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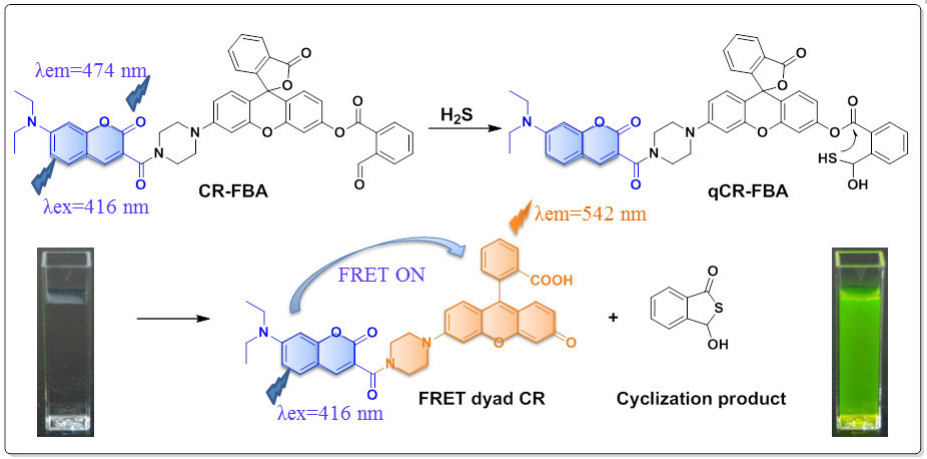

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Cascade reaction and FRET-based fluorescent probe for the colorimetric and ratiometric signaling of hydrogen sulfide

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ABSTRACT

A novel addition-elimination cascade reaction and FRET-based fluorescent probe for the colorimetric and ratiometric signaling of hydrogen sulfide was designed. Employing of 2-formylbenzoic acid as the trapper, the probe was highly selective and sensitive to H_2S over other biologically relevant species to give color change from colorless to bright orange for naked eye observation. A linear response to H_2S in the range of 0-200 μM indicated that it could detect H_2S quantitatively with a detection limit as low as 0.39 μM. Furthermore, fluorescent inverted microscope imaging experiments demonstrated that the probe could assess intracellular H_2S levels change.

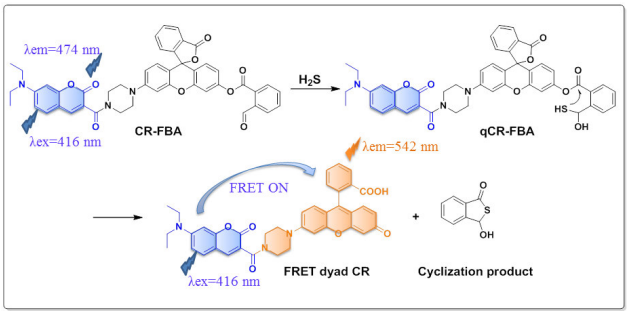
Hydrogen sulfide, along with nitric oxide and carbon monoxide, has recently emerged as an important endogenous signaling molecule.[1–2] It is well accepted that the physiological level of H_2S regulated a number of processes in cardiovascular,[3] immune,[4] endocrine,[5] as well as the gastrointestinal[6] and central nervous system.[7] On the other hand, H_2S levels are abnormal in diseases ranging from Alzheimer's disease,[8] and chronic kidney disease,[9] to liver cirrhosis[10] and Down's syndrome.[11] Thus, to better understand the physiological and pathological functions of H_2S, selective tracking and quantifying this small molecule is increasingly crucial and has attracted a wide research interest.

