Supplementary Material

Alizarin Red S –Zinc(II) Fluorescent Ensemble For Selective Detection of Hydrogen Sulphide and Assay with H_2S donor

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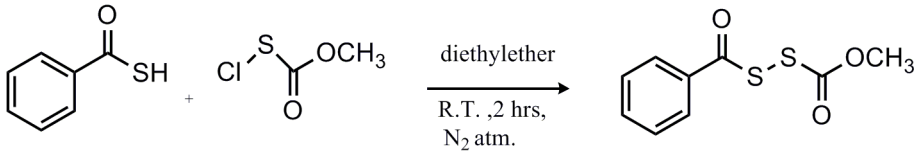
Synthesis of H_2S Donor



H_2S donor compound benzoic (methyl carbonic) dithioperoxyanhydride was synthesized by following reported procedure[1]. Accordingly in a 100 ml round bottom flask 300µl of methoxycarbonylsulfenyl chloride (3.2 mM) was dissolved in 20 ml of distilled diethylether, to this 324 µl of thiobenzoic acid (2.7 mM) was added drop wise at room temperature under stirring at N_2 atmosphere. After 2 hours, the solution was concentrated under high pressure and passed through a short silica column chromatography using Hexane/$CHCl_3$ as a solvent. After removal of the solvent in rotary evaporator, a colorless oily product was obtained. This product was stored under cold condition. Yield: 350 mg (52 %), ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 3.85 (3H), 7.54 (2H), 7.59 (1H), 7.94 (1H): ESI-MS (-ve) (m/z) = 265.2 $[M+Cl]^+$, 337.2 $[M+3Cl]^+$

UV-Visible absorption studies

Absorbance study of ARS with Zn: For the absorbance study, stock solution of **ARS** (0.5 mM) was prepared in MeOH:PBS buffer (3/1,(v/v),10 mM, pH = 7.4).For the titration 120 µl of stock solution was diluted to 2000 µL by adding 1878 µL buffer. The titration was done with different concentration of zinc perchlorate (0 – 30 µM) in MeOH:PBS buffer (3/1,(v/v),10 mM, pH = 7.4), each spectra was recorded in standard quartz cuvette cell of length 1 cm. The change in absorption was monitored at 530 nm.

Absorbance study of ARS-Zn vs H_2S: For the absorbance study stock solution of **ARS** (0.5 mM) was prepared and 120µl was diluted to 2000 µl followed by addition of 40µl of zinc perchlorate (1.5 mM) in MeOH:PBS buffer (3/1,(v/v),10 mM, pH = 7.4). The titration was done with different concentration of H_2S (0 – 44 µM). The titration was done in standard quartz cuvette cell of length 1 cm.

Fluorescence titration studies

Fluorescence study of ARS with Zn: For the emission study, stock solution of ARS (0.5 mM) was prepared in MeOH:PBS buffer (3/1,(v/v),10 mM, pH = 7.4) and for the titration 120 µL of stock solution was diluted to 2000 µL by adding 1878 µL buffer. The titration was done with different concentration of zinc perchlorate (0 - 30 µM) in MeOH:PBS buffer (3/1,(v/v),10 mM, pH = 7.4), excitation wavelength was 530 nm with a slit width of 10/10.

Fluorescence study of ARS-Zn(II) with H_2S: For the emission study stock solution of **ARS** (0.5 mM) was prepared and 120 µl was diluted to 2000 µl followed by addition of 40 µl of zinc perchlorate (1.5 mM) in MeOH:PBS buffer (3/1,(v/v),10 mM, pH = 7.4). The titration was done with different concentration of H_2S (0 - 42 µM). The excitation wavelength was 530 nm with a slit width of 10/10.

Competitive experiments

For the competitive studies with different analytes stock solution of **ARS** (0.5 mM) was prepared and 120µl was diluted to 2000 µl followed by addition of 40µl of zinc perchlorate (1.5 mM) in MeOH:PBS buffer (3/1,(v/v),10 mM, pH = 7.4) and 300 µM of different analytes such as nitrate, chloride, bromide, hydroxide, acetate, bicarbonate, glutathione, methionine, nitrite, phosphates, azide, fluoride, hydroxide, thiosulphate, cysteine (150 µM) and H_2S (30 µM) was taken in the titration. Excitation wavelength for the fluorescence titration is 530 nm and slit width was 10/10.

pH studies

105µl of ARS (0.5 mM) was diluted to 1860µl and followed by addition of 35µl of Zinc perchlorate solution prepared in MeOH:PBS buffer (3/1,(v/v),10 mM, pH = 7.4). The titration was done with H_2S (27 µM) at various pH between (4-12) using fluorescence spectrophotometer. Excitation wavelength for the fluorescence titration is 530 nm and slit width was 10/10.

Fluorescence study of ARS-Zn(II) with H_2S in presence of Human Serum and BSA

Stock solution of **ARS** (0.5 mM) was prepared in MeOH:PBS buffer (3/1,(v/v),10 mM, pH = 7.4). 120μL of this stock solution was diluted to 2 mL followed by addition of zinc perchlorate (30 μM) titrated against H_2S spiked Human serum and bovine serum albumin (6 - 30 μM). The emission intensity at 620 nm was monitored upon excitation at 530 nm. The titration was done with slit width 10/10.

Preparation of Bovine serum albumin (BSA) solution: The BSA solution was prepared by dissolving BSA (1.0 g) in 40 mL MeOH:PBS buffer (3/1,(v/v),10 mM, pH = 7.4) so that the final concentration will be 25 mg/ml. The above solution was taken for the fluorescence studies of probes.

Fluorescence study of ARS-Zn(II) with H_2S donor

For the fluorescence study, 100 μl of ARS-Zn (60 μM) was prepared in MeOH:PBS buffer (3/1,(v/v),10 mM , pH = 7.4, 0.04% THF used as a co-solvent) and diluted by adding 134μl of buffer and followed by addition of 60μl donor (0.001 M, 10 equivalent) and 6μl glutathione (0.1 M, 100 equivalent) and was monitor for 30 minutes at 35°C. The excitation wavelength was 530 nm and emission wavelength was 645/30 nm. The titration was done in 96 well- plate using multimode micro plate reader.

Temperature dependent Fluorescence study of ARS-Zn(II) with H_2S donor

For the fluorescence study , 100 μl of ARS-Zn(II) (60 μM) was diluted by adding 134μl of buffer and followed by addition of 60μl donor (0.001 M, 10 equivalent) and 6μl glutathione (0.1 M, 100 equivalent) and was monitor for 30 minutes at 30°C, 40 °C and 50°C. The excitation wavelength was 530 nm and emission wavelength was 645. The titration was done in 96 well-plate in multimode micro plate reader.

Detailed experimental procedure of cell imaging

Maintenance of C6 cell lines

Glial cell lines (C6) were cultured in RPMI-1640 growth medium supplemented with 10% (v/v) FBS (Fetal Bovine Serum), penicillin, streptomycin and gentamycin in CO_2 incubator at 37 °C, 5% CO_2 and 95% relative humidity in tissue culture flask. Medium was changed on a regular basis and cells were harvested at the log phase of growth for various analysis.

