Organic layer was separated and dried over anhydrous Na_2SO_4. The solvent was removed using a rotary evaporate to afford yellow solid (35 mg, 31%). HRMS: $C_{29}H_{18}N_2O_2S^+$ m/z, 458.1089 found for $[M + H]^+$ 459.1099 ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm): 8.34 (s, 1H), 8.16 (m, 4H, J=8.0 MHz), 7.97 (t, 3H, J=12.0 MHz), 7.91 (d, 2H, J=8.0 MHz), 7.83 (t, 1H, J=8.0 MHz), 7.68 (d, 1H, J=4.0 MHz), 7.58 (t, 1H, J=8.0 MHz), 7.48 (t, 1H, J=8.0 MHz), 7.36 (d, 1H, J=8.0 MHz), 6.7 (d, 1H, J=16.0 MHz), 6.42 (m, 1H, J=8.0, 12.0 MHz), 6.1 (d, 1H, J=8.0 MHz). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 153.53, 147.25, 144.84, 144.42, 143.40, 143.27, 143.16, 142.57, 142.00, 140.06, 132.53, 132.22, 131.40, 131.37, 131.32, 128.16, 128.02, 127.99, 127.78, 127.04, 126.88, 124.15, 121.76, 48.76

Experimental Section:

Materials and reagents:

All solvents were purchased from Spectrochem and other materials were obtained from commercial suppliers and Sigma-Aldrich (India). Silica gel (60-120 mesh) was used to perform Column Chromatography.

Instruments:

Mass spectra were carried out using a Waters QTOF Micro YA 263 mass spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Brucker 400, 100 MHz instrument. $CDCl_3$ were used as solvent and TMS as an internal standard in NMR spectra. Chemical shifts are expressed in δ ppm units. UV spectra were recorded on a JASCO V-530 spectrophotometer.

Cell line study:

In the present study Human breast cancer cell line MDA-MB 231and human normal kidney epithelial cell line NKE ware procured from the NCCS, Pune, India. All the cell lines were cultured in a T25 flask with DMEM supplemented with 10% FBS (Fetal bovine serum), 1mM sodium pyruvate, 2mM L-glutamine, non-essential amino acids, 100 units/L penicillin, 100 mg/L streptomycin, and 50 mg/L gentamycin in a 37°C humidified incubator containing 5% CO2.

Cytotoxicity assay:

MTT cell proliferation assay1,2 was performed to assess the cytotoxic effect of the ligand **ABN** in both the cancer cell line MDA-MB-231 and normal cell line NKE. In brief, cells were first seeded in 96-well plates at a concentration of 1 × 104 cells per well for 24 h and exposed to the different working concentration of ligand **ABN** (0 μM, 10 μM, 20 μM, 40 μM, 80 μM, 100μM) for 24 hrs. After incubation cells were washed with 1×PBS and MTT solution (0.5 mg/ml) were added to each well and incubated for 4 h and the resulting formazan crystals were dissolved in DMSO and the absorbance was measured at 570 nm by using a microplate reader. Cell viability was expressed as a percentage of the control experimental setup.

To envisioned the fluorescence ability of the probe ABN in the presence of cysteine (10 μM), dexamethasone (5μM) (used as viscosity enhancer inside cells) and in combination of cysteine (10 μM) + dexamethasone (5 μM), fluorescence imaging was performed in cell line MDA-MB 231. Briefly, cells were grown in coverslips for 24 hrs.in a 37°C humidified incubator containing 5% CO2 and then either mock-treated or treated with 10 μM of probe ABN in the presence of cysteine (10 μM), dexamethasone (5 μM) individually and in combination of cysteine (10 μM) + dexamethasone (5 μM), and incubated for the time period of 15 min in dark at 37ºC. The cells were then washed with PBS buffer three times to remove any unbound ABN and then they were mounted on a glass slide and detected under fluorescence microscope (Olympus).Further, to find the ability of the probe to image viscosity in live cell both MDA-MB-231 and NKE cells were incubated and either mock-treated or treated with 10 μM of probe ABN in the presence of cysteine (10 μM), dexamethasone (5 μM) and in combination of cysteine (10 μM) + dexamethasone (5 μM), and incubated for the time period of 15 min in dark at 37ºC and fluorescence level was detected under fluorescence microscope (Olympus).

Computational details:

Geometries have been optimized using the B3LYP/6-31+G(d,p) level of theory. The geometries are verified as proper minima by frequency calculations. Time-dependent density functional theory calculation has also been performed at the same level of theory. All calculations have been carried out using Gaussian 16 program.

Preparation of Solution for UV−Vis Titration:

A stock solution of the probe **ABN** (1.0 μM) was prepared in PBS buffer / DMSO (9:1 v/v, 10.0 mM, pH = 7.4, 25 ^{0}C) solution. During the titration in the glycerol as viscous medium, stock solution of the probe **ABN** (1.0 μM) was prepare in PBS (0.1% DMSO, pH = 7.4, at 25 ºC). All experiments were carried out in PBS buffer (pH = 7.4, 10 mM, 25ºC). Titration experiment was carried out by adding each time (0.1 μM) solution **ABN** was filled in a quartz optical cell of 1 cm optical path length, and the analyte stock solutions (8 μM) were added into the quartz optical cell slowly by using a micropipette.

Preparation of Solution for Fluorescence Titration:

A stock solution of the probe **ABN** (1.0 μM) was prepared in PBS buffer (pH = 7.4, 25ºC). All experiments were carried out in PBS buffer. Titration experiment was carried out by adding each time (0.1μM) solution **ABN** was filled in a quartz optical cell of 1 cm optical path length, and the analyte stock solutions (8 μM) were added into the quartz optical cell slowly by using a micropipette. In selectivity experiments, the test samples were prepared by placing appropriate amounts of the analyte, stock into 2 mL of solution of probes (1.0 μM).

