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A fast-responsive fluorescent probe based on a styrylcoumarin dye for visualizing hydrogen sulfide in living MCF-7 cells and zebrafish†

 Xu Tong,^a Liguao Hao,^b Xue Song,^a Shuang Wu,^a Na Zhang,^a Zhongtao Li,^b Song Chen^c and Peng Hou^{ib*}

As a vital antioxidant molecule, H₂S can make an important contribution to regulating blood vessels and inhibiting apoptosis when present at an appropriate concentration. Higher levels of H₂S can interfere with the physiological responses of the respiratory system and central nervous system carried out by mammalian cells. This is associated with many illnesses, such as diabetes, mental decline, cardiovascular diseases, and cancer. Therefore, the accurate measurement of H₂S in organisms and the environment is of great significance for in-depth studies of the pathogenesis of related diseases. In this contribution, a new coumarin-carbazole-based fluorescent probe, COZ-DNBS, showing a rapid response and large Stokes shift was rationally devised and applied to effectively sense H₂S *in vivo* and *in vitro*. Upon using the probe COZ-DNBS, the established fluorescent platform could detect H₂S with excellent selectivity, showing 62-fold fluorescence enhancement, a fast-response time (<1 min), high sensitivity (38.6 nM), a large Stokes shift (173 nm), and bright-yellow emission. Importantly, the probe COZ-DNBS works well for monitoring levels of H₂S in realistic samples, living MCF-7 cells, and zebrafish, showing that COZ-DNBS is a promising signaling tool for H₂S detection in biosystems.

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1. Introduction

In the process of maintaining biological homeostasis, reactive sulfur species (RSS), including hydrogen sulfide (H₂S) and biological mercaptans (Cys, Hcy, and GSH), are essential thiol-containing molecules for resisting oxidative stress and maintaining the normal physiological function of organisms.^{1–3} Numerous research works have revealed that H₂S is considered to be a third endogenous gaseous transmitter after nitric oxide (NO) and carbon monoxide (CO).^{4–6} As a vital antioxidant molecule, H₂S can make an important contribution to the regulation of blood vessels and inhibition of apoptosis when present at an appropriate concentration.^{7–9} Reports in the literature have suggested that the level of intracellular H₂S can be significantly upregulated during inflammatory events. Higher levels of H₂S can interfere with the physiological responses of the respiratory system and central nervous system carried out by mammalian cells. This is associated with many illnesses, such as diabetes, mental decline, cardiovascular diseases, and cancer.^{10–13} Therefore, the accurate measurement of H₂S in organisms and the

environment is of great significance for in-depth studies on the pathogenesis of related diseases.

Fluorogenic assays utilizing small-molecule fluorescent sensors have attracted wide attention in pharmacology and the life sciences because of the obvious advantages of non-invasive detection, excellent selectivity, rapid response, and the lack of need for pretreatment.^{14–18} To date, numerous H₂S-specific fluorescent probes have been established *via* exploiting various sensing mechanisms,^{19–24} such as the thiolysis of 2,4-dinitrobenzenesulfonamide and dinitrophenyl ether groups; the reduction of azide, hydroxylamine, and nitro groups; nucleophilic addition with C=N⁺ groups; and the high affinity of S^{2–} for Cu²⁺. Although big breakthroughs have been made, some problems still need to be overcome, such as slow responses, small Stokes shifts (<100 nm), synthetic complexity, and low sensitivity, which have restricted the application of these sensors in the fields of biochemistry and biomedicine. A fluorescent probe with a large Stokes shift is more preferred because it is relatively easy to reduce self-quenching and auto-fluorescence during fluorescence sensing.^{25–27} This method can remarkably improve the detection accuracy. Moreover, a rapid response is an important index for optical sensors used for the real-time monitoring and bio-imaging of H₂S in biological systems. Thus, the development of a H₂S-specific fluorescent probe with excellent properties is particularly necessary.

