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# A real-time ratiometric fluorescent probe for imaging of SO<sub>2</sub> derivatives in mitochondria of living cells†

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A real-time ratiometric fluorescent probe (IN-CZ) for highly selective detection of sulfite was designed and synthesized, which is based on modulating the intramolecular charge transfer (ICT) of the hemicyanine dye platform. The mechanism of using the probe is mainly through the Michael addition that occurs between IN-CZ and sulfite with a detection limit of  $2.99 \times 10^{-5}$  M. IN-CZ displays a fast response (within 1 minute) and is highly selective for SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup> over ROS, biologically relevant ions, biological mercaptans and other reactive species. More importantly, IN-CZ was suitable for ratiometric fluorescence imaging in living cells, by real-time monitoring of SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup> changes in mitochondria targeted in living cells.

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## 1. Introduction

Sulfur dioxide (SO<sub>2</sub>) is an inevitable product from the combustion of coal and fossil fuels as well as automobile exhausts, causing a serious threat to the ecological environment and human health.<sup>1</sup> In general, SO<sub>2</sub> is inhaled into the human body through the respiratory tract, and then it mainly exists in a two anions equilibrium state, sulfite and bisulfite (SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup>) in physiological systems.<sup>2</sup> Of which, sulfite has been widely used as an antioxidant in food, beverages, pharmaceuticals and preservatives.<sup>3</sup> However, prolonged exposure to excessive SO<sub>2</sub> may cause respiratory tract infection, even leading to lung cancer, cardiovascular disease and neurological disorders.<sup>4</sup> SO<sub>2</sub> and its derivatives (SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup>) can alter the intracellular thiol levels, and then destroy the redox balance in the cells leading to neuron disorders.<sup>5</sup> Mitochondria as the main place for energy supply and transformation, provide a large number of reactive oxygen species (ROS) and reactive sulfur species (RSS).<sup>6</sup> Moreover, in the process of energy supply and transformation in mitochondria of cells, SO<sub>2</sub> and its derivatives (SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup>) can also be produced from L-cysteine in reactions catalyzed by aspartate aminotransferase-2. The abnormal endogenous levels of SO<sub>2</sub> and its derivatives (SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup>) can also cause a series of pathological changes.<sup>7</sup> The

United Nations Food and Agriculture Organization (FAO) and The World Health Organization (WHO) have regulated an acceptable daily intake of sulfite to be lower than 0.7 mg kg<sup>-1</sup> of body weight.<sup>8</sup> Thus, developing methods to detect exogenous and endogenous SO<sub>2</sub> and its derivatives (SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup>) is of great significance.

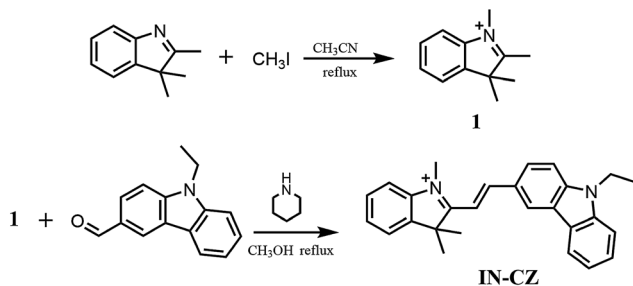
At present, there are many detection strategies for SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup>, including Monier-Williams,<sup>9</sup> electrochemical,<sup>10</sup> fluorescence spectrophotometry,<sup>11</sup> chromatography,<sup>12</sup> capillary electrophoresis,<sup>13</sup> titration<sup>14</sup> and fluorescent probe.<sup>15</sup> Among which, fluorescent indicators for SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup> have attracted much attention due to their apparent advantages over other methods, such as high selectivity and specificity, operational simplicity, as well as high space-time resolution real-time detection and imaging *in vivo*.<sup>16</sup> As SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup> is mainly expressed in mitochondria of cells, the quantitative detection of SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup> is of great importance in both chemical and mitochondria targeted in living cells.<sup>17</sup>

In this work, we designed and synthesized a new ratiometric fluorescent probe IN-CZ for real-time monitoring of SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup> changes in mitochondria targeted in living cells. The synthesis route for IN-CZ is shown in Scheme 1. The Michael addition that occurs between IN-CZ and sulfite makes the conjugate structure change (as shown in Scheme 2). The probe can not only realize ratiometric detection of SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup>, but also shows high selectivity and sensitivity for SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup> against various interferences with a detection limit of  $2.99 \times 10^{-5}$  M. IN-CZ exhibits excellent stability and localizes well in the mitochondria. Moreover, IN-CZ displayed a fast response (within 1 minute) and lower cell toxicity, which endows IN-CZ with good permeability and application to imaging SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup> in living cells.

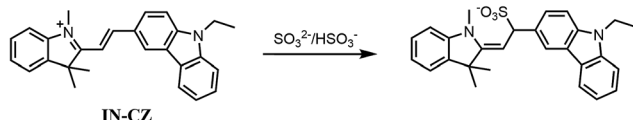
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Scheme 1 Synthesis route for IN-CZ.