Confocal imaging

Confocal imaging was done according to the method given by Ramsay et al (1988). C6 glial cells (100,000 cells/mL) were incubated in 6 well plates having coverslip in each well for 24 hours and thereafter treated with different concentration of test compound for 20 minutes. Cells washed twice with chilled phosphate buffer saline (PBS), fixed with chilled 4% paraformaldehyde and then washed with chilled PBS and treated with DAPI (10 µg/mL) for 30 minutes. Then washing of wells was done to remove the excessive dye. Coverslip were placed on slide over floromount. Finally, Slides were observed under Nikon Air Laser Scanning Confocal Microscope System.

C6 glial cells with ARS only

C6 glial cells were incubated with 30 µM of the **ARS** in buffer for 20 minutes, then confocal microscope images were taken at λ_{ex} = 543 nm with 40X objective.

C6 glial cells with ARS-Zn(II) complex

***ARS-Zn(II)** complex was prepared insitu by following method:* 6 µl of the stock solution of **ARS** (0.001 M) was added to 188 µl of buffer, to this 6 µl of Zinc perchlorate hexahydrate (0.001 M) was added and the solution allow to stand for 5 minutes. Final concentration of **ARS** was 30 µM and $Zn(ClO_4)_2$ was 30 µM. **ARS-Zn(II)** complex prepared by above procedure was added to the cell lines and incubated for 20 minutes at 37°C before recording confocal microscope images at λ_{ex} = 543 nm with 40X objective.

C6 glial cells with ARS-Zn(II) and Na_2S

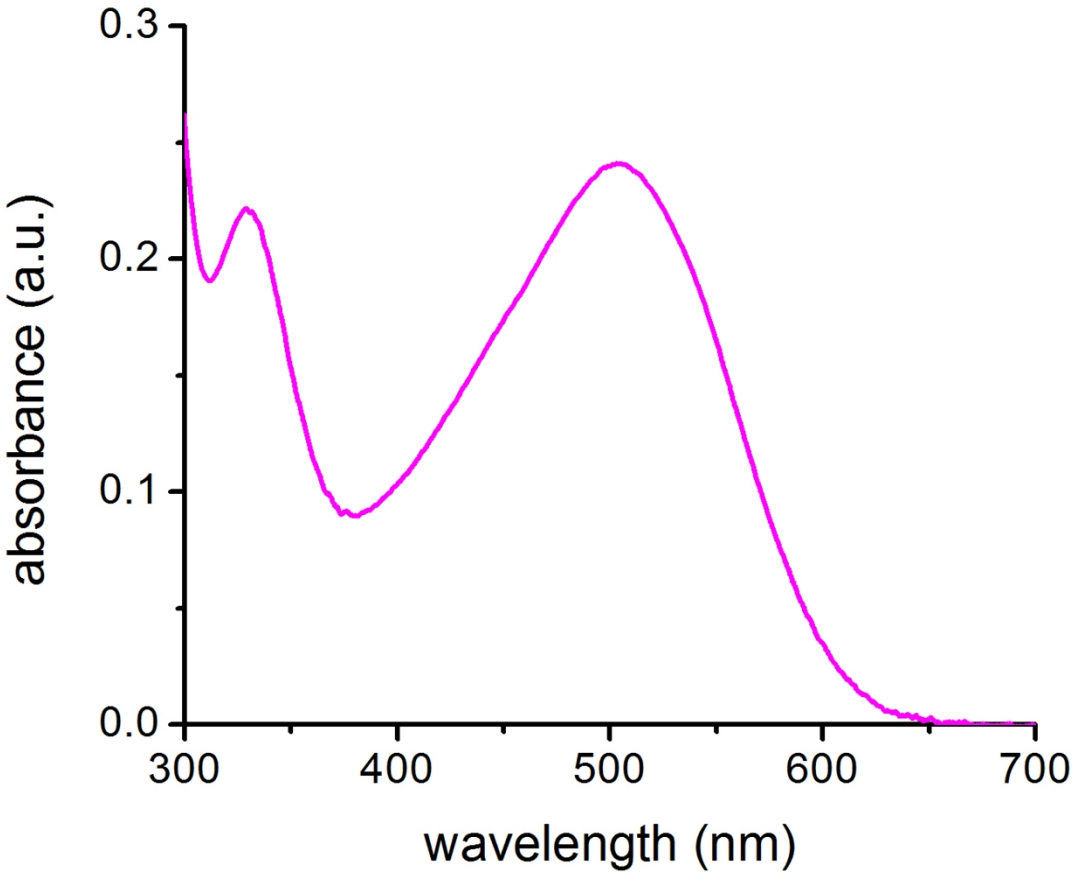
C6 cell lines were incubated with 30 µM of **ARS-Zn(II)** and then treated with 44 µM of Na_2S for 20 minutes at 37°C. The confocal microscope Images were recorded at λex = 543 nm with 40X objective.

Calculation for the detection limit:

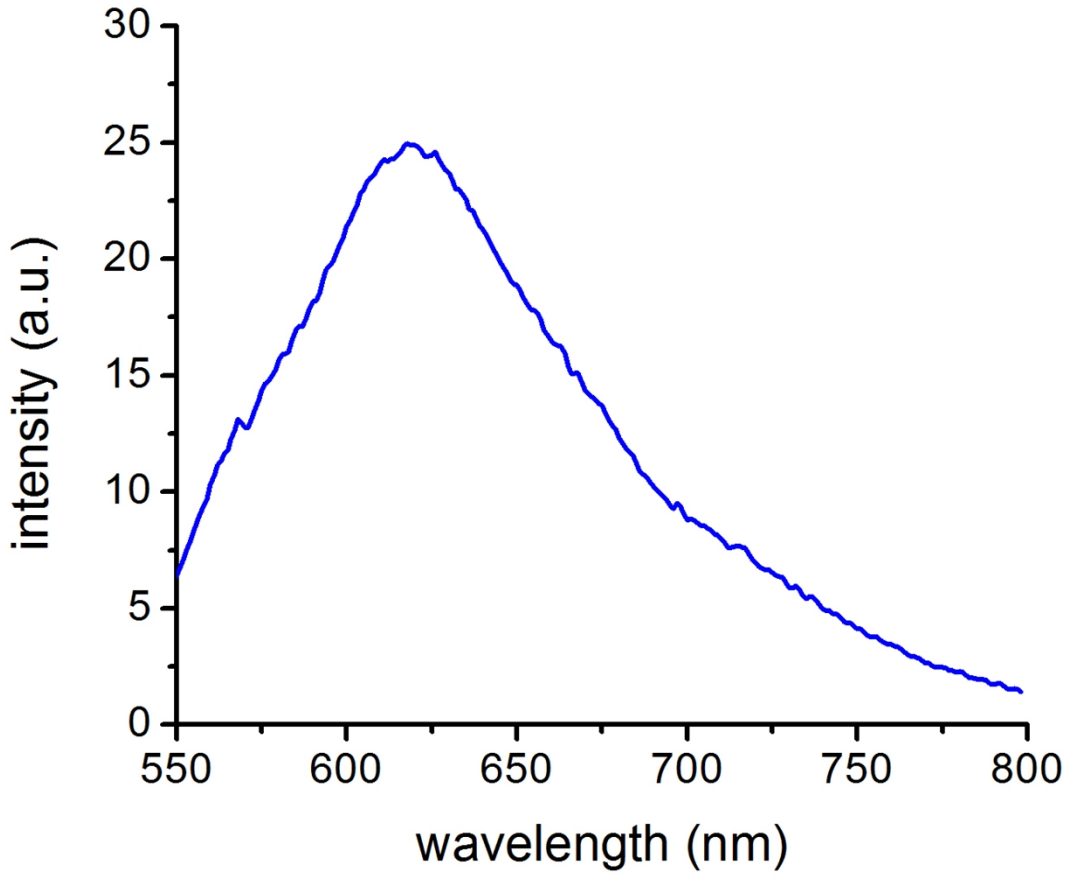
The detection limit was calculated using formula:

