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A ratiometric fluorescent probe for hydrogen sulfide imaging in living cells

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ABSTRACT

We herein report a turn-on fluorescent probe based on excited state intramolecular proton transfer (ESIPT) mechanism and self-immolative linker for hydrogen sulfide detection. The new probe exhibits high sensitivity and selectivity over other biologically relevant anions. Moreover, we show the utility of the probe for the detection of hydrogen sulfide in living cells as well.

1. Introduction

Hydrogen sulfide (H_2S) is well known as the third signaling molecules in biology along with nitric oxide and carbon monoxide [1]. In mammalian system, the endogenous H_2S is biosynthesized from a cysteine substrate or its derivatives which are catalyzed by several enzymes, such as cystathionine γ-lyase (CSE) [2], cystathionine β-synthase (CBS) [3], cysteine aminotransferase (CAT) [4] and 3-mercaptopyruvate sulfurtransferase (MST) [5]. H_2S is generated in response to regulate energy production in mitochondria of mammalian cells under stress conditions [6]. In addition, H_2S is also involved in various important physiological processes, such as relaxation of vascular smooth muscles, inhibition of apoptosis, intervention of neurotransmission, regulation of inflammation, stimulation of angiogenesis, etc. [7]. Furthermore, the levels of H_2S are associated with many diseases, such as Down syndrome and Alzheimer′s diseases [8]. Despite a number of reports have been published, our knowledge on the significance of hydrogen sulfide in biological system and the mechanism of its action is still far from complete because of the limited availability of detection methods.

There have been several types of probes reported for detecting H_2S, such as colorimetric [9], electrochemical analysis [10], gas chromatography [11] and metal-induced sulfide precipitation [12] often require post-mortem processing and/or destruction of tissues or cell lysates. Among these biological detection technologies, fluorescence spectroscopy is a powerful tool for sensing and imaging trace amounts of samples because of its simplicity, sensitivity, real-time imaging, and especially its nondestructive detection of target biomolecules in live cells or tissues [13–16]. Xian′s group, for example, designed probe containing a thiopyridine moiety to trap H_2S through a nucleophilic substitution reaction [17]. Qian and co-workers reported a ratiometric fluorescent probe using a similar method [18]. On the other hand, Chang′s group and other research groups have developed an azide-based probe, which taps into the reduction of azide group to its parent amine by H_2S [19,20]. He and co-workers developed fluorescent probes based on H_2S-induced tandem chemical reactions [21,22]. Nagano′s group and other groups have employed the displacement strategy to design off–on fluorescent probes for cellular bioimaging [23]. Despite the aforementioned advancement, further development of highly sensitive and selective fluorescent probes for H_2S detection is still intensely sought after because of the critical role of H_2S in physiological and pathological processes.

On the basis of the fact that the *p*-aminobenzyl moiety is able to self-immolate through an intramolecular 1,6-elimination, we reason that importing an H_2S-responsive group to a chromophore could make the chromophore responsive to H_2S. On the other hand, 2-(2′-hydroxyphenyl)-benzothiazole (HBT) was chosen as the chromophore

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as this ESIPT chromophore showed a large Stokes shift, good photostability and corresponding efficient ratiometric fluorescence response. If the self-immolative process was triggered by H_2S so as to release the ESIPT dye of HBT, remarkable ratiometric fluorescence signals would be obtained (Scheme 1). To the best of our knowledge, such a self-immolative linker has never been used to develop a selective fluorescent probe for H_2S previously.

2. Experimental

2.1. Reagents and chemicals

2-aminobenzenethiol, 2-hydroxybenzaldehyde, H_2O_2, HCl and K_2CO_3 were all purchased from Acros. Dulbecco's modified Eagle's medium (DMEM), PBS, fetal bovine serum (FBS), trypsin–EDTA and penicillin/streptomycin were purchased from Invitrogen. Other chemicals were of guaranteed analytical grade and solvents were of HPLC grade and used directly without further purification. Milli-Q water was used throughout all experiments.