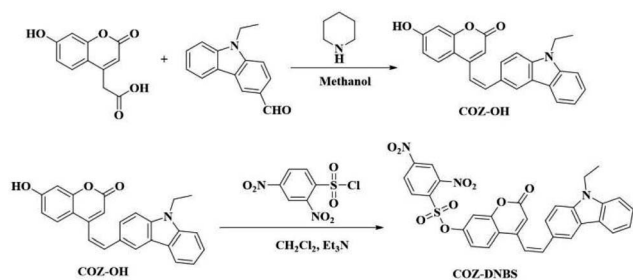
Combining all these considerations, in this work, a new coumarin-carbazole-based fluorescent probe, COZ-DNBS, with

^aThe Third Affiliated Hospital, Qiqihar Medical University, Qiqihar 161006, China

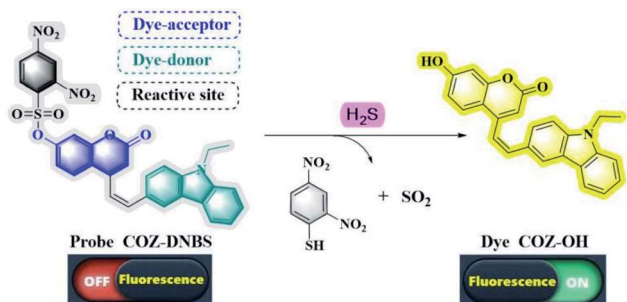
^bCollege of Medical Technology, Qiqihar Medical University, Qiqihar 161006, China

^cCollege of Pharmacy, Qiqihar Medical University, Qiqihar 161006, China. E-mail: houpeng1982@163.com

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Scheme 1 The synthetic route to the new fluorescent probe COZ-DNBS.



Scheme 2 The proposed mechanism involving the fluorescent probe COZ-DNBS for H₂S detection.

a rapid response and large Stokes shift was rationally devised and applied to effectively sense H₂S *in vivo* and *in vitro*. The concrete synthesis route for COZ-DNBS is depicted in Scheme 1, and the structures of COZ-OH and COZ-DNBS were characterized *via* HRMS and ¹³C-NMR and ¹H-NMR spectroscopy (Fig. S2–S7†). Structurally, in order to lengthen the emission wavelength, a carbazole group was modified onto the coumarin core to extend the π -conjugation structure. 2,4-Dinitrobenzenesulfonyl (DNBS), an outstanding fluorescence quenching moiety, served as the reactive site for H₂S,^{28,29} while the newly synthesized coumarin-carbazole dye COZ-OH was employed as the fluorophore (Scheme 2). When using the probe COZ-DNBS, the established fluorescent platform could detect H₂S with excellent selectivity, showing 62-fold fluorescence enhancement, a fast response (<1 min), high sensitivity (38.6 nM), a large Stokes shift (173 nm), and bright-yellow emission (Table S1†). Importantly, the probe COZ-DNBS works well when monitoring levels of H₂S in realistic samples, living MCF-7 cells, and zebrafish, showing that COZ-DNBS is a promising signaling tool for H₂S detection in biosystems.

2. Experimental

2.1 Instruments and reagents

HRMS (high-resolution mass spectroscopy) analysis was carried out using AB Sciex TripleTOF 4600 apparatus. ¹H- and ¹³C-NMR (nuclear magnetic resonance) spectra were obtained using a Bruker Avance 600 MHz spectrometer. The UV-vis absorption and fluorescence spectra data were obtained using a Shimadzu

UV-2450 spectrometer and HITACHI F-4600 fluorescence spectrophotometer. Fluorescence images of cells and zebrafish were acquired using a Zeiss LSM710 Wetzlar laser scanning confocal microscope. All chemicals were purchased from suppliers in China and used directly without further refining.

