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A novel FRET-based ratiometric fluorescent probe for highly sensitive detection of hydrogen sulfide

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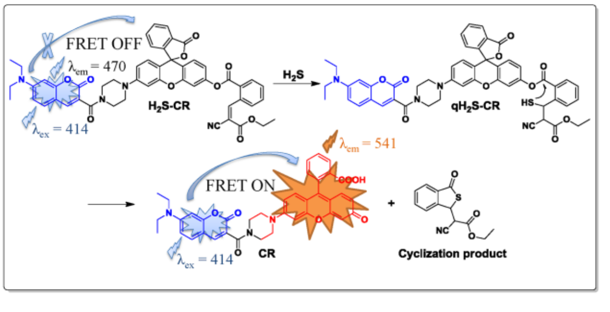
A novel FRET-based ratiometric fluorescent probe H_2S-CR for the quantitative detection of H_2S was designed and synthesized. It exhibits a response (20 min), a considerably fluorescence signal enhancement (15 folds), and an extremely low detection limit (19 nM). It can be successfully applied to imaging H_2S in living cells.

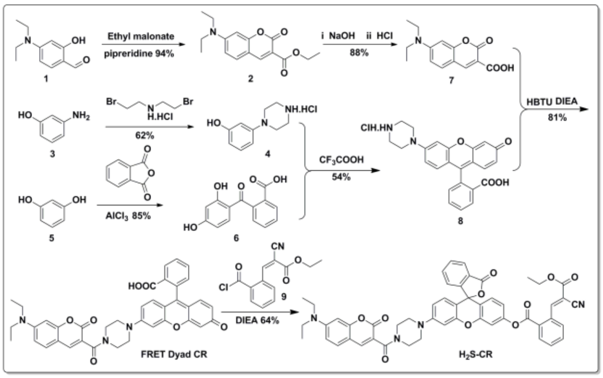
As a member of the reactive sulfur species (RSS) family, hydrogen sulfide (H_2S) has been regarded as a toxic pollutant with the typical smell of unpleasant rotten eggs for a long time. However, more recent studies on endogenous H_2S have challenged this traditional view of H_2S as a toxin and suggested that H_2S is the third member of the gasotransmitter family, in addition to nitric oxide (NO) and carbon monoxide (CO), existing in the human body and other biological systems.[1] Furthermore, H_2S at physiological concentration appears to be involved in various physiological processes, including regulation of cell growth,[2] stimulation of angiogenesis,[3] modulation of neuronal transmission,[4] the anti-inflammation effect and etc.[5] Concurrently, abnormal H_2S levels are engaged in diseases such as Alzheimer's disease,[6] Down's syndrome,[7] gastric mucosal injury,[8] diabetes,[9] liver cirrhosis, and etc.[10] Therefore, highly sensitive and selective detection of H_2S and direct indication of its concentration in living systems are crucial for the better understanding of its related physiological and pathological functions.

In the past decades, the detection of H_2S has attracted a wide research interest, and some strategies including metal-induced sulfide precipitation,[11] colorimetric assays,[12] electrochemical analysis[13] and gas chromatograph[14] have been developed. Among them, fluorescence-based detection with a fluorescent probe is extremely attractive because of its simplicity, real-time imaging, and nondestructive detection of intracellular biomolecules.[15–22] Recently, a number of fluorescent probes for H_2S have been constructed using the special properties of H_2S, such as its dual nucleophilicity,[15] good reducing property toward azides,[16] nitros[17] and hydroxyl amines,[18] high binding affinity with copper and zinc ions,[19] efficient thiolysis of dinitrophenyl ether[20] as well as specific Michael addition reaction towards unsaturated double bonds.[21] Besides, as far as we know, the probes utilizing the unique dual nucleophilic character of H_2S exhibited superior selective advantage because the potential interference from bio-thiols can be well excluded.[15] Usually, such fluorescent probes contain a potential fluorescent group and a specific H_2S trap group with two nucleophilic reaction sites.

