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Mitochondria-targeted reversible ratiometric fluorescent probe for monitoring SO_2/HCHO in living cells

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Abstract: Sulfur dioxide (SO_2) maintains a certain steady state balance in the body, high concentration SO_2 will be harmful to human health. Seeking a suitable detection method to monitor sulfur dioxide in real time becomes an urgent requirement owing to the transient nature of sulfur dioxide in organisms. Here, a novel NIR ratiometric fluorescent probe for detection of SO_2 was developed based on a conjugation of coumarin and indol salt with excellent water solubility. The probe **Mito-CI** displayed highly sensitive (69 nM), fast response time (30 s), large Stokes shift (174 nm) and the NIR fluorescence emission wavelength (655 nm). In the reversibility process of the SO_3^--probe **Mito-CI** system induced by HCHO in vitro was also detected. Besides, cell imaging showed that **Mito-CI** posseses mitochondria-targeted ability. Particularly, **Mito-CI** was proved to reversibly detect SO_2/HCHO in living cells.

Keywords: SO_2/HCHO; Near-infrared; Reversibly; Mitochondria; Cellular imaging.

1. Introduction

Formaldehyde and sulfur dioxide (SO_2) are important components to maintain the homeostasis of living organisms, the abnormal production of these components lead to some critical diseases [1]. According to the latest research, formaldehyde could deregulate the NO signaling pathway by mediating the nitrosylation of S-protein. Formaldehyde assimilation and enhancement during protein S-nitrosation may lead to extremely low local GSH concentrations. GSH is one of the main raw materials for the production of endogenous SO_2 [2-7], high concentrations of SO_2 may result in vasodilation by upregulating the NO/cGMP signaling pathway. These conclusions indicated that formaldehyde and sulfur dioxide has a close relationship. Therefore, accurate detection of sulfur dioxide in living organisms is crucial to the overall health of biological systems.

At present, many research groups have developed a series of fluorescent probes for detection of SO_2 [8-20]. However, the real-time detection of SO_2 in the body is still a challenge. First, the limitations of most fluorescent probes are quite poor water solubility [21-25]. Second, almost all reported probes caused high photodamage to live samples [26-29]. Third, there are few reports about reversible detection of fluorescent probes for SO_2/HCHO [30-34]. Thus, it is essential to exploit a fluorescent probe with good water solubility, NIR emission wavelength and reversible detection of fluorescent probes for SO_2/HCHO.

Herein, **Mito-CI** was found to be the perfect tool for solving above problems. It showed highly sensitive (69 nM), fast response time (30 s), excellent water solubility,

large Stokes shift (174 nm) and the NIR fluorescence emission at 655 nm. **Mito-CI** was successfully synthesized based on the coumarin and indol salt (Scheme 1). And due to the nucleophilicity of SO_3^{2-}/HSO_3^-, **Mito-CI** could detect SO_2 with ratiometric fluorescent emission. Moreover, cell imaging experiment showed that **Mito-CI** could be used for reversible visualization of exogenous SO_2/HCHO and mitochondria-targeted in living cells.

<Inserted Scheme 1>

2. Materials and methods

2.1 Materials

Related materials are described in the support information.

*2.2. Synthesis of **Mito-CI***

2.2.1 Compound 1

Diethylmalonate (6.08 g, 38 mmol), 4-diethylaminosalicylaldehyde (3.67 g, 19 mmol) and piperidine (2.0 mL) were mixed with ethanol (60 mL), then the mixture was refluxed for 12 hours. After the reaction ended, solvent was evaporated under reduced pressure. Next, a mixture of concentrated acetic acid (40 mL) and HCl (40 mL) was added, reactant was refluxed at 120 °C for 13 hours. The reaction mixture was poured into 200 ml of 0°C water after cooling to 25 °C. Adding NaOH solution (40 %) to improve pH up to 5, brown precipitate formed instantly. The mixture was filtered and washed with water four times, drying it in vacuum, compound 1 was obtained as a yellowish brown solid (3.92 g, 95 %); ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.82 (d, J = 9.3 Hz, 1H), 7.42 (d, J = 8.8 Hz, 1H), 6.68 (dd, J = 8.8, 2.5 Hz, 1H), 6.51

(d, *J* = 2.3 Hz, 1H), 5.99 (d, *J* = 9.3 Hz, 1H), 3.42 (q, *J* = 7.0 Hz, 4H), 1.12 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 156.80, 144.96, 129.78, 109.22, 108.62, 97.04, 44.49, 12.77 (Fig. S1).

2.2.2 Compound 2 and compound 3

Compound 2 and compound 3 was prepared according to the reported literature [35].

