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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†

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As a vital antioxidant molecule, H_2S can make an important contribution to regulating blood vessels and inhibiting apoptosis when present at an appropriate concentration. Higher levels of H_2S 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 H_2S 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 H_2S *in vivo* and *in vitro*. Upon using the probe **COZ-DNBS**, the established fluorescent platform could detect H_2S 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 H_2S in realistic samples, living MCF-7 cells, and zebrafish, showing that **COZ-DNBS** is a promising signaling tool for H_2S detection in biosystems.

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1. Introduction

In the process of maintaining biological homeostasis, reactive sulfur species (RSS), including hydrogen sulfide (H_2S) 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 H_2S is considered to be a third endogenous gaseous transmitter after nitric oxide (NO) and carbon monoxide (CO).[4–6] As a vital antioxidant molecule, H_2S 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 H_2S can be significantly upregulated during inflammatory events. Higher levels of H_2S 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 H_2S 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 H_2S-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 Cu^{2+}. 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 autofluorescence 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 H_2S in biological systems. Thus, the development of a H_2S-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

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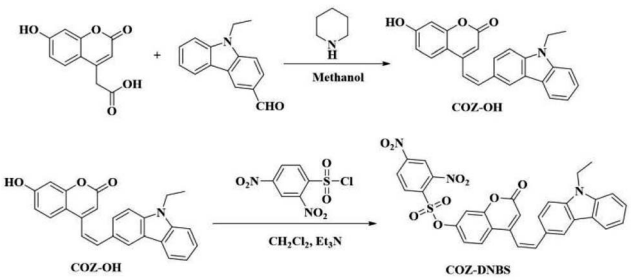
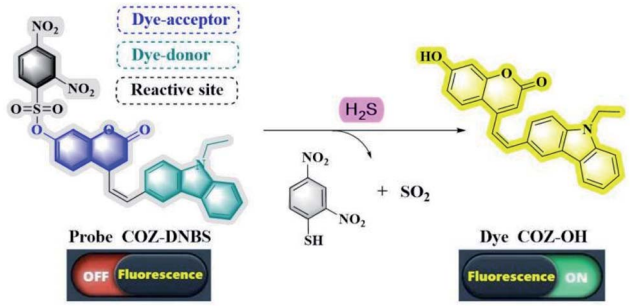


Scheme 1 The synthetic route to the new fluorescent probe **COZ-DNBS**.



Scheme 2 The proposed mechanism involving the fluorescent probe **COZ-DNBS** for H_2S detection.

a rapid response and large Stokes shift was rationally devised and applied to effectively sense H_2S *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 ^{13}C-NMR and ^{1}H-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 H_2S,[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 H_2S 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 H_2S in realistic samples, living MCF-7 cells, and zebrafish, showing that **COZ-DNBS** is a promising signaling tool for H_2S 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. ^{1}H- and ^{13}C-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 CH_3OH, and then piperidine (46.0 μL) 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 Na_2SO_4. The crude product was purified *via* column chromatography (CH_2Cl_2 : CH_3OH = 50 : 1) to give the dye **COZ-OH** (97.2 mg, 51% yield). ^{1}H 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). ^{13}C 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 $[C_{25}H_{20}NO_3]^+$, 382.1443; found, 382.1422.

2.3 Synthesis of the probe COZ-DNBS

COZ-OH (38.0 mg, 0.1 mmol) and 30.0 μL of Et_3N were mixed in 8.0 mL of CH_2Cl_2 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 Na_2SO_4. The crude product was purified *via* silica gel column chromatography using CH_2Cl_2 as the eluent to obtain **COZ-DNBS** (47.1 mg, 77% yield). ^{1}H 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). ^{13}C 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 $[C_{31}H_{22}N_3O_9S]^+$, 612.1077; found, 612.1074.