Scheme 2 Proposed sensing mechanism of IN-CZ towards  $\text{SO}_3^{2-}/\text{HSO}_3^-$ .

## 2. Experimental

### 2.1 Materials and instruments

All the chemicals and reagents were obtained from commercial suppliers and prepared in stock with standard methods before use. Silica gel (200–300 mesh, Qingdao Haiyang Chemical Co.) was used for column chromatography. NMR spectra were recorded on a Bruker Advance III at 400 MHz for  $^1\text{H}$  NMR and at 100 MHz for  $^{13}\text{C}$  NMR with chemical shifts reported as ppm (in  $\text{CDCl}_3$ , TMS as internal standard). Mass spectra (MS) were measured with a Bruker Apex IV FTMS using electrospray ionization ( $\text{ESI}^+$ ). Absorption spectra were recorded on a Purkinje TU-1901 spectrophotometer. Fluorescence measurements were taken on a Hitachi F-7000 fluorescence spectrometer with a 10 mm quartz cuvette. Excitation wavelength was 420 nm.

### 2.2. Synthesis of fluorescent probe IN-CZ

**2.2.1 Synthesis of compound 1.** In 10 mL of acetonitrile, 2,3,3-trimethylindolenine (1.0 g, 6.3 mmol) and iodomethane (4.43 g, 31.4 mmol) were added, the mixture was stirred and

refluxed overnight. Afterwards, cooling to room temperature (RT), the mixture was filtered and pink solid **1** was obtained.

**2.2.2 Synthesis of compound IN-CZ.** In 1 mL of piperidine and 25 mL of ethanol, a mixture of compound **1** (215 mg, 0.75 mmol) and *N*-ethyl-3-carbazolecarboxaldehyde (167 mg, 0.75 mmol) was stirred at 80 °C for 24 h. Afterwards, the mixture was extracted with DCM ( $30 \times 3$  mL) and washed with water ( $50 \times 3$  mL). The residue was purified by silica gel column chromatography using DCM/methanol (v/v, 30/1) as eluent to give white solid **IN-CZ**.  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ , 400 MHz, TMS),  $\delta$  (ppm): 9.10 (s, 1H), 8.64 (d,  $J = 7.82$  Hz, 1H), 8.38 (d,  $J = 7.78$  Hz, 1H), 8.24 (d,  $J = 6.5$  Hz, 1H), 7.86 (t,  $J = 7.93$  Hz, 3H), 7.72–7.68 (m, 2H), 7.63–7.58 (m, 3H), 7.38 (t,  $J = 6.23$  Hz, 1H), 4.58–4.53 (m, 2H), 4.15 (t,  $J = 6$  Hz, 3H), 1.85 (s, 6H), 1.38 (t,  $J = 8.1$  Hz, 3H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ),  $\delta$  (ppm): 181.12, 156.94, 144.09, 142.52, 141.51, 140.64, 130.47, 129.38, 128.83, 127.17, 126.57, 125.42, 124.38, 123.45, 122.44, 122.36, 121.12, 110.76, 109.85, 108.94, 51.76, 38.15, 36.72, 27.43, 13.91. MALDI-MS ( $m/z$ ): 379.216 for  $[\text{M}^+]$ .

### 2.3 General procedure for analysis

Parent stock solution of fluorescent probe **IN-CZ** (10 mM) was prepared in absolute DMSO, and stored at 5 °C in a refrigerator as standby application. The various testing species were prepared from NaBr, NaCl, NaClO, Cys, Hcy, GSH,  $\text{FeCl}_3$ ,  $\text{FeCl}_2$ ,  $\text{NaNO}_2$ ,  $\text{NaNO}_3$ ,  $\text{NaHSO}_4$ ,  $\text{NaHCO}_3$  and  $\text{NaHSO}_3$ , and the stock solution concentrations of various species (10 mM) were prepared in twice-distilled water. The test solutions of **IN-CZ** (10  $\mu\text{M}$ ) were prepared by placing 100  $\mu\text{L}$  of the corresponding stock solution into 100 mL PBS (10 mM, pH = 7.4). PBS solution was prepared with  $\text{Na}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$ , and adjusted to pH 7.4.

### 2.4 Determination of detection limit

According to the relevant literature published,<sup>18</sup> the detection limit was calculated based on the fluorescence titration. Fluorescence titration was carried out in the aqueous buffer (10 mM PBS, pH = 7.4) to determine the detection limit, which was calculated with the following equation:

$$\text{Detection limit} = 3\sigma/k$$

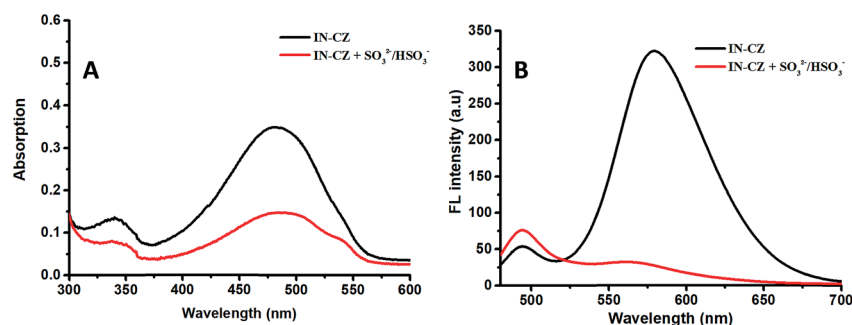
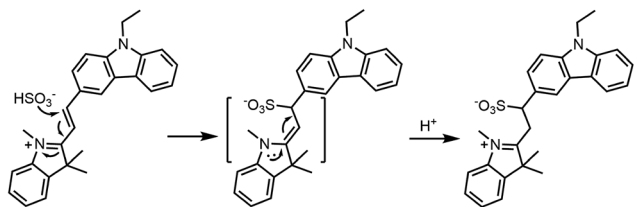


Fig. 1 Spectroscopic properties of **IN-CZ** (10  $\mu\text{M}$ ) in PBS buffer (pH = 7.4, PBS = 10 mM). (A) UV-vis absorption response of **IN-CZ** (10  $\mu\text{M}$ ) in the presence of  $\text{SO}_3^{2-}/\text{HSO}_3^-$  (10  $\mu\text{M}$ ). (B) Fluorescence emission intensity change of **IN-CZ** (10  $\mu\text{M}$ ) in the presence of  $\text{SO}_3^{2-}/\text{HSO}_3^-$  (100  $\mu\text{M}$ ). Excitation at 420 nm.





Scheme 3 Proposed reaction mechanism of IN-CZ with  $\text{SO}_3^{2-}/\text{HSO}_3^-$ .

Where  $\sigma$  is the standard deviation of blank measurements,  $k$  is the slope between the fluorescence intensity vs.  $\text{SO}_3^{2-}/\text{HSO}_3^-$  concentration.

### 2.5 Cytotoxicity

HeLa cells were cultured in the DMEM in a 5%  $\text{CO}_2$  humidity incubator at 37 °C. Cells were inoculated in 96-well plates at 5000 cells per plate. After overnight culture, the medium in each well was replaced by fresh medium containing different concentrations of IN-CZ (0, 5, 10, 15, 20, 25  $\mu\text{M}$ ). After 24 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5  $\text{mg mL}^{-1}$ , 20  $\mu\text{L}$ ) was added to each plate for 3 h. Then medium was discarded, DMSO (100  $\mu\text{L}$ ) was added to dissolve the MTT formazan crystals and cultured for another 4 h. The absorbance in proportion to viable cell count was directly measured using a Bio-Rad 680 ELISA reader at 570 nm and all the measurements were completed three times under the condition of blank control.

### 2.6 Fluorescence imaging of $\text{SO}_3^{2-}/\text{HSO}_3^-$ in living cells

HeLa cells were grown on glass-bottom culture dishes in DMEM supplemented with 10% fetal bovine serum and 1% penicillin. The cells were incubated under an atmosphere of 5%  $\text{CO}_2$  and 95% air at 37 °C for 24 h. Before use, the cells were washed with PBS buffer solution three times. For the experiment of imaging

of  $\text{NaHSO}_3$ , cells were incubated with 100  $\mu\text{M}$  of  $\text{NaHSO}_3$  for 30 min at 37 °C. Afterwards, cells were washed three times with PBS-free DMEM, the pretreated cells were incubated in culture media with IN-CZ (5  $\mu\text{M}$ ) for an additional 10 min at 37 °C, and then washed with PBS (pH = 7.4) three times. Fluorescence imaging of HeLa cells was conducted with an Olympus IX81 confocal fluorescence microscope with excitation wavelength of 405 nm.

## 3. Results and discussion

### 3.1 Spectral response of IN-CZ to $\text{SO}_3^{2-}/\text{HSO}_3^-$

The absorption and fluorescence spectra of IN-CZ in the PBS buffer (pH = 7.4, PBS = 10 mM) were studied. We first explored the spectra changes in the absence and presence of  $\text{SO}_3^{2-}/\text{HSO}_3^-$ . As shown in Fig. 1, the probe displayed a major absorption band centered at 480 nm with a corresponding fluorescence emission peak at 579 nm. With the addition of  $\text{NaHSO}_3$ , the absorption intensity of the peak at 480 nm significantly receded, revealing that the structure of IN-CZ has changed. Meanwhile the emission peak at 579 nm significantly reduced, and the corresponding fluorescence emission peak at 494 nm enhanced. The main reason for the spectra change is attributed to a 1,4-addition reaction that occurs between IN-CZ and  $\text{NaHSO}_3$ , the C=C conjugation between hemicyanine and carbazole is broken by the attack of  $\text{SO}_3^{2-}/\text{HSO}_3^-$  to obtain a blue-emission which is attributed to carbazole. These results indicate that IN-CZ has potential as a ratiometric fluorescent indicator for detection of  $\text{SO}_3^{2-}/\text{HSO}_3^-$ .