*2.2.3 Compound **Mito-CI***

Compound 2 (98.0 mg, 0.4 mmol) and compound 3 (92.9 mg, 0.4 mmol) were mixed in ethanol (40 mL) and was refluxed for 12 hours. After the reaction finished, evaporation of the solvent under reduced pressure. Next, the crude product was purified by column chromatography on silica gel with CH_2Cl_2/MeOH (25:1) to get the desired Mito-CI (102.87 mg, yield: 56 %).^{1}H NMR (600 MHz, DMSO-d_6) δ 8.82 (s, 1H), 8.21 (d, J = 13.4 Hz, 1H), 7.82 (d, J = 10.6 Hz, 2H), 7.80 (d, J = 8.0 Hz, 1H), 7.61–7.54 (m, 2H), 7.52 (d, J = 7.3 Hz, 1H), 6.98–6.87 (m, 1H), 6.71 (s, 1H), 4.60 (s, 2H), 3.56 (d, J = 6.6 Hz, 4H), 2.88 (s, 2H), 1.73 (s, 6H), 1.18 (t, J = 6.8 Hz, 6H).^{13}C NMR (151 MHz, DMSO-d_6) δ 171.76, 159.86, 158.06, 154.38, 141.50, 132.81, 129.33, 123.29, 112.84, 111.75, 109.88, 97.04, 51.85, 45.31, 32.14, 26.51, 21.55, 12.96. ESI-MS m/z: [**Mito-CI** + H]$^+$ Calcd. For 459.2278, Found 459.2283 (Fig. S2) (Scheme 2).

<Inserted Scheme 2>

2.3 Fluorescence imaging

HepG2 cells were cultivated with DMEM contain 1 % penicillin-streptomyc and

10 % fetal calf serum at 37°C. The cells were washed four times in absolute PBS buffer solution before imaging. Fluorescence imaging of HepG2 cells was performed at 460-510 nm (blue channel) and 630-680 nm (red channel). Then, colocation imaging was proceed to study the mitochondrial distribution of **Mito-CI** in cells.

3. Results and discussion

*3.1 Sensing Properties of **Mito-CI** toward SO_3^{2-}/HSO_3^-*

Herein, we detected the fluorescence intensity of **Mito-CI** in the range of pH 3-9. When SO_3^{2-} (a standard source for SO_2) was added, **Mito-CI** demonstrated the optimal response in the pH scope of 6.0−9.0 (Fig. S3), which coincided with the weakly alkaline environment of mitochondria [36]. This conclusion illustrated that **Mito-CI** is suitable for monitoring SO_2 of mitochondrial. Therefore, we chose pH 7.4 in the following spectral experiments. Then, the sensing properties of **Mito-CI** for SO_3^{2-}/$HSO_3^-$are tested. As shown in Fig. 1a, **Mito-CI** (10 μM) was added into 10.0 mM of PBS buffer, then the change of fluorescence spectrum was observed, the probe showed a near-infrared fluorescence emission at 655 nm. Thereafter, when SO_3^{2-} (0-100 μM) was gradually added, the fluorescence intensity at 655 nm was gradually decreased, with a marked enhancement in fluorescence intensity at 481 nm. These phenomena demonstrated that the π-conjugation of **Mito-CI** was interrupted. Then, UV-Vis titration experiment was carried out (Fig. 1b), the probe exhibited a major absorption band at 577 nm. Under the condition of gradually adding SO_3^{2-} (0-150 μM), the absorption band at 577 nm gradually reduced, absorption peak at 423 nm

increased. The color of solution changed from dark purple to colorless, indicating that **Mito-CI** can be used to detect SO_3^{2-}/HSO_3^- with the "naked-eye".

<Inserted Figure 1>

3.2 Sensing Properties of ***Mito-CI-*** $\boldsymbol{SO_3^{2-}}$ *toward HCHO*

After the result of **Mito-CI** as a suitable fluorescent probe for the detection of SO_3^{2-}/HSO_3^-, the reversibility of **Mito-CI-** $\boldsymbol{SO_3^{2-}}$ were further studied. As shown in Fig. 2a, under the condition of gradually adding HCHO (0-200 μM), the fluorescence intensity at 655 nm slowly raised, with a marked decrease in the fluorescence intensity at 481 nm. Then, UV-Vis titration experiment of **Mito-CI-** $\boldsymbol{SO_3^{2-}}$ toward HCHO were performed (Fig. 2b), when HCHO (0-300 μM) was gradually added, the absorption band at 577 nm slowly enhanced, with a marked decrease in the absorption band at 423 nm. The color of solution changed from colorless to dark purple monitored by the "naked eye". The absorption intensity of **Mito-CI-** $\boldsymbol{SO_3^{2-}}$ ensemble at 577 nm changed from the initial 0.59 to 0.49 (after adding HCHO). These conclusions revealed that **Mito-CI-** $\boldsymbol{SO_3^{2-}}$ can effectively reversible by addition of HCHO, the level of recovery reached 83 %.

