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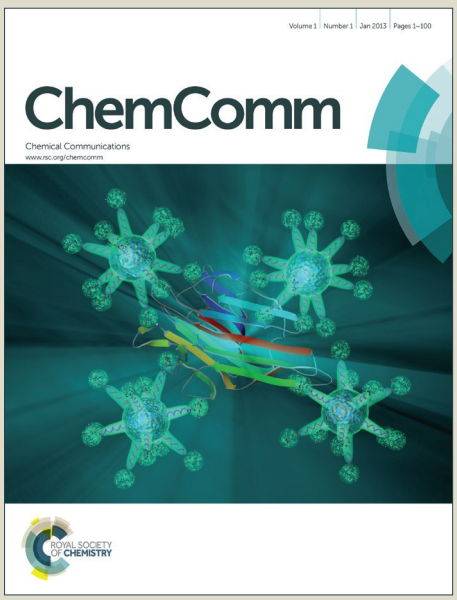

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A new ratiometric fluorescent probe for rapid, sensitive and selective detection of endogenous hydrogen sulfide in mitochondria

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We have developed a new ratiometric fluorescent probe composed coumarin–merocyanine dyad based on FRET mechanism. The probe showed clear dual-emission signal changes in blue and red spectral windows upon H_2S in a dose dependent manner under a single wavelength excitation. The probe targeted to mitochondria with high selectivity and sensitivity toward H_2S.

Hydrogen sulfide (H_2S) is an important endogenous gasotransmitter which is associated with diverse physiological processes, such as vasodilation,[1] angiogenesis,[2] apoptosis,[3] inflammation,[4] and neuromodulation.[5] It has been reported that the physiological concentration of H_2S in mammalian serum is in the 30–100 μM range, whereas those in the brain can be as high as 160 μM.[6] However, abnormal H_2S levels are implicated in many diseases such as Alzheimer's disease,[7] Down's syndrome,[8] diabetes,[9] and liver cirrhosis.[10]

Heightened research interest into the mechanism of the physiological and pathological functions of H_2S has led to the development of fluorescent probes that can easily and reliably detect and quantify H_2S in a living biological system.[11] Most of them were designed based on the strong nucleophilicity[12] and reduction capability of H_2S,[13] as well as H_2S-mediated metal sulfide precipitation from fluorophore-ligated metals.[14] Most of the probes based on the reduction of aromatic azide by H_2S showed a delayed response. The probes based on nucleophilic substitution or addition reaction suffered the possible interference from biological thiols. Therefore, a challenging issue in developing fluorescent probes for H_2S is how to secure selectivity over competing biothiols. Moreover, most of the probes based on fluorescent intensity may be influenced by many environmental effects.[15] However, ratiometric fluorescent probes, in contrast, can eliminate these shortcomings by self-calibration of two emission bands, often corresponding to the unreacted probe and its subsequent reaction product.[16a]

A number of ratiometric fluorescent probes were developed based on Förster resonance energy transfer (FRET). FRET is the interaction between two fluorophores linked by a non-conjugated spacer in the same molecule. The fluorophore with shorter wavelength emission as the energy donor may collect radiation at excitation wavelength and transfer this energy to the longer wavelength-emitting fluorophore (the acceptor). FRET-based sensing system can rule out any influence of excitation backscattering effects on fluorescence detection because of the large shift between donor excitation and acceptor emission. In addition, two well-separated emission bands with comparable intensities can ensure accuracy in determining their intensities and ratios. However, developing FRET cassettes is still limited by the requirement that the donor emission must have more overlap with the acceptor absorption. Therefore, consideration must be given to both spectral overlap and the emission shifts of FRET systems.[12b] In addition, other factors should also be considered in the design of probes for biological systems, such as high photo-stability, high quantum yield, good solubility in aqueous and lipid environments, good cell permeability, and low interference from biological environments. Xanthene dyes are usually used as acceptor in the FRET dyad.[16b] A spectral overlap between the donor emission and the acceptor absorption can be controlled by ring-opening or ring-closing of xanthene.[16c] To date, a lot of xanthene-based FRET sensors have been reported for cations, anions and biomolecules.[17] Besides, the FRET process can be controlled by the cleavage of an appropriate linker.[18] Recently, the coumarin–naphthalimide dyad has been developed as a FRET platform because of the coumarin emission well overlaps with the naphthalimide absorption.[19] However, the less emission shifts of these FRET systems seem not so satisfactory. Yuan et al reported a new FRET system with two-photon excitation in which anthocyanidin-analogue dye was an acceptor. The probe showed distinguished improvements in ratiometric response