### 3.2 Recognition mechanism of IN-CZ for $\text{SO}_3^{2-}/\text{HSO}_3^-$

The recognition mechanism of IN-CZ for  $\text{SO}_3^{2-}/\text{HSO}_3^-$  is presumably due to the 1,4-addition reaction that occurs between IN-CZ and  $\text{NaHSO}_3$  (Scheme 3). In order to verify the sensing mechanism, high resolution mass spectrometry (HRMS) was utilized to provide evidence in the absence and

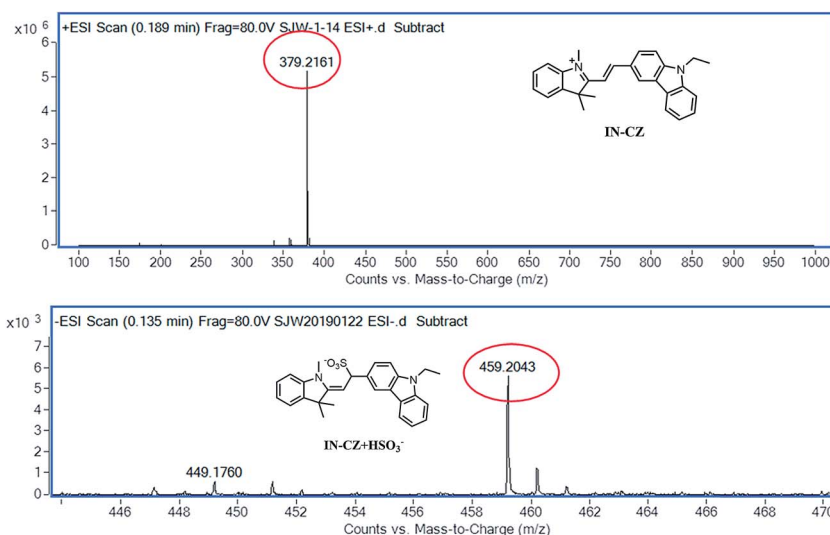


Fig. 2 HRMS spectra of probe IN-CZ treated without and with  $\text{HSO}_3^-$ .



presence of  $\text{NaHSO}_3$  (as shown in Fig. 2). In the absence of  $\text{NaHSO}_3$ , HRMS of the probe in PBS buffer (pH = 7.4, PBS = 10 mM) was conducted and the peak exhibited at  $m/z = 379.2161$ . In the presence of  $\text{NaHSO}_3$ , the HRMS of the compound in PBS buffer (pH = 7.4, PBS = 10 mM) was conducted and the peak displayed at  $m/z = 459.2043$ , which is almost identical to the molecular weight of the adduct (calcd for  $\text{C}_{27}\text{H}_{27}\text{N}_2\text{O}_3\text{S}$ ,  $m/z = 459.18$ ). Both the literature and experimental results provide strong support for the proposed mechanism.<sup>17e,f</sup> The reaction changes the intramolecular charge transfer (ICT) of **IN-CZ**, and then a ratiometric fluorescent probe for detection of  $\text{SO}_3^{2-}/\text{HSO}_3^-$  was obtained.

### 3.3 The selectivity of **IN-CZ** for various analytes

To confirm the selectivity of **IN-CZ** towards  $\text{HSO}_3^-$ , the probe was incubated with various analytes. As shown in Fig. 3A, with the addition of  $\text{Br}^-$ ,  $\text{Cl}^-$ ,  $\text{ClO}^-$ , Hcy, Cys, GSH,  $\text{HSO}_4^{2-}$ ,  $\text{HCO}_3^-$ ,  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ,  $\text{S}^{2-}$ ,  $\text{CN}^-$ ,  $\text{HS}^-$  and  $\text{S}_2\text{O}_3^{2-}$ , there were nearly no fluorescence changes or weak fluorescence changes, with only  $\text{SO}_3^{2-}/\text{HSO}_3^-$  causing an obvious fluorescence change. Moreover, the probe was incubated with various analytes and  $\text{HSO}_3^-$  together. As shown in Fig. 3B, when other analytes coexist with  $\text{HSO}_3^-$ , the response of the probe to

$\text{HSO}_3^-$  was not affected. These results demonstrated that **IN-CZ** is not affected by these species and the emission of **IN-CZ** is stable in the presence of interference species; **IN-CZ** can detect  $\text{SO}_3^{2-}/\text{HSO}_3^-$  specifically.

Through selectivity and interference experiments, the photostability of **IN-CZ** was studied by continuous laser scanning at 420 nm. The fluorescence signal loss was calculated to be less than 3% over 30 min (Fig. S1†), indicating the excellent photostability of **IN-CZ**. Then, the kinetic profile of the recognition of **IN-CZ** for  $\text{SO}_3^{2-}/\text{HSO}_3^-$  was carried out, the response time of **IN-CZ** in the presence of  $\text{NaHSO}_3$  was evaluated (Fig. S2†). When  $\text{NaHSO}_3$  (50  $\mu\text{M}$ ) was added to the solution of **IN-CZ** (10  $\mu\text{M}$ ), the relative fluorescence of  $I_{494}/I_{579}$  increased immediately. This is consistent with the fact that the 1,4-addition reaction occurred between **IN-CZ** and  $\text{NaHSO}_3$ , making the probe conjugate structure change rapidly. The results above demonstrate the excellent photostability and sensitivity of **IN-CZ**.