2.4 Procedure for optical data measurements

COZ-DNBS was dissolved using CH_3CN 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^-, NO_3^-, CO_3^{2-}, SO_4^{2-}, Zn^{2+}, H_2O_2, and NaHS) were made up in deionized water for immediate use. All solution tests were performed in pH 7.4 PBS/CH_3CN (4 : 1, v/v). The fluorescence spectra were obtained

at λ_{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 μM **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 μM, respectively) for 30 min and further treated with **COZ-DNBS** (10.0 μM) 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 μM) 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 μM H_2S for 30 min and stained with **COZ-DNBS** (10.0 μM) 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 μM), 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†). With an increase in the H_2S concentration, the fluorescence enhanced gradually and showed a 62-fold increase upon the addition of 60.0 μM 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†) has nearly the same molecular weight as **COZ-OH** (m/z = 382.1443) (Fig. S6†). Moreover, the regression equation was obtained according to the dose-dependent spectral response, and it displayed a good linear

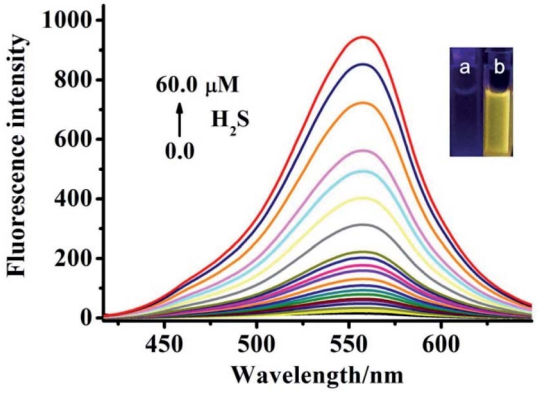


Fig. 1 Fluorescence spectra of **COZ-DNBS** (10.0 μM) in the presence of different amounts of H_2S (0.0–60.0 μM); inset: the colorimetric changes of **COZ-DNBS** without (a) and with (b) H_2S.

relationship between the fluorescence intensity and concentration of H_2S (Fig. 2). In the concentration range of 0.0–5.0 μM, the limit of detection (LOD) for 10.0 μM **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

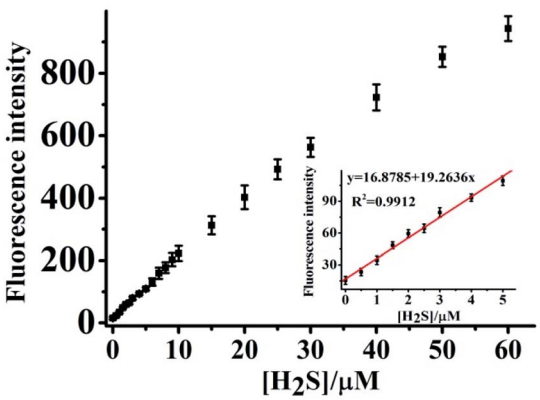


Fig. 2 The fluorescence intensity of **COZ-DNBS** at 558 nm as a function of the H_2S dose (0.0–60.0 μM); inset: the linear relationship between the concentration of H_2S (0.0–5.0 μM) and the fluorescence intensity at 558 nm.

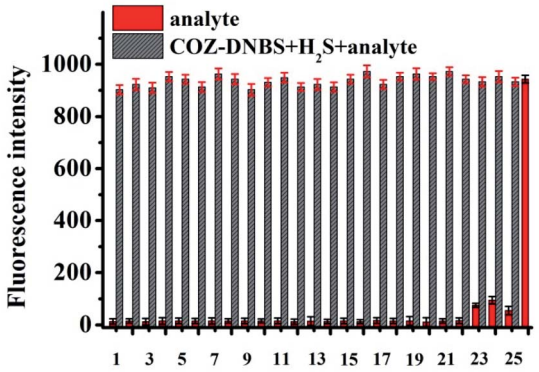


Fig. 3 The fluorescence responses of **COZ-DNBS** (10.0 μM) incubated with various analytes (red bars; 60.0 μM for H_2S (26) and 0.5 mM for 1–25, which were Ala, Asn, Arg, Glu, Gln, Met, Ser, His, Pro, Thr, Ile, Cl^-, Br^-, F^-, I^-, NO_3^-, CO_3^{2-}, SO_4^{2-}, Zn^{2+}, H_2O_2, Cys, GSH, and Hcy, respectively); and the detection of H_2S (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^-, NO_3^-, CO_3^{2-}, SO_4^{2-}, Zn^{2+}, H_2O_2, 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^-, NO_3^-, CO_3^{2-}, SO_4^{2-}, Zn^{2+}, H_2O_2, Cys, GSH, and Hcy), were performed to explore the feasibility of using **COZ-DNBS** to detect H_2S. It is found that the degree of fluorescence intensity change was similar as that in the presence of H_2S alone, demonstrating that **COZ-DNBS** could selectively respond to H_2S in complex biological environments.

