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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 H2S in a dose dependent manner under a single wavelength excitation. The probe targeted to mitochondria with high selectivity and sensitivity toward H2S.

Hydrogen sulfide (H2S) is an important endogenous gasotransmitter which is associated with diverse physiological processes, such as vasodilation, angiogenesis, apoptosis, inflammation, and neuromodulation. It has been reported that the physiological concentration of H2S in mammalian serum is in the 30–100 μM range, whereas those in the brain can be as high as 160 μM. However, abnormal H2S levels are implicated in many diseases such as Alzheimer’s disease, Down’s syndrome, diabetes, and liver cirrhosis.

Heightened research interest into the mechanism of the physiological and pathological functions of H2S has led to the development of fluorescent probes that can easily and reliably detect and quantify H2S in a living biological system. Most of them were designed based on the strong nucleophilicity and reduction capability of H2S, as well as H2S-mediated metal sulfide precipitation from fluorophore-ligated metals. Most of the probes based on the reduction of aromatic azide by H2S 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 H2S is how to secure selectivity over competing biothiols. Moreover, most of the probes based on fluorescent intensity may be influenced by many environmental effects.

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. 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. 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. A spectral overlap between the donor emission and the acceptor absorption can be controlled by ring-opening or ring-closing of xanthene. To date, a lot of xanthene-based FRET sensors have been reported for cations, anions and biomolecules. Besides, the FRET process can be controlled by the cleavage of an appropriate linker.

Recently, the coumarin–naphthalimide dyad has been developed as a FRET platform because of the coumarin emission well overlaps with the naphthalimide absorption. 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...
with a large emission shift and comparative fluorescence intensity.

Though numerous examples of fluorescence-sensing strategies in the detection of H\textsubscript{2}S are reported, FRET system remains underused for sensing H\textsubscript{2}S.\textsuperscript{12b,19b,c} Therefore, developing advanced probes with high sensitivity, selectivity and fast-response for real time visualization of low concentration H\textsubscript{2}S 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 nucelophilic addition of H\textsubscript{2}S to the acceptor (Scheme 1). The probe (CPC) exhibited a large emission shift compared with most reported probes for H\textsubscript{2}S. Prior to reaction with H\textsubscript{2}S, 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\textsubscript{2}S 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\textsubscript{2}S 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\textsubscript{2}S (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\textsubscript{2}S 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\textsubscript{2}S, 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\textsubscript{2}S 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 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\textsubscript{2}S was also evaluated by UV-vis absorption in the buffer solution. Probe CPC exhibited two major absorption bands 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\textsubscript{2}S, 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\textsubscript{2}S 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\textsubscript{2}S in the aqueous buffer. Addition of these representative interfering species induced no marked ratio increase. However, just addition of 50 μM Na\textsubscript{2}S can elicit remarkable increase in the ratio value (I\textsubscript{587}/I\textsubscript{474}) (Fig. S8, ESI). The reaction was completed within 7 min and the ratio value peaked. Thus, probe CPC could fast sense H\textsubscript{2}S in a real-time in vivo imaging (Fig. S9, ESI). In addition, the CPC ratiometric sensing ability for H\textsubscript{2}S in the presence of other biological thiol was also studied (Fig. S10, ESI). These results suggested that probe CPC has a high selectivity toward H\textsubscript{2}S over other biological species and is able to detect H\textsubscript{2}S without any distinct interference from these biological thiol.

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\textsubscript{2}S, the fluorescence intensity change was closely monitored upon the addition of H\textsubscript{2}S (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\textsubscript{587}/I\textsubscript{474}) 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\textsubscript{2}S was up to 55 μM (Fig. S12, ESI). The intensity ratio was linearly related to the concentration of H\textsubscript{2}S 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. These results suggest that probe CPC is highly sensitive to H₂S and is potentially useful for quantitative determination of H₂S. Moreover, upon addition of Na₂S, 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 (Inset 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 Na₂S 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-S + H]+ and [CPC-S + Na]+, respectively (Fig. S15, ESI).

The desirable fluorescence properties of probe CPC for H₂S prompted us to utilize it for the detection of intracellular H₂S. 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 Na₂S (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 H₂S. It is known that cysteine could be catalyzed by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) in living cells for H₂S production. 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. 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 H₂S in living cells.

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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$_2$S (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$_2$S in mitochondria, which is of great importance to clarify the physiological roles of H$_2$S 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$_2$S in a dose dependent manner. The probe revealed high selectivity and sensitivity toward H$_2$S. The probe exhibited the acceptable biocompatibility and targeted to mitochondria. Therefore, probe CPC can be used for ratiometric tracking of endogenous and exogenous H$_2$S in mitochondria and might be a promising tool for the biological research of H$_2$S.

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**Notes and references**