2.2 Synthesis of the dye COZ-OH

7-Hydroxycoumarin-4-acetic acid (110.0 mg, 0.5 mmol) and *N*-ethylcarbazole-3-carbaldehyde (116.4 mg, 0.5 mmol) were dissolved in 6.0 mL of CH₃OH, and then piperidine (46.0 μ L) was added to the above mixed solution. After stirring for 12.0 h at 75 °C, the reaction was completed. The cooled precipitate was washed with cold methanol (10.0 mL), whereafter the obtained solid was extracted using dichloromethane/brine (15.0 mL/50.0 mL) and dried over Na₂SO₄. The crude product was purified *via* column chromatography (CH₂Cl₂ : CH₃OH = 50 : 1) to give the dye COZ-OH (97.2 mg, 51% yield). ¹H NMR (600 MHz, DMSO) δ (ppm) 10.58 (s, 1H), 8.67 (s, 1H), 8.18 (dd, J = 41.8, 8.2 Hz, 2H), 7.96–7.63 (m, 5H), 7.50 (t, J = 7.6 Hz, 1H), 7.27 (t, J = 7.4 Hz, 1H), 6.87 (dd, J = 8.7, 1.8 Hz, 1H), 6.77 (d, J = 1.8 Hz, 1H), 6.58 (s, 1H), 4.48 (q, J = 7.0 Hz, 2H), 1.34 (t, J = 7.1 Hz, 3H). ¹³C NMR (151 MHz, DMSO) δ (ppm) 161.69, 161.34, 155.89, 151.00, 140.84, 140.57, 139.48, 127.45, 127.15, 126.90, 126.67, 123.10, 122.75, 120.97, 120.89, 119.81, 117.19, 113.27, 111.33, 110.01, 109.90, 103.88, 103.05, 37.63, 14.23. HRMS (ESI) m/z : calcd for [C₂₅H₂₀NO₃]⁺, 382.1443; found, 382.1422.

2.3 Synthesis of the probe COZ-DNBS

COZ-OH (38.0 mg, 0.1 mmol) and 30.0 μ L of Et₃N were mixed in 8.0 mL of CH₂Cl₂ at room temperature for 10.0 min. Then, 2,4-dinitrobenzenesulfonyl chloride (32.0 mg, 0.12 mmol) was added into the mixture and it was stirred for 6.5 h. After natural cooling, the reaction solution was filtered and dried over Na₂SO₄. The crude product was purified *via* silica gel column chromatography using CH₂Cl₂ as the eluent to obtain COZ-DNBS (47.1 mg, 77% yield). ¹H NMR (600 MHz, DMSO) δ (ppm) 9.14 (d, J = 2.3 Hz, 1H), 8.66 (s, 1H), 8.62 (dd, J = 8.7, 2.3 Hz, 1H), 8.42 (d, J = 8.9 Hz, 1H), 8.35 (d, J = 8.7 Hz, 1H), 8.19 (d, J = 7.7 Hz, 1H), 7.99–7.89 (m, 2H), 7.73–7.63 (m, 3H), 7.54–7.47 (m, 1H), 7.40 (d, J = 2.4 Hz, 1H), 7.30–7.24 (m, 2H), 6.89 (s, 1H), 4.49 (d, J = 7.2 Hz, 2H), 1.34 (t, J = 7.1 Hz, 3H). ¹³C NMR (151 MHz, DMSO) δ (ppm) 160.22, 154.56, 152.11, 150.53, 149.94, 148.63, 140.99, 140.63, 140.58, 134.19, 131.01, 128.12, 127.97, 127.29, 126.98, 126.74, 123.08, 122.71, 121.72, 121.18, 120.90, 119.90, 118.98, 118.37, 116.34, 111.27, 110.08, 109.98, 108.31, 37.65, 14.24. HRMS (ESI) m/z : calcd for [C₃₁H₂₂N₃O₉S]⁺, 612.1077; found, 612.1074.

