Chinese Chemical Letters 36 (2025) 109867

Contents lists available at ScienceDirect

Chinese Chemical Letters

journal homepage: www.elsevier.com/locate/cclet

Development of ESIPT-based specific fluorescent probes for bioactive species based on the protection-deprotection of the hydroxyl

Haixian Ren[a], Yuting Du[a], Xiaojing Yang[a], Fangjun Huo[c], Le Zhang[d], Caixia Yin[a,b,*]

[a] Department of Chemistry, Xinzhou Normal University, Xinzhou 034000, China
[b] Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Key Laboratory of Materials for Energy Conversion and Storage of Shanxi Province, Institute of Molecular Science, Shanxi University, Taiyuan 030006, China
[c] Research Institute of Applied Chemistry, Shanxi University, Taiyuan 030006, China
[d] Department of Chemistry, The University of Texas at Austin, Austin, TX 78712, United States

ARTICLE INFO

Article history:
Received 15 February 2024
Revised 2 April 2024
Accepted 7 April 2024
Available online 8 April 2024

Review

Keywords:
ESIPT
Protection-deprotection
Specificity
Fluorescence probe
Bioimaging

ABSTRACT

Excited-state intramolecular proton-transfer (ESIPT) based fluorescence probes are particularly attractive due to their unique properties including environmental sensitivity, a large Stokes shift, and potential for ratiometric sensing. In general, ESIPT-based fluorophore incorporates an intramolecular hydrogen bonding interaction between a hydrogen bond donor (–OH and NH_2 are common) and a hydrogen bond acceptor (C=N and C=O). More, protection–deprotection of hydroxyl group as hydrogen bond donor could induce an off-on switch of ESIPT-based emission. Therefore, protection–deprotection of hydroxyl group has been the widely used strategy to design fluorescent probes, where the potential key issue is selecting a protective group that can specifically leave in the presence of the target analyte. In this review, we mainly summarize the specific protecting groups (sites) and deprotection mechanisms for biologically important species (including reactive sulfur species (RSS), reactive oxygen species (ROS), enzymes, *etc.*), and analyze the advantages and disadvantages of different protection mechanisms from some aspects including probe stability, selectivity, response rate and assay system, *etc.* Based on the aforementioned, we further point out the current challenges and the potential future direction for developing ESIPT-based probes.

1. Introduction

Innovations and breakthroughs in experimental technology have vigorously promoted the research process of biochemistry and molecular biology [1]. Extensive evidence shows that diverse bioactive species are critical for cell proliferation, differentiation, aging and apoptosis, and many diseases are closely related to the abnormal expression of these species [2]. Among these bioactive species, reactive oxygen species (ROS) and reactive sulfur species (RSS) have attracted increasing interest because they are associated with the intracellular redox homeostasis and many biological events; in addition, some enzymes are widely distributed in subcellular organelles and play vital and different roles in biosystems [3]. Therefore, accurately monitoring their concentrations is beneficial for understanding the diverse physiological and pathological processes and for exploring novel strategies of regulating biological phenomena and improving diagnosis, treatment and prognosis of different diseases.

Among the diverse detection strategies, fluorescence probes represent a simple, sensitive and non-invasive technique, which enables real-time, dynamic and selective imaging of these targets in living cells [4]. As a response to the analytes, fluorescent probes can give a specific signal output generally based on such response mechanisms as fluorescence resonance energy transfer (FRET), photo-induced electron transfer (PET), intramolecular charge transfer (ICT) and excited-state intramolecular proton transfer (ESIPT) [5–7]. Of course, dual-channel signal readout under one excitation wavelength is more favorite because it could provide diverse information about the levels of the target analyte. FRET and ESIPT-based probes all satisfy the above requirements, however, the development of FRET-based probes are limited to the rare donor-acceptor pair matched in energy [8]. In comparison, ESIPT-based probes are easy to construct and modify. Therefore, increasing number of ESIPT-based probes have been developed for biological imaging [9].

In general, ESIPT-based fluorophores incorporate an intramolecular hydrogen bonding interaction between proton donor (OH/NH_2 "OH" is common) and proton acceptor (C=N and C=O). The common ESIPT fluorophores shown in Fig. 1, exist as enol form on the

* Corresponding author.
E-mail address: yincx@sxu.edu.cn (C. Yin).

https://doi.org/10.1016/j.cclet.2024.109867
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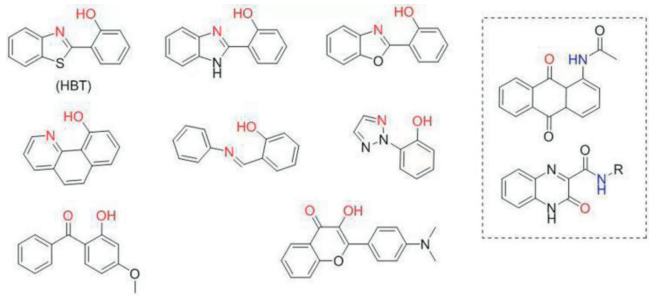

Fig. 1. The common ESIPT-based fluorophores.