LOD = (3 x SD)/slope = (3 x 2.0)/6.52273 x 10^7 = 92 nM

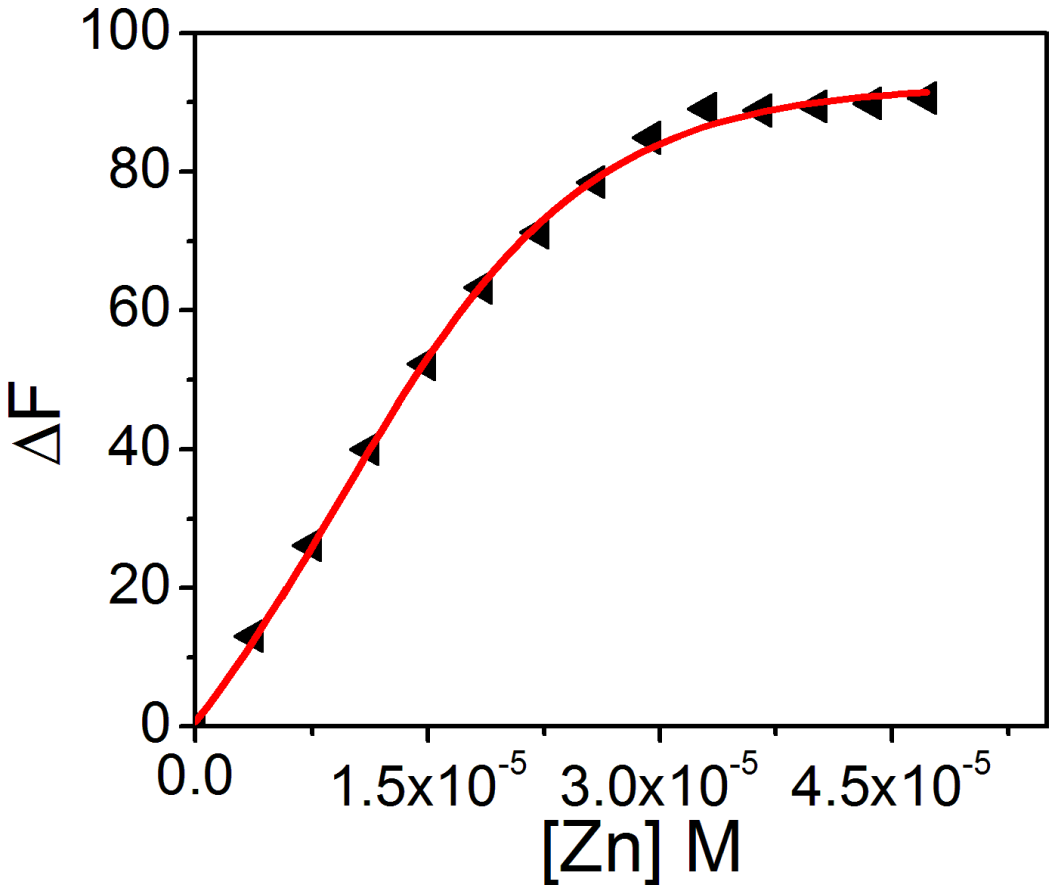
Where, LOD= Limit of detection and SD = Standard deviation of the blank



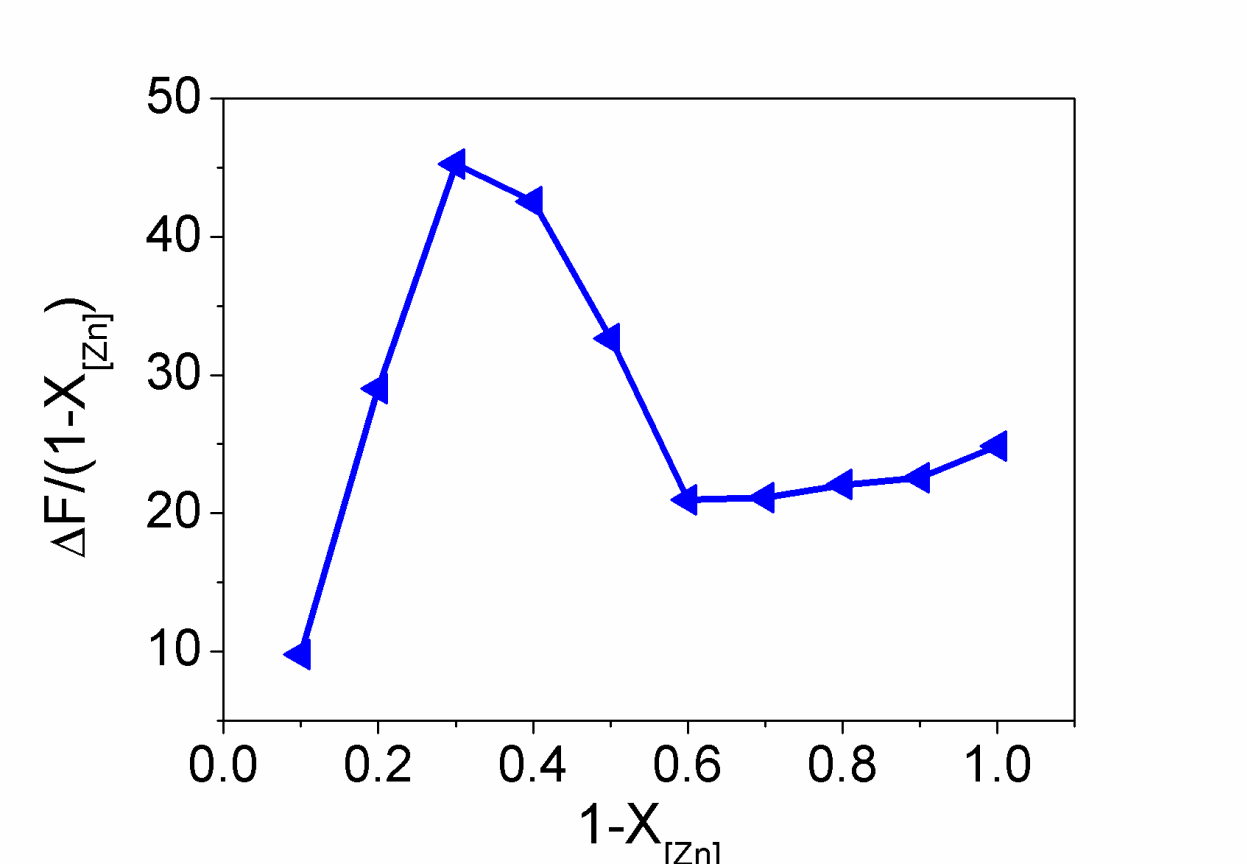
SI Figure 1: The UV/visible absorption of ARS (30μM) in MeOH:PBS buffer (3/1,(v/v),10mM,pH = 7.4).