<Inserted Figure 2>

3.3 Time-dependent and Selectivity of ***Mito-CI***

The time-dependent of **Mito-CI** toward SO_3^{2-} (10 equiv.) showed that the reaction was accomplished within 30 s (Fig. S4). The selectivity of the probe for sulfur dioxide is also crucial in practical application. In the selective test, **Mito-CI** was treated with SO_3^{2-} and various analytes each (10 equiv.). Only upon addition of

SO_3^{2-} could make the fluorescence intensity at 481nm increase and decrease at 655 nm. Then, the competition tests were performed with the presence of corresponding analytes. In Fig. S5, all the competing analytes did not interfere the detection of SO_3^{2-} .

*3.4 LOD and Fluorescence Reversibility of **Mito-CI***

In order to obtain the detection limit of **Mito-CI** for SO_3^{2-}/HSO_3^-, the relative fluorescence emission intensities (I_{481}/I_{655}) at different SO_3^{2-} concentrations (0-80 μM) was measured. We could get the linear equation I_{481}/I_{655} = 0.10c + 9.63 (R^2 = 0.9980), where c is the SO_3^{2-} concentration. The calculated result is 69 nM according to the formula (LOD=3Sb/m) [37-41], which can cover the endogenous SO_2 concentration of the organism (Fig. 3A). Next, after adding HCHO, we performed the reversible detection of **Mito-CI** toward SO_3^{2-}. As shown in Fig. 2b, the reversibility could be repeated 3 times (Fig. 3B).

<Inserted Figure 3>

3.5 Proposed mechanism

The sensing mechanism of **Mito-CI** for SO_3^{2-}/HSO_3^- was examined by mass spectrometry. **Mito-CI** displayed a characteristic peak at m/z = 459.2283 (Fig. S2). A mixture solution of **Mito-CI** with SO_3^{2-} was injected into ESI-MS analysis (Fig. S6). It was found that the peak at m/z = 539.1869 displayed a characteristic peak. A mixture of Mito-CI+SO_3^{2-} with HCHO was added in ESI-MS analysis, the new characteristic peak appeared at m / z = 459.2280 (Fig.S7)., which accorded with the value of the theoretical calculation $[M + H]^+$ (calcd = 459.2278). Therefore, the

sensing mechanism of **Mito-CI** for SO_3^{2-}/HSO_3^- was based on nucleophilic addition (Scheme 1). And the free bisulfite group is effectively captured by HCHO to drive the dissociation of the adduct **Mito-CI** -SO_3 to the formation of the probe Mito-CI [1].

3.6 Cellular Imaging

In vivo imaging experiments, we used the MTT method to test the toxicity of **Mito-CI** in cells, and the result indicated that **Mito-CI** was appropriate for living cells under 10.0 μM (Fig. S8). Next, HepG2 cells were cultured in PBS buffer with **Mito-CI** (10 μM) for 20 minutes at 37 °C (Fig. 4). Then, the cells were observed to have a distinct red fluorescence, and a weak blue fluorescence, which was consistent with the fluorescence test in *vitro*. The red fluorescence was obviously weakened and blue fluorescence was obviously enhanced after the addition of SO_3^{2-} (40 μM). These results showed that **Mito-CI** can successfully detect exogenous SO_2 in living cells. Moreover, when HCHO (40 μM) was mixed with the just stained HepG2 cells for another 20 min, it was observed that the red fluorescence enhanced and the blue fluorescence reduced, indicating that **Mito-CI** could be reversible in living cells by HCHO indeed. In a co-localization test, the combined images obtained with the mitochondrial dye Mito-Tracker Green and **Mito-CI-SO_3^{2-}** adduct displayed that the fluorescence of Mito-Tracker Green and **Mito-CI-SO_3^{2-}** co-localized good with a high Pearson coefficient of 0.81 (Fig. 5), which indicated the capability of the **Mito-CI** in detecting SO_3^{2-} in mitochondria.

<Inserted Figure 4>

<Inserted Figure 5>

4. Conclusions

In summary, a mitochondria-targeted ratiometric reversible detection of SO_2/HCHO fluorescent probe **Mito-CI** was firstly obtained in our work. **Mito-CI** displayed highly sensitivity (69 nM), fast response time (30 s), large Stokes shift (174 nm) and the NIR fluorescence emission. Besides, we carried out reversible detection of SO_2/HCHO in absolute PBS buffer solution, it could be reversible at least 3 times. Last but not least, we also successfully reversibly detected the exogenous SO_2/HCHO in HepG2 cells. These results will lead to a deeper understanding of SO_2 concentration-dependent biological effects.

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Figure captions

Scheme 1 Diagram of **Mito-CI** for SO_3^{2-}/HCHO detection.

Scheme 2 Synthesis of the probe **Mito-CI**.