Cytotoxic effect on Cells

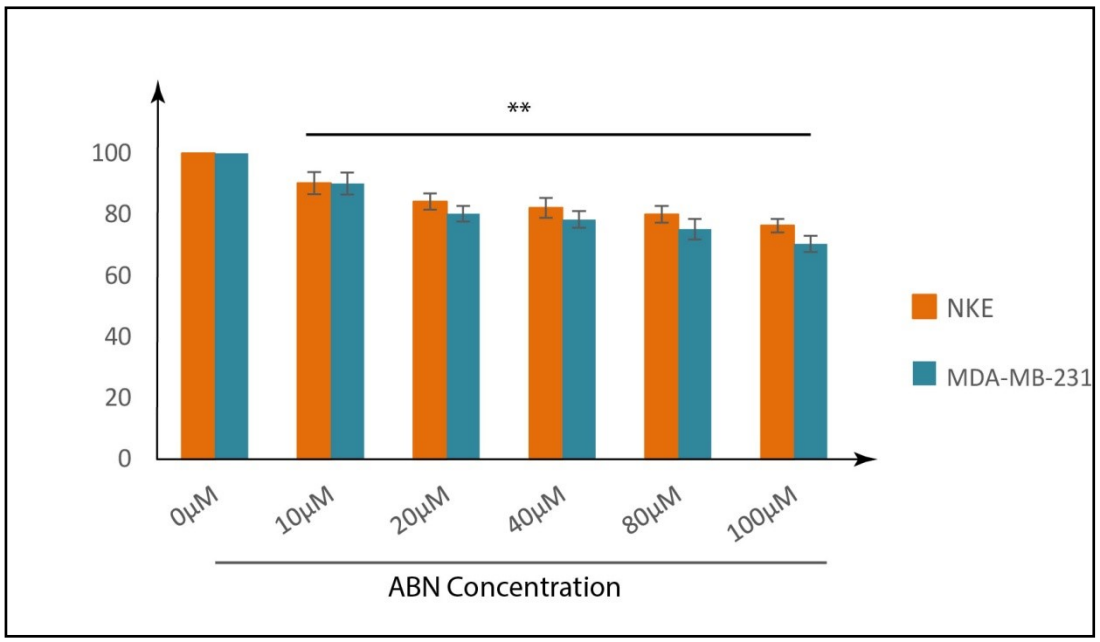


Figure S1. Cell survivability of MDA-MB 231 and NKE cells exposed to different probe **ABN** concentrations. Data are representative of at least three independent experiments and bar graph shows mean ± SEM, $**p < 0.001$ were interpreted as statistically significant, as compared with the control.

Kinetics Study:

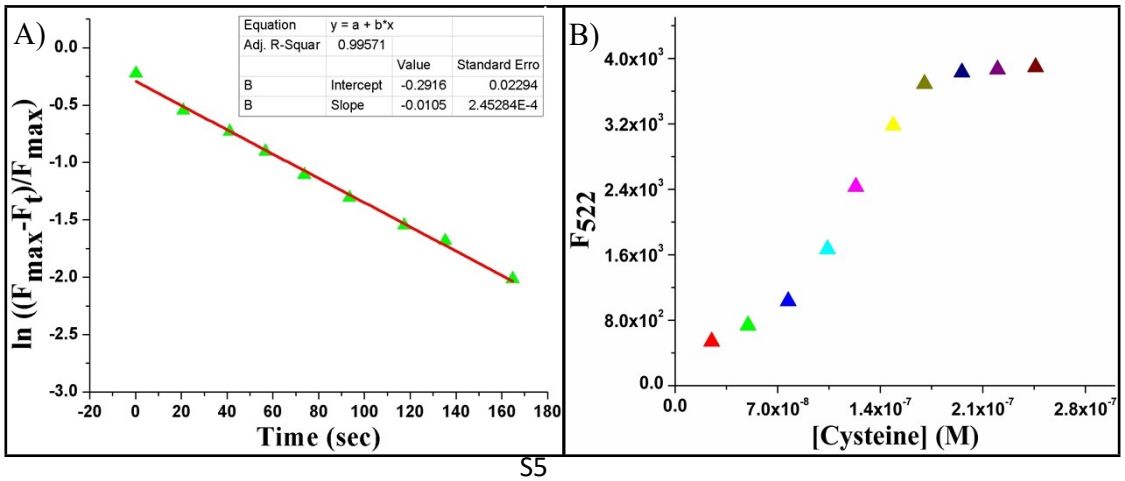


Figure S2. A) Pseudo first-order kinetic plot of reaction of **ABN** (1.0 μM) with Cys in PBS buffer / DMSO (4:1 v/v, 10.0 mM, pH = 7.4, 25 ^{0}C) k = 0.00838 sec^{-1}. Rate constant k was calculated using this eqn. ln [(Fmax - Ft)/Fmax] = - kt.Where Ft and Fmax are the fluorescence intensities at 522 nm at time t and the maximum value obtained after the reaction is complete, and k is the observed pseudo-first-order rate constant. B) Fluorescence intensity changes (F_{522}) of **ABN** (1.0 μM) upon concomitant addition of cysteine.

Competitive Study:

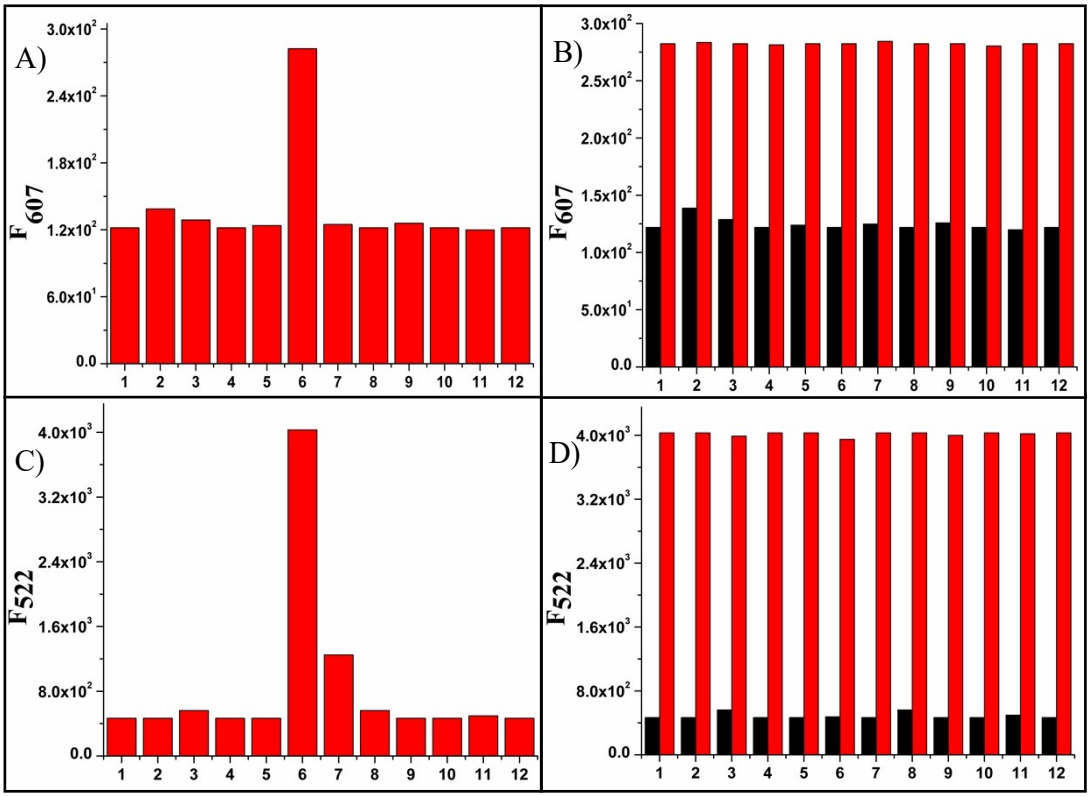


Figure S3. The fluorescence intensity values indicated the selectivity of **ABN** for Cys.10 μM, Others: 50 μM, (1) H_2S 2) ClO^- 3) Glu 4) Ala, 5) Gly, 6) Cys, 7) Hcy 8) GSH 9) Asp 10) H_2O_2 (11) SO_3^{2-}(12) HSO_3^- at A) 607 nm in the glycerol and PBS system (0.1% DMSO) and C) at 522 nm in PBS buffer solution. Selectivity profile diagram in bar representation, Changes of fluorescence of **ABN** (1.0 μM) different species (50.0 μM): red bars: **ABN**+ other species+ Cys; black bars: **ABN**+ other species. B) 607 nm in the glycerol and PBS system (0.1% DMSO) D) 522 nm in PBS buffer solution at pH 7.4 upon addition of the other species: (1) H_2S 2) ClO^- 3) NO 4) Ala, 5) Gly, 6) Glu, 7) Hcy 8) GSH 9) Asp 10) H_2O_2 (11) SO_3^{2-}(12) HSO_3^-, (λ_{ex} = 400 nm).

Calculation of Detection Limit:

The detection limit (DL) of **ABN** in PBS buffer / DMSO for Cys was determined from the following equation:
DL = K* Sb1/S

Where K = 2 or 3 (we take 2 in this case); Sb1 is the standard deviation of the blank solution; S is the slope of the calibration curve.
From graph S = 1.76022E10, Sb1 =71.38804, DL= 8.11 nM.

The detection limit (DL) of **ABN** in a glycerol and PBS buffer mixture with a volume ratio of 9: 1, for Cys was determined from the following equation:

DL = K* Sb1/S

Where K = 2 or 3 (we take 2 in this case); Sb1 is the standard deviation of the blank solution; S is the slope of the calibration curve.

From graph S = 2.21285E6, Sb1 = 0.01585, DL= 14 nM.

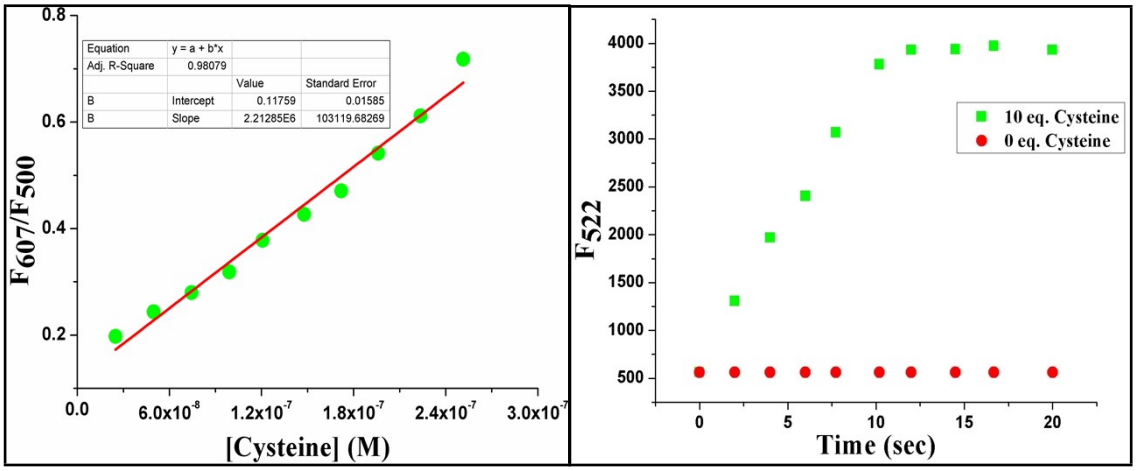


Figure S4. a) The relationship of fluorescence intensity and concentration of Cys from 0 to 0.35 μM in glycerol and PBS system (0.1% DMSO). b) Time course (0–1 min) of fluorescence enhancement at 522 nm of **ABN** (1.0 μM) in PBS buffer / DMSO (4:1 v/v, 10.0 mM, pH = 7.4, 25 ^{0}C).

Effect of pH:

We also tested the effect of pH on the fluorescence response of probe **ABN** to Cys in PBS buffer / DMSO (4:1 v/v, 10.0 mM, pH = 7.4, 25 0C) measured with and without 10 equiv. of Cys. In the absence of Cys, the free probe is stable over a wide range of pH values from 2 to 12, and displayed the obvious response for Cys in the region of 5–9. At pH 7.4, this indicates that the probe may be suitable for bio applications at the physiological pH.

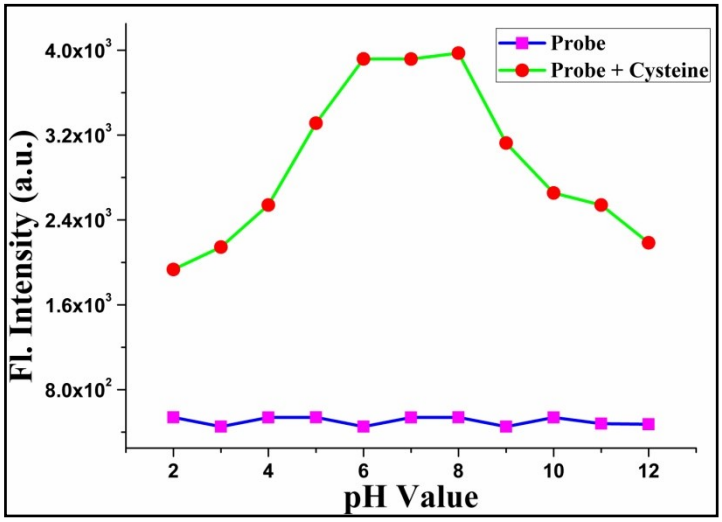


Figure S5. pH effect of **ABN** in DMSO/PBS buffer, v/v, 1:4, pH 7.4, itself (blue) and in presence of 10 equiv. of Cys (green).