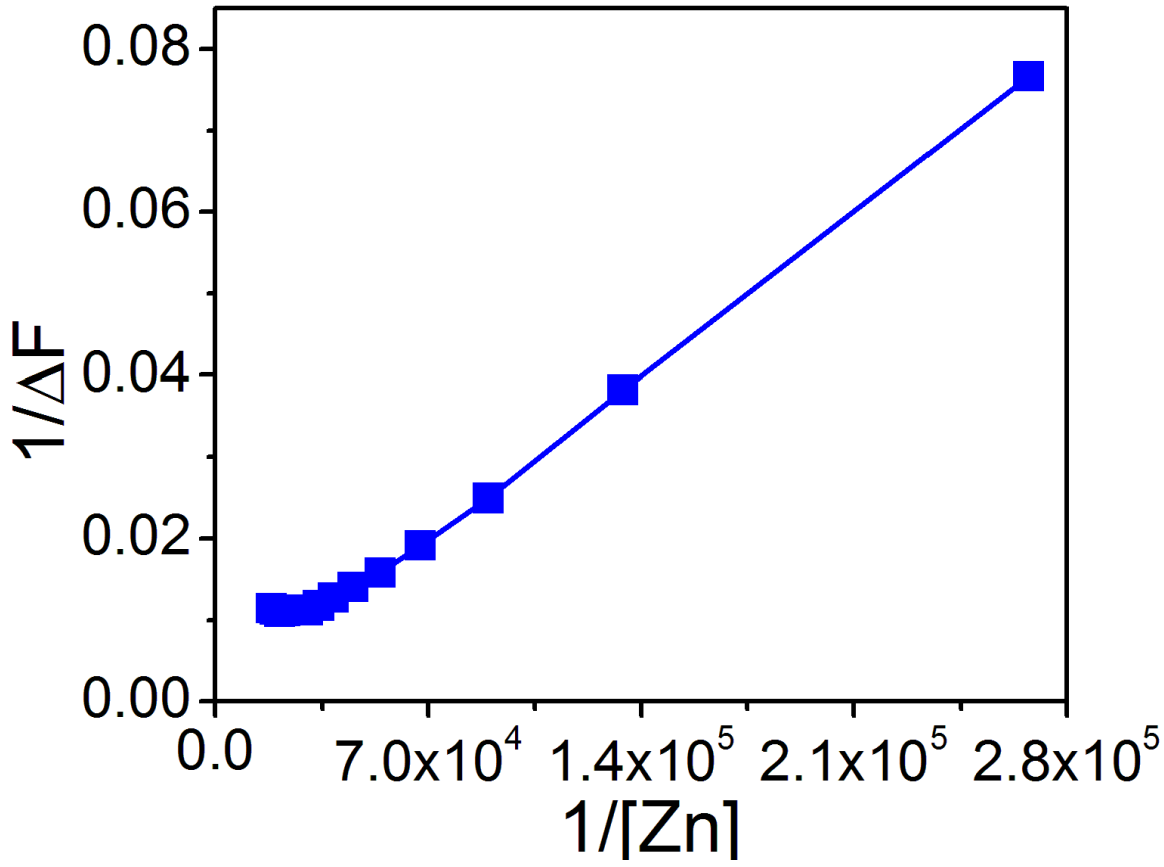
SI Figure 2: The emission spectrum of **ARS** (30μM) in MeOH:PBS buffer (3/1,(v/v),10mM,pH = 7.4)



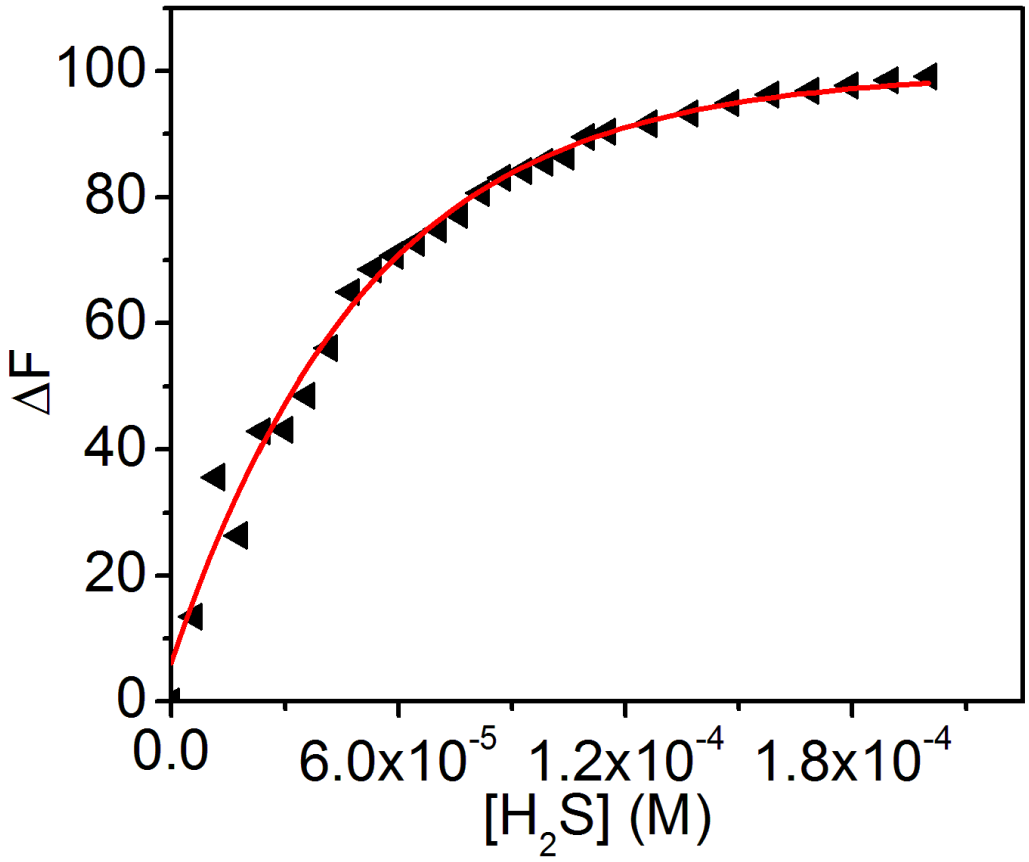
SI Figure 3: The emission titration profile of ARS with Zinc