Fig. 1 (A) The fluorescence intensity of **Mito-CI** (10 μM) with various concentrations SO_3^{2-} (0−100 μM) in absolute PBS buffer solution (10.0 mM) after 30 s equilibrium, λ_{ex} = 413 nm, slit: 5 nm/10 nm. (B) UV-vis spectrum of **Mito-CI** (10.0 μM) in the presence of different amounts of SO_3^{2-} (0−150 μM) in absolute PBS buffer solution after 30 s equilibrium, (10.0 mM, pH = 7.4).

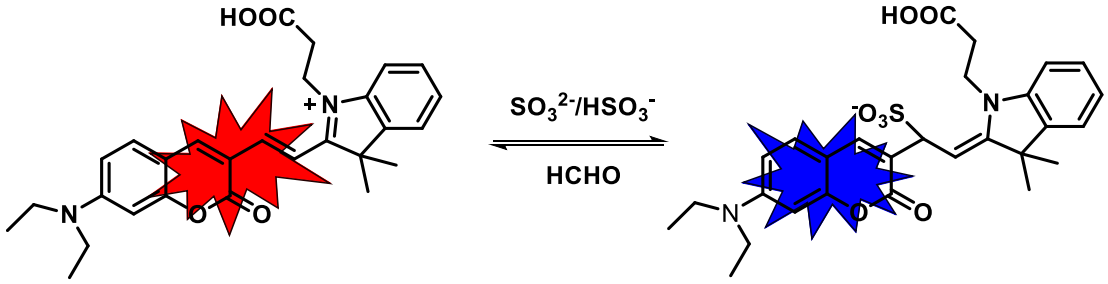
Fig. 2 (A) Fluorescent spectral changes with HCHO (0−200 μM) in absolute PBS buffer solution (10.0 mM, pH = 7.4) after 3 min equilibrium, λ_{ex} = 413 nm, slit: 5 nm/10 nm. (B) UV-vis spectrum of **Mito-CI-SO_3^-** in the existence HCHO (0−300 μM) in absolute PBS buffer solution after 3 min equilibrium, (10.0 mM, pH = 7.4).

Fig. 3 (A) Reversibility of **Mito-CI** (10 μM) toward SO_3^{2-}(100 μM) with HCHO (200 μM). (B) The linear curve obtained from relative fluorescence emission intensities (I_{481}/I_{655}) of **Mito-CI** (10 μM) with Na_2SO_3 from 0–80 μM.

Fig. 4 Cellular images of SO_3^{2-} and HCHO with dual-channel: (a1−d1): HepG2 cells were cultivated with 10 μM of **Mito-CI** for 20 min; (a2−d2): HepG2 cells were cultivated with 10 μM of **Mito-CI** for 20 min, next cultivated with 40 μM of SO_3^{2-} for 20 min; (a3−d3): HepG2 cells containing **Mito-CI** were cultivated with SO_3^{2-} (40 μM) for 20 min, next cultivated with HCHO (40 μM) for another 20 min. Red channel (λ_{em} = 630-680 nm. λ_{ex} = 560 nm), Blue channel (λ_{em} = 460-510 nm. λ_{ex} = 458 nm).

Fig. 5 Co-localization imaging of HepG2 cells. Cells were cultivated with **Mito-CI** (10 μM) and 0.2 μM Mito-Tracker Green at 37 °C for 20 min. (a) Red channel (λem = 638-747 nm. λ_{ex} = 633 nm), (b) Green channel (λ_{em} = 500-540 nm. λ_{ex} = 488 nm). (c) Combined image of (a) and (b). (d) Intensity scatter plot of Mito-Tracker Green and **Mito-CI**.