2.2. Apparatus

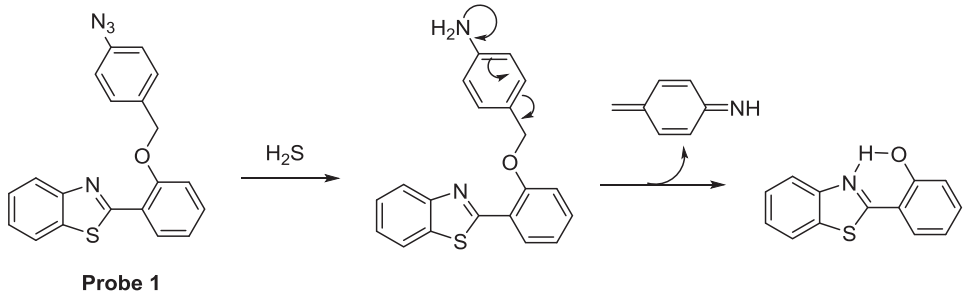
^{1}H NMR and ^{13}C NMR were recorded on a Bruker 400 NMR spectrometer. ESI-MS spectra were measured on a PC Sciex API 150 EX ESI-MS system. Absorption spectra were measured using a Shimadzu UV-1700 spectrophotometer. Fluorescence measurements were carried out with a Shimadzu RF-5301pc spectrofluorophotometer. Cells fluorescence images were acquired using a Leica TCS SP5 Confocal Scanning Microscope. HPLC was performed on an Agilent 1100 HPLC System (column: Agilent C18 5 μM, 4.6 × 250 mm).

2.3. Synthesis of HBT and 1-azido-4-(bromomethyl)benzene

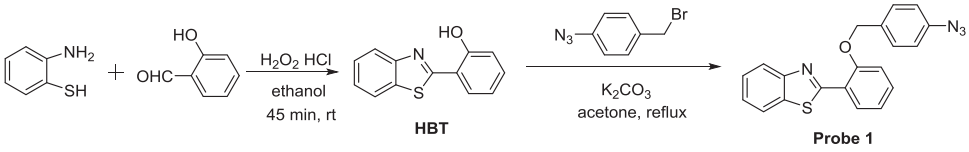
HBT and 1-azido-4-(bromomethyl) benzene were synthesized according to literature [24,25]. The synthetic detail for synthesis of these two compounds is shown in Scheme S1 (Supporting information). Their structures were confirmed by ^{1}H NMR and ^{13}C NMR (Figs. S1–S3, Supporting information).

2.4. Synthesis of probe 1

The probe can be easily obtained through the reaction between HBT and 1-azido-4-(bromomethyl)benzene (Scheme 2). To a stirred mixture of 2-(2-hydroxyphenyl)-benzothiazole (227 mg, 1.0 mmol) and anhydrous potassium carbonate (402 mg, 3 mmol) in 10 mL dry acetone under N_2 atmosphere, was added in a dropwise manner 1-azido-4-(bromomethyl)benzene (233 mg, 1.1 mmol) at room temperature. The resulting reaction mixture was allowed to stir at room temperature overnight. The reaction was quenched with 1 mL of water and the solvent was evaporated under vacuum. The resulting



Scheme 1. Possible H_2S-selective signaling mechanism.



Scheme 2. Synthesis of ratiometric probe 1 for hydrogen sulfide.

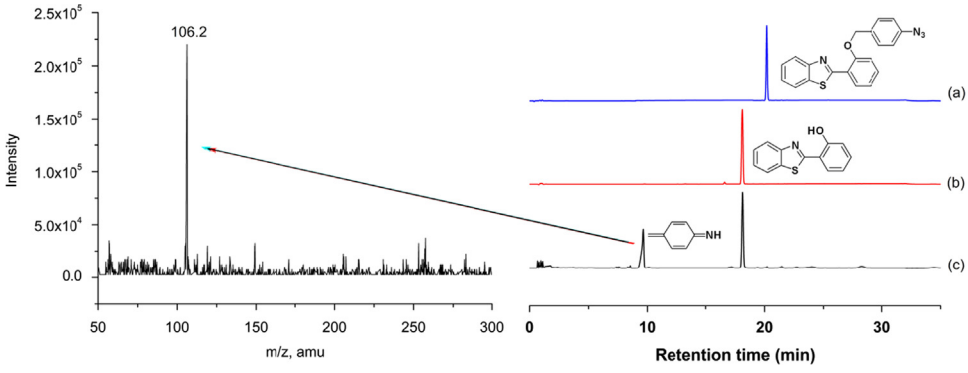


Fig. 1. Right part: HPLC of (a) probe 1 (50 μM), (b) HBT (50 μM) and (c) the reaction product of Probe 1 (50 μM) with NaHS (1 mM) after incubation of them for 1 h. Left part: Mass spectrum of intramolecular 1,6-elimination product.