NMR and Mass spectra:

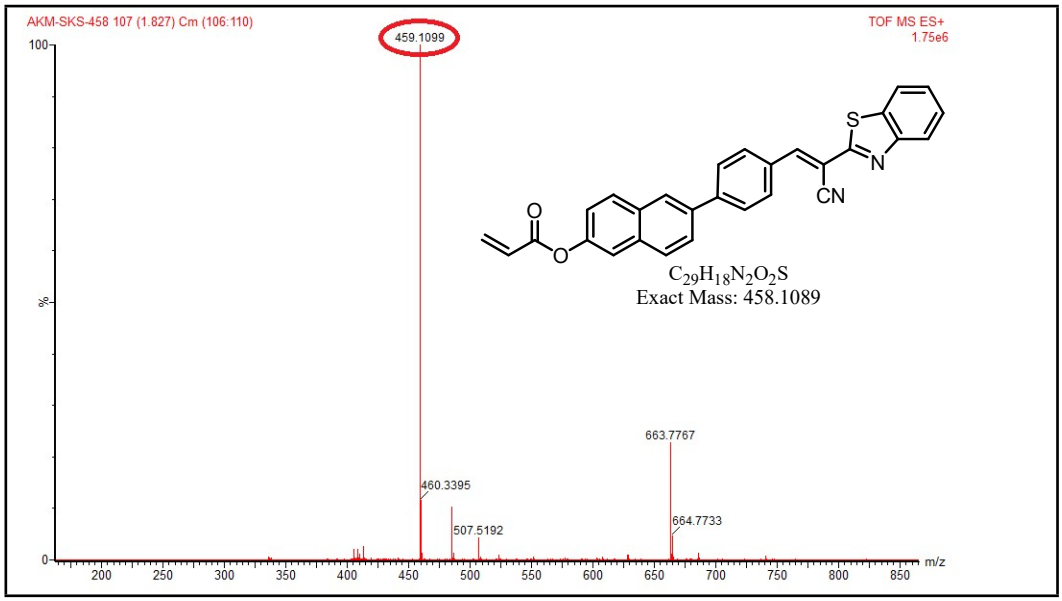


Figure S6: HR-MS mass spectrum of Compound **ABN**.

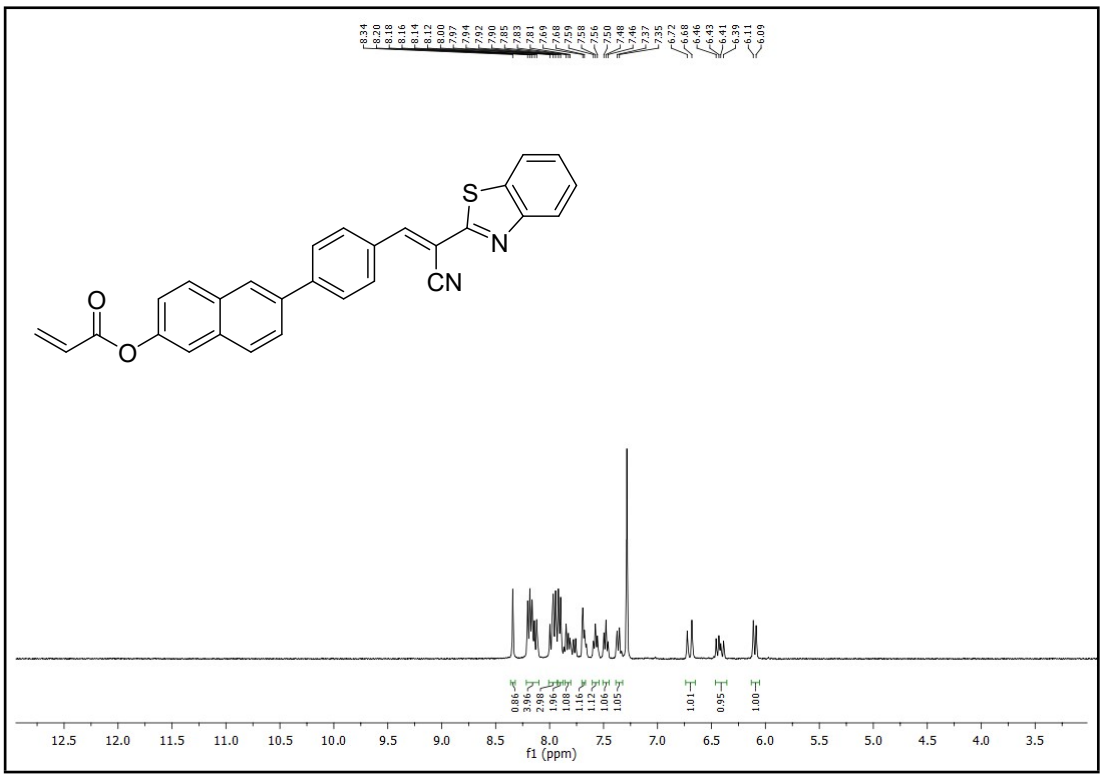


Figure S7: ^{1}H NMR of **ABN** in $CDCl_3$.

