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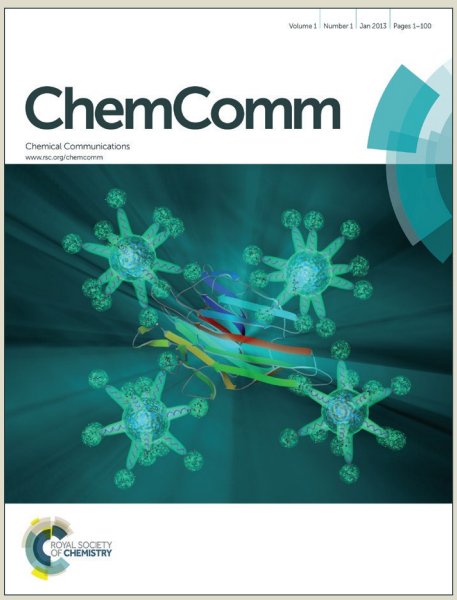

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A novel dual-emission fluorescent probe for simultaneous detection of H_2S and GSH

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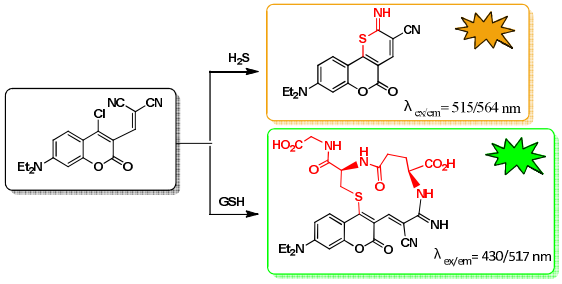
A novel chlorinated coumarin-malonitrile fluorescent probe was synthesized for simultaneous detection of H_2S and GSH from different emission channels.



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A novel dual-emission fluorescent probe for simultaneous detection of H_2S and GSH

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A novel chlorinated coumarin-malonitrile fluorescent probe with three potential reaction sites, which exhibited highly selective, rapid response, low detection limit, and simultaneous detection of H_2S ($\lambda_{ex/em}$= 515/564 nm) and GSH ($\lambda_{ex/em}$= 430/517 nm), was first proposed. The probe was successfully applied to dual-channel imaging H_2S and GSH in MCF-7 cells.

Hydrogen sulfide (H_2S) and biothiols such as Cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), play important roles in many physiological and physiological processes.[1] H_2S is involved in various physiological processes, such as antioxidation,[2] ischemia reperfusion injury,[3] anti-infammation,[4] and apoptosis.[5] Cys displays diverse functions in regulating of matrix degradation and cell motility.[6] GSH plays a central role in combating oxidative stress, maintaining redox homeostasis, and defending against toxins and free radicals.[7] Abnormal levels of H_2S or biothiols are associated with various diseases.[8] More importantly, there is a significant correlation between H_2S level and biothiols level in the living systems.[9] Hence, the determination of H_2S and biothiols in biological systems is required for better understanding their roles in biological systems and accurate diagnosis of disease.[10] Till now, a number of fluorescent probes have been developed for the detection of H_2S based on their several significant characteristic properties: dual-nucleophilicity,[11] high binding affinity towards Cu^{2+} ion,[12] reduce the azide, nitro and hydroxy-amine groups,[13] and thiolysis of dinitrophenyl ether.[14] The discrimination of Cys from Hcy/GSH was achieved based on the Cys-induced S_NAr substitution–rearrangement reaction,[15] Michael addition,[16] or cyclization reaction with –CHO.[17] And, the discrimination of GSH from Cys/Hcy was proposed based on deprotection of 2,4-dinitrobenzenesulfonyl-conjugated fluorophore,[18] selective cleavage of the selenium-nitrogen bond,[19] and GSH-induced substitution-cyclizatioin.[20] In fact, the determination of H_2S and biothiols is important to clarify their complicated relationship in various physiological processes. In spite of the amazing progress has been made in distinguish H_2S or GSH from other biothiols, to design a single fluorescent probe displaying a distinct response to H_2S and GSH simultaneously from different emission channels is highly valuable but even more challenging. In the current study, we present a novel fluorescent probe, chlorinated coumarin-malonitrile (1), which could discriminate H_2S and GSH in different emission channels. Particularly, the probe exhibits several meritorious features as follow: (i) high sensitivity and selectivity to H_2S and GSH from different emission channels; (ii) significant fluorescence enhancement based on different structures; (iii) rapid response; (iv) excellent live cell imaging.

