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Hydrogen Sulfide Triggered Self-Immolative Fluorescent Probe for Lysosomes Labeling in Live Cells

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We developed a naphthalimide-based, lysosome-targeting, and self-immolative fluorescent probe for H_2S detection. Probe LS-1 comprises a 2-formylbenzoate derivative of the 1, 8-naphthalimide fluorophore. H_2S forms a thiohemiacetal intermediate by reacting with the formyl group of the 2-formylbenzoate derivative, which subsequently undergoes intramolecular cyclization with the ester moiety to generate a free naphthalimide fluorophore (FL-1).The UV-Vis absorbance (λabs) value of probe LS-1 at 450 nm increased in the presence of H_2S. Similarly, in the presence of increasing H_2S concentrations, the emission band (λem) centered at 560 nm increased gradually.The probe was found to be highly sensitive and chemoselective towards H_2S compared with other biological analytes. Probe LS-1 was nontoxic and very stable across the physiological pH range. The probe LS-1 enables to detect intracellular endogenous H_2S formation in HT29 cells in lysosomes.

H_2S is an important endogenous gasotransmitter and reactive sulphur species. It plays pivotal role in various physiological systems, such as the cardiovascular, gastrointestinal, immune, and nervous systems. [1-2] In addition, H_2S is crucial for blood pressure modulation and the reduction of ischemia reperfusion injury. [3-5] H_2S is produced endogenously from three key enzymes, namely cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase, in a controlled manner. [6] CBS and CSE are pyridoxal phosphate-dependent enzymes that produce H_2S from the substrates, L-cysteine and homocysteine. A recent study indicated that CBS is highly active in several cancer cells, including colon, [7] ovarian, [8] prostate, [9] and breast cancer cells, [10] and produces excess H_2S compared to neighbouring noncancerous cells or tissues. It has been reported that CBS-driven H_2S overproduction in cancer cells enhance cell proliferation, migration, and invasion. [7-8] Therefore, H_2S detection is critical for understanding cancer biology and could act as a biomarker for early cancer detection.

Although colorimetric, electrochemical analysis, and gas chromatography methods are available for H_2S detection; these methods encounter difficulties in maintaining accuracy and real-time imaging because of the short lifetime of H_2S in living cells.

Currently, fluorescent probes are used for the detection of particular analytes because of their high sensitivity, specificity, and remarkable temporal resolutions. [11-14] Mostly, azide [15-24] and nitro [25-28] functionality-based fluorescent probes have been developed for H_2S detection. However, the use of formylbenzoate derivatives as self-immolative fluorescent probes for H_2S detection is relatively unknown. [29-32] Tang et al. developed an ester linker-based probe for H_2S detection in the mitochondria. [33] A few lysosome-targeting fluorescent probes for H_2S have been reported; [34-37] however, none of them can detect endogenous H_2S without adding any stimulators. In the present study, we developed a self-immolative probe, **LS-1**, by using 2-carboxybenzaldehyde as the H_2S detection unit for lysosomes labelling in living cells. The lysosome is a cell organelle that contains hydrolytic enzymes. It engulfs various biomolecules in its acidic environment (pH ≤ 5). However, its proton pumping ability can occasionally be a concern during fluorescence labelling. Therefore, to overcome this problem, we used the 1, 8-naphthalimide fluorophore to construct probe **LS-1** because of its longer emission wavelength, high quantum yield, and two-photon property. [38-40] The probe **LS-1** was synthesized in a single step from **FL-1**; **FL-1** was synthesized according to the reported literature. [41] 2-carboxy benzaldehyde was reacted with oxalyl chloride in dichloromethane to form corresponding acid chloride as a reactive intermediate which was further reacted with **FL-1** in the presence of triethylamine to obtain a crude product. The crude product was subjected to column chromatography to afford probe **LS-1** as pale yellow solid. The detail of the synthetic procedure has provided in the experimental section. The spectroscopic evidence, such as mass, ^{1}H-NMR, ^{13}C-NMR, LCMS and HRMS are available in the supplementary information (Fig. S1-Fig. S4).