2.4 Procedure for optical data measurements

COZ-DNBS was dissolved using CH₃CN and a stock solution was prepared with a concentration of 3.0 mM. 10.0 mM analyte stock solutions (including Ala, Asn, Arg, Glu, Gln, Met, Ser, His, Pro, Thr, Ile, Cys, GSH, Hcy, Cl⁻, Br⁻, F⁻, I⁻, NO₃⁻, CO₃²⁻, SO₄²⁻, Zn²⁺, H₂O₂, and NaHS) were made up in deionized water for immediate use. All solution tests were performed in pH 7.4 PBS/CH₃CN (4 : 1, v/v). The fluorescence spectra were obtained



at $\lambda_{\text{ex}} = 400.0$ nm. **COZ-DNBS** was dissolved in DMSO for cell culture and zebrafish studies.

2.5 Cell cultures and imaging

Human breast carcinoma MCF-7 cells were plated in a confocal Petri dish with DMEM culture medium to adherence for 24 h. As a control, MCF-7 cells were immersed in $10.0 \mu\text{M}$ **COZ-DNBS** at 37°C for 30 min. For the experiment groups, MCF-7 cells were pre-incubated with different concentrations of H_2S (10.0 , 20.0 , and $50.0 \mu\text{M}$, respectively) for 30 min and further treated with **COZ-DNBS** ($10.0 \mu\text{M}$) for another 30 min. After rinsing the confocal Petri dish with phosphate-buffered saline (PBS) three times to remove residue, the MCF-7 cells were imaged using a Zeiss LSM 710 laser scanning confocal microscope.

2.6 Zebrafish imaging

Four-day-old zebrafish were obtained from Eze-Rinka Company (Nanjing, China). Firstly, the zebrafish to be imaged were incubated with the probe **COZ-DNBS** ($10.0 \mu\text{M}$) at 37°C for 30 min, and they were then imaged following three cycles of PBS washing. Subsequently, other zebrafish were pretreated with $50.0 \mu\text{M}$ H_2S for 30 min and stained with **COZ-DNBS** ($10.0 \mu\text{M}$) for another 30 min. After washing with PBS three times, fluorescence imaging was performed using a confocal microscope.

2.7 Preparation of spiked samples

The river water, lake water, and tap water samples used in these experiments were obtained from the Nenjiang river, Laodong lake, and a laboratory in Qiqihar Medical University, respectively. After being filtered, the above real water samples were spiked with different concentrations of H_2S (5.0 , 10.0 , and $20.0 \mu\text{M}$), and then the fluorescence intensity changes of the mixtures were measured in triplicate.

3. Results and discussion

3.1 Spectral response

To understand the optical properties of **COZ-DNBS** when it reacted with H_2S , fluorescence titration was carried out in pH 7.4 PBS/ CH_3CN (4 : 1, v/v) with various concentrations of H_2S (Fig. 1). It was seen that the photoinduced electron transfer (PET)-based emission of **COZ-DNBS** resulted in “turn-off” fluorescence being observed in the absence of H_2S . Upon the addition of H_2S , **COZ-DNBS** showed dramatic fluorescence emission centered at 558 nm and with a large Stokes shift (173 nm) (Fig. S1^\dagger). With an increase in the H_2S concentration, the fluorescence enhanced gradually and showed a 62-fold increase upon the addition of $60.0 \mu\text{M}$ H_2S . Based on previous research,^{30,31} the above profound optical changes at 558 nm for **COZ-DNBS** in the presence of H_2S should be ascribed to the formation of the dye **COZ-OH**. This sensing strategy was also verified based on the HRMS spectra. A mixture of **COZ-DNBS** and H_2S (cal.: 382.1415) (Fig. S8^\dagger) has nearly the same molecular weight as **COZ-OH** ($m/z = 382.1443$) (Fig. S6^\dagger). Moreover, the regression equation was obtained according to the dose-dependent spectral response, and it displayed a good linear

