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Polysulfide-Triggered Fluorescent Indicator Suitable for Super-Resolution Microscopy and Application in Imaging

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A new physiologically benign and cell membrane permeable BODIPY based molecular probe, MB-S_n specifically senses intracellular hydrogen polysulfides (H_2S_n, n > 1) localized in the endoplasmic reticulum. This reagent is suitable for mapping the intracellular distribution of H_2S_n by wide-field as well as super-resolution Structured Illumination Microscopy (SIM).

Hydrogen polysulfide H_2S_n is one of the reactive sulphur species (RSS) and is primarily produced in cells from 3-mercaptopyruvate by the enzyme 3-mercaptopyruvate sulfurtransferase.[1] It is also produced by the reaction of H_2S with NO.[1] H_2S_n influences many important biological functions and activities associated with H_2S. For example, it induces sulfuration, wherein a sulphur atom is added to cysteine thiol groups within proteins, causing changes in conformation and the activity of the affected protein.[2] By activating transient receptor potential (TRP) A1 channels, H_2S_n induces Ca^{2+} influx in astrocytes more efficiently than H_2S.[3,4a] It is also a potent signaling molecule that regulates the activity of tumor suppressors and transcription factors.[3]

To develop a deeper understanding of the regulatory roles of H_2S_n, it is crucial to identify appropriate reagent and methodology for efficient detection and tracking of H_2S_n within cells. Traditional spectroscopic methods for the detection of H_2S_n rely on measuring characteristic absorbances at 290 - 300 nm and 370 nm[5]- a methodology of limited application due to its low sensitivity. Another methodology using mass spectroscopic technique requires derivatization of the sample with monobromobimane to produce chemically unstable derivatives that often lead to inaccurate results.[3] Moreover, none of these methods are suitable for studying in-vivo biological processes or mapping the intracellular distribution of H_2S_n. Thus, there is clear scope to develop an efficient methodology for detection of H_2S_n using a fluorescence-based molecular probe.[4c] There are several literature reports for detection of other biothiols such as cysteine, glutathione, and H_2S using this approach.[6] However, such reports are scarce for H_2S_n due to the lack of proper understanding about its reactivity. Recent literature reports reveal that most polysulfide probes rely on a nucleophilic substitution reaction involving the polysulfide moiety and a 2-fluoro-5-nitro benzoate derivative, while the transient species undergo spontaneous cyclization to release the fluorophore in its "on state".[7] However, such probes also participate in nucleophilic substitution reaction with other biothiols, and this limits their application as a specific reagent for H_2S_n. Another approach involves the use of H_2S_n induced aziridine ring opening reactions.[8] Such probes show good selectivity for H_2S_n, however, their utility is largely limited to cell-free studies. Barring some recent reports from Xian and co-workers, H_2S_n specific probes are still very sparse in the literature.[9] However; there is no report on an organelle specific H_2S_n probe that is suitable for Structured Illumination Microscopy (SIM).[10a,b]

Fluorescence microscopy has proved to be an invaluable tool for imaging studies as well as for studying the functions of specific analytes in living systems.[4b,c] In many instances, the applicability of this technique is limited by its relatively poor spatial resolution.[10a] However, recent advances in super-resolution microscopy (SRM) have broken the diffraction limit of conventional optical-based techniques providing hitherto inaccessible imaging capabilities.[10b,d] One such SRM technique is SIM, which provides an appreciable increase in resolutions (100 - 120 nm)[10a,b] with minimal disruption of data acquisition rate. SIM requires considerably lower illumination intensities compared to other super-resolution microscopies like STORM and STED,[10c,d] making it particularly suited to live cell imaging.

With these facts in mind, we have developed a BODIPY based probe **MB-S_n** that could exclusively detect H_2S_n in physiological conditions as well as it could be used for mapping endogenous H_2S_n localized in the endoplasmic reticulum of cells. Importantly, the probe is also compatible for SIM studies.