Fig. 2. The design of ESIPT-based probe by masking the hydroxyl group.

ground state. Upon photoexcitation, an extremely fast enol to keto phototautomerization event occurs to them along with intramolecular proton transfer, leading to dual emission from enol form (E*) and keto form (K*). Consequently, ESIPT-based fluorophores feature dual emission under one excitation wavelength, and are characterized by large Stokes shift which helps avoiding unwanted self-reabsorption and inner-filter effects. Therefore, ESIPT-based fluorophores have great attraction for biological imaging [10].

Since ESIPT-based emission (keto form K*) usually arises from the intramolecular proton transfer from OH to the nitrogen/oxygen atom participating in double bonds, protection-deprotection of hydroxyl group as proton donor could regulate the on/off switch of ESIPT process by intervening the occurrence of proton-transfer (Fig. 2). More, it has been as the widely used strategy of designing ESIPT-based probes for which the key issue is selecting an appropriate protective group that can specifically leave in the presence of the target analyte. In the last decade, significant achievements have been made in developing ESIPT-based probes specific for target analytes by masking the hydroxyl group [11].

This review mainly focuses on the specific protecting groups (sites) and deprotection mechanisms of ESIPT-based probes for bioactive species (mainly including RSS/ROS and a few common enzymes, *etc.*), and analyzes the advantages and disadvantages of different protection mechanisms from some aspects including probe stability, selectivity, response rate and assay system, *etc.* Moreover, the current challenges and the potential future directions in this field are included as well.

2. ESIPT-based sensors for RSS

RSS including biothiols, hydrogen sulfide (H_2S), sulfur dioxide (SO_2), hydrogen polysulfides (H_2S_n) and other sulfur-containing agents in living organisms, play important roles in maintaining cell health. Biothiols including glutathione (GSH), homocysteine (Hcy) and cysteine (Cys), as the main part of RSS, act as antioxidants and free-radical scavengers and function in maintaining redox homeostasis [12]. Other RSS (H_2S, H_2S_n, and SO_2) play vital roles in signal transduction and information transmission. They function independently but have a certain connection in their production, transportation, and downstream functions (Fig. 3) [13]. For example, a shortage of Cys-perhaps caused absolutely slow synthesis rate and low concentration of erythrocyte GSH for the human immunodeficiency virus (HIV) infected patients. Therefore, it is of great significance to specifically monitor the levels of each analyte for better understanding their roles in physiological and pathological processes [14].

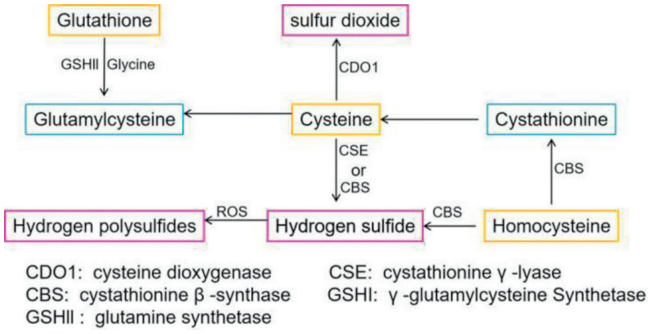

Fig. 3. The transformation process of different RSS.

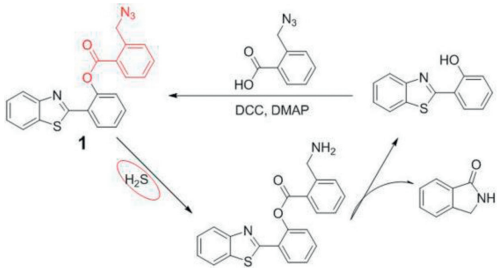

Fig. 4. The design and synthesis of probe **1** for H_2S.

2.1. H_2S

H_2S, is a key gaseous transmitter involving vascular tension mediation in blood vessels and neuroregulation in the brain [15]. In recent years, focusing on strong reducibility and nucleophilicity of hydrogen sulfide, some ESIPT-based probes have been conducted based on protection and deprotection of hydroxyl group.

Based on the recognition that azido group could act as specific reaction site for H_2S [16], Lin *et al.* creatively developed probe **1** by employing 2-(azidomethyl)benzoic acid masking the hydroxyl group in 2-(benzo[*d*]thiazol-2-yl)phenol (HBT) (Fig. 4) [17]. For probe **1**, ESIPT-based emission was quenched due to hydroxyl group being masked, but in the presence of hydrogen sulfide, the azide group was reduced to an amino group, which spontaneously cyclized with intramolecular ester unit to release the fluorophore HBT, and the quenched emission was restormed. As a result, probe **1** exhibited high selectivity and a large signal change (400-fold), except for a slow response. In view of the physiological features of H_2S including low concentration, short lifetime and high reactivity, it was difficult for probe **1** to detect the real-time levels of intracellular H_2S. Hence, it was very necessary to develop response-rapid probes for hydrogen sulfide.

Since H_2S has smaller pK_a values and a stronger nucleophilicity than thiols at physiological condition (H_2S: 7.0; biothiols $\geq$ 8.5), the S-S bond could act as a specific reaction site for H_2S [18]. For example, in 2012, Qian *et al.* developed a rapid ratiometric probe **2** based on successive nucleophilic substitution, where the hydroxyl group was protected by 2-(pyridin-2-yl-disulfanyl)benzoic acid (Fig. 5) [19]. As the disulfide was reduced by H_2S, a new disulfide was formed where the electron-poor carbonyl carbon atom was subsequently attacked by the nucleophilic sulfhydryl group forming a self-immolative benzoic acid-disulfide structure cleaved, resulting in the recovery ESIPT-based emission.

