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# A novel ratiometric fluorescent probe for "naked-eye" detection of sulfite ion: Applications in detection of biological $SO_3^{2-}$ ions in food and live cells

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#### ABSTRACT

A new "turn-on" luminescent probe **PI** has been designed and synthesized for the selective detection of sulfite ions based on the mechanism of nucleophilic addition. The designed probe **PI** owns naked eye detection, excellent selectivity, sensitivity, rapid response (150s) and low limits of detection (LOD) of  $0.57\mu$ M, which is an agreeable limit by the world wide expert food additive committees. Furthermore, the probe **PI** was used to recognize the sulfite ions level in realistic samples and live cells.

**Key words:** Ratiometric fluorescent probe, Naked eye detection, realistic samples, cell imaging studies.

Sontral

#### **1. Introduction**

Due to the vast industrial development, the release of toxic gases such as oxides of nitrogen (NO), sulfur (SO<sub>2</sub>) and carbon (CO) are predominantly increasing during current juncture. Of all these gases, SO<sub>2</sub> is a widely known pernicious environmental pollutant which generally occurs as well-known derivatives such as sulfite and bisulfite anions [SO<sub>3</sub><sup>2-</sup>/HSO<sub>3</sub><sup>-</sup>] [1-6]. There are lot of ways for humans to consume sulfite and bisulfite either through inhalation or by oral perception [7, 8]. However, SO<sub>2</sub> derivatives play a major role in biological systems to control the enzyme activity through binding, in food and beverage industries to counteract the deterioration of stored food products plus maintain the optimistic colour of the food items and in medicinal field to prepare drugs [9-13]. Besides, intake of excess amount of sulfite  $(SO_3^{2-})$  and bisulfite ions (HSO<sub>3</sub><sup>-</sup>) may lead to numerous effects such as asthma, lung cancer in respiratory system, neurological diseases, cardiovascular diseases and acute symptoms such as abdominal pain, urticarial, hypotension and allergic reaction in some individuals [14-21]. Due to all the above health effects, the expert food additive committees such as Food and Agriculture Organization (FAO)/ Joint Expert committee for Food Additives (JECFA)/ World Health Organization (WHO) have resolute that the daily consumption of sulfite ions in food products must be acceptable only when it is less than 0.7 mg/kg of an individual body weight. For instance, in China the total amount of sulfite in white granular sugar is severely delimited as < 30mg/kg and it is not permissible in USA for using sulfite in meat [22-24]. Hence, there is a demand to develop an agreeable and reliable technique to detect sulfite ions both in environment and biological systems.

Countless methods are available to detect sulfite ions including several traditional methods such as capillary electrophoresis, electrochemistry, conductivity, voltammetry, titrimetry, chromatography, flow injection analysis and others [25-28]. But all the above traditional methods detect sulfite ions only by sample pre-treatment, need expensive equipment and consume more time [29-32]. Therefore, the traditional detection techniques are not taken into account due to the above issues. At present, fluorescent chemosensors attracted researcher attention to detect sulfite ions because of its captivating characteristics such as low price equipments, simple operation, greater sensitivity, superior selectivity and real time visual detection [33-38].

As per the above mentioned significance of fluorescent chemosensors, various luminescent probes have been developed for the detection of  $SO_3^{2-}$  ions. On the other hand, the present researchers have their desire towards the syntheses of ratiometric and NIR luminescent probes for examining the derivatives of anions in organic medium [39, 40]. For instance, Cheng et al [41], had developed a fluorescent probe to detect the sulfur-di-oxide derivatives with greater selectivity in aqueous and biotic atmosphere. Zhang et al [42], used melamine and gold nanoparticles for the preparation of colorimetric probe to detect the sulfite anion. A resorufin fluorophore containing levulinate group was used for the detection of sulfite anion in water samples by Choi et al [43]. Yu et al [44], have synthesized a "turn-on" luminescent sensor for the recognition of  $SO_3^{2-}$  ion in food and beverages with suitable fluorescence emission. By comparing the normal single emission probes, the ratiometric luminescent probes holds some benefits like providing clear-cut outputs, superior selectivity and reducing the auto luminescent instabilities [45]. However, maximum number of reported probes based on "turn-on" (ratiometric) method display some shortcomings such as absorption and emission peak in the visible region (400-800 nm) with high LOD, less solubility in aqueous medium, slow response and intrusion due to other significant anions which all hold back their importance in biotic system [46, 47]. Thus, the synthesis of a new novel "turn-on" luminescent probe for the detection of  $SO_3^{2-}$  ions tempts our attention.