Scheme 1



Scheme 2

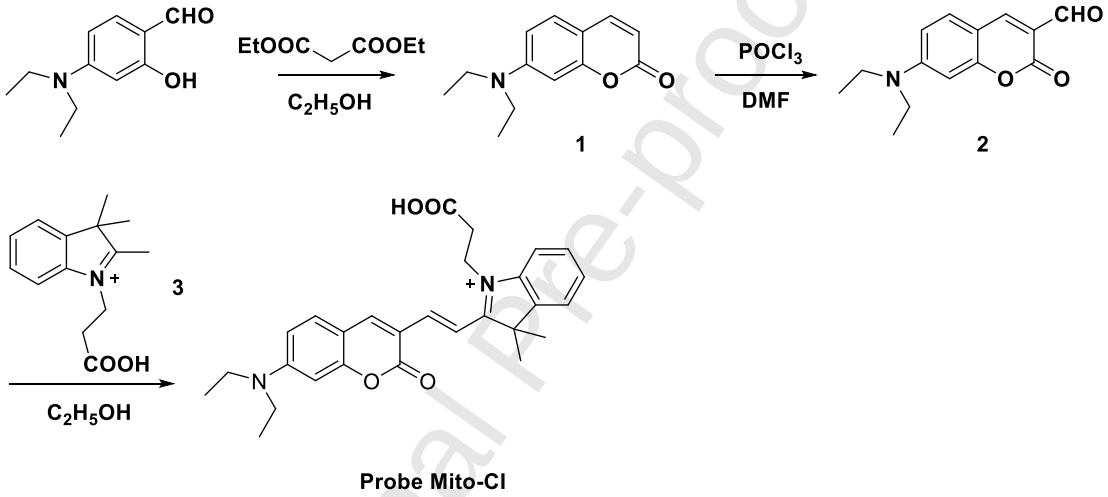
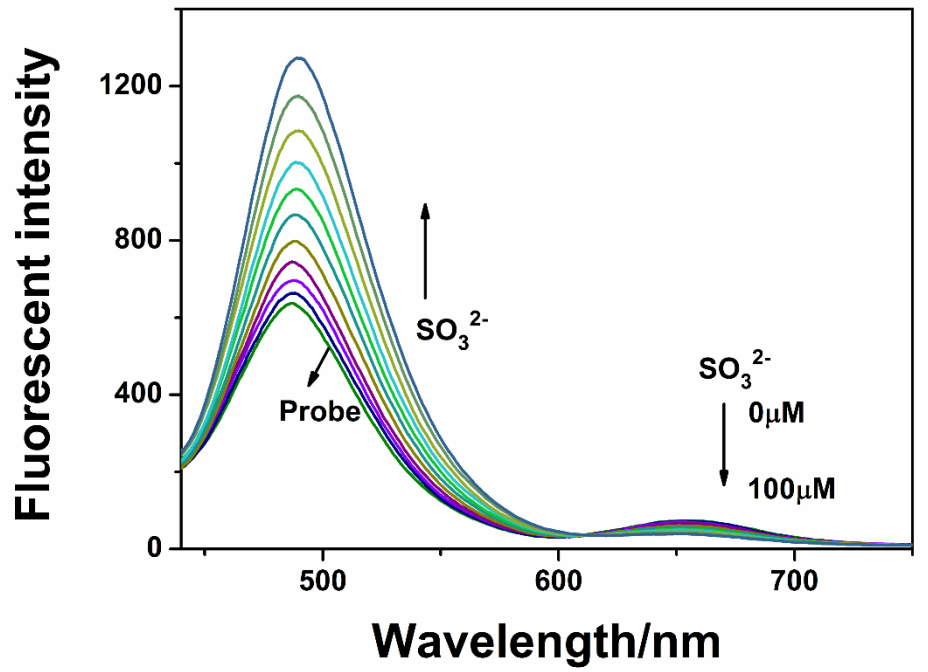


Figure 1

(a)


Fluorescent intensity
1200
800
400
0
500
600
700
Wavelength/nm
SO_3^{2-}
Probe
SO_3^{2-}
0μM
100μM

(b)