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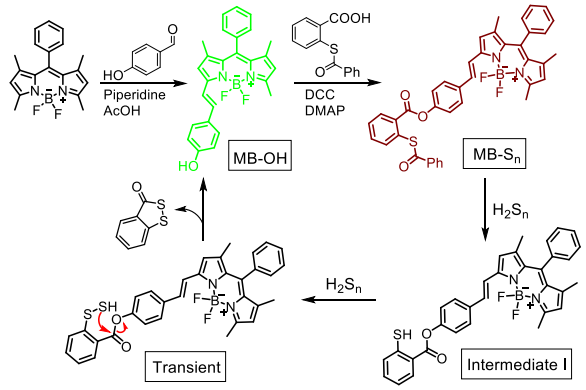
We chose difluoroborondipyrromethene (commonly known as BODIPY), as a fluorescent reporter functionality owing to its excellent photophysical properties such as high molar absorptivity, tuneable emission, excellent photostability, narrow emission bandwidth and high quantum yield.[6c,10d,11] The dual-reactivity of H_2S_n was exploited in designing this molecular probe using a BODIPY moiety functionalized with benzothioester as the polysulfide recognition site. Detailed synthetic procedures and all relevant characterization data for the molecular probe and the intermediates are provided in the supporting information (Fig. S14-17). Esterification of the phenolic functionality of **MB-OH** was achieved by reacting with thiobenzoate in the presence of DCC to yield the final chemodosimetric reagent **MB-S$_n$**. The extended conjugation of one the pyrrole arm (Scheme 1) helped in achieving shifts in the absorption and emission bands maxima toward the red region of the spectrum.



Scheme 1. The synthetic route towards **MB-S$_n$** and its reaction with H_2S_n.

With the probe in hand, we first checked its absorption and emission response in 20 mM phosphate buffer medium, pH 7.4 (9:1 Phosphate buffer: CH_3CN) containing 50 µM CTAB. Na_2S_2 is known to be highly unstable and readily decomposes in the buffer. CTAB is used to ensure the stability of Na_2S_2 for our studies.[8,9a] Absorption spectra of **MB-S$_n$** showed a band in the red region with a maximum ~570 nm (Fig. S2A & S3). Earlier reports revealed that modulation of the hydroxyl group of styryl BODIPYs results in significant quenching of the BODIPY-based fluorescence through an efficient photo-induced electron transfer.[6c,10e] Accordingly, **MB-S$_n$** is expected to be poorly fluorescent. Steady state emission studies confirmed this assumption and revealed a poor emission quantum yield ($\Phi_f^{MB\text{-}Sn}$ = 0.007, Rhodamine B is used as reference).Upon addition of Na_2S_2 (a H_2S_n donor), a significant increase in the fluorescence intensity (Figure 1A) is observed accompanied by a slight shift in the emission maximum to the longer wavelength (λ_{Max}^{Ems} ~ 584 nm). The new emission is characteristic of **MB-OH** (Fig. S2B) indicating the release of the free probe, **MB-OH** upon reaction of **MB-S$_n$** with H_2S_n. Fluorescence intensities gradually increased upon concomitant increase in the concentration of Na_2S_2 (0-20 µM, Figure 1A), resultant from a remarkable elevation in quantum yield to Φ_f = 0.125. Time-dependent luminescence studies reveal that the maximum emission intensity is attained within 10 minutes of the initial mixing time, signifying the fast response of the probe to H_2S_n (Fig. S4). Fluorescence intensity was found to increase linearly with the concentration of Na_2S_2 (0 - 10 µM) and the detection limit of the probe is evaluated as 26.01 nM following 3σ method (Fig. S7). This confirms the high sensitivity of the chemodosimetric probe **MB-S$_n$** towards H_2S_n.

