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Imaging in living cells and zebrafishes in vivo using a ratiometric fluorescent probe for hydrogen sulfide

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We have developed a novel colorimetric and ratiometric fluorescence probe for selective and sensitive monitoring hydrogen sulfide based on dicyanoisophorone platform. An excellent linear relationship of fluorescence intensity ratio (Ioff/Ion) (R² = 0.9867) versus hydrogen sulfide concentration in the range of 1–12 μM was also obtained. This probe exhibited a remarkably fluorescence response to hydrogen sulfide over other physiological thiols or biological species, which fluoresces in the red region with a large Stokes shift (172 nm). This probe was successfully utilized to monitor H2S in in vitro physiological conditions and imaging H2S in living cells and living zebrafishes in vivo.

Hydrogen sulfide as an important endogenous gasotransmitter following nitric oxide (NO) and carbon monoxide (CO), plays important roles in a number of biological processes. It has been established to regulate the intracellular redox status and mediate fundamental biological and physiological processes, including anti-oxidation, anti-inflammation, anti-apoptosis, intervention of neurotransmission, regulation of vascular pressure etc. In mammals, the production of endogenous hydrogen sulfide is mediated by the enzymatic process of cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), as well as the coordinated action of cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3-MST). Abnormal levels of hydrogen sulfide correlate with many diseases, such as Alzheimer’s disease, Down syndrome, and liver cirrhosis. Therefore, detection approach of hydrogen sulfide in a selective and sensitive manner in living biological systems is important for better understanding its biological and physiological functions.

Fluorescence imaging has attracted much attention as its excellent sensitivity, selectivity, and the capability of biomolecules detection in live biological specimens. In order to meet the urgent need of selective visualization tools for the detection of hydrogen sulfide in live biosystems, the development of fluorescent probes for hydrogen sulfide has recently made rapid advances. Early work in this area from our laboratory as well as from Xian et al. developed a serial of fluorescent turn-on probes based on dual nucleophilic reaction etc. Nagano and Zeng et al. have reported copper sulfide precipitation strategies to remove quencher from fluorescent probes for hydrogen sulfide detection.

Chang and Wang et al. exploited the selective fluorescent turn-on probes based on the hydrogen sulfide induced specific azide reduction, this approach has recently been widely adopted. These fluorescent probes have been successfully utilized for detecting exogenous or endogenous hydrogen sulfide in living cells, blood and tissue samples, and in vitro enzyme assays, and most of them display a response time from 20 min to 2 hours. However, many of these fluorescent probes need much organic cosolvent (≥10%, v/v) and short-wavelength excitation etc. Moreover, most of these probes were based on fluorescence off–on response mechanism, which is easily influenced by probe concentration, microenvironments, photobleaching etc. As a result, their biological application in the complicated living systems is limited as the strong absorption and autofluorescence of biomolecules background would lead to low signal-to-noise ratio.

We are now interested in designing and developing fluorescent probes to detect and image hydrogen sulfide in living cells and in small animal model in vivo, based on ratiometric measurements rather than simple fluorescence intensity–based off-on processes. There is increasing interest in developing ratiometric probes for hydrogen sulfide owing to their self-calibration effect which will reduce most of the aforementioned interferences. Recently, a few of ratiometric fluorescent probes for hydrogen sulfide had been reported. Nevertheless, some of these ratiometric probe needed pure organic solvent to work, or the excitation and emission wavelength of the probe were relatively short for biological application, or the low fluorescence quantum yield and poor stability would limit their application.

Thus, ratiometric fluorescent probes with good solubility, large Stokes shift, longer excitation wavelength, and high quantum yield are still urgently needed for hydrogen sulfide imaging in living cells and in vivo.

Herein, we report a novel ratiometric fluorescent probe (SFP-GR) based on dicyanoisophorone electron-accepting group for imaging hydrogen sulfide in living biosystems. In this probe, a reactive azide group was introduced into dicyanoisophorone electron-accepting group. Upon reduction of hydrogen sulfide, we anticipate that the electron-withdrawing azide group will be converted into electron-donating amine group resulting in red shift in emission, which may provide a ratiometric detection of hydrogen sulfide. As expected, a selective and sensitive fluorescent probe for hydrogen sulfide has been developed. This probe exhibits colorimetric and ratiometric fluorescence response to hydrogen sulfide over other physiological thiols or biological species, which fluoresces in the red region with a large Stokes shift (172 nm, Fig. S1). We successfully used this fluorescent probe to visualize hydrogen sulfide in living HeLa cells and in small model animal zebrafishes.