In consequence to all the above aspects, we have developed a novel fluorescent probe appended with phenanthrene imidazole fluorophore moiety (**PI**) to detect  $SO_3^{2^-}$  ions over the other competing anions. The probe **PI** possesses great selectivity and fast response. Besides, the synthesized probe **PI** could be effectively employed to monitor sulfite ions in real samples and in live (HeLa) cells.

#### 2. Experimental section

#### 2.1 Materials and methods

All chemicals taken for the syntheses were analar pure grade and are brought from Sigma-Aldrich. The solvents were delivered from commercial suppliers and are used without any further purification. The double distilled water was used all through the synthesis. Technico micro heating table was used to measure the melting points in an open capillary tube and was uncorrected. Vario EL III Elemental analyzer was used to acquire the analytical data. UV-Visible

spectra and fluorescence spectra were recorded on Shimadzu 1800 UV-Visible spectrophotometer and JASCO FP-8200 spectrophotometer respectively at room temperature using a 1cm<sup>2</sup> quartz cuvette (made of pure silica) at pH 7.4. Bruker AMX-500 spectrometer was used to obtain <sup>1</sup>H-NMR spectra in dimethyl sulfoxide-d<sub>6</sub> or duterated chloroform. Tetramethylsilane (TMS) is used as a reference to record the chemical shifts ( $\delta$ ) in ppm. By using a high voltage electro spray ionization probe (aerosol) in an advanced Q-TOF micro<sup>TM</sup> mass spectrometer, the mass spectrum was recorded. Fluorescence IX71 microscope (Japan, Olympus) was used to record and examine the cell imaging studies.

#### 2.2 Synthesis of probe PI

The precursor compound **1** was prepared according to the literature survey [48]. Using the precursor, the following procedure was used to synthesis the probe **PI**. A mixture of compound **1** (0.6486 g, 2 mmol) and 1- ethyl-2,3,3-trimethyl-2,3-dihydro-1H-indole (0.378 g, 2 mmol) was dissolved in ethanol (80 mL) in the presence of piperidine (10  $\mu$ L). Then the whole mixture was allowed to stir at 80°C and the TLC plates were used to check the progress of the reaction. The completion of the reaction takes place after 3 hours and the solvent was evaporated under rotary evaporator which leaves a brown colour solid. The crude product formed was further purified by column chromatography using silica gel with 6:3:1 ratio of petroleum ether/ethyl acetate/methanol mobile phase.

Colour: Brown; Yield: (60%, 0.8 g); M.pt: 232 °C. Anal. Calc. for  $C_{35}H_{30}IN_3$ : C, 67.85; H, 4.88; N, 6.78 %. Found: C, 67.42; H, 4.39; N, 6.35%. FT- IR (cm<sup>-1</sup>): 3391 (-NH group), 1643 (C=N, imidazole ring), UV- Vis (DMSO,  $\lambda_{max}$ , nm): 330, 490. <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, ppm): 13.02 (s, 1H), 8.73 (d, J = 4.8 Hz, 2H), 8.14 (t, 2H), 8.12 (t, J = 7.8 Hz, 2H), 7.65 (t, 2H), 7.61 (t, 1H), 7.87 (d, 2H), 7.59 (d, J = 7.7 Hz, 2H), 7.49 (d, J = 7.8 Hz, 2H), 4.29 (q, 2H, CH=CH), 2.39 (s, 6H), 2.13 (t, 2H). MS (ESI, m/z): 492.24 [M+H]<sup>+</sup>.