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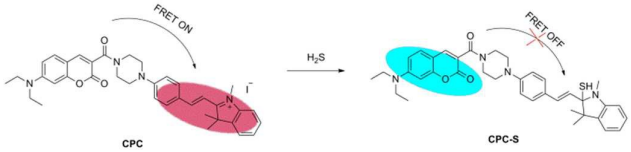
with a large emission shift and comparative fluorescence intensity.[12b]



Scheme 1 A proposed novel ratiometric fluorescent H_2S probe

Though numerous examples of fluorescence-sensing strategies in the detection of H_2S are reported, FRET system remains underused for sensing H_2S.[12b,19b,c] Therefore, developing advanced probes with high sensitivity, selectivity and fast-response for real time visualization of low concentration H_2S in living biological samples is still needed. Especially, developing a new strategy for ratiometric fluorescent probe remains challenging. In this work, we have developed a new strategy to construct a ratiometric fluorescent probe based on the FRET process. The ratio emission signal of the coumarin–merocyanine dyad is controlled through the nucleophilic addition of H_2S to the acceptor (Scheme 1). The probe (CPC) exhibited a large emission shift compared with most reported probes for H_2S. Prior to reaction with H_2S, the probe has a significant overlap of the coumarin emission with the merocyanine absorption and the FRET process occurs, and the dyad displays merocyanine fluorescence. However, interaction of merocyanine moiety with H_2S affords nucleophilic addition product **CPC-S**, which is not an acceptor fluorophore due to the destruction of conjugate system, and the FRET process does not exist. The dyad displays coumarin emission. Thus, the dyad **CPC** might detect H_2S as a ratiometric fluorescent probe based on the new FRET strategy. Probe **CPC** was synthesized by convenient coupling reactions (Scheme S1, ESI) and fully characterized (see ESI).

We first examined the emission spectra of probe **CPC** in PBS buffer (0.01 M, pH 7.4). As designed, upon excitation at 410 nm, the free probe exhibits weak coumarin fluorescence at 474 nm and strong merocyanine fluorescence at 587 nm. The distinct gap between the two bands is over 113 nm, which makes this probe favorable for the dual emission ratiometric imaging. These results are consistent with our design that the coumarin fluorescence in **CPC** is quenched through the FRET process. However, upon addition of Na_2S (a standard source for hydrogen sulfide), a significant merocyanine acceptor emission peak at around 587 nm disappeared and simultaneously the emission peak of the coumarin donor obviously enhanced (Fig. S1, ESI), suggesting that conjugate system of acceptor in **CPC** was destroyed to afford an addition product **CPC-S** in the presence of Na_2S and FRET from the coumarin unit to the merocyanine was effectively blocked.

To clarify the new FRET mechanism, we synthesized the **donor** and the **acceptor**, respectively. Upon addition of Na_2S, the fluorescence intensity of the **Donor** in the same buffer solution did not change, however, that of the **Acceptor** decreased dramatically (Fig. S2 S3, ESI). It suggested that the merocyanine moiety of probe **CPC** interacted with Na_2S while the coumarin moiety did not. Notably, the coumarin emission with the merocyanine absorption has a significant overlap (Fig. S4, ESI). Thus the FRET process occurs effectively. When **CPC** was excited at maximum absorption wavelength of the coumarin, the typical coumarin emission at about 474 nm was nearly completely quenched compared with that of the reference **Donor**. Meantime, the fluorescence intensity of the merocyanine moiety at around 587 nm appeared relative to that of the reference **Acceptor** at the same concentration (Fig. S5, ESI). This suggested that the excitation energy of the coumarin donor was efficiently transferred to the merocyanine acceptor. The intramolecular energy transfer efficiency from the donor to the acceptor in **CPC** was calculated to be 98.35%. The efficient intramolecular energy transfer was collaborated by the excellent correspondence between the absorption and excitation spectra of the probe (Fig. S6, ESI).