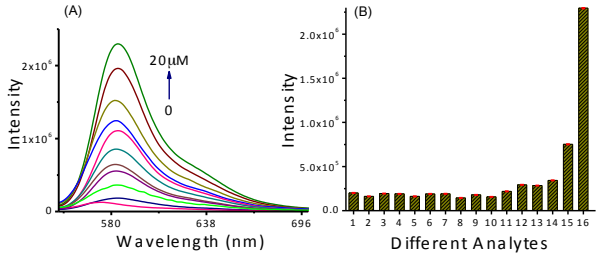


Figure 1. (A) Emission response of **MB-S$_n$** (10 µM) in the presence of varying concentration of Na_2S_2 (0-20 µM). (B) Emission response of **MB-S$_n$** (10 µM) in the presence of various analytes (1) **MB-S$_n$** only, (2) H_2O_2 (200 µM), (3) HOCl (50 µM), (4) $^{\cdot}OH$ (200 µM H_2O_2 + 50 µM Fe^{II}), (5) $^1O_2$ (200 µM H_2O_2 + 50 µM OCl^-), (6) O_2^- (50 µM), (7) NO_2^- (50 µM), (8) NO_3^- (50 µM), (9) Cys/ Hcy (100 µM), (10) GSH, (100 µM), (11) Na_2S, (100 µM), (12) $^{\cdot}OH$ + Na_2S (50 µM + 100 µM), (13) $^1O_2$ + Na_2S (50 µM + 100 µM), (14) H_2O_2+ Na_2S (200 µM + 100 µM), (15) HOCl + Na_2S (50 µM + 100 µM), (16) Na_2S_2 (20 µM). Excitation-530 nm and emission was monitored at 584 nm. Each spectrum was recorded after 10 minutes of incubation with **MB-S$_n$**.

Generation of **MB-OH** from **MB-S$_n$**, along with the turn-ON emission response, is attributed to the cleavage of the thioester group following a nucleophilic attack by H_2S_n. The resulting thiolate (Scheme 1) functionality reacts with another H_2S_n to generate a transient species that undergoes a spontaneous cyclization reaction to release benzodithiolone and **MB-OH** (Scheme 1). To confirm this, we have carried out a control reaction between **MB-S$_n$** and Na_2S_2, which reveals a distinct change in solution colour from pale pink to purple (Fig. S1). Analytical and spectroscopic (^{1}H NMR and HRMS) data for the isolated product confirmed the formation of **MB-OH** (Scheme 1 & Fig. S20, S21).

In order to evaluate the specificity of this reagent towards H_2S_n, the emission response on formation of **MB-OH** produced *in situ* is compared with the emission response of **MB-S$_n$** towards various reactive oxygen species (ROS) (e.g. •OH, H_2O_2, $^1O_2$, O_2^-, HOCl), anionic analytes (e.g. NO_2^-, NO_3^-, SO_4^{2-}, SO_3^-, $S_2O_3^-$) and biothiols like H_2S, cysteine (Cys)/ homocysteine (Hcy), Glutathione (GSH) (Figure 1B). Figure 1B shows that the chemodosimetric probe shows excellent selectivity towards H_2S_n as all other analytes failed to induce any detectable change in the observed fluorescence.

Literature reports also reveal that H_2S_n can be generated *in situ* from the reaction of H_2S with HOCl.[3,12] To verify this fact, a solution containing different ROS was added to an aqueous solution of H_2S (100 µM), and subsequently, emission spectra were recorded in the presence of **MB-S$_n$**. A significant enhancement in fluorescence intensity at ~ 584 nm was observed for a solution containing HOCl as ROS. This confirms the *in situ* generation of H_2S_n by the reaction of HOCl with H_2S (Figure 1B). The effect of pH was also examined, the results indicate that H_2S_n showed higher reactivity with **MB-S$_n$** at

physiological pH whereas its reactivity is decreased both under acidic as well as basic conditions (Fig. S5 & S6).

In general, thioester groups are known to react with the biothiols,[13] and one would expect biothiols like Cys, Hcy and GSH to compete and interfere with the H_2S_n detection process. These biothiols are present in a relatively higher concentration in human blood plasma, and a reaction with such biothiols would lead to the undesired consumption of **MB-S$_n$**. Figure 1B also confirms that biothiols fail to interfere with the detection and estimation of H_2S_n, even when present at 10 fold higher concentrations than H_2S_n. It may be argued that biothiols (Cys, Hcy, GSH) could react with **MB-S$_n$** to yield the intermediate 1 (see scheme 1) and fail to yield **MB-OH** eventually. To nullify such a possibility, we have evaluated the emission response of **MB-S$_n$** (10 μM) with higher concentrations of Cys and GSH (1 mM each) in the presence or absence of S_8 (50 μM), as reaction intermediate 1 should further react with elemental sulphur (S_8) to yield **MB-OH** with an associated fluorescence ON response. Gratifyingly, negligible change in the emission was observed (Fig. S8) when the reaction is carried out in presence and absence of S_8. This further, confirms the specificity of the chemodosimetric probe towards H_2S_n even in the presence of competing biothiols.