solid product was extracted twice with dichloromethane. After drying over $MgSO_4$, the organic phase was filtered. The crude material was purified by flash chromatography on silica gel providing probe 1 (304 mg, 85%). ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm) 8.56 (dd, J=8.0 Hz, 1.6 Hz, 1H), 8.09 (d, J=8.0 Hz, 1H), 7.88 (d, J=8.0 Hz, 1H), 7.53 (d, J=8.8 Hz, 2H), 7.48 (td, J=8.0 Hz, 1.2 Hz, 1H), 7.42 (td, J=8.0 Hz, 1.6 Hz, 1H), 7.36 (td, J=8.0 Hz, 1.2 Hz, 1H), 7.15 (t, J=8.0 Hz, 1H), 7.08 (m, 3H), 5.28 (s, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm) 163.10, 156.21, 152.23, 140.22, 136.22, 132.84, 131.82, 130.00, 129.71, 126.07, 124.75, 122.92, 122.88, 121.69, 121.42, 119.39, 113.01, 70.58. ESI-MS(m/z): 359.2$[M+H]^+$, calcd. for $C_{20}H_{14}N_4OS$=358.1 (Fig. S4, Supporting information).

3. Results and discussions

3.1. Mechanism

To verify the proposed mechanism, the purified product of the reaction of probe 1 with H_2S was then characterized by HPLC and ESI-MS. The HPLC spectra of probe 1 in the presence of NaHS showed two major peaks at 9.6 min and 18.1 min respectively, which could be assigned as HBT and intramolecular 1,6-elimination product. Further, ESI-MS spectra supported our proposed reaction mechanism strongly (Scheme 1, Fig. 1).

3.2. UV–vis and fluorescence emission

Spectroscopic properties of probes 1 were obtained under physiological conditions. First, the probe was dissolved in acetonitrile (ACN) and subsequently diluted in PBS buffer (1:1, v/v, 50 mM, pH 7.4). The UV–vis absorption spectrum of the probe 1 in aqueous solution (100 μM, PBS buffer, pH 7.4) displays a maximum at 330 nm, and the emission spectrum shows a peak maximum at 370 nm (Fig. 2). After incubation with H_2S (500 μM) for 1 h, the emission at 370 nm decreased sharply to its limiting value, while an emission band at 465 nm developed. This result clearly indicated that probe 1 could react with H_2S.

3.3. Sensitivity and detection limit

To investigate the ratiometric fluorescence response of probe 1 to H_2S, probe 1 was treated with different concentration of NaHS

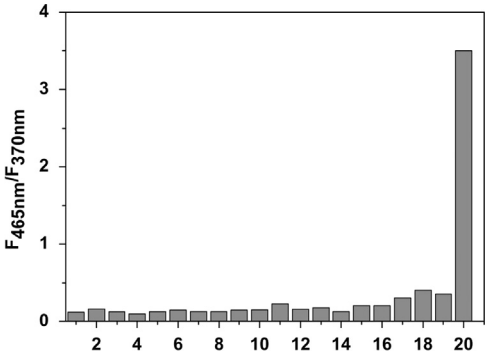


Fig. 4. Selectivity experiments of probe 1 (5 μM) with 18 different reactive sulfur, nitrogen, oxygen species (1 mM) and NaHS (100 μM) in PBS–ACN solution (1:1, v/v, 50 mM PBS, pH=7.4). 1: probe 1 only, 2: H_2O_2, 3: HOCl, 4: $ONOO^-$, 5: NO_2^-, 6: NO_3^{2-}, 7: N_3^-, 8: F^-, 9: Cl^-, 10: Br^-, 11: I^-, 12: SCN^-, 13: SO_3^{2-}, 14: $S_2O_3^{2-}$, 15: $S_2O_4^{2-}$, 16: cysteine, 17: homocysteine, 18: glutathione, 19: mercaptoethanol, and 20: HS^-. Ex=330 nm and Em=350–600 nm.

