

Recent Advances in the Application of Small-Molecule Fluorescent Probes for Bisulfite (HSO_3^-)

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Abstract. Bisulfite (HSO_3^-) serves as an effective preservative in food industry and plays an important role in endogenous cellular redox balance. However, excessive intake of bisulfite into humans may induce a series of complications such as allergic asthma, cardiovascular diseases and neurological disorders. Consequently, it is essential to develop a method to monitor the bisulfite levels in foods and cells. In recent years, small-molecule fluorescent probes for bisulfite detection have emerged as an advanced technology due to their high selectivity, sensitivity and reliability, moreover, they have been utilized in fluorescence imaging for real-time visualization of bisulfite in living tissues and organisms. This review provides a general introduction into the practical applications of these fluorescent probes from 2022 to 2026, exhibiting several advanced detection methods for bisulfite in various samples and the improvements in probe performance. Furthermore, we discuss about certain aspects in probe applications that requires optimization, which can serve as a direction for in-depth researches in the future.

Keywords: Bisulfite, Fluorescent probes, Practical applications, Fluorescence imaging

1. Introduction

Bisulfite is one of the derivatives of liquid form SO_2 , a harmful pollutant primarily derived from the combustion of sulfur-containing fuels such as coal and fossil [1]. Serving as an intermediate in the metabolic conversion of sulfur-containing amino acid like cysteine to sulfate, bisulfite itself does not have distinct and independent physiological functions in body [2]. This process is catalyzed by sulfite oxidase, ensuring that sulfur-containing amino acids can eventually be converted into sulfate ions, which are essential materials for the synthesis of mucopolysaccharides, bile acids, and certain hormones [3, 4]. However, when sulfite oxidase is compromised or inhaling excessive SO_2 , bisulfite will accumulate in body and damage protein structures or even DNA bases by breaking disulfide bonds [5]. Once serum sulfite levels (the sum of sulfite and bisulfite) exceed $9.85 \mu\text{M}$ in long-term, complications like lung injury, respiratory diseases and cancer may occur due to its accumulation in body [6, 7].

In healthy individuals, the endogenous bisulfite content is extremely low due to rapid conversion into non-toxic sulfate and then excreted from the body through urine [8]. Therefore, exogenous

bisulfite is the primary cause of certain associated diseases. With functions including bleaching, preserving, color protection and antioxidation, bisulfites such as sodium bisulfite, potassium metabisulfite are widely used in foods like sugar, wine, dried fruits and drinks [9]. To ensure food safety, the U.S. Food and Drug Administration (FDA) stipulates that the highest bisulfite levels in foods or drinks are 10 ppm (equivalent to 125 μM) [10]. Besides, some medications may also include bisulfite as preservative or antioxidant to maintain the stability of the drug components [11].

As excessive exposure to bisulfite may lead to a series of health problems, it is essential to develop a highly reliable method to monitor the bisulfite contents in food and medication samples. In recent years, fluorescent probes for bisulfite detection have drawn significant attentions in researchers due to their outstanding advantages such as high selectivity, high sensitivity and ease of operation [12-14]. Several excellent reviews also summarize the development of fluorescent probes detecting bisulfite. For example, Li et al. [15] discussed probe structures, sensing mechanisms, application progress and envision of an ideal probe performance evaluation in the future, exhibiting the advancement in this field from 2014 to 2022. Following this, another review in 2026 from Zhang et al. [16] provided a detailed and intuitive comparison of probes in the past 5 years depending on their performance in multiple dimensions, highlighting their respective advantages and disadvantages.

While these reviews mainly focus on the chemical properties of the probes, seldom concentrate on the practical applications of them. Thus, this review will exhibit the applications of the probes in food and herb samples, along with their fluorescent imaging *in vitro* and *in vivo*. Instead of explaining chemical mechanisms and synthesis of probes, we will introduce the conventional method of applying probes into practical usages and exhibit several advanced detection methods emerged in recent years. If there is no innovation in detection method in certain fields, we will exhibit the comparison of the probe performance based on response time and detection limits. Finally, we will demonstrate how bisulfite detection technology will propel the discovery of disease mechanisms and progress in clinical interventions.