When compared with sophisticated techniques for H_2S detection, fluorescence techniques are preferred by virtue of their high sensitivity/selectivity, high spatiotemporal resolution and non-destructive advantages.[12] Since 2011, several types of fluorescent probes have been designed. Among them, fluorescent probes displaying dual functions with both colorimetric and ratiometric properties could be more useful in convenient visual sensing by the "naked eye" and in quantitative fluorometric detection via their internal calibration of dual emissions.[13–18]

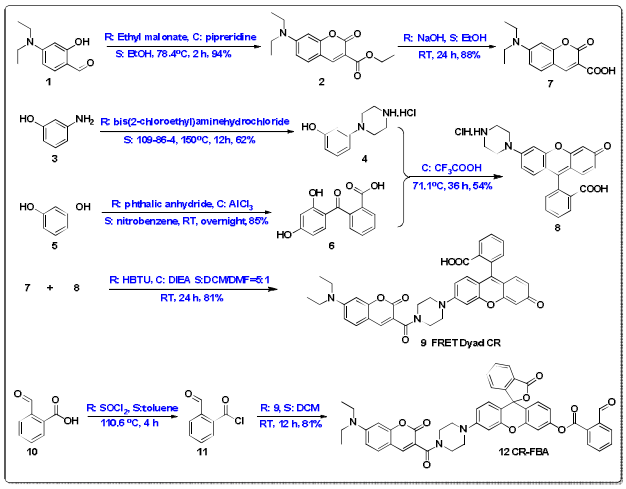
Currently, several dual-function colorimetric and ratiometric fluorescent probes for H_2S have been reported. However, there are still some limitations. For example, long response time is often required, especially for most of those probes based on the nitro reduction approach.[14] Besides, the selective detection of H_2S is still challenging due to the interference of other biothiols.[17] In addition, the defects of the probe itself could also restrict its wide application such as small Stokes' shift,[16] high detection limits,[14] excitation wavelength in the UV region[18] or employment of labile cyanine dyes and zido fluorogens.[15] Therefore, it is quite necessary and important to exploit new probes with improved properties. Herein, we reported an addition-elimination cascade reaction and FRET-based dual-function colorimetric and ratiometric fluorescent probe **CR-FBA** (as shown in Scheme 1), which featured good selectivity, high sensitivity and excellent photostability. More importantly, it was successfully applied to detect H_2S in living cells.

The synthesis of probe was outlined as shown in Scheme 2.[19] Treatment of 4-(diethylamino)-2-hydroxybenzaldehyde with diethyl malonate in the presence of piperidine afforded **2**, which was then reacted with Sodium hydroxide to yield **7**. Compound **4** and **6** could easily provide the **rhodamine/fluorescein** acceptor building block **8**. The key intermediate **FRET dyad CR** was readily synthesized by condensation reaction of carboxyl compound **7** with amide **8** in the presence of HBTU and DIEA in good yield. The target compound **CR-FBA** was achieved through the esterification reaction from **CR** and 2-formylbenzoyl chloride. The structural characterization of the probe was characterized by standard ^{1}H-NMR, ^{13}C-NMR, and HRMS.

Initially, colorimetric responses of probe (10 μM) to a range of biological species were investigated. As we expected, a distinct visible color changing from colorless to bright orange was



Scheme 1. The proposed mechanism of probe for H_2S detection.



Scheme 2. Synthesis of **CR-FBA**.

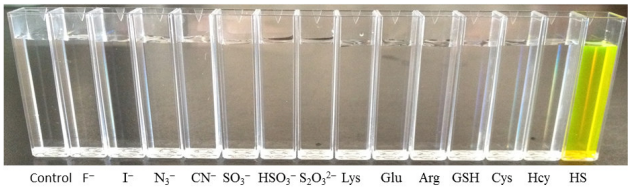


Figure 1. Changes in visible color of probe (10 μM) with NaHS (400 μM) and other various biological species (4 mM). Conditions: acetonitrile-PBS buffer solution (60 mM, pH 7.4, 1:9 v/v) at 25 °C for 25 min.

observed upon the addition of NaHS (400 μM) (Figure 1). In contrast, no significant color change was generated by treatment species (SO_3^{2-}, HSO_3^-, and $S_2O_3^{2-}$), non-thiol amino acids (Lys, Glu, Arg) and biothiols (GSH, Cys, and Hcy), even at a high concentration (4 mM). These observations clearly indicated that probe exhibited high selectivity for naked-eye detection of H_2S over other biological species.