To know the sensitivity of **LS-1** toward H_2S (Na_2S), UV-Vis-absorption and fluorescence changes of **LS-1** in the presence of variable concentrations of H_2S (Na_2S) were monitored in HEPES buffer under an artificial physiological condition (pH 7.4; 37°C).

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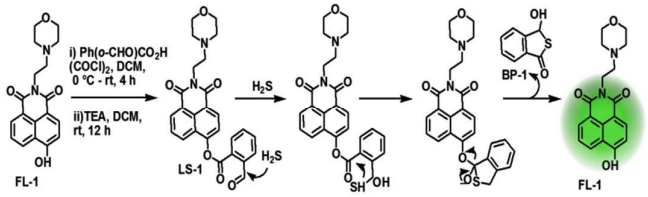

Scheme 1: Synthesis of **LS-1** and Reaction mechanism of **LS-1** with **Na_2S**.

It was observed that UV-Vis-absorption spectra (Fig. 1a) of probe **LS-1** has gradually increased with increasing concentrations of H_2S (Na_2S) at λ_{abs} 450 nm. Similarly, the fluorescence intensity at λ_{em} 560 nm (Fig. 1b) was also concomitantly increased upon addition of variable concentrations of Na_2S. Notably, the fluorescence intensity at 560 nm of **LS-1** has enhanced ~15 fold in the presence of Na_2S. By applying regression equation, the calculated detection limit toward H_2S found to be 24.0 x 10^{-9}M (Fig. S5). This result suggests that **LS-1** is highly sensitive toward H_2S and it may be capable of detecting of H_2S even at its low concentrations in the biological milieu (≤ 10 μM).[42-44]

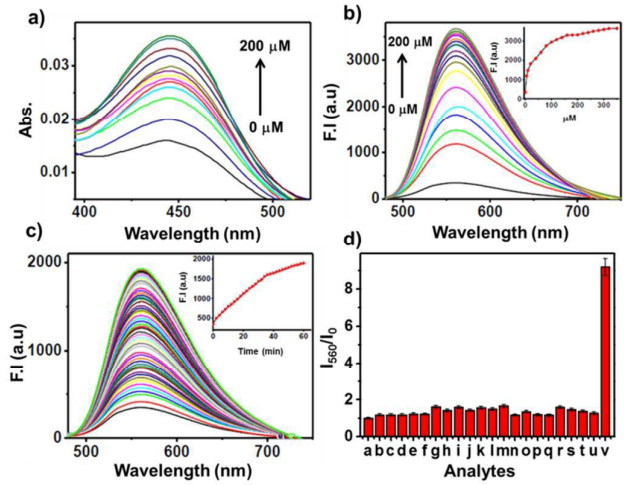

Fig.1. a) UV-Visible absorption spectrum and b) Concentration dependent fluorescence study of **LS-1**(5.0 μM) with various concentrations of Na_2S (0-200 μM) in HEPES buffer (pH -7.4, 1% DMSO).**LS-1** was incubated with Na_2S for 30 min at 37°C. c) Fluorescence response with time for the reactions of **LS-1** (5.0 μM) with 100μM Na_2S in the HEPES buffer (pH -7.4) with 1%DMSO. Spectra were acquired at 0 to 60 min after addition of Na_2S. Inset: Time vs intensity at 560 nm. d) Fluorescence responses of **LS-1** (5.0 μM) toward 200 μM a: HOCl, b: H_2O_2, c: $NaNO_2$, d: $Cu(OAc)_2$, e: $Zn(OAc)_2$, f:$Na_2S_2O_4$, g: $FeSO_4$, h:NO i: KCl j: $CaCl_2$ k: Na_2CO_3, l: GSH (1 mM), m: Ascorbic Acid (1 mM), n: Cysteine(1 mM), o: Folic Acid (1 mM), p: Histidine (1 mM), q: Lysine (1 mM),r: Trypsin (100 ng/mL), s: Lipase (100 ng/mL), t: Pepsin (100 ng/mL), u: Esterase (liver) (100 ng/mL) and v: Na_2S (200 μM). Excitation was effected at 450 nm and emission 560 nm with both slit width set at 5 nm.