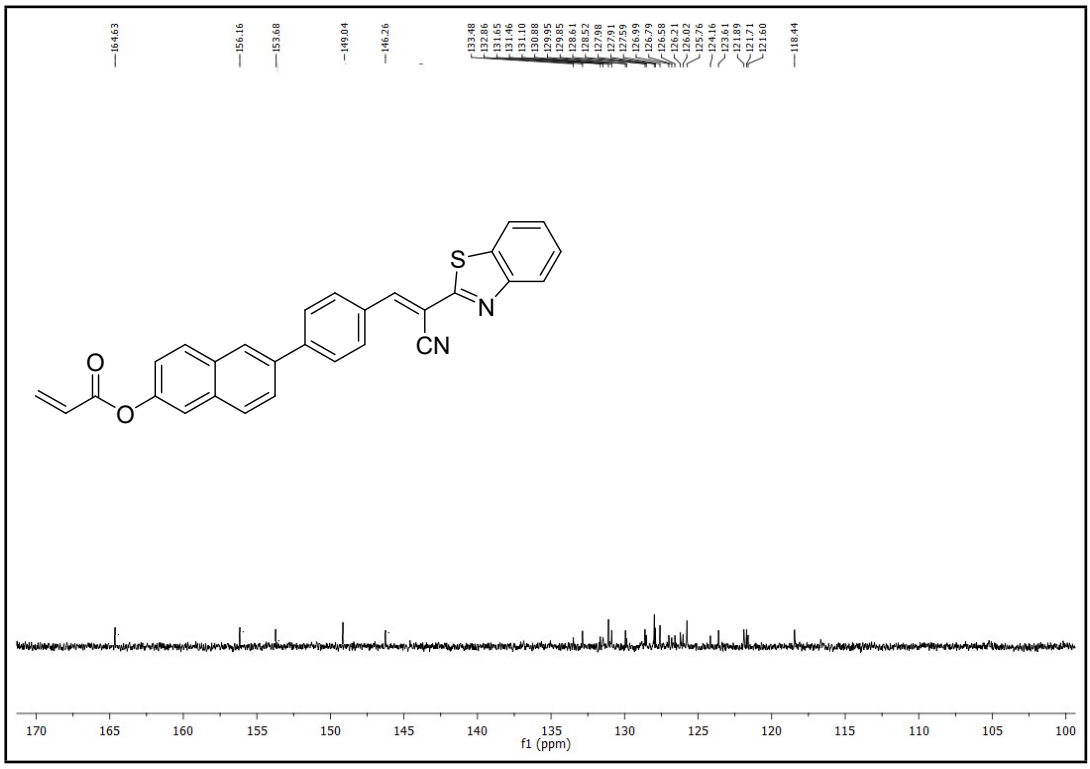


Figure S8: ^{13}C NMR spectrum of Probe **ABN** in CDCl3**.**

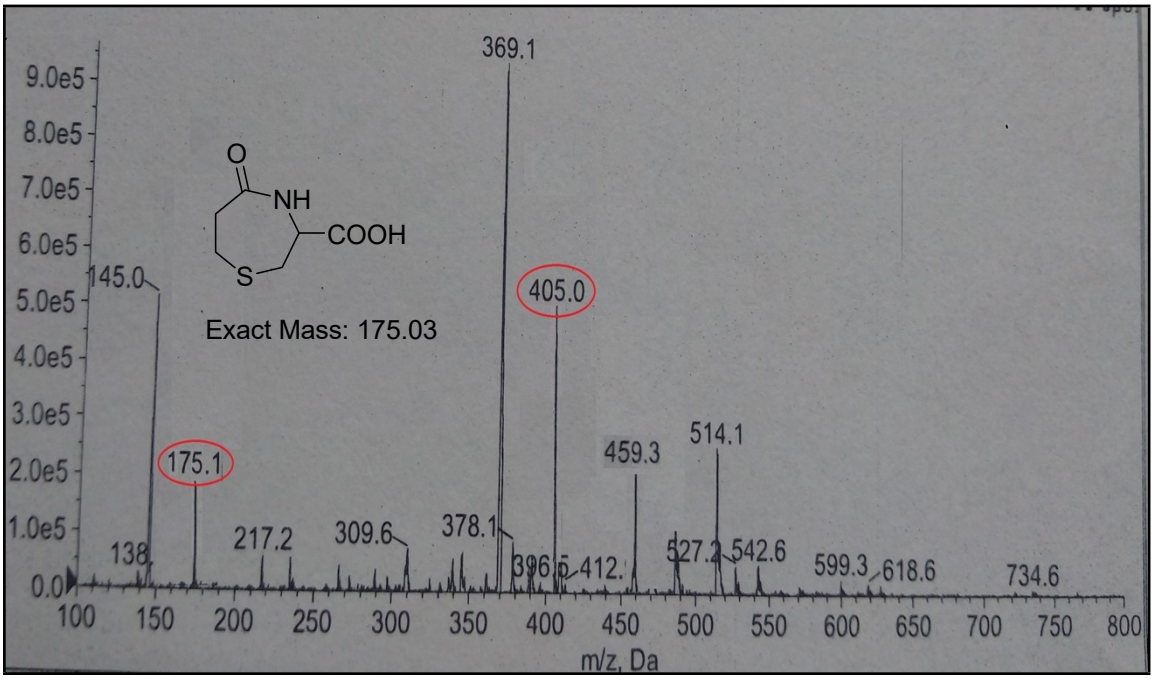


Figure S9: LC-MS mass spectrum of Compound **ABN** + Cysteine.

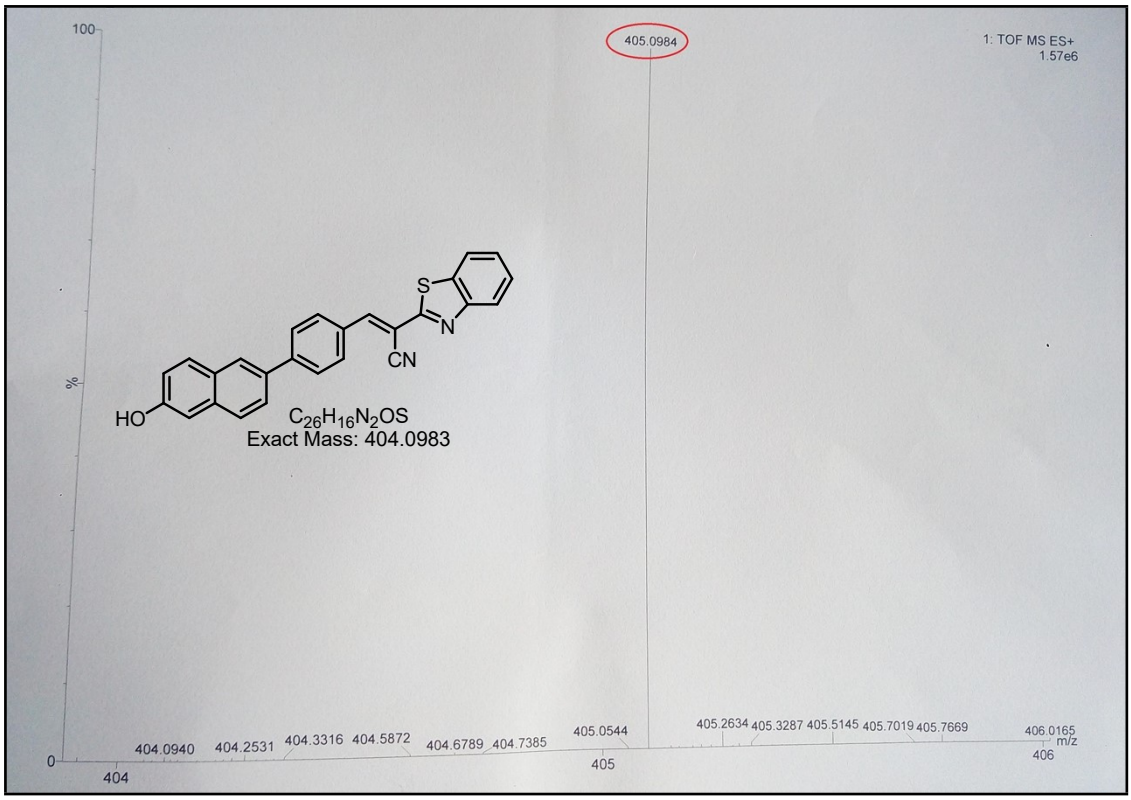


Figure S10: HR-MS mass spectrum of Compound **ABN**.