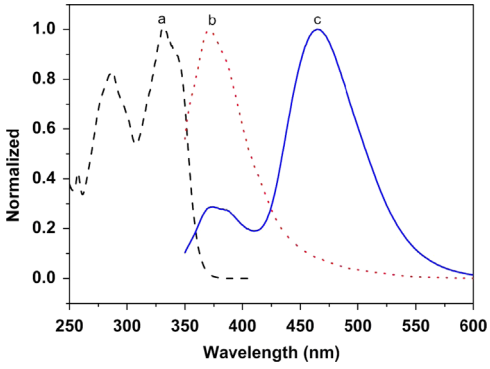


Fig. 2. (a) Absorption spectra of probe 1 (10 μM), (b) emission spectra of probe 1 (10 μM), (c) emission spectra of probe 1 and NaHS (100 μM) after incubating for 1 h.

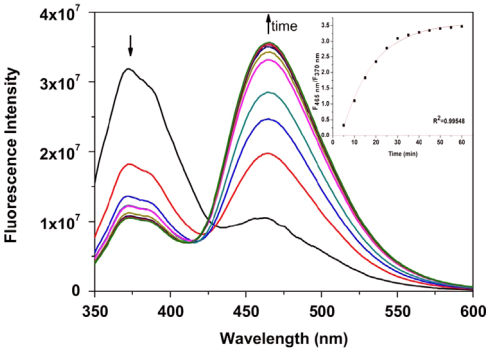


Fig. 5. Time course experiment of probe 1 (5 μM) reacting with NaHS in PBS–ACN solution (1:1, v/v, 50 mM PBS, pH=7.4) at 37 °C. Ex=330 nm and Em=350–600 nm.

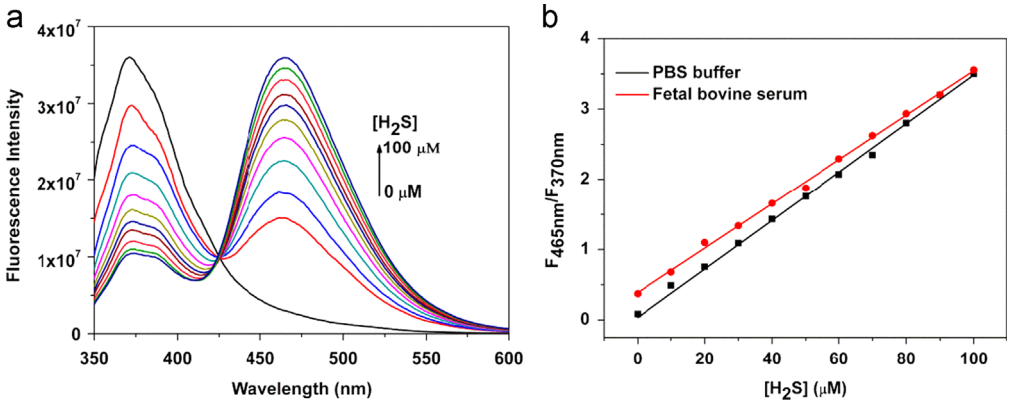


Fig. 3. (a) Fluorescence emission spectra of probe 1 (5 μM) in PBS–ACN solution (1:1, v/v, 50 mM PBS, pH=7.4) with addition of increasing concentrations of NaHS (from 0 μM to 100 μM). (b) The relationship between the ratio of $I_{465\ nm}/I_{370\ nm}$ and NaHS concentration in PBS buffer (blank) and commercial fetal bovine serum (red). (Probe 1: 5 μM, NaHS: 0–100 μM) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