2. Bisulfite detection in food samples

Food industry is one of the most significant fields for bisulfite detection. Due to their excellent antioxidant and antibacterial properties, bisulfite and its derivatives are commonly used in bleaching and quality preservation [17]. However, excessive intake of bisulfite and its derivatives may cause abdominal pain, nausea, and diarrhea. Long-term exposure to them may lead to Vitamin B1 deficiency, causing fatigue and dysfunction of the nervous system [18]. National Food Safety Standard: Standards for the Use of Food Additives has clarified the maximum usage and residue limits of sulfite (measured by the residual amount of sulfur dioxide, the unit is usually g/kg or mg/kg) in various foods: less than 0.1 g/kg in sugar and 0.4 g/L in red wine, etc. To protect public health, developing highly sensitive and selective probes for real-time detection of bisulfite concentration has become a practical and valuable work in chemistry industry.

2.1. In sugar samples

Detection to bisulfite in sugar samples share a similar process: dissolving sugar samples in buffer solution (PBS or HEPES), adding fluorescent probes, recording fluorescence changes and conducting recovery experiments at last to verify the accuracy of the method. Following the procedures above, Zhong et al. [19] developed a ratiometric probe QLP, which triggered a fluorescence enhancement at 600 nm and quenching at 515 nm. The probe exhibited excellent

recoveries ranging from 100.3% to 102.9%, along with a detection limit of 177 nM and a response time of 2 minutes. QLP demonstrates reliability in quantitative analysis for sugar samples, while its detection limit and response time still need development.

To overcome these drawbacks, Li et al. [20] introduced a turn-on probe RQB-R that had better analytical performance. With an emission wavelength at 625 nm, this probe achieved a detection limit of 53 nM and an ultrafast response within 5 seconds. In real sugar samples including white, brown, granulated, and crystal sugars, RQB-R demonstrated recoveries from 78.7% to 120.4%, highlighting its great potential in food safety surveillance. Recently, Kodiyawala et al. [21] developed another turn-on probe PI based on a Benz [c,d] indolium moiety and a phenolic hydroxyl group. In matrix sugar samples (soft sugar, crystal sugar, and powdered sugar), it exhibited recoveries from 96.04% to 99.67%. Importantly, PI also owned an extraordinary detection limit low to 8.4 nM and a rapid response time within 300 seconds, achieving an improvement of two orders of magnitude compared to QLP.

2.2. In red wine samples

For detection in red wine, performances of fluorescent probes also achieve dramatic improvement in the past 5 years. Li et al. [22] developed a benzopyran-benzothiazole-based probe DCA-Bt, which triggered a fluorescence turn-off at 710 nm. This probe exhibited recoveries ranging from 99.59% to 103.48% in red wine samples, along with a detection limit of 9.48 μ M and a response time of 135 minutes. To cut down detection limit and shorten response time in practical detection, Jiang et al. [23] introduced a turn-on probe TPA-tpd with an emission wavelength at 451 nm that had better analytical performance. This probe achieved a much lower detection limit of 0.17 μ M, a faster response time within 15 minutes and recoveries from 95.2% to 102.9% in red wine samples. However, response time still remains further shortening. Qu et al. [24] developed a turn-on probe BODIPY-Y based on a BODIPY fluorophore and an ethyl cyanoacetate group. It exhibited an emission wavelength at 514 nm and recoveries from 85.1% to 94.0% in red wine samples. The most significant advantage of BODIPY-Y was its ultrafast response time in 50 seconds and a detection limit of 0.263 μ M, which made it another notable improvement in response time compared to the previous.