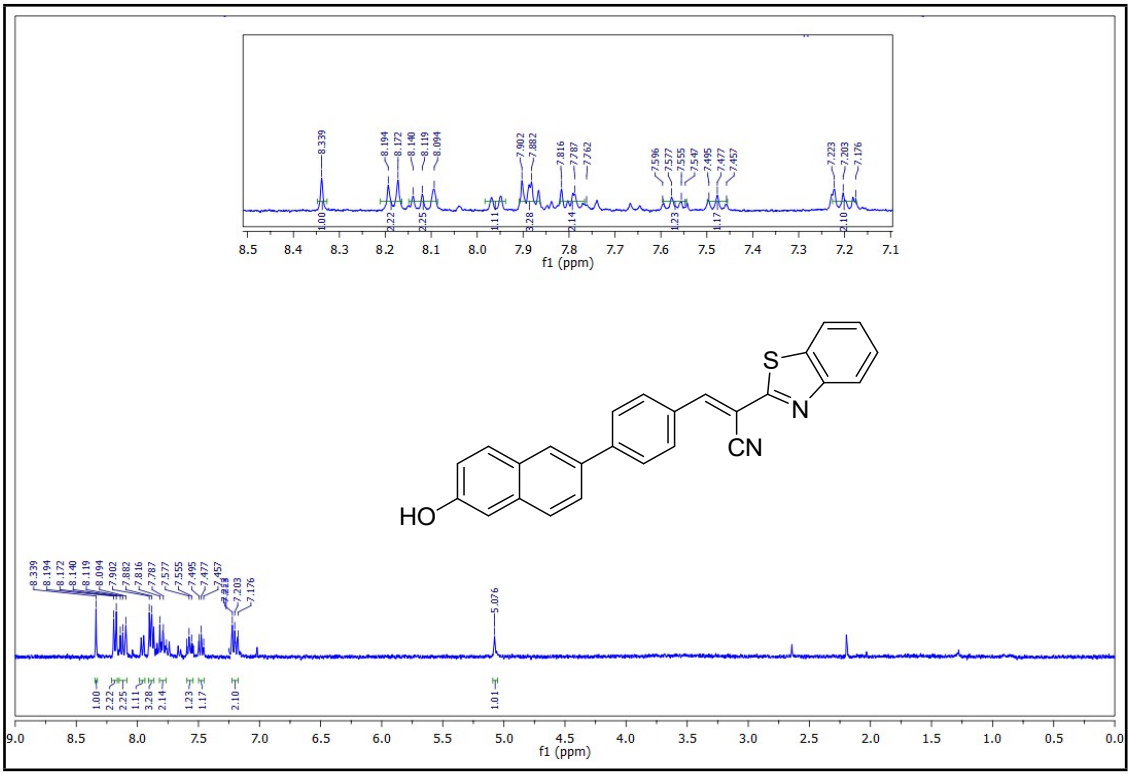


Figure S11: 1H NMR of **ABN**-OH in DMSO.

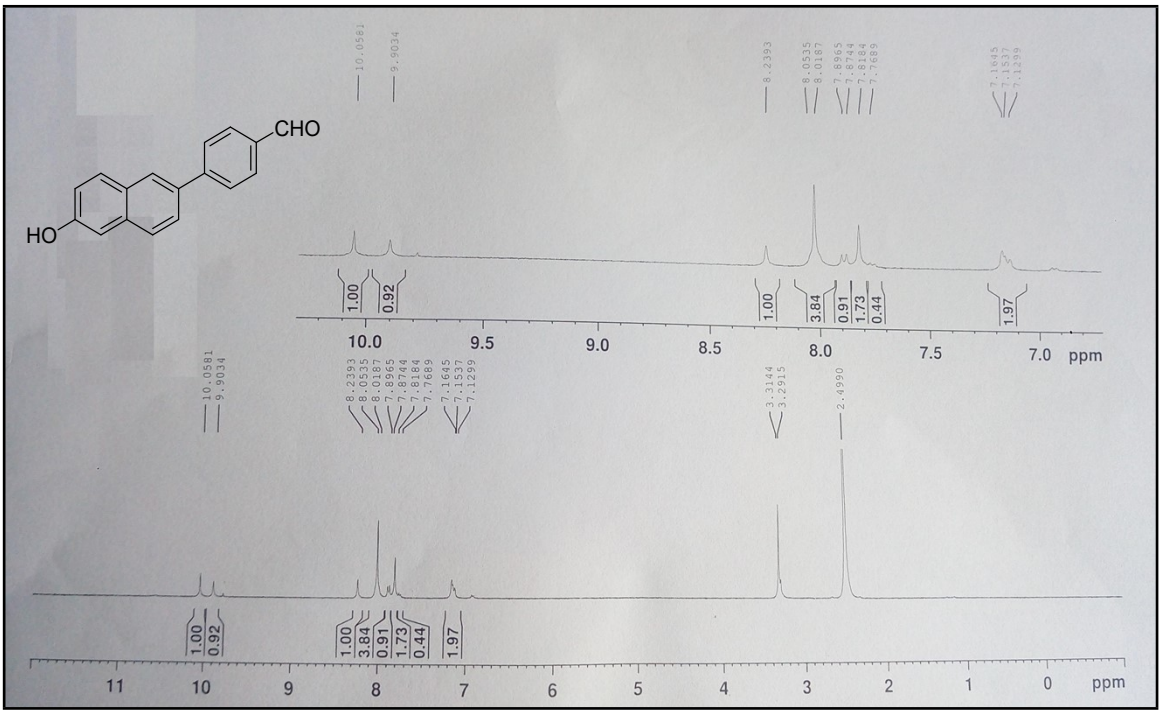
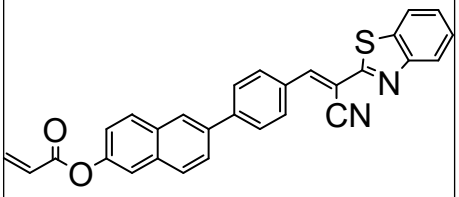
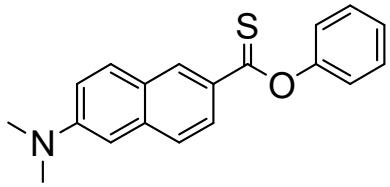
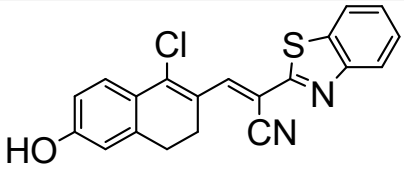
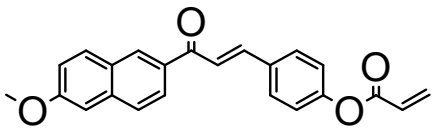
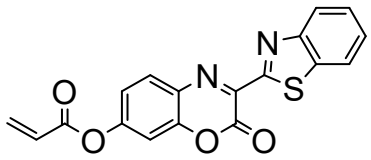
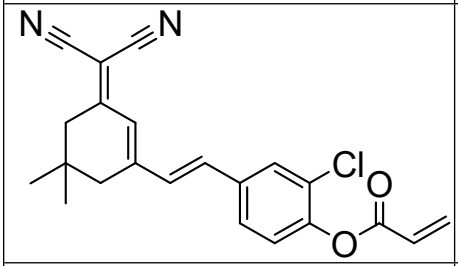
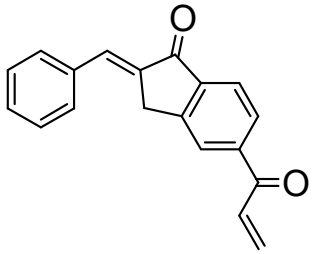