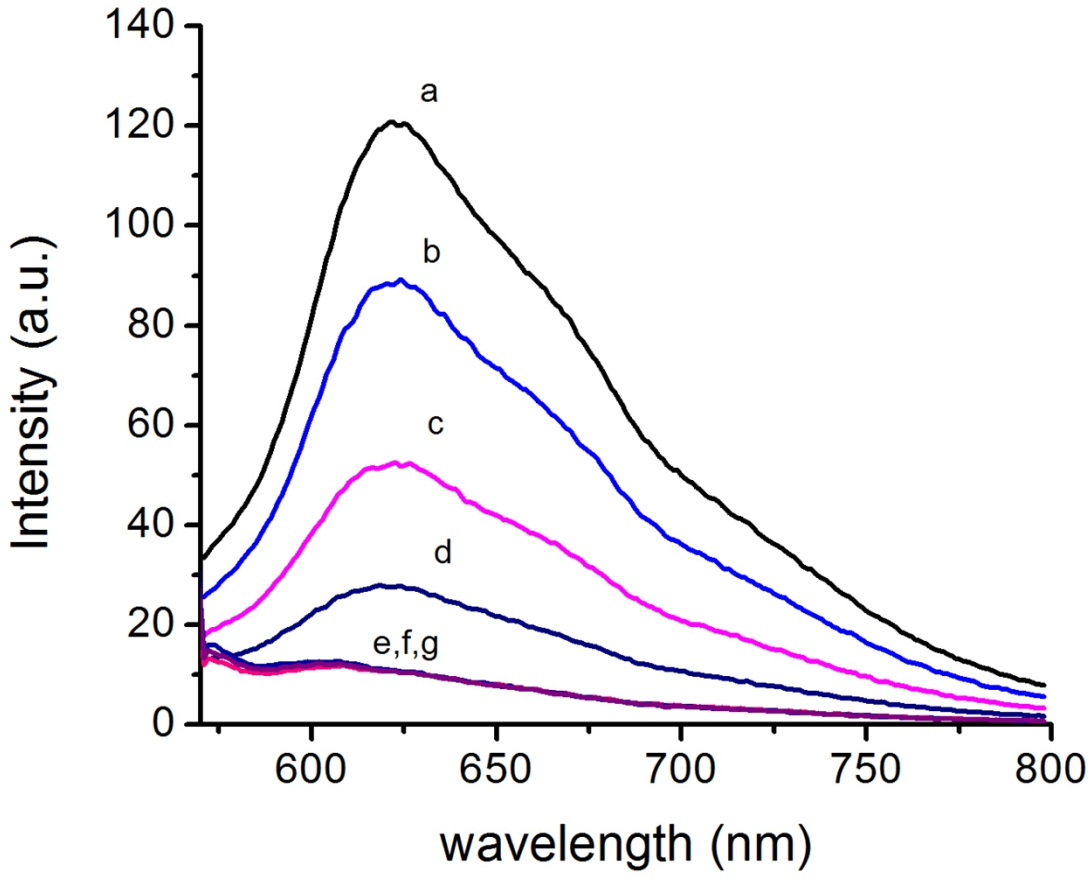
SI Figure 3: Job plot for the binding of ARS with Zinc (II) (2:1 Complex formation). Where, X is the mole fraction.



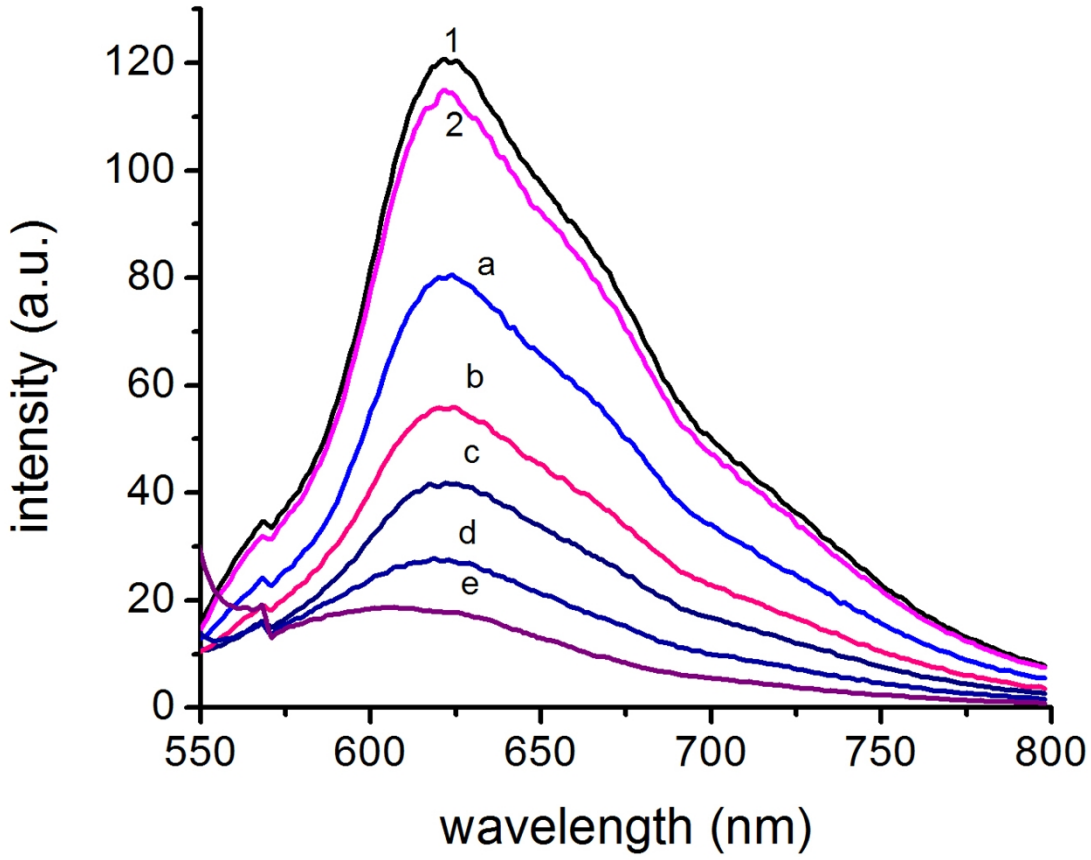
SI Figure 4: Benesi-Hildebrand plot for the fluorescent titration of ARS with Zinc ion