Although these probes utilizing the unique dual nucleophilic character of H_2S are innovative and effective, some improvements are still needed. One of the main concern using this strategy is how to avoid the probe consumption by biothiols,[15b] which would otherwise lead to high probe loading and low sensitivity. Another concern is that most of these probes exhibit a response to H_2S with changes only in fluorescence intensity at a single wavelength,[15(a, c, d)] and single increase or decrease emission detection is sometimes problematic for precise fluorometric analysis because fluorescence intensity can be affected by variables such as excitation intensity variations, environmental factors, light scattering, probe concentrations, and etc. Moreover, the development of novel fluorescent probes for H_2S detection with better optical properties and higher sensitivity is of pressing need. Herein, we reported the design and synthesize of a novel ratiometric fluorescence probe **H_2S-CR** (Scheme 1) based on a H_2S induced Michael addition-



Scheme 1 The proposed mechanism of ratiometric fluorescent probe **H_2S-CR** for H_2S detection.



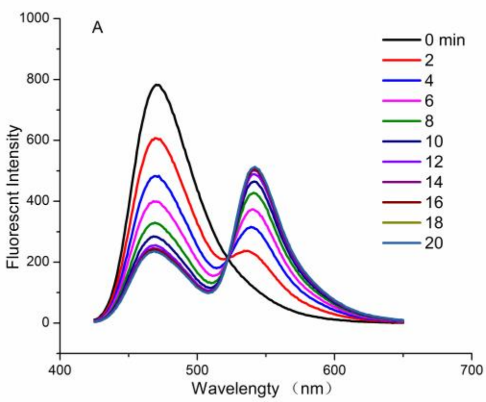
Scheme 2 The synthesis of the probe **H_2S-CR**.

cyclization cascade reaction and the FRET modulated fluorescence process. It exhibits a response time (20 min), a considerably fluorescence signal enhancement (15-fold), and an extremely low detection limit (19 nM) toward H_2S. Moreover, we successfully applied it to image the change of H_2S level in living cells.

As shown in Scheme 2, probe **H_2S-CR** can be conveniently prepared from 4-(diethylamino)-2-hydroxybenzaldehyde by a four-step procedure under mild conditions with a good yield. The **coumarin** donor building block **7** and **rhodamine/fluorescein** acceptor building block **8** were synthesized by a reported synthetic procedure.[23] The **FRET dyad CR** was prepared by the condensation of carboxyl compound **7** with amide **8**. Finally, we transformed the compound **CR** to probe **H_2S-CR**. The structural characterization of the probe was characterized by standard ^{1}H NMR, ^{13}C NMR, HRMS, and etc (Fig. S3).

We first evaluated the effect of buffer solution to the fluorescence of the probe. As shown in Fig. S5, the probe could work well in phosphate buffered solutions. The photophysical properties of the probe (10 μM) were investigated under simulated physiological conditions (30 mM, pH 7.4, 1:9 v/v CH_3CN/PBS). Prior to reaction with NaHS, probe presented a fluorescence maximum at 470 nm (Fig. 1A) with a corresponding major absorption band centered at 408 nm (Fig. 1B) (Φ = 0.132). With the addition of NaHS (80 μM) into the solution of probe, the fluorescence emission intensity at 470 nm gradually decreased within 12 min, along with a time-dependent fluorescence emission intensity increase centred at 541 nm (Fig. 1A) (Φ = 0.100). Simultaneously, there emerged a new absorption peak at 511 nm, accompanied by a dramatic change in the probe solution from colorless to bright orange. Furthermore, we performed the time-dependent fluorescent spectra studies. As shown in Fig. 2, although 80 μM NaHS could cause the reaction to be completed within 12 min, the low concentrations of NaHS needed the longer reaction time (20 min) to reach the fluorescence intensity ratio (I_{541}/I_{470}) saturation. Thus, the time point after the addition of NaHS was selected to be 20 min in the subsequent experiments.

To gain insight into potential of **H_2S-CR** as a probe for H_2S, a titration experiment was performed under simulated physiological conditions. Accordingly, as shown in Fig. 3A, upon excitation at 414 nm, the fluorescence intensity around 470 nm decreases along with the incremental addition of NaHS, and simultaneously a new emission band around 541 nm gradually increased. The fluorescence



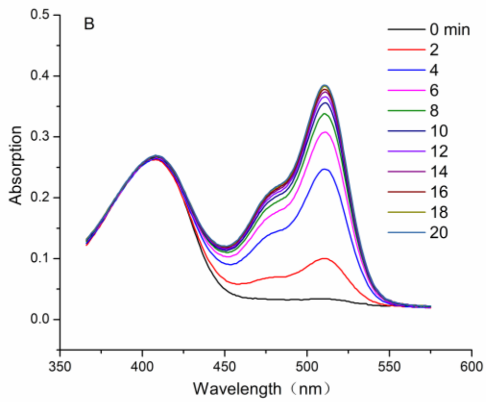


Fig. 1 (A) Fluorescence responses of 10 μM probe to 80 μM NaHS. (B) Time-dependent absorption spectra of probe (10 μM) in the presence of 80 μM NaHS. Conditions: excitation wavelength is 414 nm, acetonitrile-PBS buffer solution (30 mM, pH 7.4, 1:9 v/v) at 25 °C.