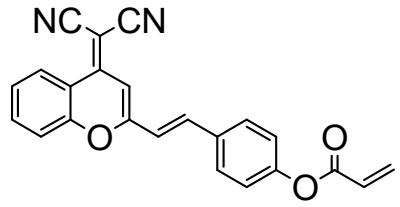
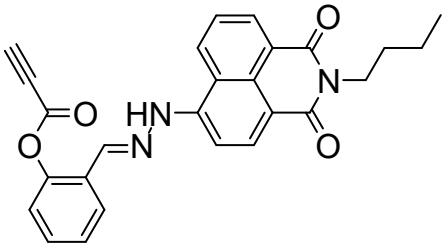
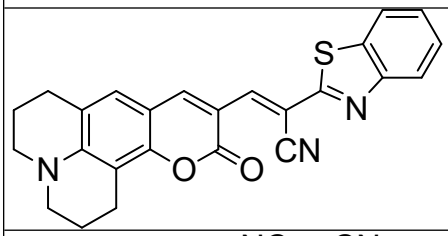
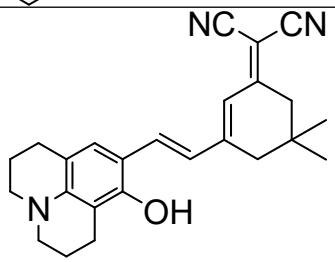
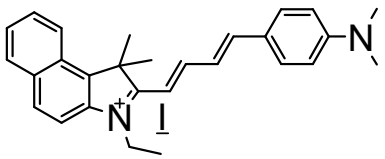
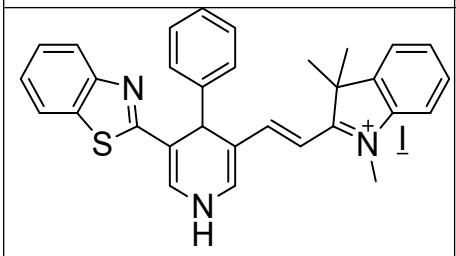
Figure S12: 1H NMR in DMSO.

Table S1. Energies of the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO)

Species	**E_{HOMO} (a.u)**	**E_{LUMO} (a.u)**	**ΔE(a.u)**	**ΔE(eV)**	**ΔE(kcal/mol)**	**f[b]**	**(composition) %**
ABN (0^0)	-0.22325	-0.10563	0.11762	3.20060	73.8	1.2831	98.0
ABN (90^0)	-0.22444	-0.09623	0.12821	3.48877	80.4	0.0002	89.1
ABN-OH(0^0)	-0.21748	-0.11628	0.1012	2.75378	71.3	1.0534	98.9
ABN-OH(90^0)	-0.21595	-0.09541	0.12054	3.28008	75.6	0.0016	95.8

[a] Only selected excited states were considered. The numbers in parentheses are the excitation energy in wavelength. [b] Oscillator strength. [c] H stands for HOMO and L stands for LUMO.

Probe	Detection of Cysteine	Detection of Viscosity	Detection of Cysteine in Viscous medium	Ratiometric	Detection Limit	Reference
	Yes	Yes	Yes	Yes	8.11 nM	This Work
	Yes	No	No	No	0.0005 μM	1
	Yes	No	No	No	2.1×10^{-8}M	2
	Yes	No	No	No	2.2×10^{-8}M	3
	Yes	No	No	No	124 nM	4
	Yes	No	No	No	173 nM	5
	Yes	No	No	No	80 nM	6

Probe	Detection of Cysteine	Detection of Viscosity	Detection of Cysteine in Viscous medium	Ratiometric	Detection Limit	Reference
	Yes	No	No	No	0.2 μM	7
	Yes	No	No	No	9.06 nM	8
	No	Yes	No	No	-	9
	No	Yes	No	No	-	10
	No	Yes	No	No	-	11
	No	Yes	No	No	-	12

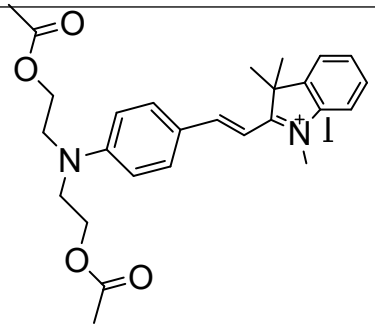
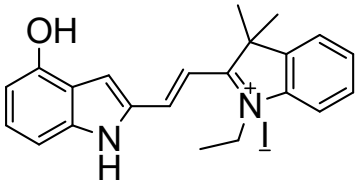
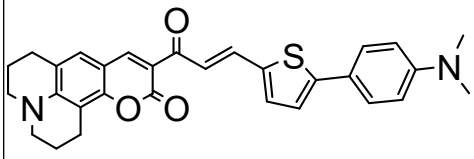
Probe	Detection of Cysteine	Detection of Viscosity	Detection of Cysteine in Viscous medium	Ratiometric	Detection Limit	Reference
	No	Yes	No	No	-	13
	No	Yes	No	No	-	14
	Yes	Yes	No	No	156 nM	15

Table S2. Comparison table for the detection of cysteine and viscosity.