3.3 Kinetics and pH studies

To obtain the optimal fluorescence sensing set-up for H_2S detection, it is important to optimize the pH to obtain the highest response of **COZ-DNBS** towards H_2S. 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 H_2S, the fluorescence intensity of **COZ-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 H_2S detection under neutral conditions. Subsequently, the time-dependent fluorescence changes of **COZ-DNBS** in the presence of H_2S were studied (Fig. 5). In the pH 7.4 PBS/CH_3CN (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 H_2S, and the bright fluorescence of the reaction product between **COZ-DNBS** and H_2S was insensitive to the incubation time. This clearly indicates that **COZ-DNBS** has the real-time capability to detect H_2S.

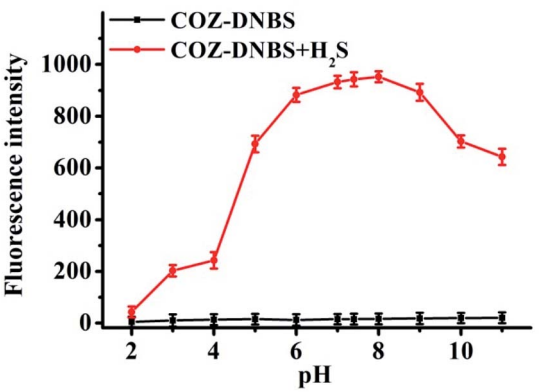


Fig. 4 The pH-dependence (2.0–11.0) of the fluorescence intensity of **COZ-DNBS** (10.0 μM) in H_2O/CH_3CN (4 : 1, v/v) without (black line) and with (red line) H_2S (60.0 μM).

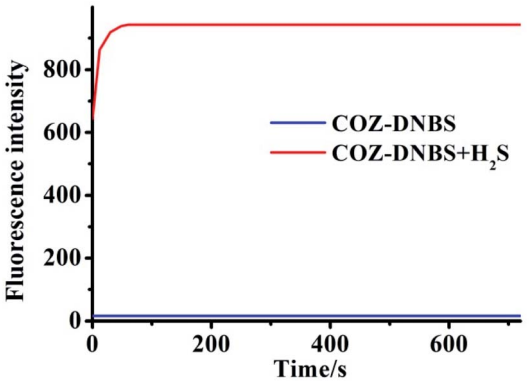


Fig. 5 The fluorescence intensity of **COZ-DNBS** (10.0 μM) over time at 558 nm before (blue line) and after (red line) the addition of H_2S.

3.4 Imaging of H_2S in living MCF-7 cells

Prompted by the favorable properties of **COZ-DNBS**, the bioimaging 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

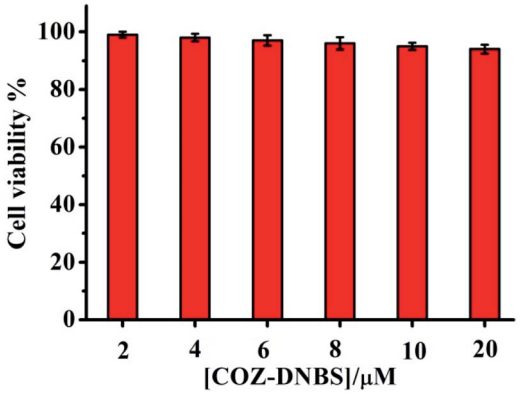


Fig. 6 The viability of MCF-7 cells incubated with different concentrations of **COZ-DNBS** (2.0–20.0 μM).