The design rationale is depicted in Fig 1. The probe 1 has three potential reaction sites, utilizing coumarin as a fluorophore, chloro atom lies in 4-position of coumarin moiety as a leaving group (Site I), α,β–unsaturated malonitrile as a Michael acceptor (Site II), and cyano group as an electrophile (Site III) and a quencher. It displays an extremely low background signal for the electron withdrawing of α,β–unsaturated malonitrile owing to intramolecular charge transfer (ICT) and photoinduced electron transfer (PET) process. We speculate that probe 1 discriminates H_2S from biothiols and the other biologically related species based on the dual-nucleophilicity of H_2S. Probe 1 reaction with H_2S provided thio-coumarin-malonitrile (1a) by undergoing rapid thiol-halogen nucleophilic aromatic substitution (S_NAr). Subsequently, the intramolecular nucleophilic-addition adduct (iminocoumarin, 1b, Fig 1A) was constructed by intramolecular nucleophilic attack of the thiophenol to site 3,[21] which resulted in blocking the ICT and PET process, and increased the π-conjugation system. Now, let us turn our attention to the reaction of 1 with GSH. Similar initial thio-halogen S_NAr would lead to thio-coumarin-malononitrile 2a (Fig 1B). However, due to the unstable 10-membered macrocyclic transition state, it is difficult for 2a to undergo the ring intramolecular

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rearrangement to generate amino-coumarin-malononitrile.[16e] Alternatively, it is possible that the free thiol group in 2a would attack site 2 or 3 to produce the 12- or 14-membered ring product glutathione-coumarin-malononitrile 2b or 2c. In fact, the speculation could be partially supported by recent publications on large ring products.[16e,16f, 20] To confirm the final product (2b), we choose DODT as a model compound. ^{1}H NMR (Fig. S12, ESI†) of product obtained by adding DODT to 1 indicated that the free thiol group attacked site 3 to produce the corresponding ring product (Fig. S8E, ESI†). Overall, if our hypothesis is rational, it would be promising to realize the selective discrimination of H_2S and GSH in terms of the different chemical structures and photophysical properties of the corresponding 1b and 2b. As for Cys/Hcy (Fig S8C and S8D, ESI†), site 1 of 1 was attacked by thiol group of Cys/Hcy to produce thio-coumarin-malononitrile 3a/4a, and the following rearrangement would lead to generate amino-coumarin-malononitrile 3b/4b, and the following Michael addition reaction of the thiol group with site 2 form a seven/eight-membered cyclized adduct 3c/4c. Meanwhile, the site 3 reactions with excessive Cys/Hcy generates condensation product 3d/4d with similar photophysical properties. So, it is difficult to discriminate between Cys and Hcy with different fluorescence signals.