### 3.4 Quantification of $\text{SO}_3^{2-}/\text{HSO}_3^-$ and detection limit

The optical response validity of **IN-CZ** toward  $\text{SO}_3^{2-}/\text{HSO}_3^-$  was investigated. The fluorescence spectra of **IN-CZ** with various concentrations of  $\text{NaHSO}_3$  (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50  $\mu\text{M}$ ) are shown in Fig. 4A.

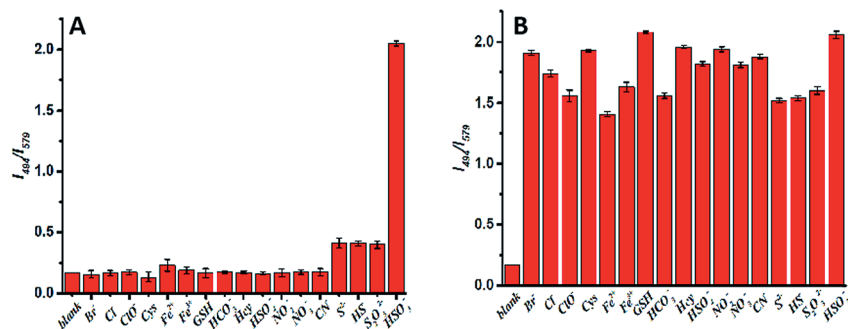


Fig. 3 (A) Relative fluorescence of **IN-CZ** ( $I_{494}/I_{579}$ ) in the presence of various analytes. (B) Relative fluorescence of **IN-CZ** ( $I_{494}/I_{579}$ ) towards  $\text{NaHSO}_3$  (50  $\mu\text{M}$ ) when coexisting with various other analytes. (100  $\mu\text{M}$ ):  $\text{Br}^-$ ;  $\text{Cl}^-$ ;  $\text{ClO}^-$ ; Hcy; Cys; GSH;  $\text{HSO}_4^{2-}$ ;  $\text{HCO}_3^-$ ;  $\text{Fe}^{2+}$ ;  $\text{Fe}^{3+}$ ;  $\text{NO}_2^-$ ;  $\text{NO}_3^-$ ;  $\text{CN}^-$ ;  $\text{S}^{2-}$ ;  $\text{HS}^-$ ;  $\text{S}_2\text{O}_3^{2-}$ ;  $\text{SO}_3^{2-}/\text{HSO}_3^-$  (50  $\mu\text{M}$ ) (except: GSH 1 mM, Hcy 1 mM, Cys 1 mM) in PBS buffer solution (pH = 7.4, PBS = 10 mM, **IN-CZ** = 10  $\mu\text{M}$ ). Excitation at 420 nm.

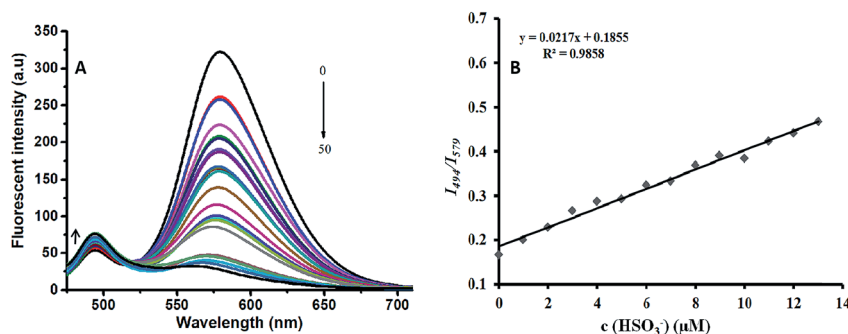


Fig. 4 (A) Fluorescence spectra of **IN-CZ** (10  $\mu\text{M}$ ) upon addition of  $\text{NaHSO}_3$  (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50  $\mu\text{M}$ ). The reaction was performed in PBS buffer (pH = 7.4, 10 mM). (B) The linear relationship between the fluorescence intensity ratio of  $I_{494}/I_{579}$  and the  $\text{SO}_3^{2-}/\text{HSO}_3^-$  concentration (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13  $\mu\text{M}$ ). The reaction was performed in PBS buffer (pH = 7.4, 10 mM). Excitation at 420 nm.



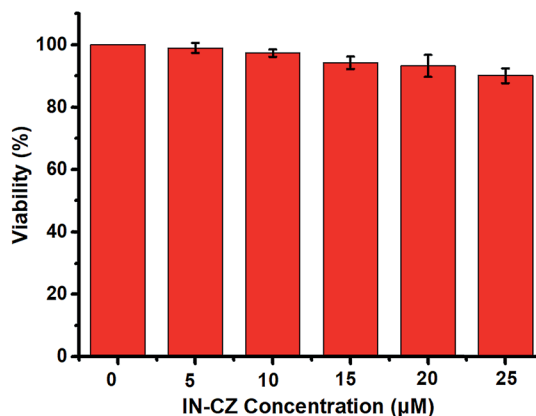


Fig. 5 Cytotoxicity assays for IN-CZ in HeLa cells. Cells were treated with different concentrations of probe IN-CZ for 24 h. Concentrations of IN-CZ (0 μM, 5 μM, 10 μM, 15 μM, 20 μM, 25 μM).