The preparation of fluorescent probe (SFP-GR) and the possible reaction mechanism of SFP-GR toward hydrogen sulfide were shown in Scheme 1. We synthesized SFP-GR conveniently by three simple steps. The detailed synthetic steps and
characterization of SFP-GR were described in the electronic supplementary information (ESI).

Scheme 1. Design and synthesis of fluorescent probe (SFP-GR) for H$_2$S detection.

With this fluorescent probe in hand, we firstly examined the spectral properties and reactivities of SFP-GR (5 µM ($\Phi = 0.47$)) with Na$_2$S (20 µM) as an aqueous sulfide source in HEPES buffer (20 mM, pH 7.4, 0.5% DMSO) with 1 mM CTAB (Fig. 1 and Fig. S2). The free SFP-GR displayed one major absorption peak at around 413 nm. Upon addition of hydrogen sulfide, the azide group of probe was reduced to amine. Consequently, the maximum absorption peak exhibited a 64 nm red shift to 477 nm and the color of the solution changed from colorless to tan (Fig. 1 inset). So SFP-GR could serve as a visual probe for hydrogen sulfide, which would allow the colorimetric detection of hydrogen sulfide directly by naked-eyes.

We next elaborated the fluorescence emission spectra. In the absence of hydrogen sulfide, SFP-GR only exhibited a weak emission peak at around 558 nm. Interestingly, SFP-GR showed a fast and significant increase of the fluorescence intensity at 637 nm when excited at 466 nm after addition of hydrogen sulfide, which was completed within 5 min (See ESI, Fig. S3). With this large red shift (79 nm) in emission, the color changed from colorless to red under irradiation with a UV (365 nm) lamp. We also monitored the change of this chemical reaction before and after addition of hydrogen sulfide in SFP-GR solution by Liquid chromatography-mass spectrometry (HPLC-MS), and the results revealed that the reaction of SFP-GR with hydrogen sulfide would reduce the azide group of probe to an amine group (see ESI, Fig. S4). The pH effect studies suggested that the significant fluorescent signals could be observed between pH 7.0 and 10.0, indicating the good suitability of SFP-GR under physiological conditions (see ESI, Fig. S5).

To evaluate the sensitivity of SFP-GR for hydrogen sulfide, we then investigated the change of fluorescence spectral via varying concentrations of Na$_2$S (1–40 µM) under simulated physiological conditions (Fig. 2a). With the continuous increasing concentration of sulfide in SFP-GR (5 µM) solution, the fluorescence intensity decreased at 558 nm and gradually increased at around 637 nm (excitation wavelength at 466 nm), resulting in the fluorescence intensity ratio ($I_{637}/I_{558}$) increased from 0.72 to 10.6. Moreover, an excellent linear relationship of fluorescence intensity ratio ($I_{637}/I_{558}$) ($R^2 = 0.9867$) or emission intensity at 637 nm ($R^2 = 0.9977$) versus hydrogen sulfide concentration in the range of 1–12 µM was also obtained (Fig. 2b and Fig. S6). The detection limit of SFP-GR for hydrogen sulfide was 77 nM (see ESI). These results demonstrated that SFP-GR not only could monitor hydrogen sulfide selectively but also provided a sensitive and quantitative detection method for hydrogen sulfide based on its excellent linear relationship.