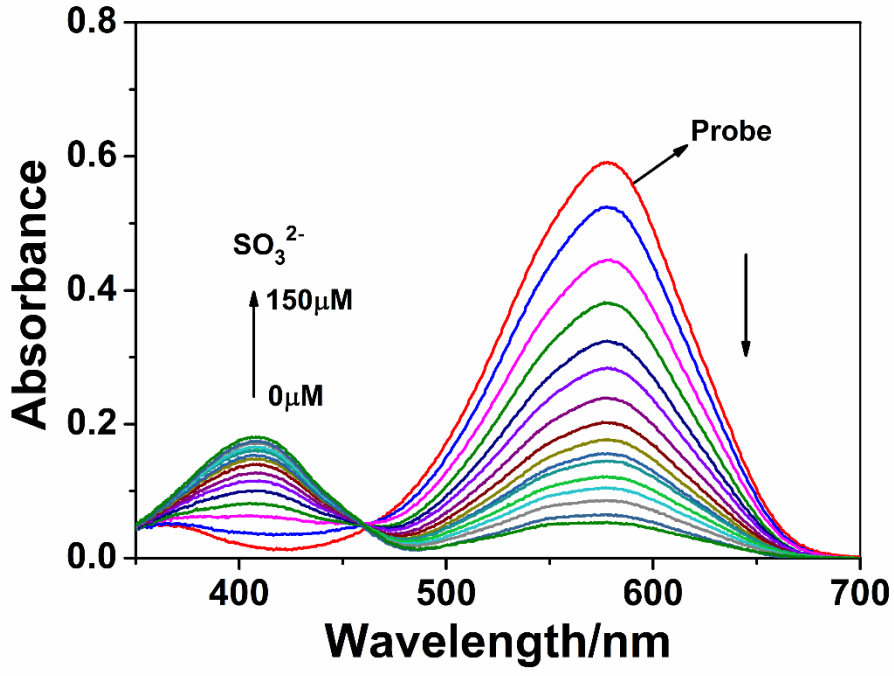
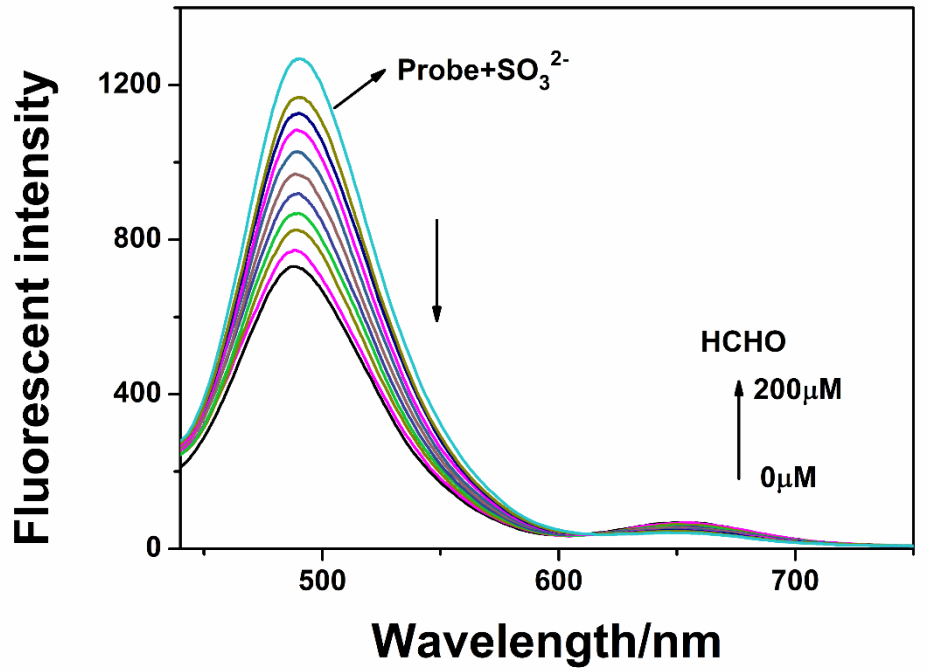

Absorbance
0.8
0.6
0.4
0.2
0.0
400
500
600
700
Wavelength/nm
Probe
SO_3^{2-}
150μM
0μM

Figure 2