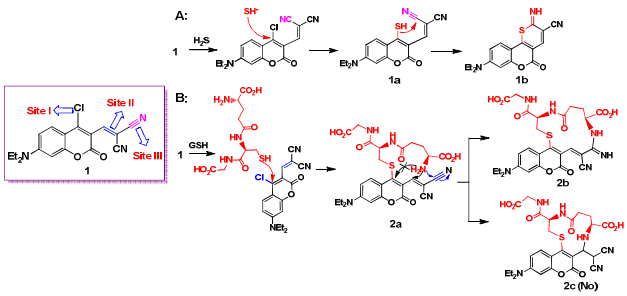

Fig. 1 Sensing scheme of 1 with H_2S and GSH

Initially, we synthesized probe 1 (Scheme S1, ESI†), and examined the optical sensing behaviour by utilization time-dependent UV–vis spectrum of 1 reaction with H_2S or GSH and Cys/Hcy in DMF/phosphate buffer (*V/V*: 3/7, 10 mM, pH 7.4) at r.t. (Fig. S1, ESI†). Upon addition of H_2S or GSH/Cys/Hcy to probe 1, UV-vis spectra are dramatically changed, a peak at λ_{max}= 505 nm disappeared while new peaks around 350 to 470 nm appeared. The profound blue-shift indicated that the conjugation between the coumarin and the malonitrile is broken due to the Michael addition of GSH/Cys/Hcy to 1. Treated with 10 equiv of H_2S in PBS buffer (10 mM, pH 7.4) at 25 °C, the product mixture exhibited a significant fluorescence enhancement at $\lambda_{ex/em}$= 515/564 nm (Fig. 2A). As a result, the new emission peak appeared and reached a plateau after 6 min, indicating the completion of the reaction. In this case, an approximate 16-fold increase in fluorescence signal could be observed. As shown in Fig. 2B, upon addition of GSH to the solution of 1 leaded to a fluorescence enhancement of 7.5-fold at 517 nm (λ_{ex} = 430 nm). These results indicated that the probe 1 could reaction with H_2S and GSH, offered different products and exhibited different optical properties. Furthermore, the absolute fluorescence quantum yield of 1b and 2b was determined to be 23.84% and 5.94% at the concentration of 3×10^{-6} M in PBS at 515 and 430 nm excitations. These results indicate that 1b possesses high fluorescence properties in water at low concentrations. So, probe 1 could selectively detect H_2S and GSH in the respective detection channels.

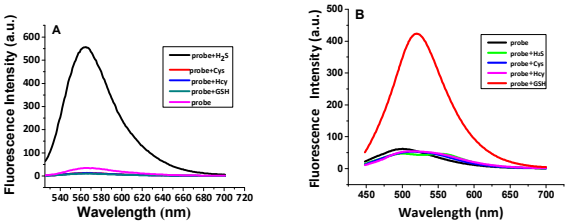

Fig. 2 Fluorescence response of probe (5 μM in PBS buffer) to 10 equiv. of H_2S (**A**, λ_{ex}= 515 nm, Slits: 5/5 nm), Cys, Hcy and GSH (**B**, λ_{ex}=430 nm, Slits: 10/10 nm) at r. t.

To apply this probe in more complicated systems, we also tested the effect of pH on the fluorescence response of 1 to H_2S and GSH (Fig. S5, ESI†). It was found that this probe is stable enough in pH range of 6-9, and displayed the obvious response for H_2S and GSH in the neutral or weak base condition at 515 and 430 nm excitation. Due to the neutral or weak base condition enhancement the nucleophilic property of H_2S and GSH that led to speed up the thio-chlorin S_NAr and intramolecular nucleophilic addition of 1 with H_2S and GSH. The stable fluorescence of 1 at neutral condition is favourable for the selective detection of H_2S and GSH in biological samples.

Subsequently, we examined the emission behaves of 1 towards the mixture of H_2S, Cys, Hcy and GSH (Fig. S3**,** ESI†). Meanwhile, the time-dependent fluorescence response of 1 to Cys and Hcy in the same conditions was investigated (Fig. S4**,** ESI†). It was found that Cys/Hcy/GSH hardly elicited any significant fluorescence changes of 1 response to H_2S. As well, H_2S/Cys/Hcy hardly elicited any significant fluorescence changes of 1 response to GSH. The above results confirmed that probe 1 exhibited the capability of highly selective detection of H_2S and GSH from different emission channels.