To examine the selectivity of SFP-GR, we subsequently treated various biologically relevant species with SFP-GR, including various anions, metal ions, amino acids, and thiols. As exhibited in Figure 3, SFP-GR showed high selectivity for hydrogen sulfide over other competing analytes. Specially, no obvious change in fluorescence signal was detected in the presence of these biological relevant thiols such as glutathione (1 mM), cysteine (1 mM), and homocysteine (10 mM) (Fig. 3 and Fig. S7). As comparison, upon the addition of Na$_2$S (20 µM) with the probe, a large fluorescence signal (13-fold) enhancement at 637 nm and a notable change of fluorescence intensity ratio ($I_{637}/I_{558}$) (10.6) was observed (Fig. S7). To further demonstrate the ability to monitor hydrogen sulfide in the presence of other competitive biological thiols, the anti-interference of SFP-GR was also investigated (Fig. 3b and Fig. S8). As their physiological concentrations of thiols were around millimolar (0.1-0.25 mM Cys, 5-10 mM GSH, and 0.010-0.012 mM Hcy), therefore, the selectivity of SFP-GR would be checked at 0.2 mM Cys, 10 mM GSH, and 0.01 mM Hcy. As shown in Figure 3b, the fluorescence emission enhancement induced by other thiols is very limited comparing to that induced by hydrogen sulfide. Additionally, exposing the probe to a mixture of other thiols and Na$_2$S still yield a significant fluorescence signal increase. The above results indicated that SFP-GR is able to detect hydrogen sulfide without any distinct interference from other biological thiols. Taken together, the in vitro selectivity experiments demonstrated that this selective fluorescent probe SFP-GR can be used for...
fluorescence detection of hydrogen sulfide at physiological conditions.

Fig. 3. Fluorescence response of SFP,GR (5 µM) to various biologically relevant species in HEPES buffer (20 mM, pH 7.4, 0.5% DMSO) with 1 mM CTAB. (a) Control (probe alone); AcO− (200 µM); Cl− (200 µM); CO3^2− (200 µM); F− (200 µM); HCO3− (200 µM); HSO3− (200 µM); NO2− (200 µM); NO− (200 µM); SCN− (200 µM); SO2− (200 µM); Al3+ (1 mM); Ca2+ (1 mM); K+ (1 mM); Mg2+ (1 mM); Na+ (1 mM); Cys (1 mM); GSH (1 mM); Lys (1 mM); Pro (1 mM); Hcy (10 mM); Na2S (20 µM). Excitation: 466 nm; emission: 637 nm. (b) Gray bars correspond to free SFP,GR or with Na2S (20 µM) or other biological thiols (0.2 mM Cys, 10 mM GSH, 0.01 mM Hcy); Purple bars correspond to SFP,GR in the presence of both Na2S (20 µM) and the other biological thiols (0.2 mM Cys, 10 mM GSH, 0.01 mM Hcy).

Next, we investigated the potential application of SFP,GR in living biosystems. We tested the ability of SFP,GR to detect hydrogen sulfide in live HeLa cells using confocal microscopy imaging (Fig. 4). Cells were incubated with 10 µM SFP,GR for 30 min, and then a green fluorescence was observed, suggesting the good cell permeability of SFP,GR. Interestingly, HeLa cells were treated with 20 µM Na2S for an additional 30 min after cells were incubated with 10 µM SFP,GR for 30 min at 37 °C led to an obviously increase in red channel fluorescence. Distinct changes of ratiometric fluorescence responses in living cells were observed (Fig. 4d and h), these results indicated that this novel developed SFP,GR indeed could be used for the ratiometric fluorescence imaging hydrogen sulfide in living cells. Furthermore, we also performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays in HeLa cells to evaluate cytotoxicity of SFP,GR (10 - 50 µM). The results were further indicated that SFP,GR was of low toxicity or non-toxic to cultured cells under the experimental conditions at the concentration of 50 µM for 24 hours (Fig. S9).

Fig. 4. Confocal microscopy images of H2S detection in live HeLa cells using SFP,GR. (a-d) HeLa cells incubated with SFP,GR (10 µM) for 30 min at 37 °C; (e-h) HeLa cells after treatment with SFP,GR (10 µM) for 30 min and subsequent treatment of the cells with Na2S (20 µM) for another 30 min at 37 °C. (a, e) Bright-field images; (b, f) Green channel images were collected in the range of 520-560 nm; (c, g) Red channel images were collected in the range of 620-660 nm; (d, h) Ratio images of generated from (b) and (c), (f) and (g), respectively. Excitation at 488 nm. Scale bars represent 20 µm.

In summary, we have rationally designed and synthesized a novel fluorescent probe SFP,GR of high selectivity and sensitivity for hydrogen sulfide. This colorimetric and ratiometric probe can be used for monitoring hydrogen sulfide in physiological conditions in vitro and could also successfully be applied for imaging hydrogen sulfide in living cells and in zebrafishes in vivo. Current efforts are focused on developing real-time in situ fluorescent probes for imaging hydrogen sulfide in living cells, tissues, living animals as well as the application of these fluorescent probes to investigate the biological functions and physiological processes of hydrogen sulfide.

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