2.3. In other foods

Sugar and red wine are two typical samples in bisulfite detection. In recent years, variety of detected samples has developed along with the improvement of detection method and probe performance. For example, Shang et al. [25] developed a ratiometric, dual-channel probe ZTX with a detection limit of 61 nM, a fast response time of 60 seconds and an emission wavelength altering from 849 nm to 475 nm, which was a large Stokes shift. Although visual changes from colorless to purplish red refer to a general increase in bisulfite concentration, it highly depends on the naked eyes to distinguish this shift so exhibits unsatisfactory accuracy. To precise the bisulfite detection, researchers then introduced a smartphone APP Color Recognizer that could digitize solution color to accurate values. The Color Recognizer performed digital color analysis on captured photos, extracting the R (red), G (green), and B (blue) channel values from samples. A standard curve could be made based on the known bisulfite concentration (X-coordinate) and RGB values (Y-coordinate), which was applied to detect bisulfite in canned fruit, jasmine tea drinks and white wine with recoveries of 92.41%, 90.54% and 92.35%, respectively. Satisfying recoveries demonstrate the reliability and precision of this new detection method.

Usage of smartphone APP is not the only innovation in bisulfite detection method. Ji et al. [26] introduced a turn-on probe DPQ-2 with an emission wavelength at 600 nm, an ultralow detection limit of 3.11 nM and a rapid response time within 5 seconds. Researchers then developed a kit comprising colorimetric reference card and a detection tube preloaded with DPQ-2 solution. Kit was then applied to detect bisulfite in Lily samples, exhibiting Pass in Lily-1 sample and Fail in Lily-2 and Lily-3 samples (regulatory limit is <100 mg/kg). To examine the accuracy of these results, comparative analyses depending on the titration method of National Standards of the People's Republic of China were conducted and calculation results were 113 ± 5.1 mg/kg in Lily-2 and 139 ± 4.7 mg/kg in Lily-3, which exhibited consistency with the results from the kit. Compared to the smartphone APP method above, this kit method is more affordable and offers greater ease of use, making it better suited into practical applications.

Not only the detection method has expanded, detection targets have also varied along with the development of probe design. Liu et al. [27] developed a triple-channel and multifunctional probe MPIM that could detect bisulfite, hypochlorite and 1, 1-dimethylhydrazine residue in mushroom products. For the bisulfite detection, its emission wavelength was 389 nm (purple) with a detection limit of 19 nM and a response time of 130 seconds. It was applied to three kinds of mushroom samples: Shiitake, Donko shiitake and *Agrocybe aegerita* and the recoveries were from 96.5% to 109.5%. For the hypochlorite and 1,1-dimethylhydrazine detection, it also exhibited excellent detection limit (7.9 nM and 17.3 nM, respectively) and rapid response time (180 seconds and 150 seconds, respectively). Multiple mushroom samples were detected then and both results of hypochlorite and 1,1-dimethylhydrazine had satisfying recoveries ranging from 97.3% to 108.3%. Overall, the triple-channel probe MPIM is cost-effective for detection and evaluation of these three residues in mushroom products, which demonstrates promising prospects in quality control.

3. Bisulfite detection in Chinese traditional herbs

Sulfur fumigation is the most direct source of bisulfite in Chinese traditional herbs. During the processing of herbs, sulfur burns to produce sulfur dioxide, which then contact with the moisture in the herbs. This will form sulfurous acid and the sulfite ion will convert into bisulfite under acidic conditions. The main purposes of sulfur fumigation are killing pests, inhibiting bacteria and mold, bleaching or enhancing color and assisting drying [28]. According to the Chinese Pharmacopoeia, unless otherwise specified, the residue of bisulfites in Chinese medicinal materials and decoction pieces (calculated as sulfur dioxide) must not exceed 150 mg/kg. Therefore, developing probes to detect bisulfite in Chinese traditional herbs has large and promising prospects.