Concerning that the reaction rate of diselenides with H_2S was much faster than that of disulfides, in 2019, Guo *et al.* reported a diselenides-contained probe **3**, which could generate a 47-fold

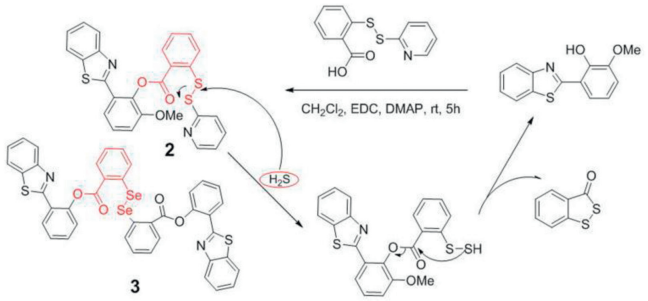

Fig. 5. The design and synthesis of probes **2** and **3** for H_2S.

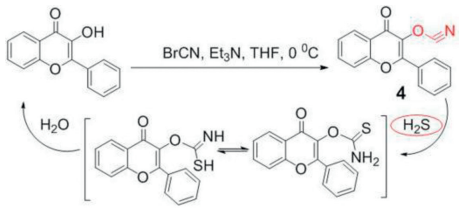

Fig. 6. The design and synthesis of probe **4** for H_2S.

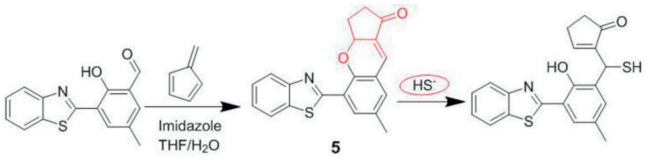

Fig. 7. The design and synthesis of probe **5** for H_2S.

fluorescence enhancement immediately after the addition of H_2S [20]. The nucleophilic attack by H_2S at diselenides bond was more favorable both kinetically and thermodynamically than at disulfide bond. Although probe **3** could sensitively detect the H_2S levels in cells or *in vivo*, the large masking groups were released to the biological system as organic waste, raising a concern on toxicity. As a response, Emrullahoğlu *et al.* reported an excellent probe **4** with a cyanate (O−CN) unit as a mask of flavonoid hydroxyl in 2016 (Fig. 6) [21].

Due to the stronger nucleophilicity of H_2S than that of cysteine and glutathione, H_2S more readily added to the electrophilic carbon atom in cyanate unit to form a thiocarbamate derivative which could rapidly hydrolyse in physical condition to release the highly emissive fluophore. Probe **4** was confirmed to have the exceptional selectivity and rapid response to H_2S, also successfully employed to detect the levels of introcellular H_2S.

HBT-based fluorophores as the classic ESIPT-based groups, usually are excited by short-wavelength light which tended to cause cellular autofluorescence, photodamage and artifactual generation of ROS. Based on the fact that two photon excitation fluorescence (TPEF) could be excited by long-wavelength light with minor damage to living organism and that HBT unit possessed TP cross-sections for TPEF imaging, Yoon *et al.* developed TP fluorescent probe **5** with obvious aggregation-induced emission (AIE) effect, which was prepared through a cascade type Baylis-Hillman and followed intramolecular Michael addition reaction (Fig. 7) [22].

The nucleophilic addition response of H_2S to probe **5** would lead to the opening of chromene ring thereby the formation of ESIPT-based fluorophore. Experiments results illustrated that probe **5** could detect intracellular H_2S levels under the excitation of two near-infrared (NIR) photons (740 nm) with negligible light damage and background interference by using two-photonmicroscopy (TPM).

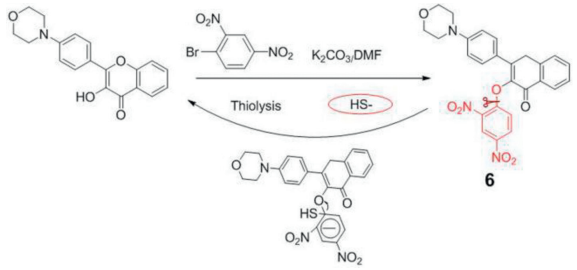

Fig. 8. The design and synthesis of probe **6** for H_2S.

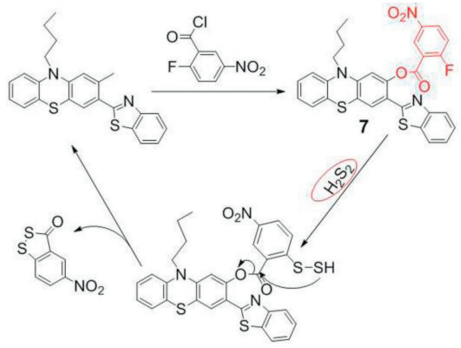

Fig. 9. The design and synthesis of probe **7** for H_2S_2.