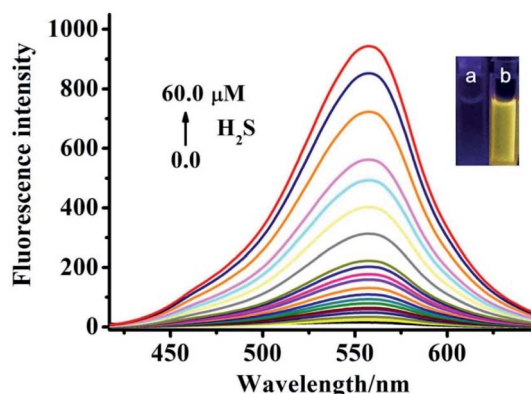


Fig. 1 Fluorescence spectra of **COZ-DNBS** ($10.0 \mu\text{M}$) in the presence of different amounts of H_2S (0.0 – $60.0 \mu\text{M}$); inset: the colorimetric changes of **COZ-DNBS** without (a) and with (b) H_2S .

relationship between the fluorescence intensity and concentration of H_2S (Fig. 2). In the concentration range of 0.0 – $5.0 \mu\text{M}$, the limit of detection (LOD) for $10.0 \mu\text{M}$ **COZ-DNBS** was estimated to be 38.6 nM, suggesting that the probe **COZ-DNBS** had the potential to detect H_2S qualitatively and quantitatively.

3.2 Selectivity study

To prove the sensitivity and specificity of **COZ-DNBS** for identifying H_2S , a series of analytes was employed and monitored in aqueous media. As depicted in Fig. 3, the fluorescence intensity of **COZ-DNBS** hardly changed upon adding various amino acids, ions, and biologically active materials (including Ala, Asn, Arg, Glu, Gln, Met, Ser, His, Pro, Thr, Ile, Cl^- , Br^- , F^- , I^- , NO_3^- , CO_3^{2-} , SO_4^{2-} , Zn^{2+} , H_2O_2 , Cys, GSH, and Hcy). However, a remarkable fluorescence response with an approximately 62-fold enhancement ratio at 558 nm was seen in the presence of H_2S compared with the slight fluorescence behavior induced by biothiols (Cys, Hcy and GSH), which indicated that **COZ-DNBS** had good sensing abilities towards H_2S . In addition, competition experiments, involving the coexistence of the

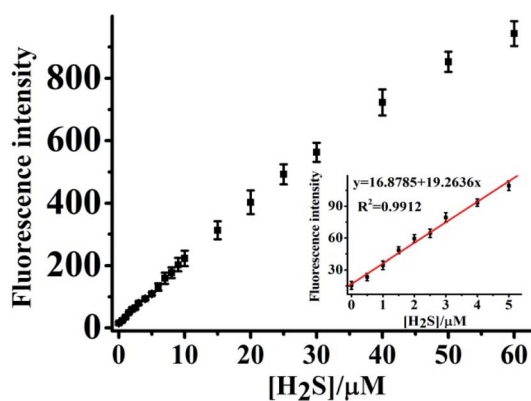


Fig. 2 The fluorescence intensity of **COZ-DNBS** at 558 nm as a function of the H_2S dose (0.0 – $60.0 \mu\text{M}$); inset: the linear relationship between the concentration of H_2S (0.0 – $5.0 \mu\text{M}$) and the fluorescence intensity at 558 nm.