#### 3. Results and discussion

#### 3.1 Design and synthesis

The schematic representation for the synthesis of the probe **PI** was charted in scheme 1 and the procedure for the preparation of probe **PI** was stated in experimental part. Furthermore,

several detection techniques were used to characterize the designed probe **PI** and the resultant spectrum, plots and graphs were provided as supportive information (Fig. S1, S2).



Scheme 1: Synthesis of probe (PI)

3.2 Influence of sulfite  $(SO_3^{2})$  ions on absorption spectrum of the probe (PI)

The sensing properties and performances of the newly designed probe **PI** (5  $\mu$ M) was primarily analyzed by absorption spectra in DMSO/H<sub>2</sub>O (1:9 (v/v), HEPES=50mM, pH=7.4). The UV-Vis spectra of the probe **PI** with addition of various anions and biothiols (100 $\mu$ M) including F<sup>-</sup>, Cl<sup>-</sup>, CN<sup>-</sup>, Br<sup>-</sup>, T, S<sup>2-</sup>, OAc<sup>-</sup>, SCN<sup>-</sup>, HCO<sub>3</sub><sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, SO<sub>3</sub><sup>2-</sup>, Cys, GSH and Hcy were recorded and the spectrum of the probe **PI** displays two main peaks at  $\lambda_{max}$  318 and 495 nm. Upon addition of sulfite ions (100  $\mu$ M), the peak at 318 nm shows a slight shift towards red region at 330 nm. Furthermore, the absorption peak at around 490 nm was completely disappeared (Fig. 1). The appearance of new peak specifies that the SO<sub>3</sub><sup>2-</sup> ions induce addition reaction which leads to the breakdown of the  $\pi$ - conjugation of probe **PI**. In contrast, the addition of various anions (100  $\mu$ M) other than sulfite anion to the probe **PI** results in minor responses. In addition to the selectivity study, the UV-Vis titration experiment was carried out to show the enhancement of absorption intensity of sulfite anion (0-100  $\mu$ M) with the probe **PI** (5  $\mu$ M). The result displayed in Fig. 2 shows that the intensity of peak around 490 nm gradually decreases and new peak appears around 330 nm with increase in the intensity. The isobestic point appears at 385 nm shows the binding of  $SO_3^{2^-}$  ion to the probe PI. The mechanism of interaction of probe PI with sulfite ions was further studied and discussed by fluorescence, <sup>1</sup>H-NMR and ESI-Mass spectral studies and the results were provided below.

< Insert Fig.1>

< Insert Fig.2>

## 3.3 Influence of sulfite $(SO_3^{2^2})$ ions on emission spectrum of the probe (PI)

After getting positive results from the absorption spectra, the recognition property of the PI was confirmed by emission studies. The emission spectra of the probe **PI** (5  $\mu$ M) in DMSO/H<sub>2</sub>O (1:9 (v/v), HEPES=50mM, pH=7.4) in the presence of other anions and biothiols namely, F<sup>-</sup>, Br<sup>-</sup>, Cl<sup>-</sup>, CN<sup>-</sup>,  $\Gamma$ , SCN<sup>-</sup>, OAc<sup>-</sup>, HCO<sub>3</sub><sup>-</sup>, S<sup>2-</sup>, SO<sub>4</sub><sup>2-</sup>, Cys, Hcy and GSH shows very weak peak at 460 nm upon excitation at 390 nm. However, addition of SO<sub>3</sub><sup>2-</sup> induces a drastic increase in the fluorescence intensity of **PI** (Fig. 3). Interestingly, stepwise addition of SO<sub>3</sub><sup>2-</sup> (0-100  $\mu$ M) to the **PI** results in systematic enhancement in the emission intensity at 460 nm and when the concentration of sulfite ion reaches 100  $\mu$ M, the saturation in the emission intensity was occurred (Fig. 4a). On the other hand, the excitation at 495 nm displays broad emission band for the free probe at 595 nm. Further addition of cumulative quantities of SO<sub>3</sub><sup>2-</sup> ions leads to the progressive reduction in the broad emission peak at 595 nm until the sulfite ion concentration reaches 100  $\mu$ M (Fig. 4b). As a result, the emission intensities of the probe **PI** were changed from red (595 nm) region to blue (460 nm) region. Thus, fluorescence spectra result show that the detection of sulfite ions by the probe **PI** involves ratiometric method.