Fluorescence responses of **CR-FBA** to different biological species were also studied as shown in Figure 2. Notably, the probe revealed no significant responses toward common anions (F^-, I^-, N_3^-, and CN^-), reactive sulfur species (SO_3^{2-}, HSO_3^-, and $S_2O_3^{2-}$), non-thiol amino acids (Lys, Glu, Arg) and biothiol (GSH). And only two selected biothiols (Cys and Hcy)) induced small fluorescence changes. By contrast, upon treatment of NaHS with the probe, the most significant fluorescence changes were observed, which demonstrated its high selectivity toward H_2S.

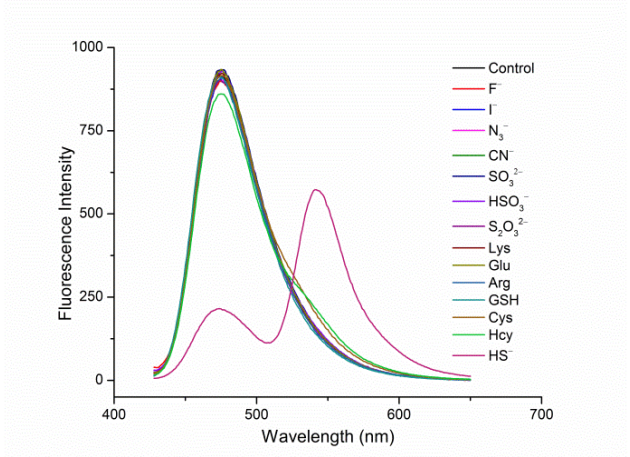


Figure 2. Fluorescence spectra of **CR-FBA** (10 μM) upon the addition of NaHS (400 μM) and other various biological species (4 mM). Conditions: excitation wavelength is 416 nm, acetonitrile-PBS buffer solution (60 mM, pH 7.4, 1:9 v/v) at 25 °C for 25 min.