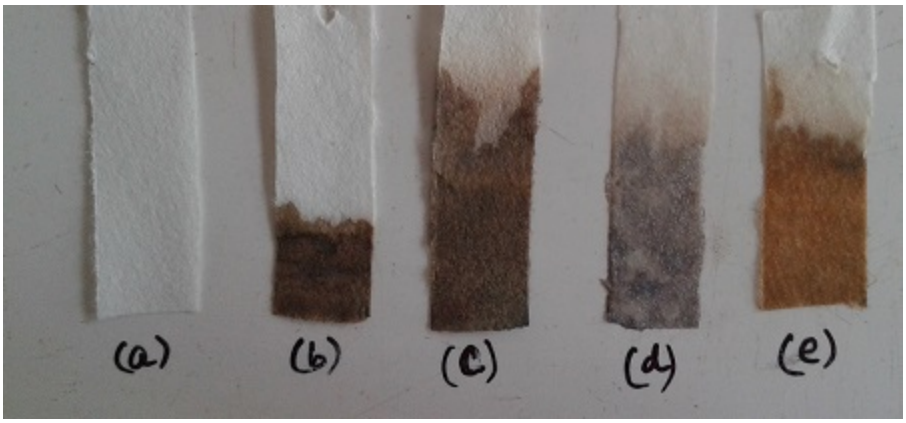
SI Figure 5: Titration profile for the binding of **ARS-Zn(II)** with H_2S in MeOH-PBS buffer at RT



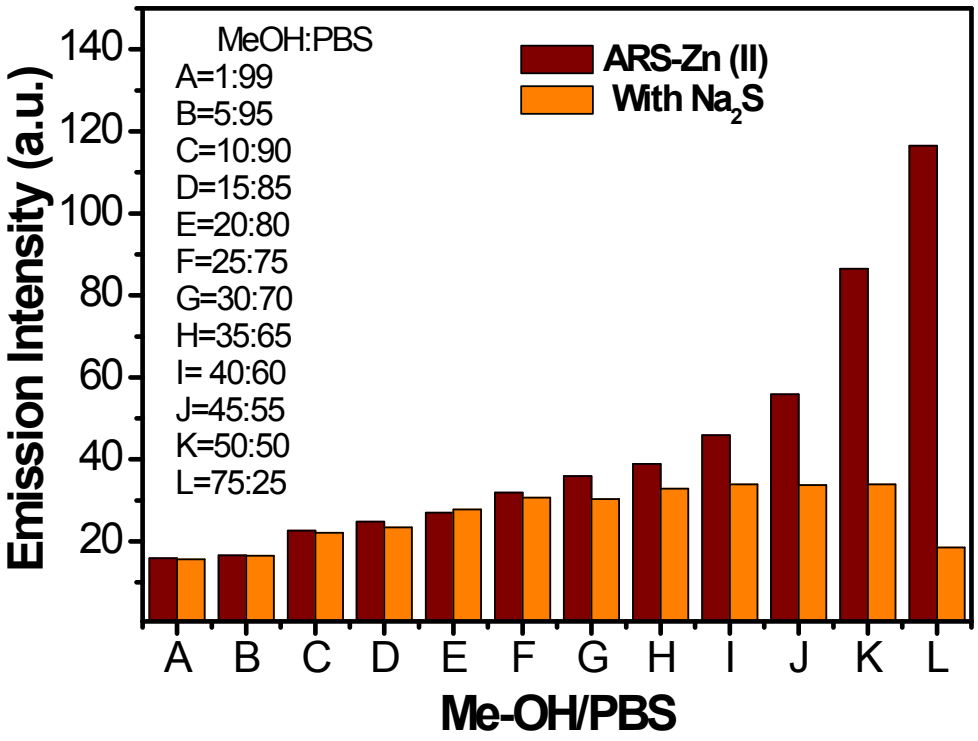
SI Figure 6: Fluorescent titration of **ARS-Zn(II)** with H_2S (0-30μM) spiked human serum (a) ARS-Zn (blank), (b) blank+ human serum without spiking H_2S, (c) 6 μM H_2S spiked, (d) 12 μM H_2S spiked (e) 18 μM H_2S spiked (f) 24 μM H_2S spiked (g) 30 μM H_2S spiked in Human Serum.



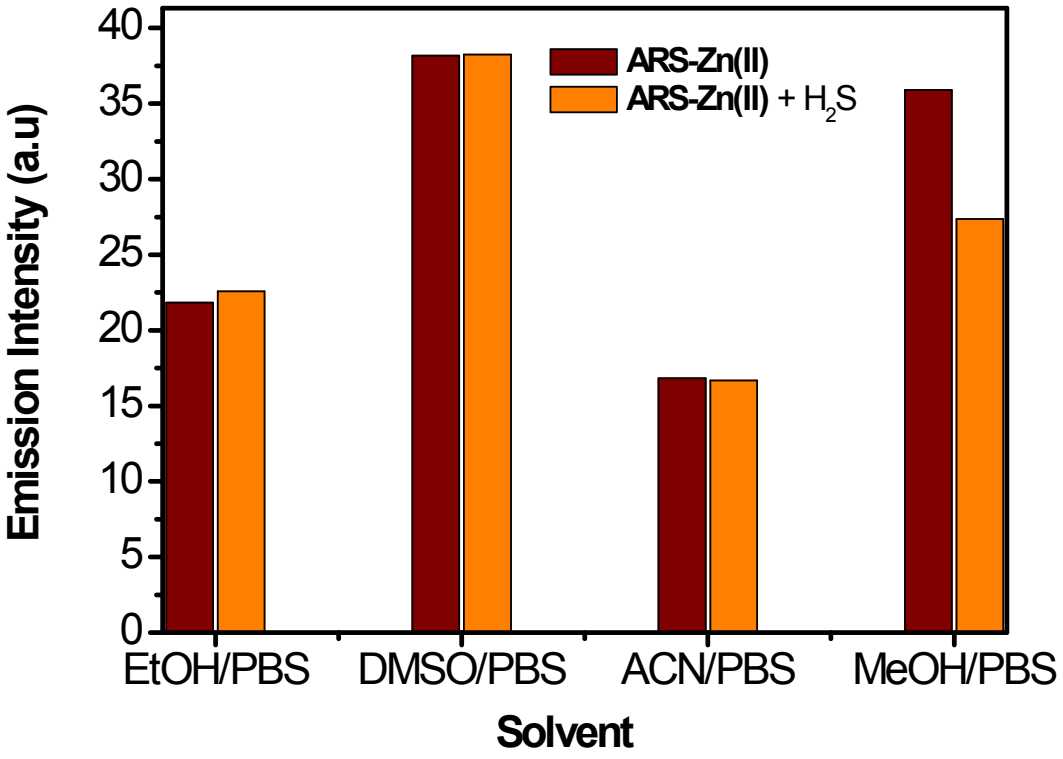
SI Figure 7: Fluorescent titration of **ARS-Zn(II)** with H_2S (0-30μM) spiked BSA (1) ARS-Zn (blank), (2) blank+ BSA without spiking H_2S, (a) 6 μM H_2S spiked BSA, (b) 12 μM H_2S spiked BSA (c) 18 μM H_2S spiked BSA(d) 24 μM H_2S spiked BSA (e) 30 μM H_2S spiked in BSA.