- < Insert Fig.3>
- < Insert Fig.4>

Furthermore, the ratio of emission intensities of the probe **PI** ( $I_{460}/I_{595}$ ) was constantly interrelated with the sulfite ions concentration ranging from 0-100  $\mu$ M (Fig. 5). The limit of detection (LOD) was found to be 0.57  $\mu$ M from the formulae  $3\sigma/S$  (where,  $\sigma$  is the standard deviation and S = slope). The LOD for the probe **PI** was lower when compared to the recently reported ratiometric sulfite ions sensors [49-52].

< Insert Fig.5>

#### 3.4 Selectivity and interference studies

Furthermore, the selectivity studies for the probe **PI** towards sulfite ions  $(SO_3^{2^-})$  over all other ecologically important anions and biothiols  $(S^{2^-}, C\Gamma, Br, \Gamma, CN, CO_3^{2^-}, HCO_3^-, OAc, NO_3^{2^-}, SO_4^{2^-}, F, SCN, GSH, Hcy and Cys) were carried out using fluorescence spectra. In the competitive experiment, sulfite ions mixed with other interfering anions and the emission spectra were recorded in presence of the probe$ **PI**. The results from the selectivity studies reveal that probe**PI**shows selective response towards sulfite ions only. The presence of other anions did not alter the fluorescent intensity of the probe (Fig. 6). In addition, "naked–eye" responses of the probe**PI** $with various anions (Fig. S3) also provides an evident for the selective recognition of sulfite (<math>SO_3^{2^-}$ ) ions by the probe **PI**.

< Insert Fig.6>

#### 3.5 Time-dependent study

For real time detection and to predict the amount of sulfite ions in live cells, the response time of the probe **PI** towards  $SO_3^{2-}$  ions is very essential. Hence, variation in the fluorescence response from time to time were measured for the probe **PI** and probe with sulfite ions (100  $\mu$ M) vs time (0-260 s) and the results were charted in Fig. S4. The emission intensity of probe **PI** with sulfite ions shows gradual improvement with increasing time and maintains a linear response at about 150 s. The time-dependent studies results suggested that the probe **PI** possesses fast analytical response towards sulfite ions.

#### 3.6 Effect of pH

For an effective chemosensor, detection ability at the physiological pH is very important. Hence, the effect of pH for probe **PI** and probe containing sulfite ion has been investigated in DMSO/H<sub>2</sub>O (1:9 (v/v) medium at various pH from 3 to 11 (Fig. S5). The result show that optimum pH condition for detection of sulfite ions was in the range 5 to 8. Therefore, the pH sensitivity study of the probe warrants its application under physiological conditions.

#### 3.7 Reaction mechanism of sensing of sulfite anions by the probe PI

Based on all the above outcomes, a plausible mechanism has been proposed and was shown in scheme 2. The probe **PI** undergoes nucleophilic addition reaction to recognize the  $SO_3^{2-}$  anion. The electron withdrawing unit [1H-benzo[e]indolium] in the probe **PI** is attached

through a vinylene bridge with phenanthrene imidazole fluorophore. The presence of  $SO_3^{2^2}$  ion forms a bond by nucleophilic addition on the vinylene carbon and hence  $\pi$ -conjugation was blocked in probe **PI** and thus hinders the ICT (Internal Charge Transfer) process. As a consequence, increase in the fluorescence intensity occurred. In addition to this, the 1:1 stoichiometry of the probe PI with  $SO_3^{2^2}$  ion was proved by Job's plot measurement (Fig. S6).