(a)


Fluorescent intensity
1200
800
400
0
500
600
700
Wavelength/nm
Probe+SO3 2-
HCHO
200μM
0μM

(b)

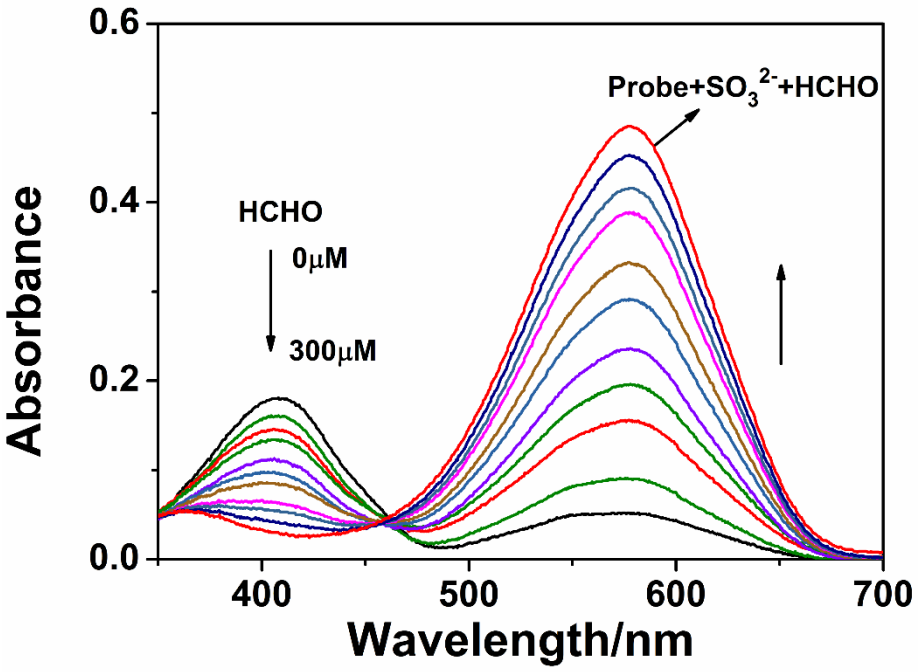
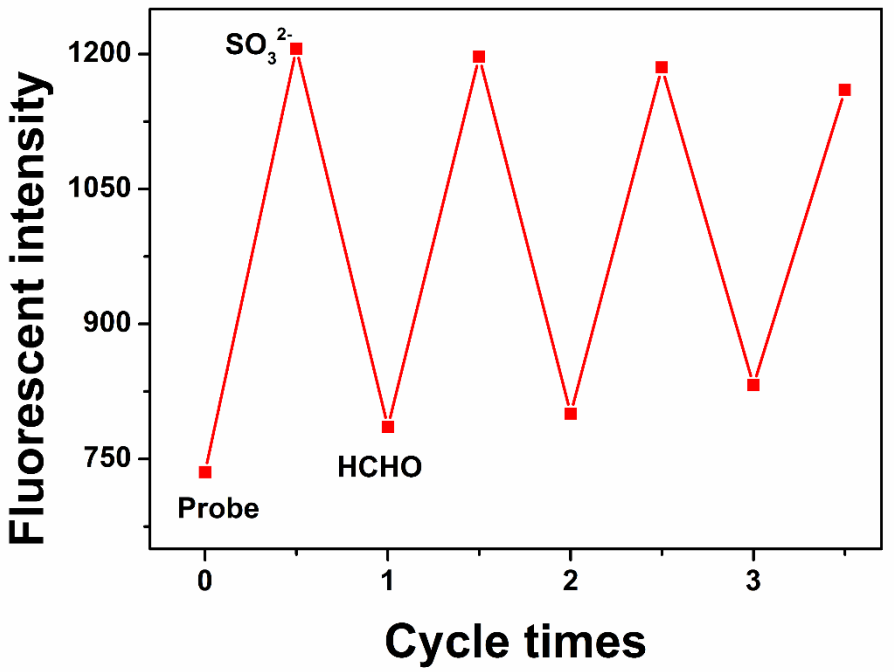