To study the time-dependent reactivity of **LS-1**toward H_2S, we recorded fluorescence changes at λ_{em} 560 nm of **LS-1** in the HEPES buffer solution upon addition of Na_2S (20 eq). The results presented in Fig. 1c provided information that as the time increased fluorescence intensity also increased and it reached to the saturation within~40 min. From this data, the calculated rate of reaction between **LS-1** and H_2S was found to be 5.0 X 10^{-3} S^{-1}(Fig. S6). This observation further confirmed that **LS-1** is suitable for real-time monitoring of H_2S in cellular milieu.

Further, we checked chemoselectivity of **LS-1** toward H_2S over other competitive analytes. Thus, **LS-1** was incubated under the artificial physiological condition in buffer solution with other biological analytes such as HOCl, H_2O_2, $NaNO_2$, $Cu(OAc)_2$, $Zn(OAc)_2$, $Na_2S_2O_4$,$FeSO_4$, $FeCl_3$, KCl, $CaCl_2$, Na_2CO_3, GSH, ascorbic acid, cysteine, folic acid, histidine, lysine, trypsin, pepsin, lipase and esterase. From Fig. 1d, it is observed that **LS-1** didn't show any fluorescence change in the presence of various analytes. Also, there was no significant fluorescence change was seen even in the presence of esterase enzymes which are known for ester hydrolysis. This result firmly justifies our proposed mechanism of H_2S mediated formation of the fluorophore (**FL-1**) and confirmed the ability of probe **LS-1** for tracking H_2S in the biological system.

Next, we evaluated the stability and reactivity of probe **LS-1** in the absence and presence of Na_2S (200 μM) in biologically relevant pH scale (≥4.5 ≤ 8.5). Results shown in Fig. 2a suggested that **LS-1** is stable across the biologically relevant pH range (≥4.5 ≤ 8.5); whereas, upon addition of Na_2S to the **LS-1**, the fluorescence intensity increased with the increase of pH. We can conclude that the probe **LS-1** is very stable within the biologically relevant pH scale and remains reactive toward H_2S. This finding supports that probe **LS-1**could be a useful tool for tracking of H_2S formation in living cells.

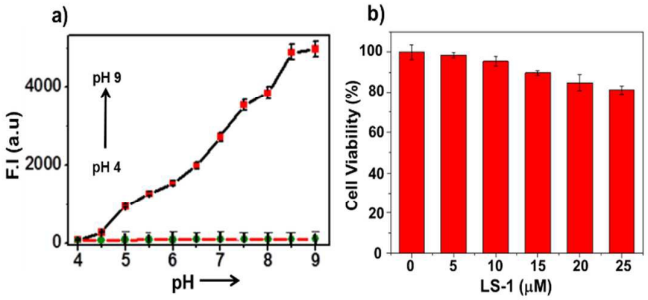

Fig.2. (a) Average fluorescence intensity changes of **LS-1** (5.0 μM) at λ_{em} 560 nm presence and absence of Na_2S (200 μM) at various pH (4-9) in HEPES buffer(1% DMSO) for 30 min at 37°C. Excitation was effected at λ_{abs} 450 nm with the excitation and emission slit widths both set at 5 nm. (b) MTT assay of MCF-7 cells incubated with **LS-1** (0–25μM) at 37 °C for 24 h.

Next, we established the mechanism for fluorescence-on in the presence of H_2S as proposed in Scheme 1. Na_2S treated **LS-1** solution in DMSO was subjected to MS analysis. MS value for **FL-1**(M+1 =327.146) as well as for **BP-1** (M+1 =167.018) (Scheme-1) have appeared in MS-spectra (Fig. S7). It indicates that H_2S has reactedwith the formyl group of the 2-formylbenzoate moiety in **LS-1** to form a hemithioacetal type intermediate; subsequently, it has gone through intramolecular nucleophilic displacement reaction to form **BP-1** as well as **FL-1**. This experimental evidence strongly supports that **LS-1** is a self-immolative probe to provide information on cellular H_2S formation through fluorescence imaging.