**Scheme 2.** Plausible mechanism for the interaction of  $SO_3^{2-}$  ion with probe (**PI**) 3.8 *Real sample analysis, fluorescence cell imaging and cytotoxicity studies* 

The sulfite ions are extensively used as an additive in food and beverage industries. Although, these anions play an essential role as an antibacterial and antifungal agent but intake of excessive level of sulfite ions containing food causes severe illness to the strength of humans. Therefore, considering the severe health issues, the sulfite ions detection in beverages and food products is very much important. Thus, the synthesized novel ratiometric probe **PI** which possesses fascinating sensing properties was further used to recognize sulfite ions in real samples. To the studies, samples like jam and soft sugar were purchased from departmental store and were well liquefied and diluted using double distilled water [53, 54]. The adulterated food samples were initially treated with 5  $\mu$ M concentration of the probe PI and then several concentration levels (2-10  $\mu$ M) of sulfite ions. The recoveries of the SO<sub>3</sub><sup>2-</sup> ions in the abovementioned food samples were analyzed by the fluorescence spectra and the retrieval results (Table S1) were ranging from 98.4% to 100.4%. Thus, the real sample analysis of foods containing sulfite ions has been validated by the novel fluorescent probe **PI**.

Inspired by the above significances, the practical efficiency of probe **PI** for sulfite anion detection in live HeLa cells was investigated. Initially, cytotoxicity of probe **PI** was tested

using MTT assay and the result reports that the probe has low toxic to the cultured cells (Fig. S7). Therefore, the probe **PI** was further subjected to cell imaging studies. Initially, the treatment of cells with probe **PI** (10  $\mu$ M) shows no fluorescence in the blue channel when it was imaged through microscope. However, after treating the cells containing probe **PI** (10  $\mu$ M) with sulfite ions (20  $\mu$ M) the cells exhibit strong luminescence in the blue region and on addition of various biothiols such as GSH, Cys, Hcy (20  $\mu$ M) to the probe **PI** (10  $\mu$ M) + sulfite ions (20  $\mu$ M) does not interfere the blue luminescence (Fig. 7). The bio labelling results strongly propose that the probe **PI** has superior capacity in the detection of sulfite ions in live cells on the basis of ratiometric approach.

#### < Insert Fig.7>

The synthesized probe **PI** was compared to the previously designed probes towards sulfite ion detection (Table S2). Even though the previously designed probes retain some benefits like selective and sensitive response towards detection but the probe **PI** reported in this work possesses best properties such as rapid, selective, sensitive and real time detection as well as no interruption from other ions. Hence, the overall summary of this work proves that the probe **PI** has superior ability to detect sulfite ions level even at smaller concentrations.

#### 4. Conclusions

A novel fluorescent probe **PI** was synthesized and characterized by analytical and spectral techniques. The probe **PI** was successfully applied for recognition of sulfite anion over other competitive anions in physiological medium through fluorescence enhancing mechanism. A nucleophilic addition mechanism which blocks the  $\pi$ -conjugation in the probe and thus hinders the ICT (Internal Charge Transfer) process was proposed for the recognition of sulfite anion. Moreover, the sensor is effectively used to detect sulfite ions up to the concentration of 0.57  $\mu$ M. Owing to its excellent features such as suitable sensitivity, greater specificity, the probe **PI** is well suited for the detection of sulfite ions in real samples and live cells and under ideal conditions.