According to that the electron-withdrawing 2,4-dinitrophenyl ether was easily cleaved by H_2S over biothiols, quite a few ESIPT-based probes were developed based on the recognition site, including probe **6** for which the relative response mechanism was shown in Fig. 8 [23].

2.2. H_2S_2

H_2S_n ($n > 1$) as an important signal molecullar, performs the function of activating ion channels, transcription factors and tumor suppressors, which could be endogenously generated by H_2S under the oxidation of ROS like HClO [24]. Based on aromatic nucleophilic substitution reaction and reduction reaction, some probes for H_2S_n have been developed. For example, Chen and co-workers selected the 2-fluoro-5-nitro-benzoic moiety as the mask of hydroxyl group to construct ESIPT-based probe **7** (Fig. 9), where the nucleophilic reaction occured in presence of H_2S_2 then the spontaneous intermolecular cyclization reaction released the dye resulted in an on-off emmision [25]. The related experiments results indicated that probe **7** could sense H_2S_2 by a remarkable fluorescence enhancement (328-fold) at 534 nm with a low detection limit of 26 nmol/L.

2.3. Thiols

Biothiols including GSH, Hcy and Cys have similar structures and properties, which independently function in the biological system and metabolically related. It has been confirmed that Cys and Hcy can metabolize to produce GSH, and GSH as the most abundant small molecule thiol, is responsible for maintaining intracellular redox homeostasis [26]. For Hcy, its content in plasma is about 15 μmol/L, and its abnormal increase can easily lead to thrombosis. As for Cys, normal cells do not have a high demand for its concentration, and its abnormally elevated levels can easily change iron homeostasis further lead to mitochondrial decline [27]. Therefore, it is of great significance for prevention and diagnosis of related diseases and understanding of related physiological and pathological processes to develop fluorescent probes that can specifically detect these thiol molecules.

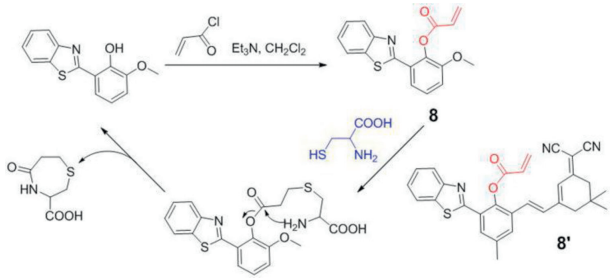

Fig. 10. The design and synthesis of probes **8** and **8′** for Cys.

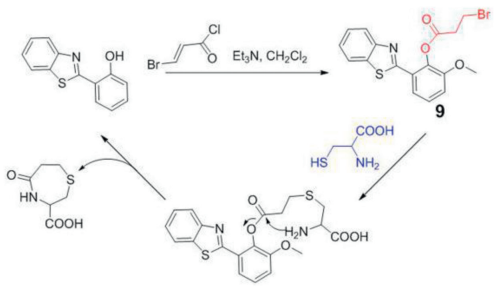

Fig. 11. The design and synthesis of probe **9** for Cys.

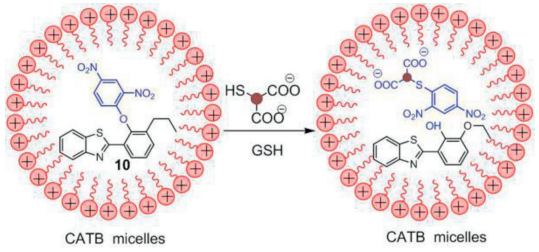

Fig. 12. The reaction of probe **10** with GSH.

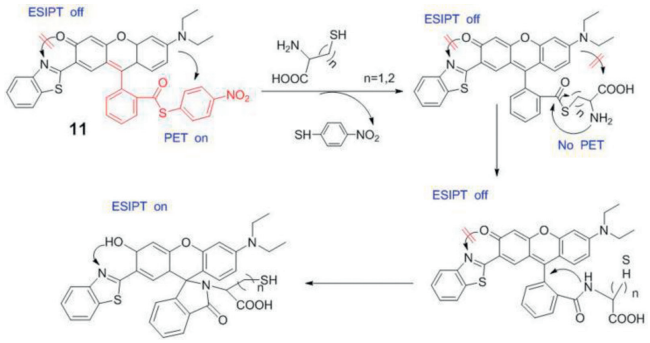

Fig. 13. The response of probe **11** to thiols.

Although it was hard that these thiols were selectively recognized due to their similarity in structure and property, a big breakthrough came in 2011 when Strongin's group designed first ESIPT-based probe **8** by masking hydroxy group in 2-(2′–hydroxy-3′-methoxyphenyl)benzothia-zole (HMBT) with α,β-unsaturated carbonyl moiety, which could discriminate Cys from Hcy based on their different reaction rates (Fig. 10) [28].

The sulfhydryl group of Cys is nucleophilically added to the C=C double bonds of the acrylate to generate thioethers, which undergoes subsequent intramolecular cyclization to release the dye. Hcy similarly could generate thioether, but subsequent intramolecular cyclization reaction to form eight-membered ring would not be kinetically favored compared to the formation of seven-membered ring resulted from Cys. Therefore, based on different circularization rates, the designed fluorescent probe 8 could distinguish Cys from Hcy.