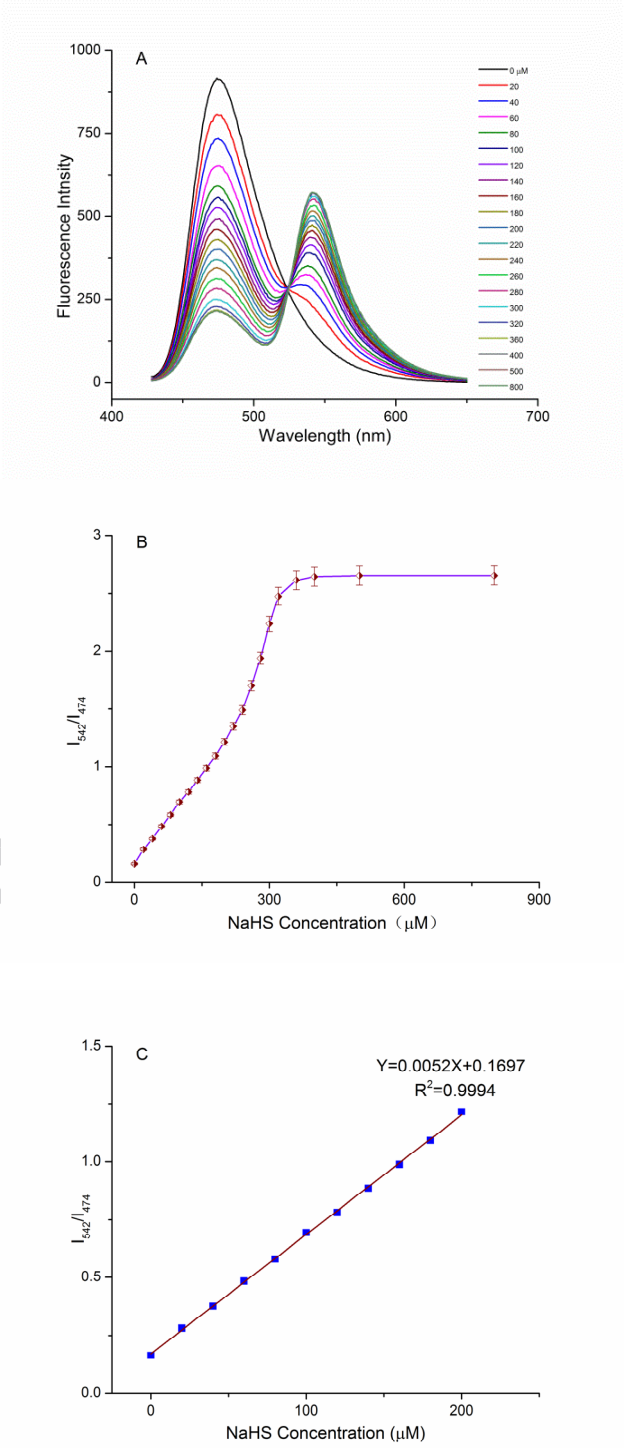


Figure 3. Fluorescence responses of probe (10 μM) in the presence of 0-80 equiv of NaHS. Conditions: excitation wavelength is 416 nm, acetonitrile-PBS buffer solution (60 mM, pH 7.4, 1:9 v/v) at 25 °C for 25 min.

The concentration-dependent fluorescence spectra were further examined. As shown in Figure 3A, in the absence of NaHS, the emission spectrum of probe exhibited an emission band with maxima at 474 nm that are typical of the **coumarin** moiety, demonstrating that there was no FRET in the free probe. Upon the gradually addition of NaHS, such emission band gradually decreased along with a new red-shift emission band at 542 nm and a well-defined isoemission point at 525 nm. The intensity ratios (I_{542}/I_{474}) reached the saturation state when adding 40 equiv NaHS. An obvious increase in I_{542}/I_{474} changing from 0.16 to 2.65 (Figure 3B) was also observed. These results confirmed the existence of FRET, as the conversion of **rhodamine/fluorescein**

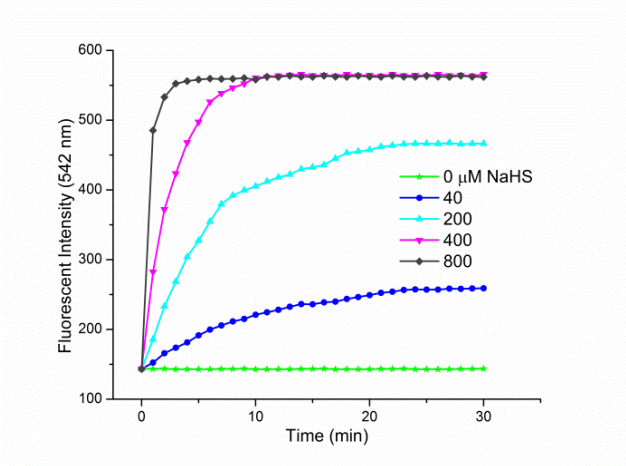


Figure 4. Time-dependent fluorescence intensity changes of probe (10 μM) at 542 nm upon addition of varied concentrations of NaHS. Conditions: excitation wavelength is 416 nm, acetonitrile-PBS buffer solution (60 mM, pH 7.4, 1:9 v/v) at 25°C .

from the spirolactam ring to open-ring form. Besides, a good linearity between the fluorescence intensity ratios and the concentrations of NaHS was obtained in the range of 0-200 μM (Figure 3C). According to fluorescence titration data, the limit of detection was calculated to be 0.39 μM.

The time-dependent fluorescence responses of probe were monitored (Figure 4). The results showed that probe was quite stable under the test conditions, and emission intensity at 542 nm gradually increased against time until reached a plateau at about 10 min when 40 equiv of NaHS was added. Although the longer time (25 min) was required to meet the signal saturation while treated with the lower concentration of NaHS, distinct fluorescence signal changes could be observed within 15 min.

It is well known that the performance of a fluorescent probe is highly dependent on the pH of the medium, thus, a pH-dependent fluorescent responses was carried out. As shown in Figure 5, I_{542}/I_{474} of probe was hardly affected between broad range of pH from 1.0 to 11.0. Upon addition of NaHS, it could respond to H_2S with a remarkable I_{541}/I_{474} enhancement from pH 6.0 to 11.0. These results indicated the good fluorescence responses of probe for physiological environment application.