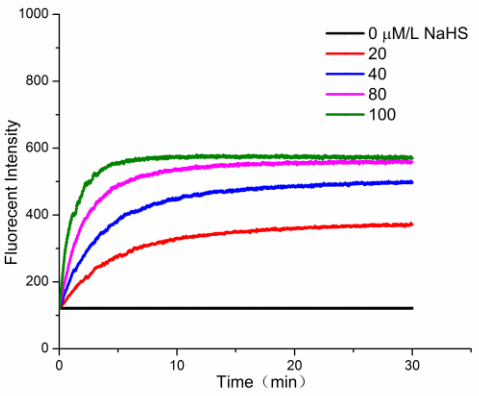


Fig. 2 Time-dependent fluorescence intensity changes of probe (10 μM) at 541 nm upon addition of varied concentrations of NaHS. Conditions: excitation wavelength is 414 nm, acetonitrile-PBS buffer solution (30 mM, pH 7.4, 1:9 v/v) at 25 °C.

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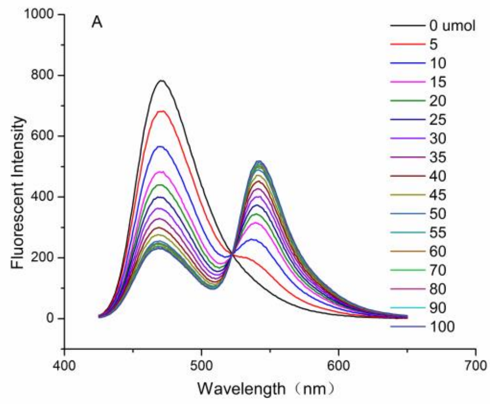

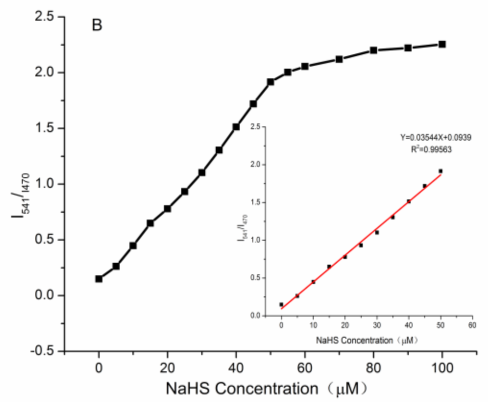

Fig. 3 (A) Fluorescence response of 10 μM probe in the presence of 0-10 equiv of NaHS. (B) Fluorescence ratio (I_{541}/I_{470}) of probe (10 μM) in the presence of 0-10 equiv of NaHS. Conditions: excitation wavelength is 414 nm, acetonitrile-PBS buffer solution (30 mM, pH 7.4, 1:9 v/v) at 25 °C for 30 min.

intensity could reach saturation when the addition amount of NaHS reached 9 equiv. of the probe (Fig. 3B). At this amount, the ratio of the fluorescence emission intensities at 541 and 470 nm (I_{541}/I_{470}) exhibited a drastic change from 0.15 in the absence of NaHS to 2.3 after complete conversion, a 15-fold enhancement in the I_{541}/I_{470} ratios. This suggested that the excitation energy of the **coumarin** donor is efficiently transferred to the **rhodamine/fluorescein** acceptor. The intramolecular energy transfer efficiency from the **coumarin** donor to the **rhodamine/fluorescein** acceptor in **H_2S-CR** was calculated to be 70.7% (in supporting information). The detection limit (*S/N*=3) of the ratiometric probe was determined to be 19 nM (in supporting information). Additionally, a standard curve between emission intensity ratio (I_{541}/I_{470}) and NaHS concentration was set up. To our satisfaction, as shown in Fig. 3B, a good linearity between the fluorescence intensity ratio (I_{541}/I_{470}) and the NaHS concentration in the range of 0-50 μM was observed, suggesting that **H_2S-CR** is potentially useful for quantitative determination of NaHS.