Further, we evaluated the changes in the emission spectra of probe upon addition of different concentrations of H_2S and GSH were in DMF/phosphate buffer (3/7: *V/V*, 10 mM, pH 7.4) solution at room temperature. As show in Fig 3, it was found that approximate 2 equiv of H_2S (equiv of GSH) could complete the reactions. In addition, the fluorescent intensity was linearly proportional to the amount of them from 0 to 2 equiv and the linear correlations (R^2 = 0.99788 for H_2S, and 0.99577 for GSH) were found between the observed fluorescence intensity and concentrations. The detection limits (DL, S/N= 3) for H_2S and GSH were determined to be 42 and 87 nM, respectively. In addition, the sensing reactions can be completed within 6 min (Fig. S2, ESI†). Because the intracellular concentration of H_2S and GSH are in the range of l0-l00 μM [22, 23] and 1–10 mM,[24] respectively. So, probe 1 is sensitive enough to image H_2S and GSH in cells.

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To evaluate the specific nature of 1 for sensing of H_2S and GSH, we then examined the fluorescence enhancement of 1 incubated with various species (Fig. S6, ESI†), all of which are biologically related. At 515 nm excitation, this probe exhibited a very limited fluorescence enhancement to various amino acids (eg. Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Gly, Ser, Thr, Glu, Lys, Arg, Tyr, His, Asp, Glu, and Asn), glucose, ascorbic acid, and thiols (Cys, Hcy, and GSH); only H_2S elicited significant fluorescence turn-on of probe 1 at 564 nm. At 430 nm excitation, various amino acids, glucose, ascorbic acid, H_2S, Cys, and Hcy triggered almost no fluorescence intensity changes, and only GSH caused significant fluorescence turn-on at 517 nm. These results demonstrate that this probe is high selective for H_2S and GSH over other competitive species at different excitation.

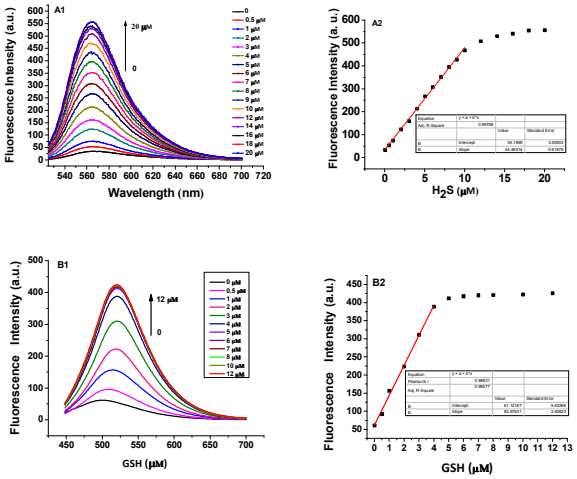

Fig. 3 Fluorescence spectra of 1 (5 μM in PBS buffer) upon addition of varied concentrations of H_2S (**A**, λ_{ex}= 515 nm, Slits: 5/5 nm), and GSH (**B**, λ_{ex}=430 nm, Slits: 10/10 nm) at r. t.

Cytotoxicity is an important indicator for applicability of probe in biological systems. Therefore, the effect of 1 on cell proliferation was determined by MTT assay in MCF-7 cells. Probe 1 showed no cytotoxicity at a detection range concentration (0.1-10 μM) (Fig. S7, ESI†). These results implied that this probe was suitable for H_2S and GSH detection in live cells.

Furthermore, the products of probe 1 with H_2S and GSH were evidenced by HRMS. The mixture of H_2S and probe 1 in CH_3CN-H_2O (1/1, *V/V*) was separated, and showed a major signal at *m/z* 348.07791 (calcd. 348.07772; Fig. S14, ESI†) assigned to $[1b+Na]^+$ ($C_{17}H_{15}N_3NaO_2S$). The formation of 1b was also evidenced by ^1H NMR study. Upon addition of NaHS to 1, an imine proton (Ha) was appearance at 11.1 ppm, and the vinyl proton (Hb) was obvious upfield shifts, as well as the benzol protons (Hc, Hd) (Fig. S11, ESI†). The product mixture of GSH with probe 1 in CH_3CN-H_2O (1/1, *V/V*) solution, exhibits a peak at *m/z*: 597.1784 correspond to $[2c-H]^-$ (calcd. 597.1768 for $C_{27}H_{29}N_6O_8S$, Fig. S17, ESI†). In addition, the products of probe 1 with Cys and Hcy were also investigated. A prominent peak at *m/z*: 517.1217 corresponding to $[3d+H]^+$ (calcd 517.1215 for $C_{23}H_{25}N_4O_6S_2$) was clearly observed in the HRMS data of reaction probe 1 with Cys in CH_3CN-H_2O (1/1, *V/V*) solution (Fig. S15, ESI†). A similar result of probe 1 with Hcy was observed, a peak at *m/z*: 545.1520 attributing to $[4d+H]^+$ (calcd. 545.1528 for $C_{25}H_{29}N_4O_6S_2$, Fig. S16, ESI†). These results are in good agreement with the designed strategy. Above all results would be promising the selective discrimination of H_2S and GSH in terms of the different photophysical properties.