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Solution

#### **Declaration of interests**

 $\boxtimes$  The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

□The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



Some



**Fig. 1.** UV-Vis spectra of the probe **PI** (5  $\mu$ M) in DMSO/H<sub>2</sub>O (1:9 (v/v), HEPES=50 mM, pH=7.4) with 100  $\mu$ M of various anions such as F<sup>-</sup>, Cl<sup>-</sup>, CN<sup>-</sup>, HCO<sub>3</sub><sup>-</sup>, S<sup>2-</sup>, SCN<sup>-</sup>, Br<sup>-</sup>, Γ, SO<sub>3</sub><sup>2-</sup>, OAc<sup>-</sup>, SO<sub>4</sub><sup>2-</sup> and biothiols such as Hcy, GSH, Csy.



**Fig. 2.** UV-Vis spectra of the probe **PI** (5  $\mu$ M) in DMSO–H<sub>2</sub>O (1:9 v/v 50 mM HEPES buffer solution) at pH 7.4 towards 0-100  $\mu$ M of SO<sub>3</sub><sup>2-</sup> ion.



**Fig. 3.** Fluorescence response of the probe **PI** (5  $\mu$ M) in DMSO/H<sub>2</sub>O (1:9 (v/v), HEPES=50 mM, pH=7.4) with 100  $\mu$ M of various anions such as F<sup>-</sup>, Cl<sup>-</sup>, CN<sup>-</sup>, HCO<sub>3</sub><sup>-</sup>, S<sup>2-</sup>, SCN<sup>-</sup>, Br<sup>-</sup>, I<sup>-</sup>, SO<sub>3</sub><sup>2-</sup>, OAc<sup>-</sup>, SO<sub>4</sub><sup>2-</sup> and biothiols such as Hcy, GSH, Csy ( $\lambda_{ex}$ =390 nm).



**Fig. 4.** Fluorescence responses of probe PI (5  $\mu$ M) in DMSO–H<sub>2</sub>O (1:9 v/v 50 mM HEPES buffer solution) at pH 7.4 towards 0-100  $\mu$ M of SO<sub>3</sub><sup>2-</sup>. a)  $\lambda_{ex}$ =390 nm, b)  $\lambda_{ex}$ =495 nm.



**Fig. 5.** Detection limit plot of  $SO_3^{2-}$  by probe **PI**.



**Fig. 6.** Fluorescence response of probe **PI** (5  $\mu$ M) in the presence of 100  $\mu$ M of SO<sub>3</sub><sup>2-</sup> and one different other species (100  $\mu$ M). (1=S<sup>2-</sup>, 2=Cl<sup>-</sup>, 3=Br<sup>-</sup>, 4=l<sup>-</sup>, 5=CN<sup>-</sup>, 6=CO<sub>3</sub><sup>2-</sup>, 7=HCO<sub>3</sub><sup>-</sup>, 8=CH<sub>3</sub>COO<sup>-</sup>(OAc), 9=NO<sub>3</sub><sup>2-</sup>, 10=SO<sub>4</sub><sup>2-</sup>, 11=F<sup>-</sup>, 12=SCN<sup>-</sup>, 13=GSH, 14=Cys and 15= Hcy).



Fig. 7. Bright field image and fluorescence images of HeLa cells treated with probe PI (10  $\mu$ M), sulfite ion (20  $\mu$ M) and biothiols such as GSH, Cys and Hcy (20  $\mu$ M) [ $\lambda_{ex}$ =390 nm].

Sontal



#### Highlights

- A novel ratiometric luminescent probe **PI** containing phenanthrene imidazole moiety have been designed and synthesized based on nucleophilic addition mechanism.
- The probe **PI** shows an excellent selectivity towards sulfite (SO<sub>3</sub><sup>2-</sup>) anion rather than other anions.
- The probe **PI** detects sulfite ion with low detection limit of  $0.57\mu M$  (LOD) and fast response time of 150s
- The probe **PI** was successfully applied to detect sulfite ion in realistic samples and in live HeLa cells.

Sontal