Supplementary Material

Alizarin Red S –Zinc(II) Fluorescent Ensemble For Selective Detection of Hydrogen Sulphide and Assay with \( \text{H}_2\text{S} \) donor

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Synthesis of \( \text{H}_2\text{S} \) Donor

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\begin{align*}
\text{CH_3COCl} + \text{C_6H_4SH} & \rightarrow \text{CH_3COOC_6H_4S} \\
\text{R.T., 2 hrs, N}_2 \text{ atm.}
\end{align*}
\]

\( \text{H}_2\text{S} \) donor compound benzoic (methyl carbonic) dithioperoxyanhydrate was synthesized by following reported procedure\(^1\). Accordingly in a 100 ml round bottom flask 300\( \mu \)l of methoxycarbonylsulfenyl chloride (3.2 mM) was dissolved in 20 ml of distilled diethylether, to this 324 \( \mu \)l of thiobenzoic acid (2.7 mM) was added drop wise at room temperature under stirring at \( \text{N}_2 \) 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\(_3\)): \( \delta \) (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 \( \text{Zn} \): 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 \( \mu \)l of stock solution was diluted to 2000 \( \mu \)L by adding 1878 \( \mu \)L buffer. The titration was done with different concentration of zinc perchlorate (0 – 30 \( \mu \)M) 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 H₂S:** 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 H₂S (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 H₂S:** 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 H₂S (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 H₂S (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 H₂S (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$_2$S 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$_2$S 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$_2$S 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$_2$S 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 $\lambda_{ex} = 543$ 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 $\lambda_{ex} = 543$ nm with 40X objective.

**C6 glial cells with ARS-Zn(II) and Na$_2$S**
C6 cell lines were incubated with 30 µM of ARS-Zn(II) and then treated with 44 µM of Na$_2$S for 20 minutes at 37°C. The confocal microscope Images were recorded at $\lambda_{ex} = 543$ nm with 40X objective.

**Calculation for the detection limit:**
The detection limit was calculated using formula:

$$\text{LOD} = \frac{(3 \times \text{SD})}{\text{slope}} = \frac{(3 \times 2.0)}{6.52273 \times 10^7} = 92 \text{ nM}$$

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$_2$S in MeOH-PBS buffer at RT.
**SI Figure 6:** Fluorescent titration of ARS-Zn(II) with H₂S (0-30μM) spiked human serum (a) ARS-Zn (blank), (b) blank + human serum without spiking H₂S, (c) 6 μM H₂S spiked, (d) 12 μM H₂S spiked (e) 18 μM H₂S spiked (f) 24 μM H₂S spiked (g) 30 μM H₂S spiked in Human Serum.
**SI Figure 7:** Fluorescent titration of ARS-Zn(II) with H$_2$S (0-30µM) spiked BSA  
(1) ARS-Zn (blank), (2) blank + BSA without spiking H$_2$S, (a) 6 µM H$_2$S spiked BSA, (b) 12 µM H$_2$S spiked BSA (c) 18 µM H$_2$S spiked BSA (d) 24 µM H$_2$S spiked BSA (e) 30 µM H$_2$S spiked in BSA.
SI Figure 8: (a) Blank lead acetate strip paper (b) 0.05 M Na$_2$S, (c) 0.005 M Na$_2$S, (d) 0.0005 M Na$_2$S, (e) 0.0005 M Na$_2$S. The black color shows the presence of H$_2$S species in the MeOH-PBS (3:1, v/v, pH 7.4).
**SI Figure 9:** Effect of methanol percentage in H$_2$S sensing of ARS-Zn(II) in Me-OH/PBS mixture.
**SI Figure 10:** Effect of different solvent in probe ARS-Zn(II) with of H₂S (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 H₂S in MeOH:PBS buffer (3/1,(v/v),10mM, pH = 7.4)
SI Figure 12: Plot of spiked $\text{H}_2\text{S}$ 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 H₂S 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$_2$S donor molecule benzoic (methyl carbonic) dithioperoxyanhydride.

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