The specific nature of probe **CPC** for H_2S was also evaluated by UV-vis absorption in the buffer solution. Probe **CPC** exhibited two major absorption band centered at 418 and 518 nm, which were assigned to the donor and the acceptor, respectively. Upon addition of different representative interfering species, no clear change in the absorption maximum was observed. However, upon addition of H_2S, the absorption at around 518 nm disappeared while the absorption at around 418 nm remains almost no change (Fig. S7, ESI). The changes in the absorption spectra were in good agreement with the variations in the emission profile. These results indicated that probe **CPC** had clear selectivity in absorption toward H_2S than other tested analytes.

To examine the selectivity, probe **CPC** (5 μM) was treated with various biologically relevant species including the representative anions, reactive oxygen species, reducing agents, small molecule thiols, and Na_2S in the aqueous buffer. Addition of these representative interfering species induced no marked ratio increase. However, just addition of 50 μM Na_2S can elicit remarkable increase in the ratio value (I_{474}/I_{587}) (Fig. S8, ESI). The reaction was completed within 7 min and the ratio value peaked. Thus, probe **CPC** could fast sense H_2S in a real-time in vivo imaging (Fig. S9, ESI). In addition, the **CPC** ratiometric sensing ability for H_2S in the presence of other biological thiols was also studied (Fig. S10, ESI). These results suggested that probe **CPC** has a high selectivity toward H_2S over other biological species and is able to detect H_2S without any distinct interference from these biological thiols. Moreover, an increase in fluorescence signal ratios was observed at physiological pH (Fig. S11, ESI).

To obtain further insight into the sensitivity of probe **CPC** for H_2S, the fluorescence intensity change was closely monitored upon the addition of H_2S (0–11 equiv.) into the probe (Fig. 1). Fluorescence titration demonstrated that its emission band at 587 nm decreased distinctly, meanwhile the band at 474 nm increased obviously. The intensity ratio of the two emission bands (I_{474}/I_{587}) increased from 0.73 to 40.93, and the final enhancement factor is about 56-fold. The fluorescence intensity remained changeless after the concentration of H_2S was up to 55 μM (Fig. S12, ESI). The intensity ratio was linearly related to the concentration of H_2S and the detection limit was

calculated to be as low as 40 nM (Fig. S13, ESI), which is superior to some reported probes suffering from the limitation of poor detection limit.[12h-k, 13b, 13e, 18b, 20] These results suggest that probe **CPC** is highly sensitive to H_2S and is potentially useful for quantitative determination of H_2S. Moreover, upon addition of Na_2S, the absorption peak at 518 nm decreased gradually and another peak located at 418 nm changed little (Fig. S14, ESI). The colour changed from red to light yellow (Insert of Fig. S14, ESI).

Although our attempt to separate HS^- nucleophilic addition product **CPC-S** was failed, the Thermo LCQ Fleet mass spectrum of **CPC** solution in the presence of excess Na_2S showed one major signal with m/z of 623.15 and another minor peak with m/z of 645.98, which can be assigned as $[CPC\text{-}S + H]^+$ and $[CPC\text{-}S + Na]^+$, respectively (Fig. S15, ESI).