Absorbance
0.6
0.4
0.2
0.0
400
500
600
700
Wavelength/nm
Probe+SO3 2-+HCHO
HCHO
0μM
300μM

Figure 3

(a)



(b)

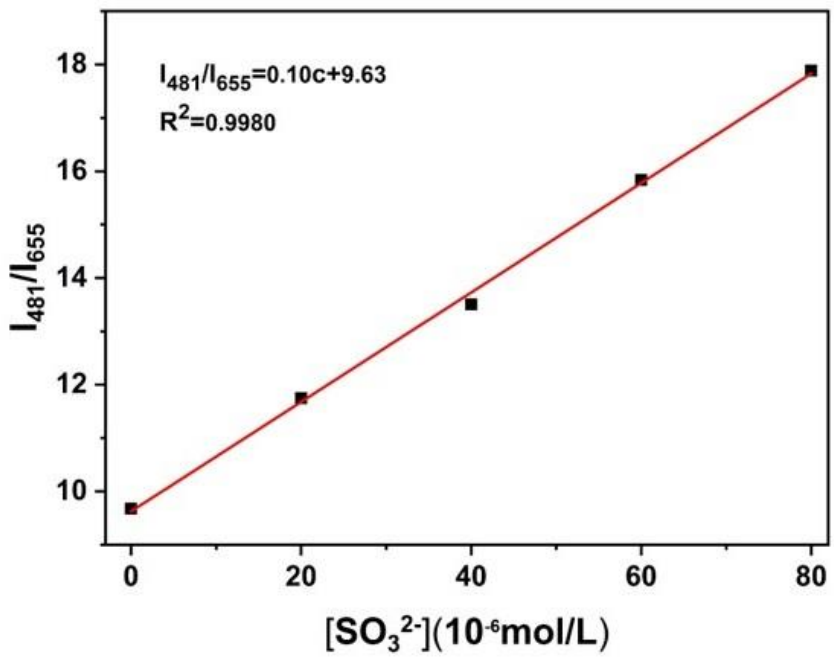


Figure 4

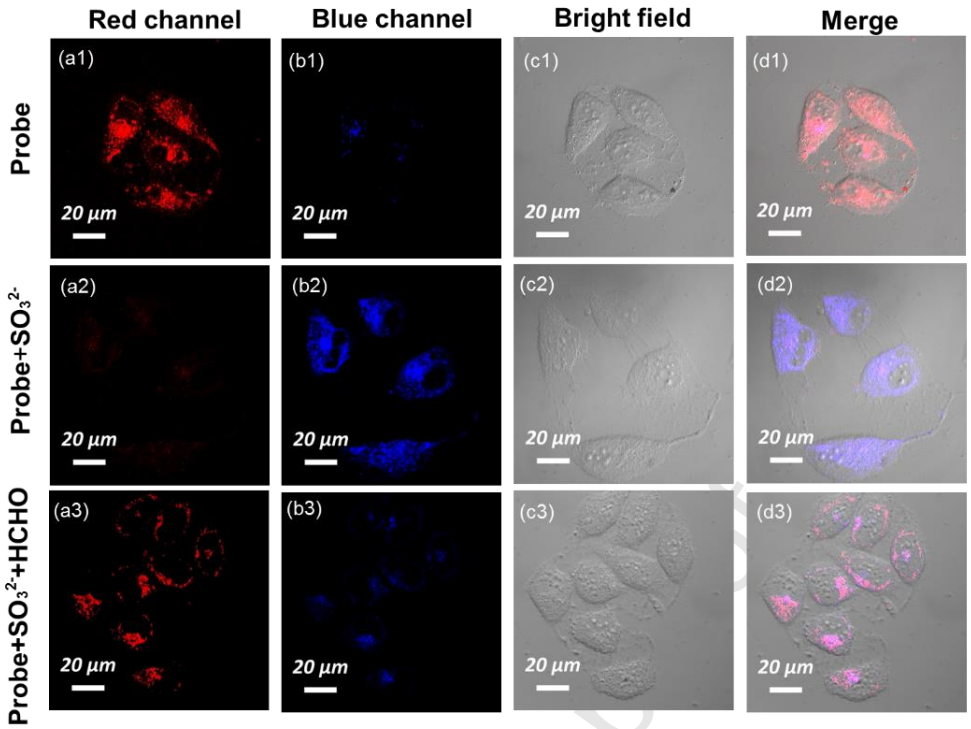
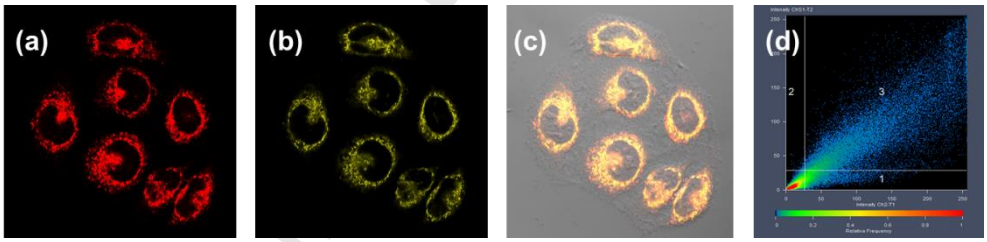

Red channel
Blue channel
Bright field
Merge
Probe
Probe+SO_3^{2-}
Probe+SO_3^{2-}+HCHO
(a1)
(b1)
(c1)
(d1)
(a2)
(b2)
(c2)
(d2)
(a3)
(b3)
(c3)
(d3)
20 μm

Figure 5


(a)
(b)
(c)
(d)
1
2
3

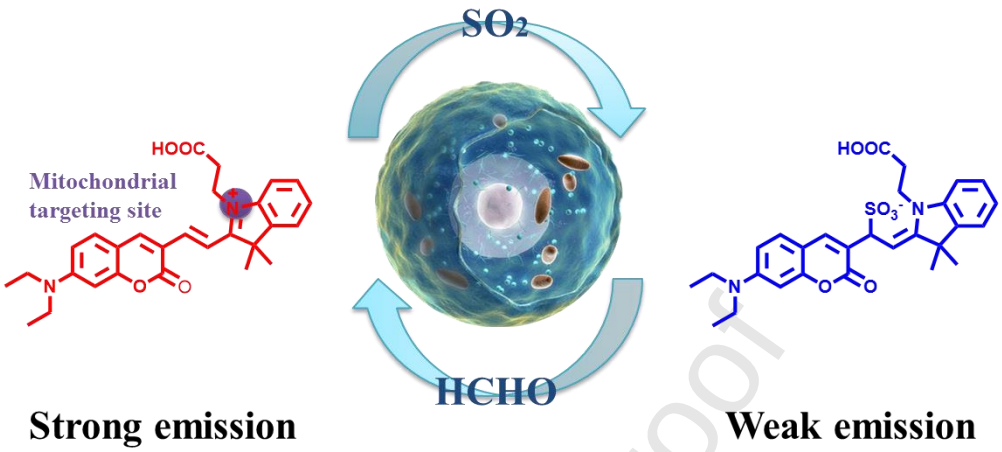
Author contributions

Tao zhang carried out synthesis and spectrometry Caixia yin and Fangjun huo's idea and writing
Jianbin Chao's NMR
Yongbin Zhang's sample detection and cell imaging
Guangming Wen support for experiments.

Declaration of interest statement

We have no any interest conflict.

Graphical abstract



Highlights

a) A near-infrared and ratiometric fluorescent probe for SO_2 was reported.

b) Probe **Mito-CI** showed highly sensitivity (68 nM), excellent water solubility.

c) Probe **Mito-CI** was successfully applied to mitochondria-targeted and reversible for monitoring SO_3^{2-}/HCHO in living cells.