which is a commonly employed H_2S donor in the same conditions. Probe 1 exhibited a characteristic emission maximum at 370 nm without NaHS. Treatment of probe 1 with NaHS in a time period of 1 h resulted in remarkable red shifts of 95 nm in fluorescence spectra. These spectral changes clearly demonstrate that the self-immolative process was triggered by H_2S (Fig. 3a). The ratio of emission intensities ($I_{465\ nm}/I_{370\ nm}$) varied from 0.08 to 3.50 (43 fold) when the concentration of H_2S increased from 0 to 100 μM with good linearity. As shown in Fig. 3b, the fluorescent signal ratio was linearly related to the concentration of NaHS in the given concentration range. The regression equation was $F=0.03383[H_2S]\ \mu M+0.08361$, with $R^2=0.99790$. To further establish the utility of probe 1 for the determination of sulfide in a biological complex sample, probe 1 (5 μM) was evaluated with NaHS in commercially available fetal bovine serum. We prepared fetal bovine serum which contained H_2S at different concentrations (0–100 μM). Using the same method, we got another standard curve between the ratio of emission intensities ($I_{465\ nm}/I_{370\ nm}$) and NaHS concentrations. The regression equation was $F=0.03157[H_2S]\ \mu M+0.38789$, with $R^2=0.99822$. These results revealed that probe 1 could test H_2S in complex biological systems both qualitatively and quantitatively. Specifically, the detection limit of the method for H_2S was determined to be 2.4 μM (3σ/slope) under the test conditions [26].

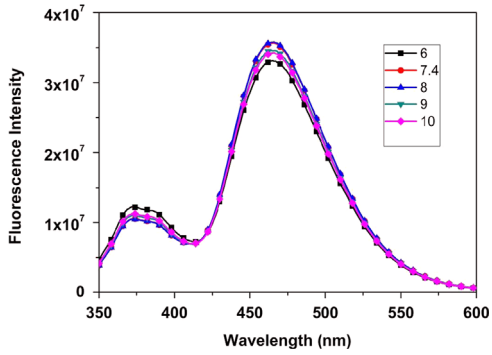


Fig. 6. pH dependent experiments of probe 1.

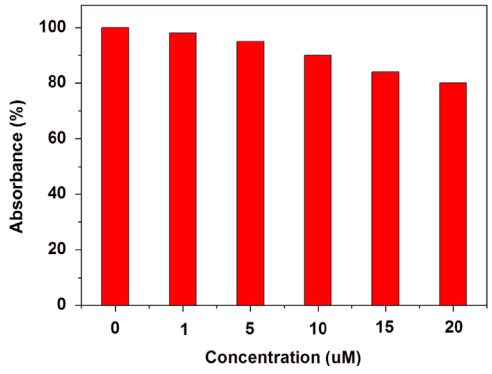


Fig. 7. MTT assay with different concentration of probe 1 (0, 1, 5, 10, 15, and 20 μM).

3.4. Selectivity

As shown in Figs. 4 and S5, upon addition of other biologically relevant reactive oxygen species (H_2O_2, HOCl, and $ONOO^-$), reactive nitrogen species (NO_2^-, NO_3^{2-}, and N_3^-), reactive sulfur species (cysteine, homocysteine, glutathione, mercaptoethanol, SCN^-, SO_3^{2-}, $S_2O_3^{2-}$, and $S_2O_4^{2-}$) and other anions (F^-, Cl^-, Br^-, and I^-) did not lead to any obviously fluorescence intensity fluctuation. The result demonstrates that probe 1 can be used to detect H_2S with high selectivity over other biologically relevant anions.

3.5. Kinetics of probe

We further investigated the reaction kinetics of probe 1 with H_2S. As shown in Fig. 5, the fluorescence signal at 465 nm increased rapidly and the fluorescence signal at 370 nm decreased rapidly. The ration of $I_{465\ nm}/I_{370\ nm}$ reached steady state at around 30 min. Under pseudo-first-order kinetic conditions, k_{obs} was found to be $1.92\times10^{-3}\ S^{-1}$. These results suggest that probe 1 is comparable with other H_2S probes.