The optical changes of **CR-FBA** in the presence of NaHS suggested that H_2S specifically triggered the nucleophilic addition-cyclization cascade reaction, simultaneously; the compound **FRET dyad CR** was released. To confirm this,

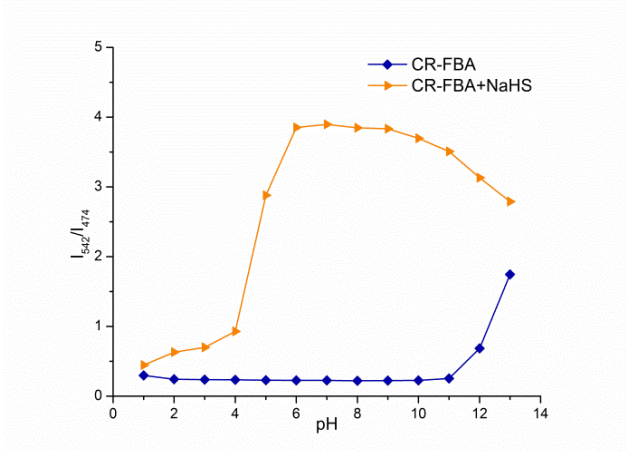


Figure 5. Effects of pH on the fluorescence of **CR-FBA** (10 μM) in the absence or presence (400 μM) of NaHS. Conditions: excitation wavelength is 416 nm, acetonitrile-PBS buffer solution (60 mM, 1: 9 v/v) at 25 °C for 25 min.

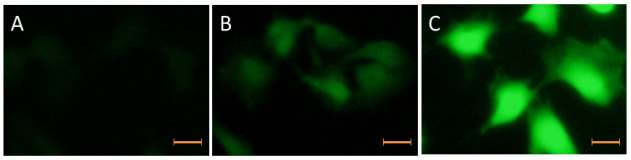


Figure 6. Fluorescence response of **CR-FBA** with NaHS in living H9C2 cells. The cells were pre-treated with the probe (10 μM) for 20 min, and then incubated with NaHS (A) 0 μM, (B) 100 μM, (C) 400 μM for 25 min. Blue excitation (420-485 nm), green emission (> 515 nm), Scale bars: 10 μm.

HPLC-MS measurements were carried out (Figure S7). The results demonstrated that the reaction between probe and NaHS proceeded as illustrated in Scheme 1.

We thereafter sought to apply whether **CR-FBA** can sense H_2S in living cells. The cell line H9C2 was selected as a bioassay model. Incubation of H9C2 cells with probe (10 μM) for 20 min at 37 °C was followed by the addition of NaHS for another 25 min. Subsequently, fluorescence imaging experiments were carried out by a fluorescent inverted microscope. As shown in Figure 6, H9C2 cells showed almost no fluorescence with only probe, while in the presence of probe and NaHS, displayed enhanced fluorescence. And more, with the increase of NaHS concentration, the fluorescence intensity increased as well. Therefore, these experiments results indicated that **CR-FBA** has the potential to be used to detect hydrogen sulfide in living cells.

In summary, we have developed a new colorimetric and ratiometric fluorescent probe **CR-FBA** for H_2S based on FRET mechanism and a novel addition-elimination cascade reaction. With the employing of 2-formylbenzoic acid as the trapper, **CR-FBA** was highly selective and sensitive to H_2S over other biologically relevant species to give color change from colorless to bright orange under naked eye observation. A good linearity between the fluorescence intensity ratios and the concentrations of NaHS in the range of 0-200 μM suggested that it can detect H_2S quantitatively with a great limit as low as 0.39 μM. Furthermore, preliminary fluorescence imaging experiments showed that probe has potential to assess intracellular H_2S levels change in H9C2 cells. In view of the increasing interest for biological research of H_2S, we expect that the probe has great potential for *in vitro* and *in vivo* applications as a functional and elucidative tool.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi. ·····.

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