Zheng et al. [29] developed a ratiometric probe InB with an enhanced emission wavelength at 474 nm and a quenching wavelength at 556 nm. This probe was successfully applied to *Dioscorea opposita* Thunb and *Gastrodia elata* Bl, achieving recoveries from 101.6% to 108.2% and 96.14% to 108.3%, respectively, exhibiting a detection limit of 4.39 nM and a response time of 300 seconds. Similar to InB, Dong et al. [30] also reported a ratiometric probe MIB with an enhanced emission wavelength at 339 nm and a quenching wavelength at 526 nm, along with a detection limit of 63.15 nM and a response time within 60 seconds. This probe exhibited recoveries from 102.3% to 104.3% in *Yam* samples and 91.67% to 94.83% in *Astragalus* samples. Both InB and MIB imply the excellent feasibility in quantitatively monitoring the concentration of bisulfite in practical herb samples.

However, the performance of probe can be further improved with advanced design. Huang et al. [31] designed a novel turn-on probe N-3-I with an emission wavelength at 488 nm. Unlike the previously reported probes, N-3-I was applied to rapid and sensitive monitoring of sulfite residues

in Chinese traditional herbs like *Angelica dahurica*, *Liquorice root*, and *American ginseng* with recoveries from 101.35% to 103.40%, 98.44% to 100.80% and 89.21% to 103.42%, respectively. The notable point of this probe was its ultralow detection limit of 4.54 nM and an ultrafast response time within 60 seconds. In summary, this promising assay can successfully and rapidly capture the sulfur dioxide contents in the Chinese traditional herbs. Compared to other probes, N-3-I uses less sample than the national standard method, demonstrating cost-effective and time-saving.

4. Bisulfite fluorescence imaging *in vitro*

In vitro refers to living cells cultured artificially outside the organisms. *In vitro* fluorescence imaging of a probe is a pivotal step that linking preceding selectivity and sensitivity evaluation and subsequent *in vivo* imaging performance [32]. Serving as a derivative of the gaseous signaling molecule SO₂, bisulfite fluorescence imaging *in vitro* has specific significance such as verifying probe performance in living cells, observing dynamic changes of bisulfite, exploring intracellular mechanisms and laying the foundation for *in vivo* applications [33].

4.1. In onion epidermal tissues

Onion epidermal cells have several advantages in fluorescence imaging: large cell size, distinct rectangular morphology, optically transparent monolayer architecture and ease of microscopic visualization. Besides, they can be directly imaged without chemical modification since this procedure may introduce unexpected interference and potentially misleading fluorescence responses. Uttam Samanta et al. [34] introduced a turn-on, single-channel probe BSP with an emission wavelength at 515 nm. In fluorescence imaging, N-ethylmaleimide (NEM) was pretreated with onion epidermal cells to eliminate endogenous bisulfite and suppress background fluorescence. When exogenous bisulfite was added, a significant fluorescence enhancement in green-channel could be observed though it was slightly lower than samples without NEM added.

However, with advanced probe design, NEM is actually not essential in fluorescence imaging. Chen et al. [35] designed a ratiometric, dual-channel probe SX-TPA with enhanced fluorescence at 439 nm and quenching at 698 nm. Herein, onion epidermal tissues were directly incubated with 20 μM SX-TPA, obtaining a strong red fluorescence and a weak blue fluorescence. However, after pre-incubating with increasing bisulfite concentration of 400 μM, a decrease in red fluorescence intensity accompanied by an increase in blue fluorescence were observed. Dual-channel probes can effectively counteract background interference caused by endogenous generated bisulfite such as GSH. Following the similar principle and procedures above, Kong et al. [36] introduced another dual-channel, coumarin–benzindolium system-based probe 1 that had better performance. With only 10 μM probe 1 and 50 μM bisulfites, the red fluorescence (655 nm) intensity was weakened importantly while the blue fluorescence (495 nm) intensity was enhanced remarkably, which demonstrates good permeability for fluorescence imaging to plant tissue.