The desirable fluorescence properties of probe **CPC** for H_2S prompted us to utilize it for the detection of intracellular H_2S. HeLa cells stained by probe **CPC** (3 μM) alone showed very dim fluorescence in the blue channel and a strong fluorescence in the red channel. Incubation with Na_2S (0.5, 1 and 5 mM) for 1 h led to a distinct increase in blue channel, accompanied by the dramatic drop of red channel fluorescence, and a drastic enhancement of emission ratio (blue/red) in a dose dependent manner can be observed (Fig. 2).

Encouraged by these promising results, we evaluated the feasibility of probe **CPC** for detecting endogenous H_2S. It is known that cysteine could be catalyzed by cystathionine β-synthase (CBS) and cystathionineg γ-lyase (CSE) in living cells for H_2S production.[19b] As shown in Fig. 3, without addition of cysteine, strong fluorescence in the red channel and faint fluorescence in the blue channel were observed. However, after the cells were incubated with cysteine, a fluorescent emission in the red channel decreased and a significant emission in the blue channel increased. The ratios of emission intensity (blue/red) markedly increased in the presence of cysteine. The inhibition assay was also carried out by inhibiting CBS and CSE.[12e,21] When the cells were incubated in a sequence with propargylglycine (PAG) or aminooxyacetic acid (AOAA) or PAG/AOAA and Cys, followed by incubation with probe **CPC**, almost no fluorescence change was observed compared to the control group. Therefore, probe **CPC** is capable of detecting extraneous and endogenous H_2S in living cells.

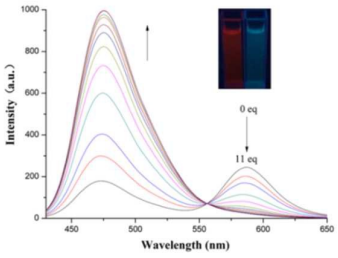


Fig. 1 Fluorescence spectra of the probe (5 μM) with the addition of Na_2S (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 equiv.) inPBS buffer (pH 7.4, 0.01 M) : DMF (6: 4, v/v). (λ_{ex} = 410 nm , slit: 12 nm/10 nm). Insert: a visual fluorescence change photograph under illumination with a 365 nm UV lamp.

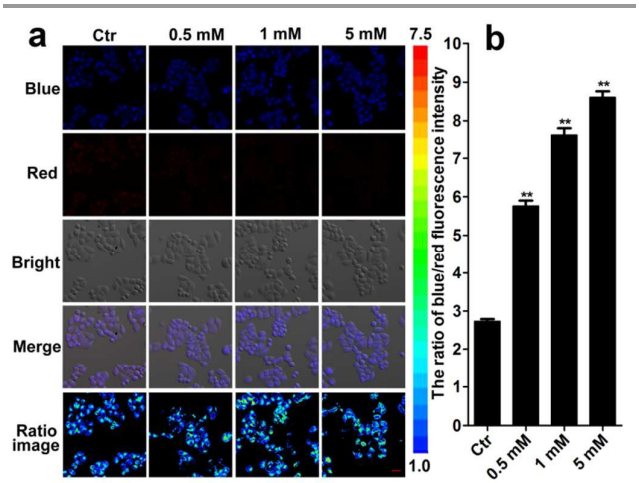


Fig. 2 (a) Fluorescence and bright field images and (b) the ratio of fluorescence intensity (blue/red) of HeLa cells incubated with probe **CPC** (3 μM) for 1 h and then with Na_2S (0.5, 1 and 5 mM) for 1 h. The ratio images were all obtained as F_{blue}/F_{red}. Excitation: 405 nm; emission channels: 405-560 nm (blue) and 560-700 nm (red). ** $P < 0.01$; (n = 3). Scale bar = 20 μm.