Based on the well-established dual nucleophilicity mechanism, the mechanism has been proved by reaction between **H_2S-CR** and NaHS, and reaction product coumarin-rhodamine/fluorescein and cyclization product were confirmed by HPLC-MS experiments (Fig. S6).

To study the specificity of **H_2S-CR** towards NaHS, an important test was performed to determine whether biological species other than NaHS could potentially introduce signal response (Fig. 4). As expected, **H_2S-CR** was considerably inert to the common cations and anions, such as Na^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Cu^+, K^+, Fe^{3+}, and Fe^{2+} (4 mM for each) (Fig. 4A); F^-, Cl^-, I^-, AcO^-, N_3^-, CO_3^{2-}, NO_3^-, NO_2^-, SO_4^{2-}, and HCO_3^- (4 mM for each) (Fig. 4A). The results revealed that the NaHS-induced ratiometric fluorescence response is hardly affected in response to reactive oxygen/nitrogen species (ROS/RNS), such as H_2O_2, NO, ROO·, $\cdot O^{2-}$, ClO^-, ·OH, $^1O_2$, and $CNOO^-$ (4 mM for each) (Fig. 4B), reducing condition, such as sodium ascorbate (4 mM) (Fig. 4B), and non-thiol amino acids, such as Lys, Ser, Glu, Pro, Phe, Arg, Thr and Asn (4 mM for each) (Fig. 4B). Moreover, CN^- (4 mM) (Fig. 4B), reactive sulfur species (SO_3^{2-}, HSO_3^-, and $S_2O_3^{2-}$ (0.8 mM for each)) (Fig.4B) and biothiols(GSH, Cys, and Hcy (0.8 mM for each)) (Fig. 4B) underwent limited fluorescence response. However, the fluorescence ratio (I_{541}/I_{470}) increase was far weaker than that caused by NaHS. By contrast, NaHS induced a robust increase in the fluorescence intensity ratio

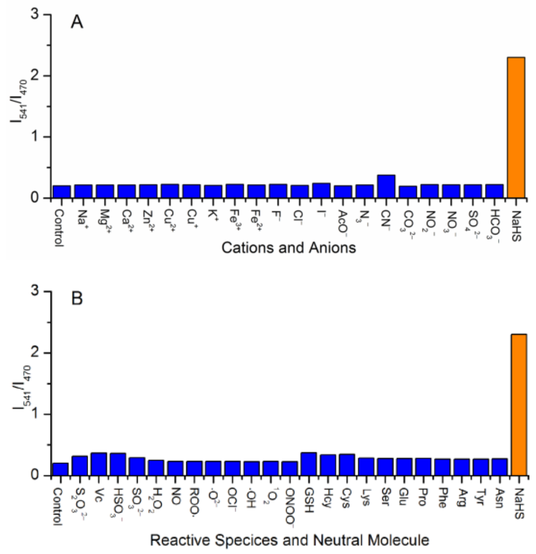

Fig. 4 Fluorescence ratio (I_{541}/I_{470}) of 10 μM probe in an acetonitrile-PBS buffer solution (30 mM, pH 7.4, 1:9 v/v) towards hydrosulfide and potential interferences for 30 min. Bars represent fluorescence ratio (I_{541}/I_{470}) to each compound. (A) The fluorescence emission of probe spiked with selected cations, such as Na^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Cu^+, K^+, Fe^{3+}, and Fe^{2+} (4 mM for each) and selected anions, such as F^-, Cl^-, I^-, AcO^-, N_3^-, CN^-, CO_3^{2-}, NO_3^-, NO_2^-, SO_4^{2-}, and HCO_3^- (4 mM for each). (B) Fluorescence responses of probe supplemented with reactive oxide species, such as H_2O_2, ROO ; O^{2-}, ClO^-, OH, and $^1O_2$ (4 mM for each), reactive nitrogen species, such as NO and $CNOO^-$ (4 mM for each), reactive sulfur species, such as SO_3^{2-}, HSO_3^-, and $S_2O_3^{2-}$ (0.8 mM for each), sodium ascorbate (4 mM) non-thiol amino acids, such as Lys, Ser, Glu, Pro, Phe, Arg, Thr and Asn (4 mM for each) and biothiols, such as GSH, Cys, and Hcy (0.8 mM for each).