4.2. In immortalized cell lines

Prior to confocal imaging, biocompatibility evaluation is conducted to investigate the cytotoxicity of the probes. The standard MTT assay is the major choice due to its intuitiveness, convenience and low-cost [37]. After incubating MTT with immortalized cells like Hela cells, HepG2 cells and A549, obtaining absorbance with a microplate reader. More living cells indicates higher absorbance so better biocompatibility of the probes. Other assays like CCK-8 and SRB share the same principle

despite their mechanisms and protocols are distinct. Only when the cell viability exceeds 80% is the cytotoxicity of the probe considered negligible [38].

Confocal imaging can be carried out in various immortalized cell lines. Li et al. [39] conducted imaging in Hela cells with 10 μM dual-channel probe RS that had enhanced emission wavelength at 522 nm and quenching wavelength at 705 nm. With increasing bisulfite concentrations from 0 to 200 μM , the green channel fluorescence intensity enhanced gradually while the red channel fluorescence intensity was quenched correspondingly. Similarly, Xin et al. [40] introduced a turn-on probe Ir-CN whose emission wavelength was 600 nm and performed imaging in A549 with 10 μM Ir-CN. The single-channel fluorescence intensity of the probe exhibited continuously rising with the increase of bisulfite concentration. Besides, the scope of intracellular staining regions also increased consistently, indicating that the probe also had high sensitivity to bisulfite content range in living cells. In summary, both RS and Ir-CN can sensitively monitor the concentration fluctuations of exogenous bisulfite in living cells through fluorescence imaging.

However, immortalized cells cannot represent the physiological environment *in vivo* so fluorescence imaging in normal cells like L-02 cells can assist researchers better understanding the interference of the microenvironment to the probe response. Li et al. [41] conducted imaging in L-02 cells and Hela cells with 2.5 μM dual-channel probe NTC that owned an enhanced emission wavelength at 550 nm and a quenching wavelength at 650 nm. Interestingly, after pre-treated with 500 μM NaHSO_3 , the green-to-red fluorescence ratio (I_{550}/I_{650}) in L-O2 cells increased to a greater extent than that in HeLa cells. This was speculated to be result from the distinctive microenvironment of cancer cells such as lower intracellular pH and higher viscosity, which might interfere with the probe's reaction to bisulfite.

Co-localization to organelles in cell imaging allows the probe to associate dynamics changes to organelle-specific functions, thereby providing mechanistic insights into the pathogenic roles of bisulfites. Lin et al. [42] introduced a turn-on probe CIVP with emission wavelength at 465 nm and conducted co-localization imaging to the mitochondria. HeLa cells were incubated with 5mM CIVP and 100nM commercial mitochondrial probe, respectively. The colocalization coefficient of them was 0.93, indicating that the probe had excellent localization ability to mitochondrial. Following the similar principles, Li et al. [43] carried out co-localization to lipid droplets in Hela cells, cultured by probe CBC (ratiometric, enhanced emission wavelength at 485 nm and quenching wavelength at 650 nm) and HCS LipidTOX Deep Red (1:300). The probe successfully targeted lipid droplets with high colocalization coefficient 0.93, therefore, it is an effective biological tool to study the physiological processes involved in lipid droplets.

5. Bisulfite fluorescence imaging *in vivo*

In vivo refers to intact living organisms. *In vivo* fluorescence imaging signifies the development of a probe from a well-controlled environment to a complex and uncontrolled environment, demonstrating that probes are effective not only in isolated living cells but also function properly under the interference of intact metabolism and immune systems. Bisulfite fluorescence imaging *in vivo* usually initiates from simple models and gradually transitions to complex mammals like mice, which is a critical step before probes' applications into human diseases [44].

5.1. In zebrafish

Zebrafish is an ideal model for *in vivo* fluorescence imaging due to its embryonic optical transparency, convenience of fluorescent labeling and low-cost husbandry. Luo et al. [45] applied

their turn-on probe TPN-DP that owned emission wavelength at 430 nm, to zebrafish fluorescence imaging. 10 μM probes were cultivated with zebrafish in 100 μM bisulfites, exhibiting significant enhanced fluorescence in the blue channel compared to the probe-only control. However, fluorescence imaging may be disturbed due to movement of embryonic zebrafish. To get fixed image, tricaine (MS-222) can be applied to anesthesia zebrafish and obtain higher-quality fluorescence imaging [46].