Upon gradually increasing the concentration of NaHSO<sub>3</sub>, the fluorescence band centered at around 579 nm decreased immediately, and the fluorescence band centered at around 494 nm increased. The relative fluorescence intensity of  $I_{494}/I_{579}$  vs. the concentration of NaHSO<sub>3</sub> in the range of 1–13 μM was linear with a detection limit of 299 nM, as shown in Fig. 4B. Therefore, the probe IN-CZ is capable of the identification and quantification of SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup>.

We also investigated the pH effect on the fluorescence spectrum of IN-CZ in the absence and presence of NaHSO<sub>3</sub> under a wider pH range (1–12). As shown in Fig. S3,† the spectral response of IN-CZ was evaluated in PBS buffer (pH = 7.4, 10 mM). The fluorescence intensity ratio ( $I_{494}/I_{579}$ ) of the probe was barely affected in the pH range 5.0–10.0, which suggested that the probe was stable within this pH range. Afterwards, in the presence of NaHSO<sub>3</sub>, the probe could respond to NaHSO<sub>3</sub> in the pH range 5.0–10.0, indicating that IN-CZ could be used for the detection of SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup> under physiological conditions.

### 3.5 Cellular fluorescence imaging and cytotoxicity

To explore the application of IN-CZ in living cells, its cytotoxicity was evaluated in HeLa cells, using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay under different probe concentrations. As displayed in Fig. 5, no significant change in the cell viability was observed when the cells were cultured in the presence of a high concentration of IN-CZ of 25 μM for 24 h. This indicates that IN-CZ shows no cytotoxicity and good biocompatibility in live cells.

To confirm the specific stain of mitochondria in living cells, we carried out a colocalization experiment (Fig. 6). HeLa cells were incubated with IN-CZ (5 μM) and Mito-tracker Red (100 nM, a commercial red-fluorescent mitochondrial dye) for 15 min at 37 °C, and the excess dye was washed away with

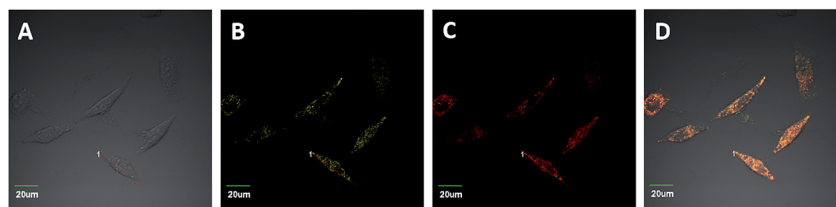


Fig. 6 HeLa cells were incubated with IN-CZ (5 μM) and Mito-Tracker Red (100 nM): (A) bright-field; (B) yellow channel of IN-CZ; (C) red-channel of Mito-Tracker Red; (D) merged images (B) and (C) in bright-field. Excitation at 405 nm. Scale bar: 20 μm.

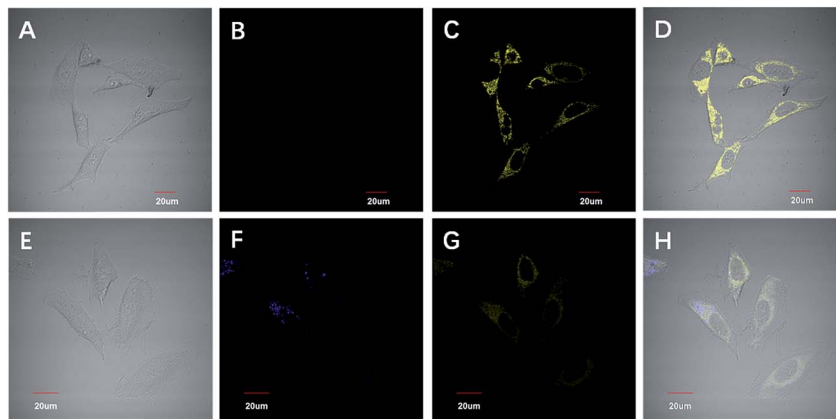


Fig. 7 Images of HeLa cells after incubation with IN-CZ (5 μM) in the absence and presence of NaHSO<sub>3</sub> (100 μM). (A and E) Bright-field; (B–D) are the images in the absence of NaHSO<sub>3</sub>, blue channel, yellow channel and merged images in bright field; (F–H) are the images in the presence of NaHSO<sub>3</sub>, blue channel, yellow channel and merged images in bright field. Excitation at 405 nm. Scale bar: 20 μm.



phosphate buffered saline. The images of yellow channel and red channel merged well in the HeLa cells. Along the red line, the intensity trends of Mito-tracker Red and **IN-CZ** have a high degree of overlap. Moreover, the Pearson's correlation coefficient was calculated to be 0.838, indicating that **IN-CZ** could localize in the mitochondria, which is one of the main organelles for generation of endogenous  $\text{SO}_2$  *in vivo*. These results imply the potential capacity of **IN-CZ** for monitoring endogenous  $\text{SO}_2$  in living cells.