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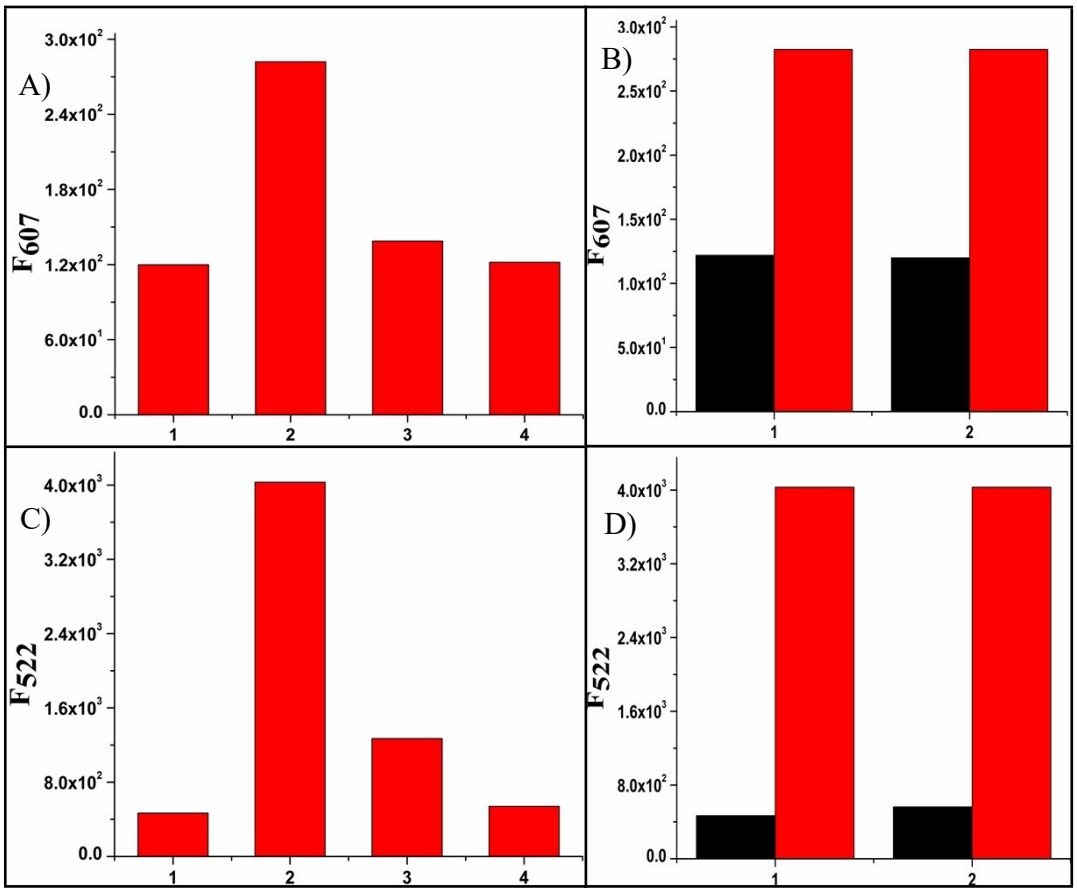

Figure S13. The fluorescence response of **ABN** (1 mM) for Cys.20 mM, Others: 50 mM, (1) **ABN** 2) Cys 3) Hcy 4) GSH A) at 607 nm in the glycerol and PBS system (0.1% DMSO, λ_{ex} = 400 nm).C) at 522 nm in PBS buffer solution. Selectivity profile diagram in bar representation, Changes of

fluorescence of **ABN** (1 mM) at B) at 607 nm in the glycerol and PBS system (0.1% DMSO, λ_{ex} = 400 nm) D) at 522 nm in PBS buffer solution upon addition of the different species (50.0 μM): red bars: **ABN**+ other species+ Cys; black bars: **ABN**+ other species: (1) Hcy 2) GSH.

The detection limit (DL) of **ABN** in a glycerol and PBS buffer system for Cys at 607 nm was determined from the following equation:

DL = K* Sb1/S

Where K = 2 or 3 (we take 2 in this case); Sb1 is the standard deviation of the blank solution; S is the slope of the calibration curve.

From graph S = 5.79128E8, Sb1 = 4.1764, DL= 14.4 nM.

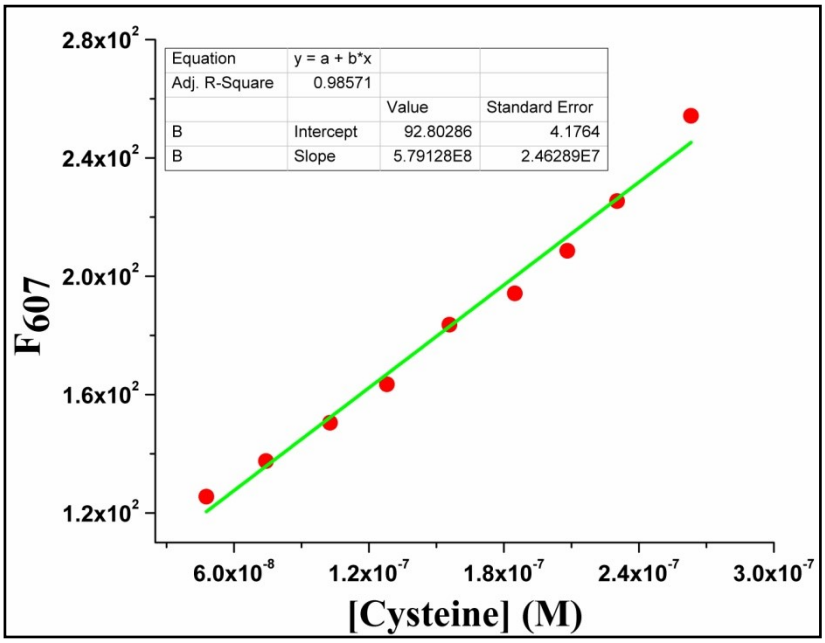

Figure S14. The relationship of fluorescence intensity and concentration of Cys from 0 to 0.35 μM in glycerol and PBS system (0.1% DMSO) at 607 nm wavelength.