Based on similar strategy, bromopropionyl or chloropropionyl groups were also used as the masker of hydroxy group to design ESIPT-based fluorescent probes for Cys. Also, the bromopropionyl group is expected to show a faster nucleophilic substitution rate with Cys than chloropropionyl as it incorporated a better leaving group. Churchill *et al.* report a new HBT-based probe **9** involving bromopropionyl group [29]. Probe **9** underwent nucleophilic substitution reaction with Cys to form the intermediate product similar to the first-step reaction product of probe **8** with Cys, then underwent intramolecular cyclization to release the fluorophore (Fig. 11).

However, the response rate of probe **9** to Cys was slower than that of probe **8** possibly because the sulfhydryl group of Cys was nucleophilically added to the C=C double bonds of the acrylate to generate thioethers more easily than it nucleophilically substituted bromine atom of bromopropionyl group. Therefore, acrylate group is more attractive as specific recognition site for Cys than bromopropionyl group. In 2020, using acrylate group as reaction site we also reported a NIR Cys probe **8′** by coupling dicyanoisophorone with HMBT, which turned on the 686 nm emission at the presense of Cys when excited by 423 nm light [30].

Relative to probes **8** and **9**, probe **8′** well performed Cys detection *in vivo*, because visible light as excitation and NIR emission caused less damage to the tissues and less interference from biological autofluorescence than short-wavelength excitation and emission.

Due to slightly stronger acidity of Cys than Hcy and GSH, Cys was more nucleophilic thereby ready to react with related probes at physical condition. Therefore, many thiol probes could generate a strong response to Cys/Hcy over GSH, as a result, developing GSH-specific probes especially based on ESIPT mechanism remains a challenge [31]. It is well known that cetyltrimethylammonium bromide (CTAB) micelles can not only enrich hydrophobic probes but also tend to electrostatically combine with GSH which is more negatively charged than Cys or Hcy due to its contained two carboxyl groups. In 2016, Chen *et al.* employed dinitrophenyl ether as masker of hydroxy group to construct probe **10** which could selectively detect GSH catalyzed by CTAB micelles (Fig. 12) [32].

The excellent selectivity of probe **10** to GSH over Cys and Hcy is mainly attributed to one more carboxyl groups in GSH. Probe **10** was readily aggregated in positively charged CTAB micelles through hydrophobic and hydrogen bonding interaction. GSH with two carboxyl groups easily generates negative charges thereby shows a stronger affinity to CTAB micelles than Cys/Hcy. Therefore, GSH could likely attack the probe inside the micellar aggregates, resulting in the release of fluorophores.

In addition, a general strategy to discriminate Cys/Hcy from GSH was two-step tandem reaction permitted only by Cys/Hcy which contained nucleophilic substitution of sulfhydryl groups and *S* and *N*-acyl intromolecular transfer [33]. Combining this strategy with "quinone-phenol" transduction of rhodol dye related to ESIPT process in HBT unit, in 2014, Strongin's group reported a rhodol thioester probe **11** discriminating Cys/Hcy from GSH (Fig. 13) [34]. Probe **11** reacted with Cys/Hcy through a series of tandem (nucleophilic substitution of sulfhydryl groups, *S* and *N*-acyl intromolecular transfer and spirocyclization) to form the corresponding deconjugated spirolactam, which converted the structure of rhodol dye from quinone type to phenol type thereby turned on the ESIPT-based emission at 454 nm. For the tripeptide GSH, only the nucleophilic substitution reaction of the sulfhydryl group occurred to probe **11**, arising a large enhancement of rhodol-based emission at 587 nm due to the removement of the PET process caused by 4-nitrobenzene. In the above strategy, the process of *S* and *N*-acyl

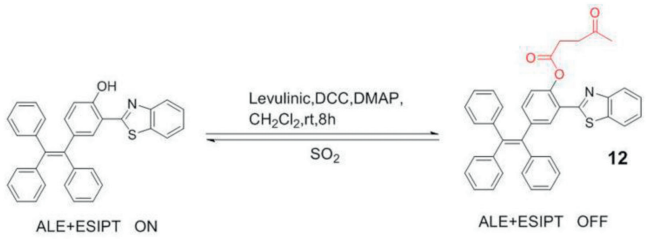

Fig. 14. The design and synthesis of probe **12** for SO_2.

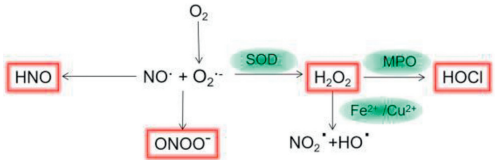

Fig. 15. The transformation relationship among different reactive oxygen species.

intramolecular transfer was the key to discriminate Cys/Hcy with GSH by probe **11**.

2.4. SO_2

Many studies claim that SO_2 can be produced endogenously by sulfur-containing amino acids in mammals, which as an active small molecule play a vital role in regulating cardiovascular functions [35]. In addition, epidemiological studies have illustrated that sulfur dioxide is also related to many diseases, including cardiovascular diseases, neurological disorders respiratory diseases even lung cancer [36]. Lin *et al.* developed first generation AIE + ESIPT ratiometric SO_2 probe **12** by introducing a thiazole group and a levulinate group into the tetraphenylethene scaffold (Fig. 14) [37]. In the pure aqueous solution, probe **13** only exhibited a strong enol-form emission at 422 nm due to the protection of the hydroxyl group by levulinate moiety, and upon the deprotection of levulinate by SO_2, the strong keto-form emission at 565 nm was turned on and the enol-form emission disappeared. Relative to pure organic solution, probe **12** could ratiometricly monitor SO_2 better due to AIE effect in pure aqueous solution.