To determine the favorable properties of the **IN-CZ** for monitoring  $\text{SO}_2$  derivatives, we then evaluated the potential application of **IN-CZ** in living cells. As shown in Fig. 7, cells were incubated with **IN-CZ** (5  $\mu\text{M}$ ) for 10 min at 37 °C. There were nearly no fluorescence signals in the blue channel; obvious fluorescence signals can be observed in the yellow channel, which indicates the remarkable membrane permeability of the probe. Afterwards, cells were incubated with 100  $\mu\text{M}$  of  $\text{NaHSO}_3$  for 30 min at 37 °C, then the cells were incubated in culture media with **IN-CZ** (5  $\mu\text{M}$ ) for an additional 10 min at 37 °C. As we expected, the blue fluorescence channel gradually became bright and the yellow fluorescence channel became darkened. The obvious changes in ratiometric fluorescence responses generated from the blue channel and yellow channel in HeLa cells were observed. These results imply that **IN-CZ** could monitor  $\text{SO}_3^{2-}/\text{HSO}_3^-$  changes through the ratiometric fluorescence imaging in living cells.

## 4. Conclusion

In summary, we have presented a novel ratiometric fluorescent probe **IN-CZ** based on a hemicyanine dye platform. The probe could be used as a ratiometric fluorescent sensor for  $\text{SO}_3^{2-}/\text{HSO}_3^-$  anions in aqueous environment based on a Michael addition reaction. The probe displayed a fast reaction time, good sensitivity, and high selectivity for  $\text{SO}_3^{2-}/\text{HSO}_3^-$  over other reactive species, ROS, biologically relevant ions and biological mercaptans. Moreover, **IN-CZ** is a ratiometric fluorescent probe for the real-time monitoring of  $\text{SO}_3^{2-}/\text{HSO}_3^-$  changes through the ratiometric fluorescence imaging with mitochondria targeted in living cells with good permeability and low cytotoxicity. We expect that this design will be further developed as a good probe for  $\text{SO}_2$  derivatives in realistic samples and biological applications.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

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## Notes and references

- (a) T. M. Chen, W. G. Kuschner, J. Gokhale and S. Shofer, *Am. J. Med. Sci.*, 2007, **333**, 249–256; (b) M. Kampa and E. Gastanas, *Environ. Pollut.*, 2008, **151**, 362–367.
- M. H. Stipanuk and I. Ueki, *J. Inherited Metab. Dis.*, 2011, **34**, 17–32.
- (a) S. Iwasawa, Y. Kikuchi and Y. Nishiwaki, *J. Occup. Health*, 2009, **51**, 38–47; (b) M. Reist, P. Jenner and B. Halliwell, *FEBS Lett.*, 1998, **423**, 231–234.
- (a) G. Li and N. Sang, *Ecotoxicol. Environ. Saf.*, 2009, **72**, 236–241; (b) G. L. Li, R. J. Li and Z. Q. Meng, *Eur. J. Pharmacol.*, 2010, **645**, 143–150.
- (a) N. Sang, Y. Yun, H. Y. Li, L. Hou, M. Han and G. K. Li, *Toxicol. Sci.*, 2010, **114**, 226–236; (b) N. Sang, Y. Yun, G. Y. Yao, H. Y. Li, L. Guo and G. K. Li, *Toxicol. Sci.*, 2011, **124**, 400–413.
- (a) Y. Liu, K. Li, M. Y. Wu, Y. H. Liu, Y. M. Xie and X. Q. Yu, *Chem. Commun.*, 2015, **51**, 10236–10239; (b) W. Xu, C. L. Teoh, J. J. Peng, D. D. Su, L. Yuan and Y. T. Chang, *Biomaterials*, 2015, **56**, 1–9.
- (a) F. Y. Zhang, X. W. Liang, W. Z. Zhang, Y. L. Wang, H. L. Wang, Y. H. Mohammed, B. Song, R. Zhang and J. L. Yuan, *Biosens. Bioelectron.*, 2017, **87**, 1005–1011; (b) W. Z. Zhang, J. M. Zhang, H. L. Zhang, L. Y. Cao, R. Zhang, Z. Q. Ye and J. L. Yuan, *Talanta*, 2013, **116**, 354–360.
- W. J. FAO, in *WHO food additives series*, World Health Organization, Geneva, 60th edn, 2009.
- G. W. Monier-Williams, *Analyst*, 1927, **52**, 343–344.
- (a) T. Yilmaz and G. Somer, *Anal. Chim. Acta*, 2007, **603**, 30–35; (b) C. S. Pundir and R. Rawal, *Anal. Bioanal. Chem.*, 2013, **405**, 3049–3062.
- (a) P. W. West and G. Gaeke, *Anal. Chem.*, 1956, **28**, 1816–1819; (b) M. A. Segundo, A. O. Rangel, A. Cladera and C. Victor, *Analyst*, 2000, **125**, 1501–1505; (c) T. Williams, S. Mcelvany and E. Ighodalo, *Anal. Chim. Acta*, 1981, **123**, 351–354.
- (a) P. Laura, D. L. Giuseppe and Q. Enrica, *Food Chem.*, 1998, **63**, 275–279; (b) R. F. McFeeters and A. O. Barish, *J. Agric. Food Chem.*, 2003, **51**, 1513–1517.
- (a) D. Zydrunas and P. Audrius, *Electrophoresis*, 2002, **23**, 2439–2444; (b) J. Giedre, D. Zydrunas and P. Audrius, *J. Chromatogr. A*, 2001, **934**, 67–73.
- L. Denise and B. Mauro, *Food Addit. Contam.*, 2001, **18**, 773–777.
- (a) L. Tan, W. Lin, S. Zhu, L. Yuan and K. B. Zheng, *Org. Biomol. Chem.*, 2014, **12**, 4637–4643; (b) Y. Q. Sun, J. Liu, J. Zhang, T. Yang and W. Guo, *Chem. Commun.*, 2013, **49**, 2637–2639; (c) X. F. Yang, M. Zhao and G. Wang, *Sens. Actuators, B*, 2011, **152**, 8–13; (d) G. Y. Li, Y. Chen, J. Q. Wang, J. H. Wu, G. Gasser, L. N. Ji and H. Chao, *Biomaterials*, 2015, **63**, 128–136; (e) W. Q. Chen, X. J. Liu, S. Chen, X. Z. Song and J. Kang, *RSC Adv.*, 2015, **5**, 25409–25415; (f) S. Paul, K. Ghoshal, M. Bhattacharyya and D. K. Maiti, *ACS Omega*, 2017, **2**, 8633–8639; (g) M. G. Choi, J. Y. Hwang, S. Y. Eor and S. K. Chang, *Org. Lett.*, 2010, **12**, 5624–5627.