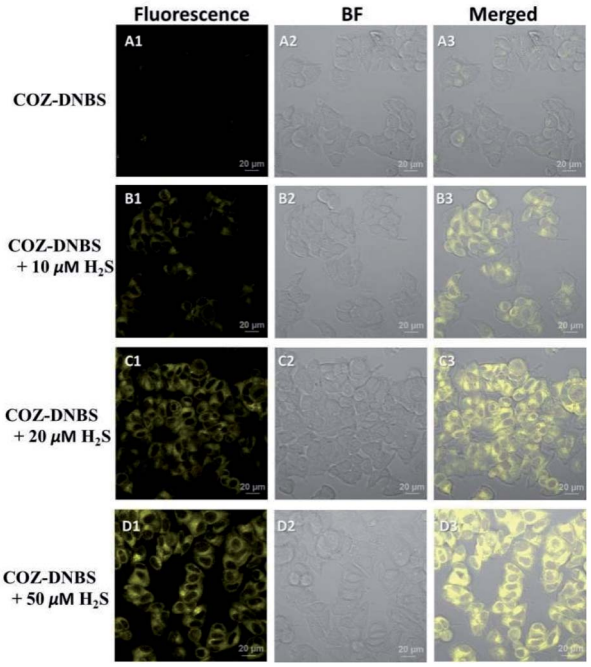


Fig. 7 Confocal imaging of MCF-7 cells: (A) MCF-7 cells incubated with 10.0 μM **COZ-DNBS** for 30 min; and (B–D) MCF-7 cells incubated with different concentrations of H_2S (10.0, 20.0, and 50.0 μM), followed by treatment with 10.0 μM **COZ-DNBS**.

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 H_2S 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 maybe arise from the presence of a very small amount of H_2S in the cells. However, the appearance of remarkable fluorescence was observed in the presence of H_2S (Fig. 7B1–D1), and the fluorescence signal increased gradually with an increase (10.0–50.0 μM) in the H_2S concentration. The above findings demonstrated that **COZ-DNBS** could be used to quantify the concentration of H_2S based on the relative fluorescence intensity from images of live cells.

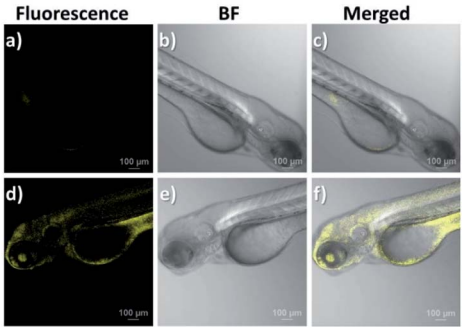


Fig. 8 Fluorescence imaging of H_2S in zebrafish using **COZ-DNBS**: (a–c) zebrafish incubated with **COZ-DNBS** (10.0 μM) for 30 min; and (d–f) zebrafish incubated with H_2S (50.0 μM) before staining with **COZ-DNBS** (10.0 μM).

3.5 Imaging of H_2S in living zebrafish

The potential application of imaging H_2S 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 H_2S already present in the zebrafish in the yellow channel. In contrast, the fluorescence signal became much brighter after the addition of H_2S (50.0 μM). It was advantageously confirmed that **COZ-DNBS** could be suitable for the detection of H_2S in living zebrafish, showing excellent membrane permeability.

3.6 Detection of H_2S 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 H_2S 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 H_2S, 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 H_2S in environmental samples.

4. Conclusions

In conclusion, we have achieved a new fluorescent probe, **COZ-DNBS**, for H_2S detection based on the novel coumarin–carbazole fluorescent dye **COZ-OH**. Upon reaction with H_2S, **COZ-DNBS** exhibited high sensitivity (38.6 nM), excellent selectivity, and extraordinary fluorescence enhancement (62-fold) in the yellow region ($\lambda_{maxem} = 558$ nm). Moreover, benefiting from the fast response (<1 min) and huge Stokes shift (173 nm), **COZ-DNBS** was successfully applied to the detection of H_2S in realistic samples, living MCF-7 cells, and zebrafish, and it displayed great potential for H_2S 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.

Acknowledgements

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