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(I_{541}/I_{470}), and only the probe solution turned from colorless to bright orange when treated by NaHS in the selective experiment. High selectivity toward H_2S in the presence of other competitive species is a very important feature to evaluate the performance of the fluorescent probe. Therefore, the competition experiments were also conducted when CN^-/biothiols/(reactive sulfur species) and NaHS co-existed in the system. To our delighted, when NaHS and these competitive species coexisted, almost the same I_{541}/I_{470} signal enhancement was observed as that only treated by NaHS (Fig. S7). Taken together, **H$_2$S-CR** can selectively respond to NaHS independently of negligible disturbance from the interference of other biological species, and it can serve as a "naked-eye" probe for colorimetric detection of H_2S (Fig. S7).

To verify whether the probe is suitable for the physiological detection, we evaluated the effect of pH on the fluorescence of the probe. As shown in Fig. S8, in the absence of NaHS, almost no change in fluorescence ratio (I_{541}/I_{470}) was observed in the free probe over a wide pH range of 2-11 indicating excellent pH stability. Furthermore, upon treatment with NaHS, the maximal fluorescence ratio (I_{541}/I_{470}) displayed constant in the pH range of 6-11. Thus, the observation that **H$_2$S-CR** had the maximal sensing response at physiological pH, suggested that **H$_2$S-CR** is promising for biological applications.

Having demonstrated the selectivity and sensitivity of **H$_2$S-CR** for H_2S *in vitro*, we next evaluated the potential utility of **H$_2$S-CR** as a probe for H_2S within living cells (Fig. 5). H9C2 cells were incubated with 10 μM **H$_2$S-CR** for 20 min at 37 °C. After washed three times with physiological saline to remove the remaining probes, the cells were then incubated with buffer containing different concentrations of NaHS (10, 20, 30, 40 and 80 μM) for 30 min. As for the control experiment, the cells untreated with NaHS were examined. The optical imaging was carried out by a fluorescent inverted microscope. A faint fluorescence was observed in the control experiments, and the lever changes were depended on the concentration of NaHS (Fig. 5). It is worth noting that the inverted fluorescence images grew brighter as the concentrations of NaHS increased from 10 to 80 M (Fig. 5 E-A). These results demonstrated that **H$_2$S-CR** is cell membrane permeable and has potential in visualizing H_2S levels change of living cells.

Conclusions

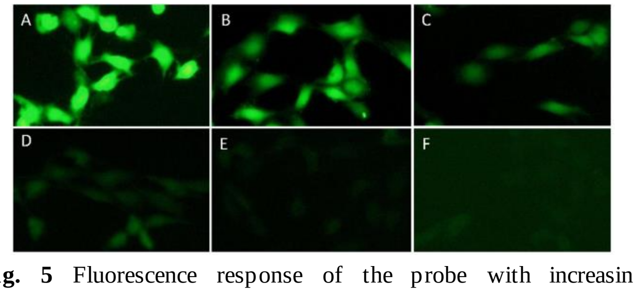

Fig. 5 Fluorescence response of the probe with increasing concentrations of NaHS in living H9C2 cells. The cells were pre-treated with the probe (10 μM) for 10 min, and then incubated with NaHS (A) 80 μM, (B) 40 μM, (C) 30 μM, (D) 20 μM, (E) 10 μM, (F) 0 μM, for 20 min.

In conclusion, with recognition of the biological significance of H_2S, we have developed a unique FRET-based ratiometric fluorescence probe **H$_2$S-CR**. Based on a H_2S induced Michael addition-cyclization cascade reaction, the probe exhibited a high selectivity and sensitivity for H_2S over other biologically relevant species, a 15-fold fluorescence signal enhancement, and an obvious colour change from colourless to bright orange. Moreover, **H$_2$S-CR** can detect H_2S quantitatively with a low detection limit up to 19 nM. Fluorescent inverted microscope images indicated that this probe can detect the level changes of H_2S in living cells. In addition, this ratiometric fluorescence probe has the potential to be a useful tool for the fast and real-time detection of H_2S in more types of biological samples.

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Notes and references

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