- 16 (a) J. Chan, S. C. Dodani and C. J. Chang, *Nat. Chem.*, 2012, **4**, 973; (b) Y. M. Yang, Q. Zhao, W. Feng and F. Y. Li, *Chem. Rev.*, 2013, **113**, 191–270; (c) J. J. Du, M. M. Hu, J. L. Fan and X. J. Peng, *Chem. Soc. Rev.*, 2012, **41**, 4511–4535; (d) F. Zhou, Y. Sultanbawa, H. Feng, Y. L. Wang, Q. T. Meng, Y. Wang, Z. Q. Zhang and R. Zhang, *J. Agric. Food Chem.*, 2019, **67**, 4375–4383; (e) W. Q. Chen, Q. Fang, D. L. Yang, H. Y. Zhang, X. Z. Song and J. Foley, *Anal. Chem.*, 2015, **87**, 609–616; (f) Y. Y. Ma, Y. H. Tang, Y. P. Zhao, S. Y. Gao and W. Y. Lin, *Anal. Chem.*, 2017, **89**, 9388–9393.
- 17 (a) J. Yang, K. Li, J. T. Hou, L. L. Li, C. Y. Lu, Y. M. Xie, X. Wang and X. Q. Yu, *ACS Sens.*, 2016, **1**, 166–172; (b) X. L. Zheng, H. Li, W. Feng, H. C. Xia and Q. H. Song, *ACS Omega*, 2018, **3**, 11831–11837; (c) G. Chen, W. Zhen, C. Y. Zhao, Y. X. Liu, T. Chen, Y. L. Li and B. Tang, *Anal. Chem.*, 2018, **90**, 12442–12448; (d) D. Y. Li, X. W. Tian, Z. Li, J. H. Zhang and X. B. Yang, *J. Agric. Food Chem.*, 2019, **67**, 3062–3067; (e) C. C. Gao, Y. Tian, R. B. Zhang, J. Jing and X. L. Zhang, *New J. Chem.*, 2019, **43**, 5255–5259; (f) Y. F. Wang, Q. T. Meng, R. Zhang, H. M. Jia, X. H. Zhang and Z. Q. Zhang, *Org. Biomol. Chem.*, 2017, **15**, 2734–2739.
- 18 (a) H. Chen, B. L. Dong, Y. H. Tang and W. Y. Lin, *Chem.–Eur. J.*, 2015, **21**, 11696–11700; (b) B. P. Guo, X. Z. Pan, Y. F. Liu, L. X. Nie, H. Z. Zhao, Y. Z. Liu, J. Jing and X. L. Zhang, *Sens. Actuators, B*, 2018, **256**, 632–638; (c) C. Y. Liu, C. X. Shao, H. F. Wu, B. P. Guo, B. C. Zhu and X. L. Zhang, *RSC Adv.*, 2014, **4**, 16055–16061.