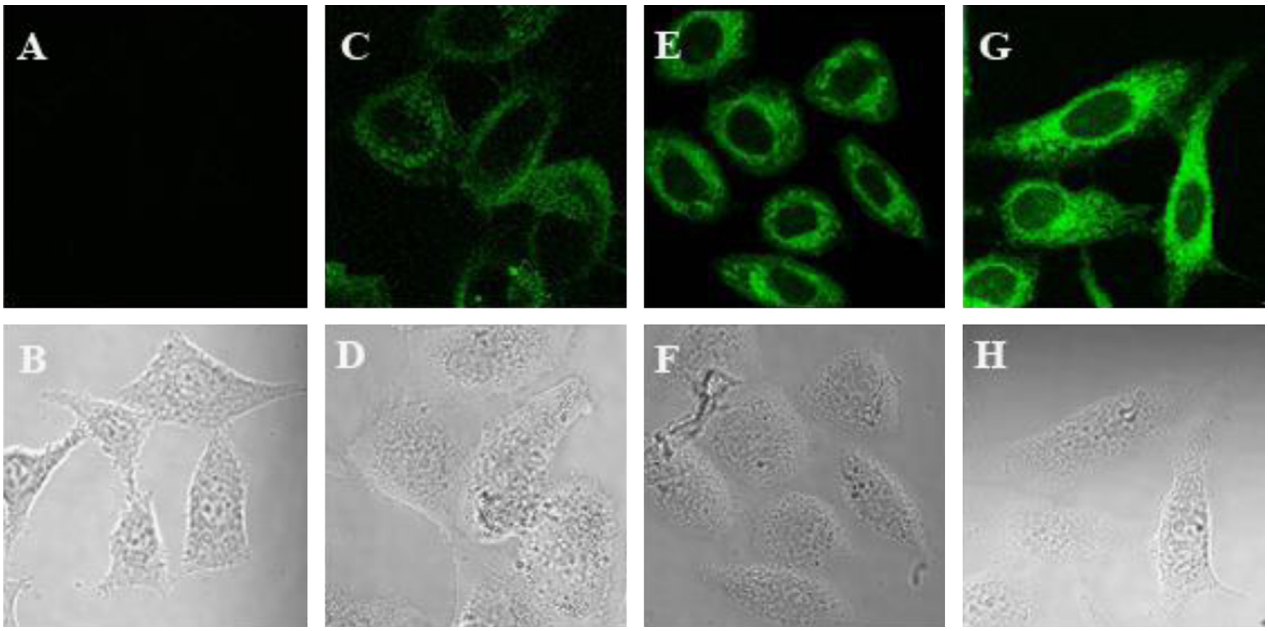


Fig. 8. Fluorescence microscopy experiments of imaging H_2S with probe 1 (5 μM) in HeLa cells. (A) Fluorescent image of HeLa cells incubated with probe 1 for 60 min and (B) brightfield image of HeLa cells in (A). Fluorescent images (top) and brightfield images (down) of HeLa cells incubated with probe 1 for 30 min followed by incubation with different concentrations of NaHS, 20 μM (C and D), 50 μM (E and F), 100 μM (G and H), for another 30 min. Ex=330 nm and Em=450–600 nm.

3.6. Effect of pH

In order to verify the influence of pH on the fluorescence of probe 1, we also measured the fluorescence spectra of probe 1 at different pH values. The fluorescence intensity ratio ($I_{465\ nm}$/$I_{370\ nm}$) was barely affected when the pH ranged from 6.0 to 10.0 (Fig. 6). This evidence indicates that probe 1 can be used for the detection of H_2S at a wide pH range.

3.7. Detection of H_2S in living cells

To investigate the potential biological applications of probe 1, we decided to further investigate the suitability of the probe to visualize H_2S in living cells. First, the MTT assay for probe 1 was conducted, and the results showed that probe 1 with a concentration at 5 μM have only minimal cytotoxicity after 24 h (Fig. 7). Thus, the probe at 5 μM was selected for imaging experiments in living cells. Incubation of HeLa cells with probe 1 (5 μM) for 30 min at 37 °C was followed by the addition of different concentrations of NaHS (20 μM, 50 μM, and 100 μM) and then incubation for another 30 min. The cells were subsequently imaged using a confocal fluorescence microscopy. As shown in Fig. 8, HeLa cells with only probe 1 show very low fluorescence. In the presence of probe 1 and NaHS, however, HeLa cells show strong fluorescence. And more, with the increasement of NaHS concentration, the fluorescence intensity increased as well. This result clearly demonstrates that probe 1 has potential in visualizing H_2S in living cells.

4. Conclusions

In this study, we have developed a new ratiometric fluorescence probe 1 for H_2S based on ESIPT and self-immolative linker. The probe exhibited high sensitivity and selectivity for H_2S detection. The potential for biological applications of probe was confirmed by employing it for fluorescence imaging of H_2S in living cells.

Acknowledgment

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.01.001.

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