3. ROS

ROS are endogenous oxidants with vital functions such as signal transduction and host defense, and are associated with stem cell differentiation, aging and cancer [38]. ROS are mainly composed of H_2O_2/HClO/HNO/$ONOO^-$ and free radicals OH/O_2^-/NO·, and they are transformed into each other in the body (Fig. 15) [39]. These transient species may endanger human health when their levels are higher than normal. To understand their effects and impede oxidative damage, developing fluorescent probes for ROS is necessary [40].

3.1. H_2O_2

H_2O_2 is one of the most studied ROS, which maintains the redox homeostasis in the organism together with GSH [41]. The monitoring of hydrogen peroxide level is helpful for the assessment of human health. Chang and his colleagues pioneered the development of a borate-based hydrogen peroxide sensor and applied it to the detection of hydrogen peroxide in biological systems [42]. On this basis, some hydrogen peroxide probes were developed based on ESIPT mechanism through masking hydroxyl groups with *p*-phenylborate benzyl bromide. For example, probe **13** reported by Tang's group, could respond to hydrogen peroxide ac-

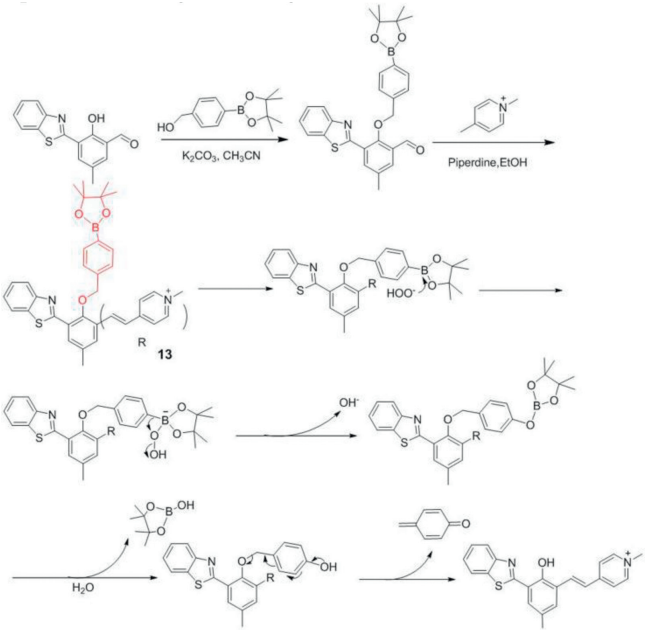

Fig. 16. The design and synthesis of probe **13** for H_2O_2.

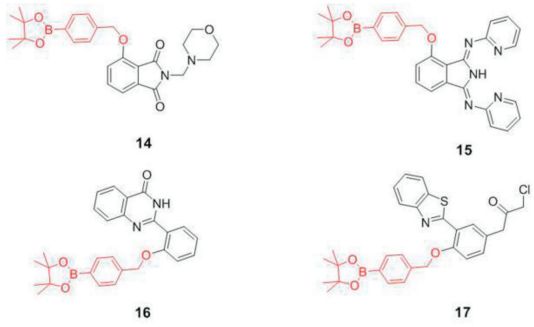

Fig. 17. The structures of other probes **14**–**17** for H_2O_2.

cording to the mechanism proposed in Fig. 16 [43]. For probe **13**, phenylboronate as the sensing group and ESIPT blocking group, was oxidized by H_2O_2 and a 1,6-rearrangement elimination reaction was followed to free the phenolic OH group and recover the ESIPT process. It exhibited high selectivity toward H_2O_2 over other ROS/RNS and biological species with a NIR emission. Similar H_2O_2 probes were also reported including that in Fig. 17 [44].

3.2. Peroxynitrite ($ONOO^-$)

$ONOO^-$ as a highly reactive nitrogen species, is more commonly known for its deleterious effects including causing irreversible damage to lipids, proteins and DNA [45]. However, it also acts as a signaling molecule *in vivo* for a number of pathways. Therefore, the development of powerful tools for the real-time detection of $ONOO^-$ is essencial to understand the role of $ONOO^-$ in different biological system and pathway process [46]. Utilizing the stronger oxidability of $ONOO^-$, aryl borate would be oxydized by $ONOO^-$ orders of magnitude faster than by H_2O_2. Based on this, Sedgwick *et al.* reported an ESIPT-based ratiometric fluorescence probe **17**, which was able to selectively detect low concentrations of ONOO (limit of detection: 21.4 nmol/L) within a few seconds [47]. In addition, Shen *et al.* developed a simple AIE-ESIPT fluorescent probe **18** for monitoring $ONOO^-$ using diphenylphosphinate group as the recognition site and salicylaldehyde azine as the fluorophore, which displayed weakly emissive in aqueous so-

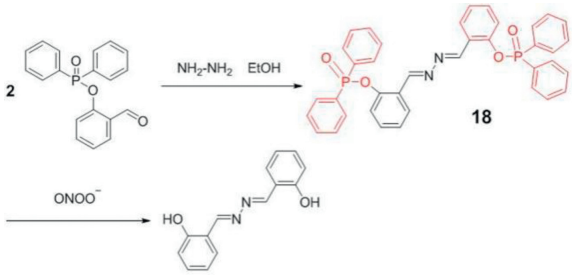

Fig. 18. The design and synthesis of probe **18** for $ONOO^-$.