After ensuring the specificity and high sensitivity of **MB-S$_n$** towards H_2S_n, the efficacy of this probe molecule as an imaging reagent for mapping the intracellular H_2S_n in living cells was examined.

The cytotoxicity of **MB-S$_n$** towards RAW 264.7 macrophages, evaluated using a conventional MTT assay, revealed negligible toxicity, even when present at higher doses (Fig. S9). Next, widefield fluorescence microscopy experiments were performed to check whether **MB-S$_n$** could be used for visualizing exogenous H_2S_n in the RAW 264.7 macrophages. Cells are first incubated with **MB-S$_n$** (10 μM) for 25 minutes, and no significant fluorescence was observed from these cells (Fig. S10). Then, these cells were further incubated with different concentrations of Na_2S_2 (2.5, 5 and 12.5 μM, respectively) and widefield microscopic images were recorded. While a bright intracellular fluorescence is observed and this indicates that **MB-S$_n$** is cell permeable and capable of detecting H_2S_n inside the living cells (Fig. S10). This result motivated us to explore the possibility of using **MB-S$_n$** in super-resolution imaging, particularly, SIM. SIM experiments with **MB-S$_n$** for cells treated with Na_2S_2 produce clear-cut images (Figure 2). Interestingly, the emission intensities from the cells found to vary linearly as a function of [H_2S_n]. This signifies that **MB-S$_n$** is capable of monitoring the relative change in H_2S_n levels inside the RAW 264.7 macrophages (Figure 2). These studies also confirm that **MB-S$_n$** is stable enough to withstand the relatively prolonged laser irradiation needed for SIM studies and suggest that **MB-S$_n$** has the potential for being used as an efficient chemodosimetric probe for super-resolution microscopy.

Intracellular luminescence due to the *in-situ* generation of **MB-OH** on the reaction of **MB-S$_n$** and H_2S_n as a function of exposure time is also examined. Results reveal that bright intracellular fluorescence is observed within 2 minutes after incubating the pre-treated cells with Na_2S_2 (Fig. S13) and this further indicates the fast response of the chemodosimetric probe.

We then explored whether **MB-S$_n$** could be used to map endogenous H_2S_n. Literature reports have demonstrated that over-expression of cystathionine γ-lyase (CSE) in cells leads to significant elevation in polysulfide levels,[4a] accordingly, we stimulated the cells with lipopolysaccharides (LPS) to induce the over-expression of CSE. Living cells are incubated with different concentration of LPS (0 - 2.5 μg/mL) and maintained for 16 hours in DMEM culture medium with 10% FBS, and then these pre-treated cells are further incubated with **MB-S$_n$** (10 μM) for 25 minutes.

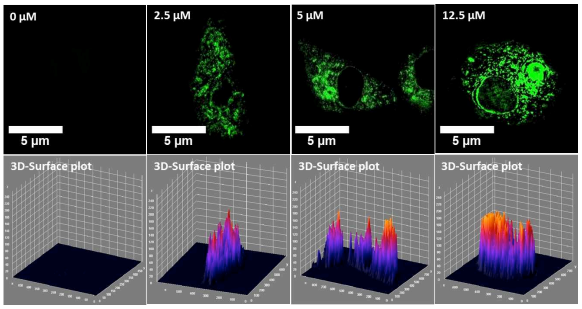


Figure 2. SIM images of exogenous H_2S_n in RAW 264.7 macrophages. Cells were incubated with **MB-S$_n$** (10 μM) for 25 minutes and then incubated with different Na_2S_2 concentrations for 20 minutes. Bottom row- corresponding 3D profile plots.