5.2. In mice

Owing to their highly comparable genetic background to humans and ideal body size which is perfectly suited for near-infrared fluorescence imaging, mice have become the predominant mammalian model for *in vivo* fluorescence imaging [47]. Moreover, bisulfite fluorescence imaging in mice provides a significant basis for discovering the mechanisms of human diseases and developing clinical interventions. Shang et al. [48] introduced a bi-functional probe QZB that could respond to both bisulfite and hypochlorous acid, exhibiting fluorescence quenching at 655 nm after reacting with bisulfite. The normal mice displayed no obvious fluorescence in near-infrared fluorescence imaging. However, mice injected with QZB (20 μM , 100 μL) in hind limb revealed an intense fluorescent signal. After further injecting bisulfite (100 μM , 40 μL), the fluorescence intensity of the injected area decreased continuously, demonstrating the feasibility of the probe for the imaging of bisulfite *in vivo*. Similarly, Cui et al. [49] also conducted near-infrared fluorescence imaging in mice with ratiometric probe IPB-RL-1 that owned enhanced fluorescence wavelength at 470 nm and quenching wavelength at 760 nm. Herein, the probe responded to sulfite and obvious signals were observed after the probe injection (100 μL , 50 μM) for 5 minutes. While the fluorescence intensity gradually weakened with the increase in sulfite concentration. As the response time is less than 5 minutes, the probe has promising prospects in monitoring the sulfites in mice.

Except imaging in normal mice, Hou et al. [50] carried out imaging in tumor-bearing mice with another ratiometric probe CPH@ZIF-90 that responded to ATP and SO_2 in sequence (enhanced emission wavelength at 580 nm and quenching wavelength at 685 nm after reacting with SO_2).

After injection of CPH@ZIF-90 (4 mg/mL) to tumor-bearing mice, fluorescent signals accumulated in the tumor area with the extension of injection time. The fluorescence of red channel reached the maximum at 3 hours and then attenuated until 6 hours, while this fluorescence kept stronger than that in normal mice during whole period. This result indicates that this probe can be used to identify and differentiate tumors *in vivo* due to overexpression of ATP and SO_2 in tumors.

6. Conclusion

This review provides an overview of the applications for the small- molecule fluorescent probes detecting bisulfite from 2022-2026. We classify the probes according to their practical application scenario and assess practicality through their performance in detection or fluorescence imaging. From our analysis, we notice that several advanced detection methods such as usage of smartphone APP and self-made detection kit, have emerged in recent years, which are excellent innovations compared to the conventional naked-eyes recognition. These detection methods may become a popular stream in the future due to their developed sensitivity and accuracy. Besides, more multifunctional probes that can detect several ions or molecules also emerged lately, which is a promising direction because the practical detection environment will not be a single bisulfite

solution, but the mixture of various ions and molecules. Depending on their sophisticated designs, these probes can better fit into the detection with complex environment.

Nevertheless, problems occur with advancement. Nowadays, conventional methods such as titration and chromatography still remain the mainstream depending on their low-cost and ease of operation [51]. Cutting down the cost on the synthesis of probes, amplifying the detection environment from controlled solution to complex matrix and establishing a series of unified detection standards may promote the adoption of fluorescent probes.

Regarding to *in vivo* fluorescence imaging, most co-localizations to organelles concentrate on mitochondria so the metabolic pathways of bisulfite in other organelles such as endoplasmic reticulum and Golgi apparatus are overlooked. Visualization of bisulfite in these organelles can provide a more comprehensive understanding to the mechanisms of diseases associated with bisulfite and its derivatives. Attaching chemical targeting groups to direct the probe to specific organelles may be a strategy worthing further investigation.

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