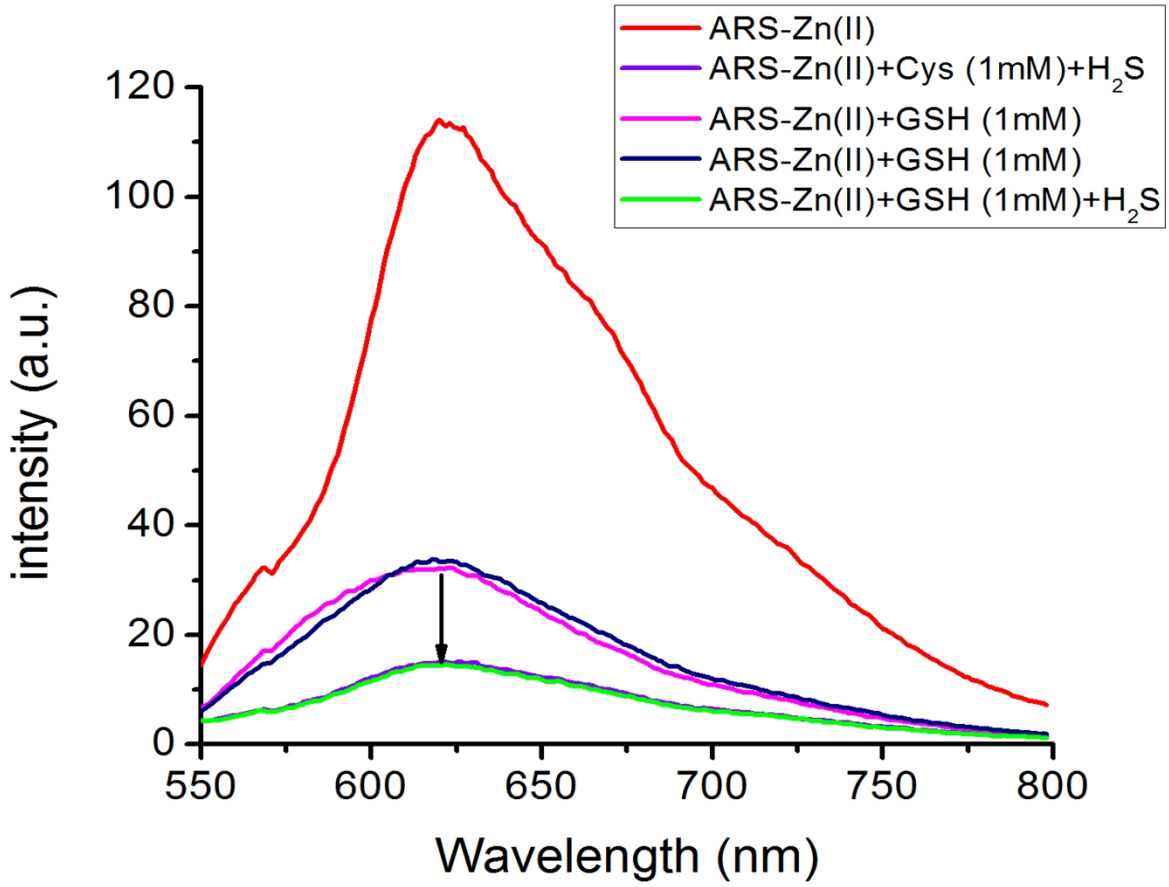
SI Figure 8 : (a) Blank lead acetate strip paper (b) 0.05 M Na_2S, (c) 0.005 M Na_2S, (d) 0.0005 M Na_2S, (e) 0.0005 M Na_2S. The black color shows the presence of H_2S species in the MeOH-PBS (3:1, v/v, pH 7.4).



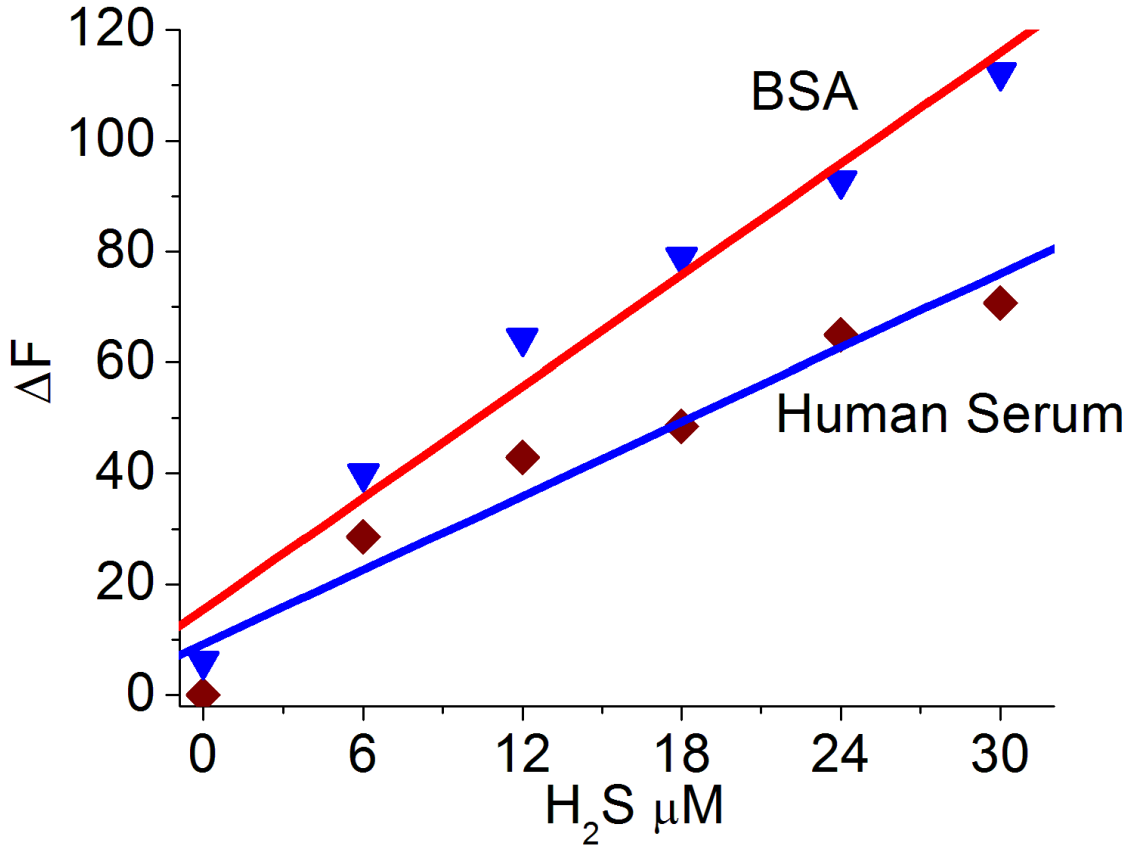
SI Figure 9: Effect of methanol percentage in H_2S sensing of **ARS-Zn(II)** in Me-OH/PBS mixture.