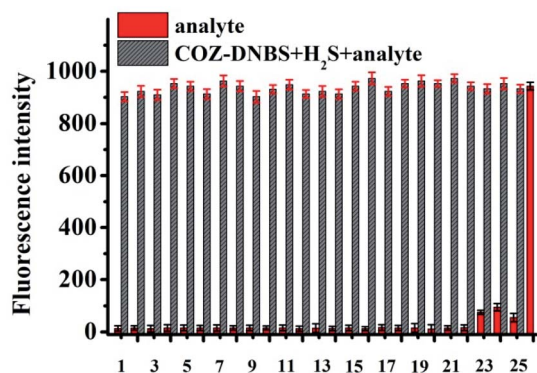


Fig. 3 The fluorescence responses of COZ-DNBS (10.0 μM) incubated with various analytes (red bars; 60.0 μM for H₂S (26) and 0.5 mM for 1–25, which were Ala, Asn, Arg, Glu, Gln, Met, Ser, His, Pro, Thr, Ile, Cl⁻, Br⁻, F⁻, I⁻, NO₃⁻, CO₃²⁻, SO₄²⁻, Zn²⁺, H₂O₂, Cys, GSH, and Hcy, respectively); and the detection of H₂S (60.0 μM) upon the addition of diverse coexisting competing analytes (gray bars; 0.5 mM for Ala, Asn, Arg, Glu, Gln, Met, Ser, His, Pro, Thr, Ile, Cl⁻, Br⁻, F⁻, I⁻, NO₃⁻, CO₃²⁻, SO₄²⁻, Zn²⁺, H₂O₂, Cys, GSH, and Hcy (1–25, respectively)). The incubation time was 60 s.

aforementioned analytes (including Ala, Asn, Arg, Glu, Gln, Met, Ser, His, Pro, Thr, Ile, Cl⁻, Br⁻, F⁻, I⁻, NO₃⁻, CO₃²⁻, SO₄²⁻, Zn²⁺, H₂O₂, Cys, GSH, and Hcy), were performed to explore the feasibility of using COZ-DNBS to detect H₂S. It is found that the degree of fluorescence intensity change was similar as that in the presence of H₂S alone, demonstrating that COZ-DNBS could selectively respond to H₂S in complex biological environments.

3.3 Kinetics and pH studies

To obtain the optimal fluorescence sensing set-up for H₂S detection, it is important to optimize the pH to obtain the highest response of COZ-DNBS towards H₂S. In the present study, the effects of pH were explored in the range of pH 2 to 11. As shown in Fig. 4, the free probe COZ-DNBS exhibited a very weak emissive nature at 558 nm in diverse pH environments. After the addition of H₂S, the fluorescence intensity of COZ-

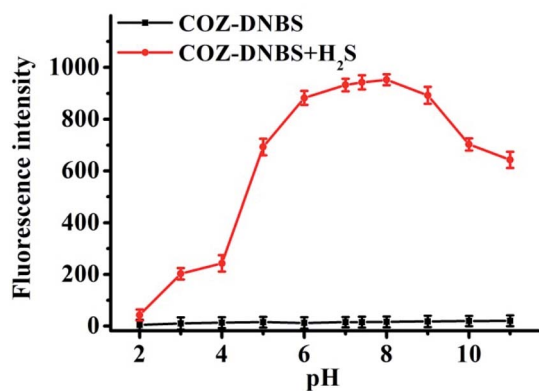


Fig. 4 The pH-dependence (2.0–11.0) of the fluorescence intensity of COZ-DNBS (10.0 μM) in H₂O/CH₃CN (4 : 1, v/v) without (black line) and with (red line) H₂S (60.0 μM).

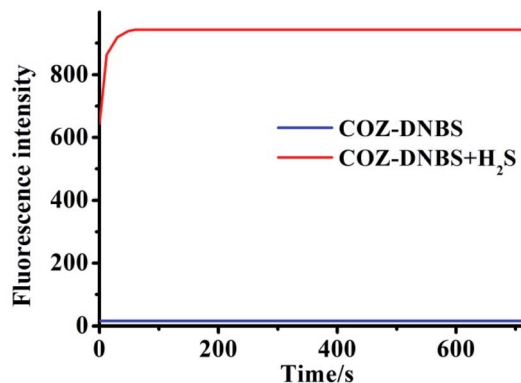


Fig. 5 The fluorescence intensity of COZ-DNBS (10.0 μM) over time at 558 nm before (blue line) and after (red line) the addition of H₂S.