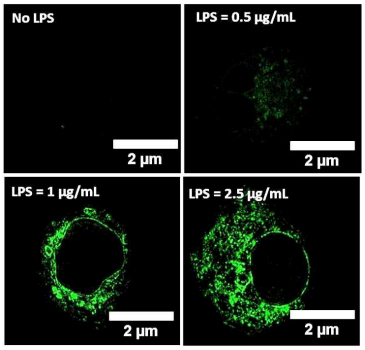


Figure 3. Endogenous H_2S_n detection by SIM. RAW 264.7 cells were treated with different concentration of LPS for 16 hours followed by incubation with **MB-S$_n$** (10 μM) for 25 minutes.

SIM images show bright intracellular fluorescence, and this confirms the generation of polysulfides and the efficacy of **MB-S$_n$** in detecting endogenous H_2S_n (Figure 3). Figures 2 and 3 also reveal that **MB-S$_n$** is not localized in the nucleus. To ascertain its exact localization, co-staining experiments are carried out. Results of the microscopic studies reveal a wide distribution of the probe in the endoplasmic reticulum of RAW 264.7 cells. Co-staining experiment with the commercial ER tracker green probe was also performed. Further analysis helps us to evaluate Pearson's correlation coefficient of 0.944 confirming the precise localization of **MB-S$_n$** within the ER of the cells (Figure 4). We have further utilized **MB-S$_n$** for 3D-SIM imaging - see figure 4 and video provided in the ESI.

As multicolour imaging is a powerful tool for simultaneous monitoring of various cellular processes in different cell

organelles.[14] We have explored the possibility of using **MB-S$_n$** as a probe molecule for dual colour imaging.

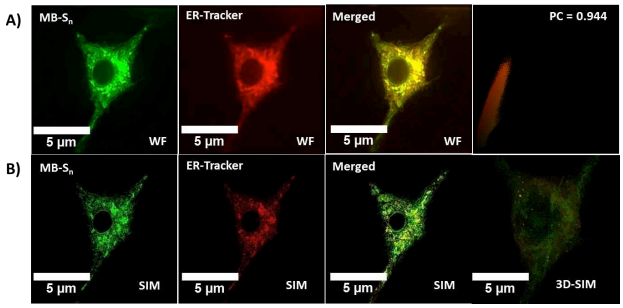


Figure 4. Colocalization experiments (Cells were pre-treated with LPS, 2.5 µg/mL before **MB-S$_n$** and ER-Tracker incubation): (A) Wide-field (WF) images of **MB-S$_n$**, ER-tracker green, merged image and Pearson's profile plot indicating maximum colocalization (0.944), confirming the preferential colocalization in the Endoplasmic reticulum. (B) Corresponding SIM images of **MB-S$_n$**, ER-tracker green, merged image and 3D-SIM image.

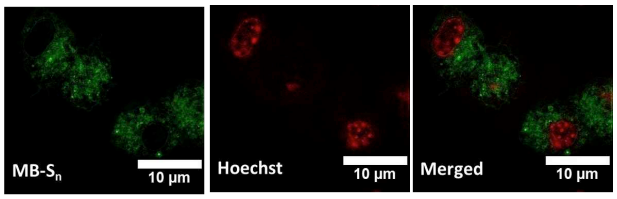


Figure 5. Dual colour SIM using **MB-S$_n$** in the presence of LPS and Hoechst-33342

To realize this, we performed both widefield (Fig. S12) and SIM imaging (Figure 5) with a nuclear staining dye Hoechst-33342 in LPS treated cells. Images shown in Figure 5, confirm the usefulness of the probe molecule for dual-colour imaging.

In summary, we have designed a BODIPY based chemodosimetric probe **MB-S$_n$** that specifically detects H_2S_n in physiological condition. H_2S_n mediated thioester cleavage followed by spontaneous cyclization led to the generation of free **MB-OH** with a turn-ON emission response. Its non-toxic nature and cell membrane permeability allowed us to utilize it for imaging exogenous and endogenous H_2S_n in living cells. Co-localization studies with ER tracker green confirmed the precise localization of the probe in the ER region of cells. We also demonstrated the utility of the probe in SIM, 3D-SIM, and dual-colour imaging. We believe this method will open up a new window for developing SIM compatible probes for other biologically important analytes.

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