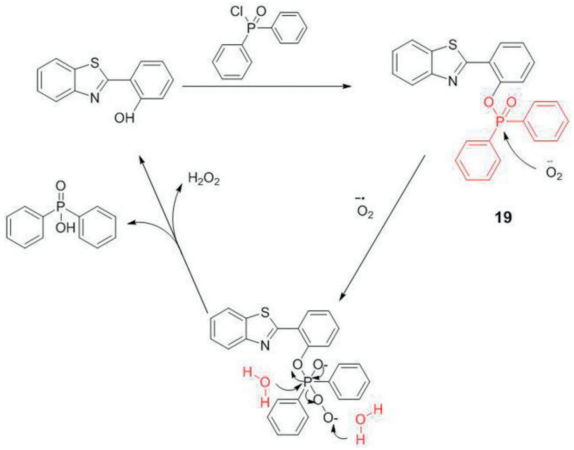

Fig. 19. The design and synthesis of probe **19** for $O_2^{\bullet-}$.

lution because of protection of OH group in salicylaldehyde azine by diphenylphosphinate group (Fig. 18) [48]. Oxidized by $ONOO^-$, probe **18** split diphenylphosphinate group away to produce salicylaldehyde azine, resulted in an enhanced emission in aqueous solution induced by a combination of the AIE-ESIPT mechanism.

3.3. KO_2

$O_2^{\bullet-}$ as both an anion and a free radical, has been identified as potential messenger to regulate the cell-signaling network. Abnormal concentrations of $O_2^{\bullet-}$ ultimately correlate with the aetiology of disease [49]. Owing to its short half-life, extremely low concentration, and high reactivity, it is challenged to develope the probes that could detect $O_2^{\bullet-}$ *in vitro* or *in vivo*. Churchill *et al.* firstly presented an ESIPT-based superoxide sensor **19** with phophinates [$P(O)Ph_2$] as the site of $O_2^{\bullet-}$ and the mask of the hydroxyl group in HBT [50]. Superoxide permits an addition elimination reaction of phophinates and hydrolysis (Fig. 19) to give free HBT.

3.4. Hypochlorous acid (HOCl)

HOCl is a biologically important ROS, which partially dissociates to form its hypochlorite anion (ClO^-) under physiological conditions. In biological systems, myeloperoxidase as an enzyme found in leukocytes, produces HOCl/ClO by catalysing the reaction between Cl and H_2O_2. This vital ROS functions in immune defence systems due to its microbicidal properties. However, excessive production of HOCl/ClO can lead to the damage of a range of biological targets such as amino acids, proteins, carbohydrates and lipids.

Sedgwick *et al.* reported the ESIPT-based fluorescence probe **20** by using a dimethylthiocarbamate protecting group [51], which could detect HClO/ClO within 10 s and have an excellent selectivity towards other ROS/RNS and amino acids (Fig. 20).

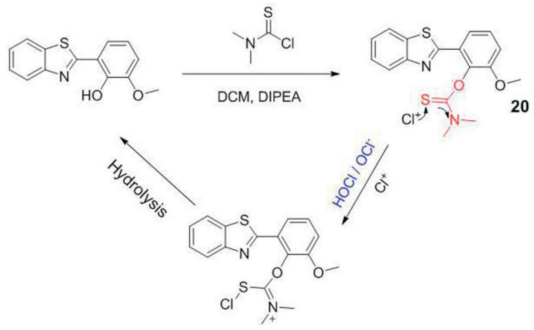

Fig. 20. The design and synthesis of probe **20** for HOCl.

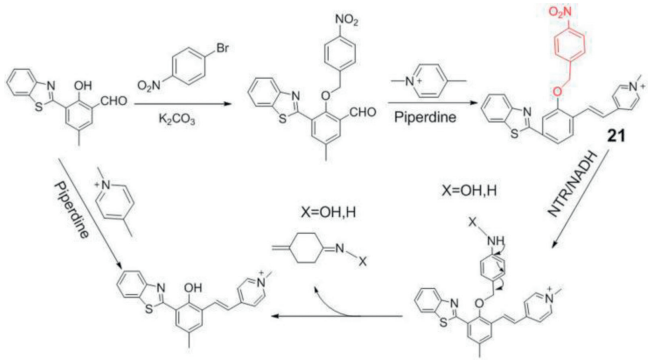

Fig. 21. The design and synthesis of probe **21** for NTR.

4. Enzymes

Enzymes are a class of important substances for life, and their abnormal levels are associated with many diseases. In recent years, some ESIPT-based enzymes-related fluorescent probes have been developed, including nitroreductase (NTR).