It is necessary to investigate cytotoxicity of probe **LS-1** before proceeding for cell imaging. Thus, we performed the cell viability assay of **LS-1** in MCF-7 cells. The MTT assay results in Fig. 2b indicated that **LS-1** is nontoxic and excellent biocompatible towards cells.

We then investigated whether probe **LS-1** can be used for detecting endogenous H_2S in live cells; thus, two different cell lines *e. g.* MCF-7 cells and HT-29 cells were incubated with **LS-1** (5 μM)

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for 1h. The images presented in Fig. 3d proved that our probe could track endogenous H_2S without adding any external inducers. In contrast, **LS-1** is failed to fluorescence label MCF-7 cells based on endogenous H_2S. However, Na_2S (H_2S) pretreated MCF-7 cells uponincubation with **LS-1** became fluorescent (Fig. S8). It indicates that endogenous H_2S formation in lysosomes of MCF-7 cells is relatively low. The previous report supports our observation that H_2S producing enzyme CBS is overexpressed in colon cancer cells (HT29).[45]

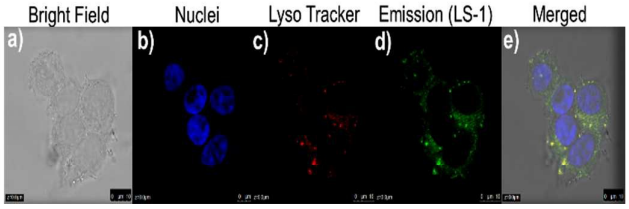

Fig.3. Confocal fluorescence images of HT-29 cells. a) Bright field image. b) Nuclei stained with DAPI ($\lambda_{ex}/\lambda_{em}$ =340/450 nm). c) Image of LysoTracker Red DND-99 ($\lambda_{ex}/\lambda_{em}$ =600/630 nm). d) Fluorescence image of probe **LS-1**($\lambda_{ex}/\lambda_{em}$ =450/540-600 nm). e) merged images of b)- d). The HT-29 cells were incubated with **LS-1** (5.0 µM) alone for 1h.Scale bar: 10 µm

To assess the lysosome targeting ability of **LS-1**, both HT-29 and MCF-7 cells were co-stained with lysosome targeting dye LysoTracker Red DND-99 and **LS-1**. The bright fluorescence image at the green channel (Fig.3d and Fig.4i) for **LS-1** and imaging of lysosomes by the LysoTracker Red DND-99 (Fig. 3c and Fig. 4h) at red channel were merged well (Fig. 3e and Fig. 4j). It confirmed that **LS-1** could especially target the lysosomes in living cells. Further Fig. 4d, confirms that the ester bond of **LS-1** is not cleaved by esterase enzymes since there is no fluorescence image was observed without addition of H_2S (Na_2S) to **LS-1** incubated MCF-7 cells. Also, the intensity profiles of linear regions of interest (Fig. 4C and Fig.4D) across HT-29 cells/MCF7 cells stained with **LS-1** and LysoTracker Red DND-99 also showed close coexistence. Altogether these findings indicate that probe **LS-1** could be specifically validated endogenous/exogenous H_2S formation in lysosomes.