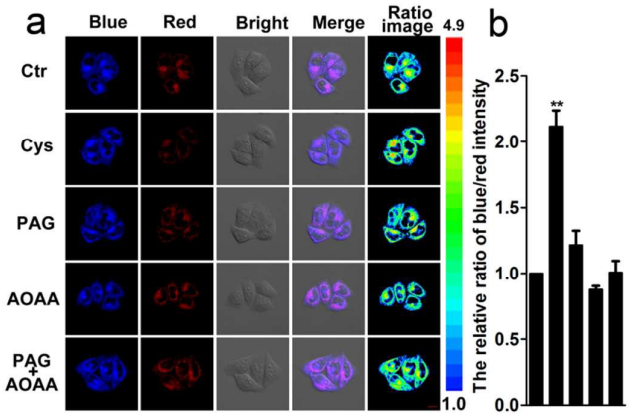


Fig. 3 (a) First line: cells incubated with **CPC** (1 μM) for 1 h; second line: cells incubated with Cys (200 μM) for 1 h followed by **CPC** (1 μM) for 1 h; third to fifth line: cells incubated with PAG (1 mM), AOAA (1 mM) and both PAG and AOAA for 1 h, respectively, followed by incubation with Cys (200 μM) for 1 h, then incubated with **CPC** (1 μM) for 1h. (b) From left to right: the relative ratio of fluorescence intensity of line 1, 2, 3, 4 and 5 in (a). The ratio images were all obtained as F_{blue}/F_{red}. Excitation: 405 nm; emission channels: 405-560 nm (blue) and 560-700 nm (red). ** $P < 0.01$; (n = 3). Scale bar = 20 μm.

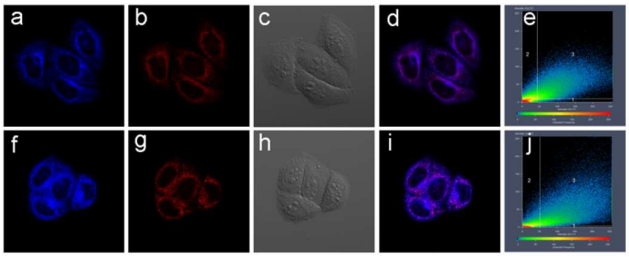


Fig. 4 Colocalization of the probe in HeLa cells. (a-e) probe **CPC** (1 μM, 1 h) and Mito Tracker Deep Red (0.3 μM, 0.5 h); (f-j) cells incubated with Na_2S (1 mM) for 1 h, followed by incubation with **CPC** (1 μM) for 1 h, then incubated with Tracker Deep Red (0.3 μM) for 0.5 h. (a, f) The cell image of **CPC**, λex = 405 nm, λem = 405-560 nm. (b, g) The cell image of Mito Tracker Deep Red, λex = 635 nm, λem = 635-700 nm. (c, h) Bright field image. (d, i) Merged image. (e, j) Quantitation of co-localization coefficients (Pearson's coefficient): 0.924 and 0.891.

Furthermore, a colocalization assay with Mito Tracker Deep Red and probe **CPC** displayed that fluorescence of probe **CPC** was colocalized with that of Mito Tracker Deep Red, implying a preferential distribution of probe **CPC** in mitochondria. In addition, the probe could rest on mitochondria after sensing H_2S (Fig. 4). Moreover, photostability of the probe was evaluated by the fluorescence images of HeLa cells exposed for different time. The ratio of blue/red fluorescence intensity indicated that the probe possesses good photostability during 720 S (Fig. S16, ESI). The SRB assay showed that probe **CPC** had little cytotoxicity in the 1-5 μM range for living cells (Fig. S17, ESI). This preliminary imaging study suggested that probe **CPC** can be used for ratiometric tracking of H_2S in mitochondria, which is of great importance to clarify the physiological roles of H_2S in the organelle.

In summary, a new ratiometric fluorescent probe composed coumarin–merocyanine dyad based on FRET mechanism was rationally designed and synthesized. The probe showed clear dual-emission signal changes in blue and red spectral windows upon H_2S in a dose dependent manner. The probe revealed high selectivity and sensitivity toward H_2S. The probe exhibited the acceptable biocompatibility and targeted to mitochondria. Therefore, probe **CPC** can be used for ratiometric tracking of extraneous and endogenous H_2S in mitochondria and might be a promising tool for the biological research of H_2S.

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