DNBS increased gradually from pH 2.0 to 5.0; a high fluorescence signal value emerged between 6.0 and 8.0, and this slightly decreased under alkaline conditions. The above results strengthen the possibility of using COZ-DNBS as a candidate probe for H₂S detection under neutral conditions. Subsequently, the time-dependent fluorescence changes of COZ-DNBS in the presence of H₂S were studied (Fig. 5). In the pH 7.4 PBS/CH₃CN (4 : 1, v/v) system, the observed fluorescence intensity of COZ-DNBS alone remained unchanged, meaning that COZ-DNBS possessed excellent stability in the liquid state. However, the fluorescence signal of COZ-DNBS immediately increased (in less than 1 min) in this buffer system after adding H₂S, and the bright fluorescence of the reaction product between COZ-DNBS and H₂S was insensitive to the incubation time. This clearly indicates that COZ-DNBS has the real-time capability to detect H₂S.

3.4 Imaging of H₂S in living MCF-7 cells

Prompted by the favorable properties of COZ-DNBS, the bio-imaging abilities of COZ-DNBS were studied *via* living cell analysis. Before intracellular imaging, the cytotoxicity of COZ-DNBS toward MCF-7 cells was assessed *via* MTT assays. The results showed that COZ-DNBS displayed low cytotoxicity in the

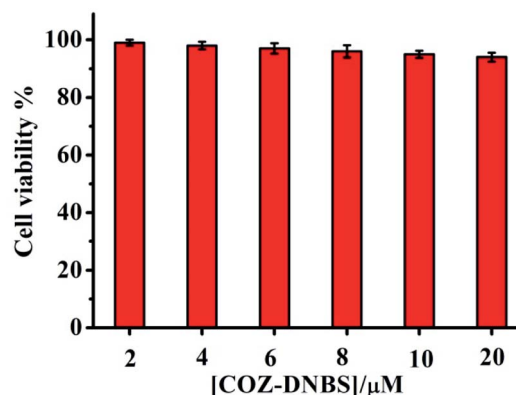


Fig. 6 The viability of MCF-7 cells incubated with different concentrations of COZ-DNBS (2.0–20.0 μM).



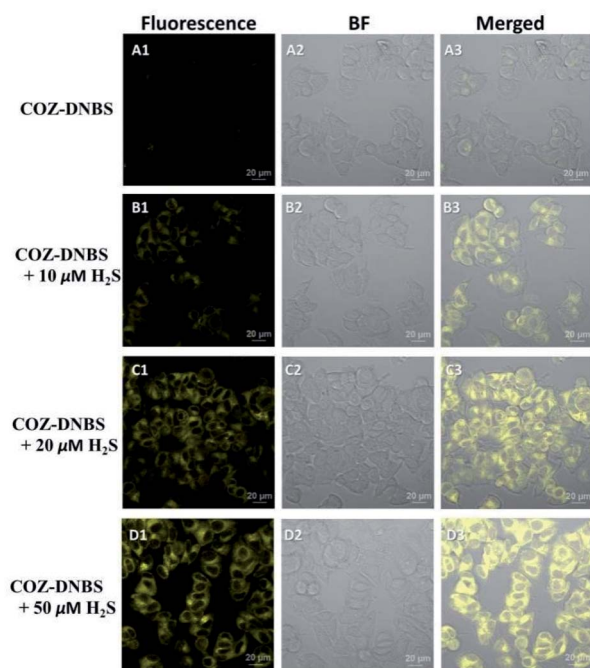


Fig. 7 Confocal imaging of MCF-7 cells: (A) MCF-7 cells incubated with 10.0 μM COZ-DNBS for 30 min; and (B–D) MCF-7 cells incubated with different concentrations of H₂S (10.0, 20.0, and 50.0 μM), followed by treatment with 10.0 μM COZ-DNBS.