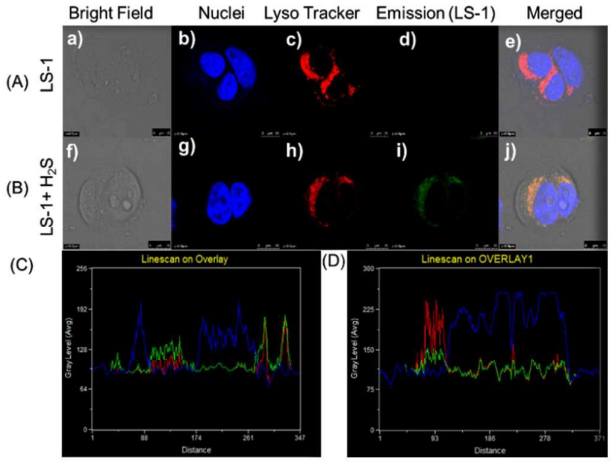

Fig. 4. Confocal fluorescence images of MCF-7 cells.(A) The cells were incubated with **LS-1** (5.0 µM) alone for 45 min. (B) Cells incubated with **LS-1** (5.0 µM) for 30 min and then treated with H_2S (150 µM) for 30 min. Images were taken at λ_{ex} = 450 nm.(C) Intensity profile of regions of interest (ROI) across HT-29 cells and (D) MCF-7 cells. Scale bar: 10 µm

Finally, we validated two-photon imaging ability of probe **LS-1**, which may overcome fluorescence interference from ubiquitous biological entities such as green fluorescence proteins.[46] **LS-1** (5.0 µM) pretreated HT-29 cells were imaged under two-photon laser scanner upon two-photon excitation at 900 nm. The results in Figure 5A, reveals that there was no autofluorescence due to cellular riboflavin and flavoproteins as cells were nonfluorescent without **LS-1**; whereas the **LS-1** treated HT29 cells (Fig. 5B) were strongly fluorescence labeled. This finding confirms that **LS-1** can provide two-photon images without interference from omnipresent biological entities, riboflavin, and flavoproteins.

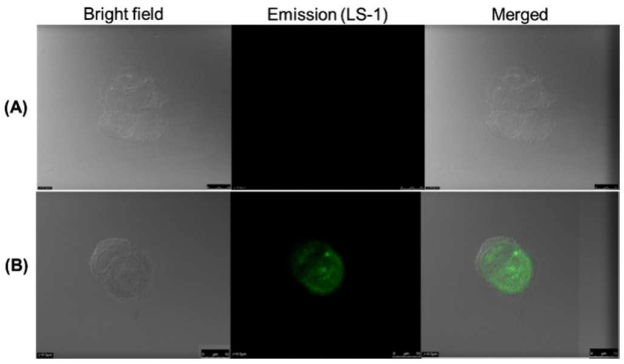

Fig.5. Two-photon microscopy images of HT-29 cells; A) without **LS-1** and **B)** with **LS-1**, cells were incubated with the **LS-1** (5.0 µM) for 1h.Images was acquired using 900 nm as excitation wavelength and fluorescence was collected 540-600 nm.Scale bar: 10 µm

Conclusions

We incorporated α-formylbenzoate functionality in a naphthalimide fluorophore to develop a self-immolative fluorescent probe (**LS-1**) for H_2S detection in the lysosomes. Various spectroscopic studies were conducted to confirm the chemical structure of probe **LS-1**. The UV-Vis absorbance values and fluorescence intensity of probe **LS-1** increased concomitantly on H_2S exposure in HEPES-buffered saline under an artificial physiological condition at 37°C. Probe **LS-1** had approximately 15-fold enhanced fluorescence intensity at 560 nm on exposure to 200 µM of H_2S. Probe **LS-1** enabled H_2S detection at concentrations as low as 24.0 × 10^{-9} M. It reacted with H_2S at a considerably high rate of 5.0 × 10^{-3}s-^{1}. The probe was found to be highly chemoselective towards H_2S, and it is highly stable against a broad pH range. High MCF-7cell viability indicated that probe **LS-1** could be a useful tool for H_2S detection in the cellular milieu. Despite the acidic environment in the lysosomes, low concentration of probe **LS-1** (3.0–5.0 µM) was used to detect H_2S and provided highly bright fluorescence images. Further, the cell imaging experiment revealed that **LS-1** could be used to identify endogenous H_2S formation in lysosomes. Finally, its two-photon imaging ability encourages being a useful tool in clinical diagnosis without interference from fluorescent biological entities.

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