Finally, we investigated the potential use of probe 1 in live cell imaging. When MCF-7 cells were incubated with it for 30 min, bright fluorescence in blue and red channels was observed simultaneously (Fig. 4). Furthermore, when the cells were pre-treated with *N*-ethylmaleimide (NEM, a scavenger of H_2S and biothiols) and then treated with 1, the fluorescence in the red and blue channels was both decreased dramatically, conforming that probe 1 is a specific tool for the detection of endogenous H_2S and GSH in actual living cells. These results showed that probe 1 not only discriminates H_2S and GSH at the blue and red channels, but also can be employed to monitor endogenous H_2S and GSH level in living cells.

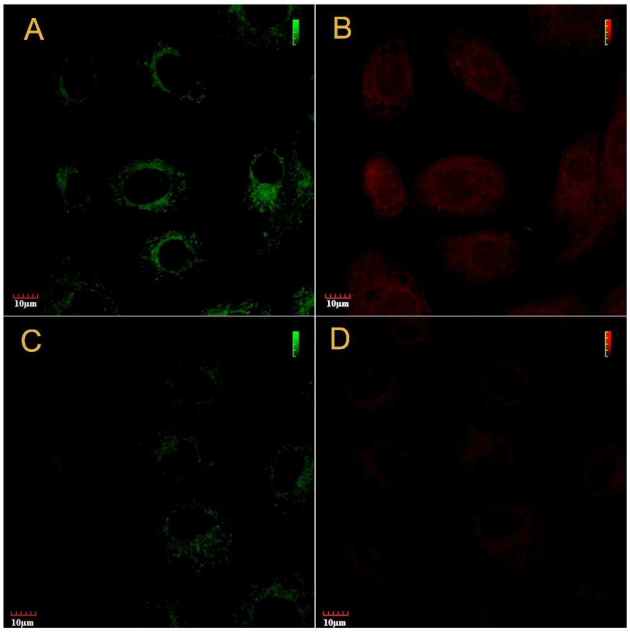

Fig. 4 Imaging H_2S and GSH in MCF-7 cells by probe 1 from different emission channels, emission was collected at 450–510 nm for green channel assigned to GSH (λ_{ex}=405 nm), and 550–610 nm for red channel assigned to H_2S (λ_{ex}= 515 nm).
Cells in A and B were incubated with 10 μM probe 1 for 30 min.
Cells in C and D were first incubated with NEM for 30 min, and then were incubated with 10 μM probe for 30 min.

In summary, we presented a novel chlorinated coumarin-malononitrile fluorescence probe with three potential reaction sites for discrimination H_2S and GSH from different emission channels (H_2S: $\lambda_{ex/em}$= 515/564 nm with 16-fold increase in fluorescence, GSH: $\lambda_{ex/em}$= 430/517 nm with 7.5-fold increase in fluorescence) based on different chemical structures with distinct photophysical properties. This probe exhibits a high selectivity for H_2S and GSH, low limit of detections (DL for H_2S and GSH were 42 and 87 nM), and fast response speeds. And,

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the probe could simultaneously monitoring endogenous H_2S and GSH in MCF-7 cells with only minimal cytotoxicity. We hope that the novel strategy could inspire the exploration of new systems to reveal H_2S and GSH levels in biological systems.

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