range of 2–20.0 μM, with above 94% cell viability (Fig. 6), indicating that COZ-DNBS was biocompatible for performing imaging in living organisms. Subsequently, the probe COZ-DNBS was applied to monitor intracellular H₂S in MCF-7 cells. As shown in Fig. 7, a slight fluorescence background signal emerged when MCF-7 cells were treated with COZ-DNBS alone, which may arise from the presence of a very small amount of H₂S in the cells. However, the appearance of remarkable fluorescence was observed in the presence of H₂S (Fig. 7B1–D1), and the fluorescence signal increased gradually with an increase (10.0–50.0 μM) in the H₂S concentration. The above findings demonstrated that COZ-DNBS could be used to quantify the concentration of H₂S based on the relative fluorescence intensity from images of live cells.

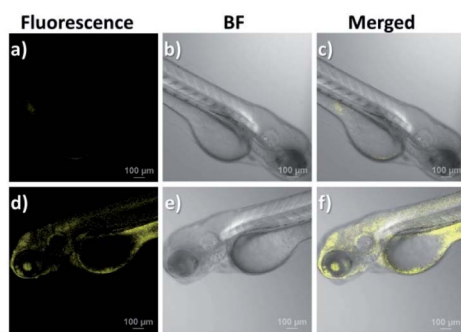


Fig. 8 Fluorescence imaging of H₂S in zebrafish using COZ-DNBS: (a–c) zebrafish incubated with COZ-DNBS (10.0 μM) for 30 min; and (d–f) zebrafish incubated with H₂S (50.0 μM) before staining with COZ-DNBS (10.0 μM).

3.5 Imaging of H₂S in living zebrafish

The potential application of imaging H₂S in living zebrafish upon incubation with COZ-DNBS was then validated. As depicted in Fig. 8, incubation with COZ-DNBS (10.0 μM) led to only a weak fluorescence signal from H₂S already present in the zebrafish in the yellow channel. In contrast, the fluorescence signal became much brighter after the addition of H₂S (50.0 μM). It was advantageously confirmed that COZ-DNBS could be suitable for the detection of H₂S in living zebrafish, showing excellent membrane permeability.

3.6 Detection of H₂S in spiked samples

To further gauge the feasibility of using the probe COZ-DNBS for practical applications, the quantitative analysis of real samples was investigated. In three different water samples (river water, lake water, and tap water), spiked H₂S concentrations were measured using COZ-DNBS. As manifested *via* recovery experiments (Table S2[†]), moderate to good recovery rates were obtained when using COZ-DNBS to sensitively detect H₂S, within the range of 95.6–103.4%, and the relative standard deviation (RSD) values were less than 3.0%. The above data showed that the proposed COZ-DNBS sensor exhibited excellent accuracy in actual water samples, proving the capacity of COZ-DNBS to act as a valid tool for the detection of H₂S in environmental samples.

4. Conclusions

In conclusion, we have achieved a new fluorescent probe, COZ-DNBS, for H₂S detection based on the novel coumarin-carbazole fluorescent dye COZ-OH. Upon reaction with H₂S, COZ-DNBS exhibited high sensitivity (38.6 nM), excellent selectivity, and extraordinary fluorescence enhancement (62-fold) in the yellow region ($\lambda_{\text{maxem}} = 558 \text{ nm}$). Moreover, benefiting from the fast response (<1 min) and huge Stokes shift (173 nm), COZ-DNBS was successfully applied to the detection of H₂S in realistic samples, living MCF-7 cells, and zebrafish, and it displayed great potential for H₂S detection in biosystems.

Ethical statement

All animal procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Qiqihar Medical University and experiments were approved by the Animal Ethics Committee of Qiqihar Medical University (QMU-AECC-2020-63).

Conflicts of interest

There are no conflicts of interest to declare.

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