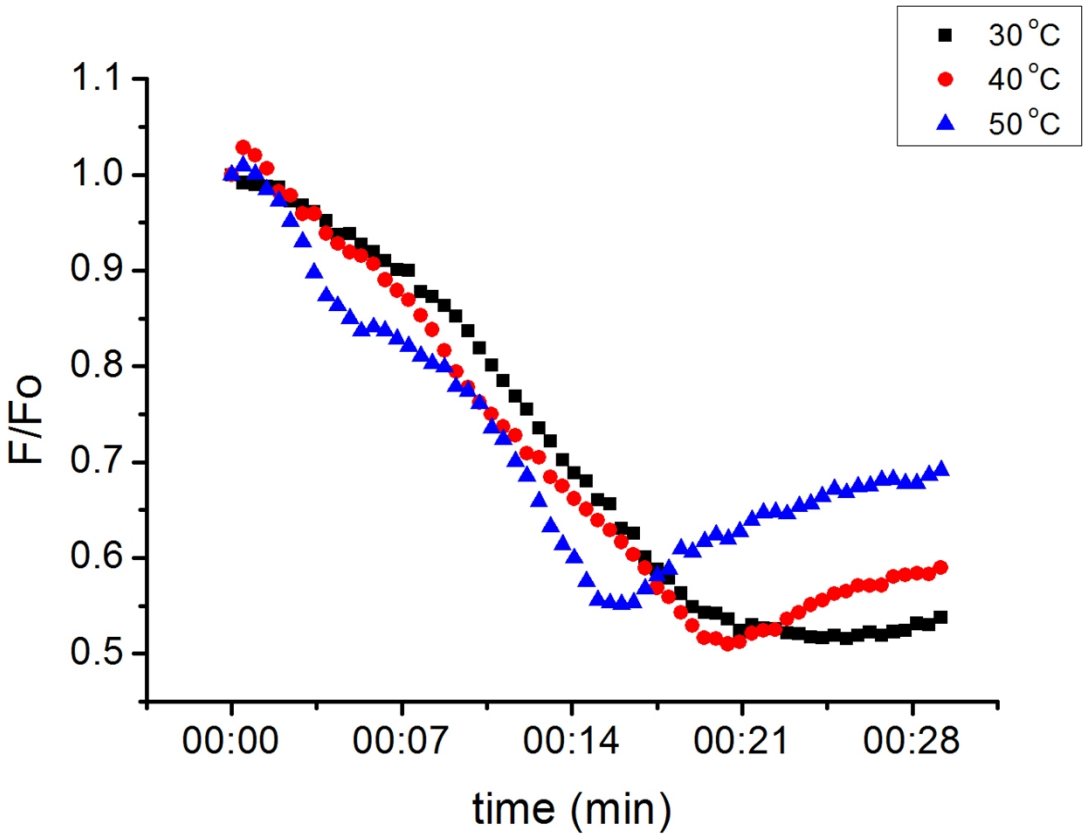
SI Figure 10: Effect of different solvent in probe **ARS-Zn(II)** with of H_2S (44 μM). Ratio of different solvent and PBS is (30/70, v/v)



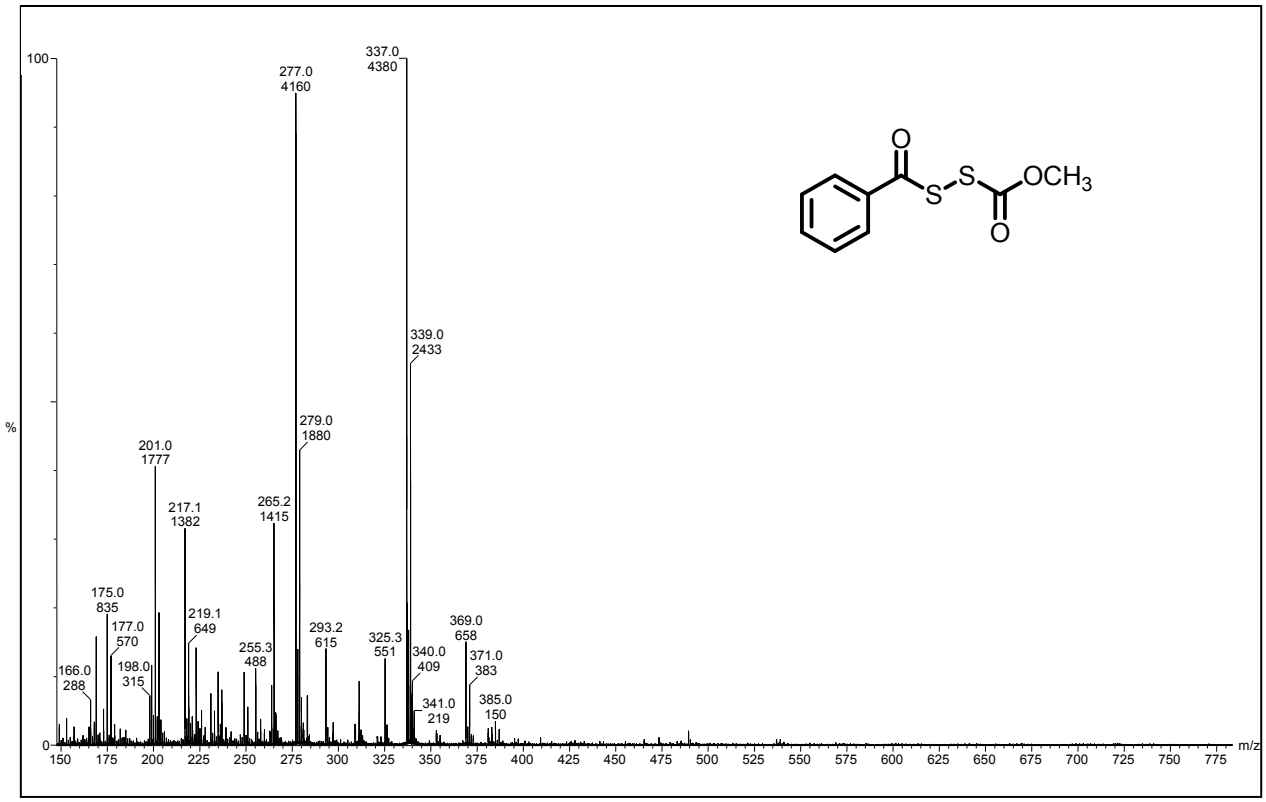
SI Figure 11: Emission spectra **ARS-Zn(II)** with GSH (1mM) and Cysteine (1 mM) and Interference of GSH (1mM) and Cysteine (1 mM) with H_2S in MeOH:PBS buffer (3/1,(v/v),10mM, pH = 7.4)



SI Figure 12: Plot of spiked H_2S in Human serum and BSA versus change in emission intensity at 620 nm.



SI Figure 13: Temperature dependant study of **ARS-Zn(II)** (60 μM) with H_2S donor (0.001 M, 10 equivalent) and glutathione (0.1 M, 100 equivalent) for 30 minutes at 30°C, 40°C and 50°C.



SI Figure 14: ESI-Mass spectrum of H_2S donor molecule benzoic (methyl carbonic) dithioperoxyanhydride.

[1] T. Roger, F. Raynaud, F. Bouillaud, C. Ransy, S. Simonet, C. Crespo, M.-P. Bourguignon, N. Villeneuve, J.-P. Vilaine, I. Artaud and E. Galardon, *ChemBioChem*, 2013, **14**, 2268-2271.