4.1. NTR

NTR can reduce nitro group into the corresponding amines or hydroxyl amines in the presence of reduced nicotinamide adenine dinucleotide (NADH), and therefore, its recognition moiety often contains the nitro group as an essential part. 4-Nitrobenzyl alcohol is another commonly used recognition moiety for NTR. Shao *et al.* developed probe **21** by incorporating 4-nitrobenzyl unit on HBT-contained derivatives (Fig. 21) [52]. The chromophore was yielded along with the reduction, rearrangement and elimination of 4-nitrobenzyl unit. Probe **21** displayed a wide linear range (0.1–1.5 mg/mL) and low detection limit (2.8 ng/mL) response to NTR.

4.2. Esterases

Esterases widely existed in tissue cells of various organisms, and catalyzed the hydrolysis of various esters, and mediated the metabolism of various tissues and cells. More importantly, esterase plays a significant role in cell viability and cytotoxicity assays. Therefore the quantitation of esterase activity could be used to evaluate cellular status. Tong *et al.* developed the esterase probe **22** with the hydroxyl group covered by acetoxy [53], as shown in Fig. 22. It could sense the esterase quantitatively in the range of 0.01–0.15 U/mL.

In view of that esterase was active in live cells but deactivated in dead cells, Lin's group developed an fluorescent probe **23** by acetylization of 3-hydroxyflavone [54], which can be hydrolyzed into 3-hydroxyflavone by active esterase with ESIPT process recovered (Fig. 23). Therefore, it displays blue emission in dead cells and

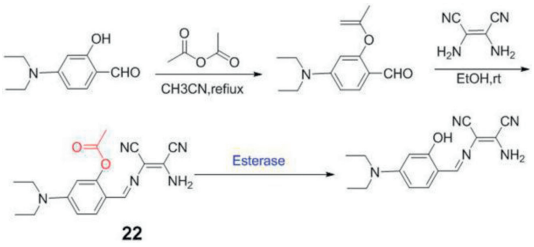

Fig. 22. The design and synthesis of probe **22** for esterases.

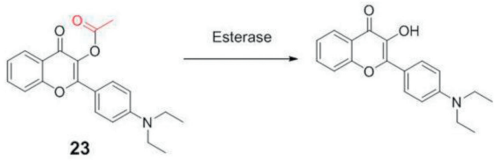

Fig. 23. The design and synthesis of probe **23** for esterases.

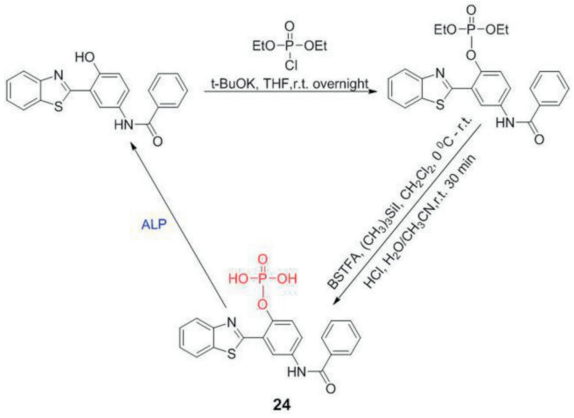

Fig. 24. The design and synthesis of probe **24** for ALP.

orange one in live cells, discriminating live cells from dead cells clearly in two emission colors.

4.3. Alkaline phosphatase (ALP)

ALP is widely distributed in human bone, intestine, liver, placenta and other tissues, which is a crucial biomarker for the diagnosis of hepatobiliary and skeletal diseases. The abnormal level of ALP is also associated with some other diseases, such as extrahepatic biliary obstruction, intrahepatic space occupying lesions, rickets and cancers. In recent years, to understand the roles of ALP in these diseases, various fluorescent probes have been developed to detect ALP activity in serum, and image ALP in cells and tumor tissues.

Yang *et al.* presented an ALP probe **24** (4-benzamio-2-(benzo[*d*] thiazol-2-yl)phenyl dihydrogen phosphate by transforming the hydroxyl group of ESIPT-based fluorophore into the corresponding phosphate group [55]. In the presence of ALP, probe **24** was hydrolyzed and the fluorophore was released, which exhibited large emission spectral red-shift (120 nm) because of the recovery of ESIPT process (Fig. 24).

4.4. β-Galactosidase

β-Galactosidase is overexpressed in primary ovarian cancers and abnormally accumulated in senescent cells, that is an important biomarker for senescence and ovarian cancers.

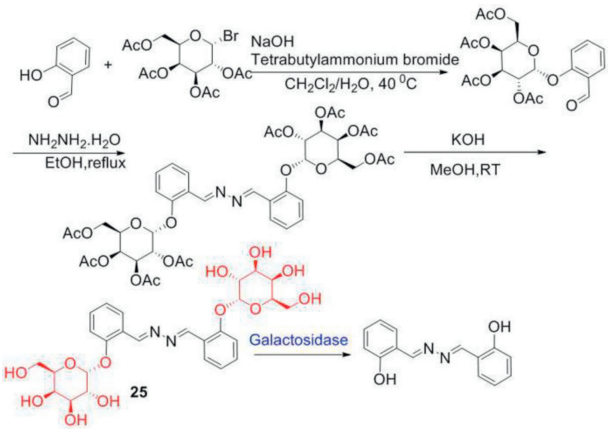

Fig. 25. The design and synthesis of probe **25** for β-galactosidase.

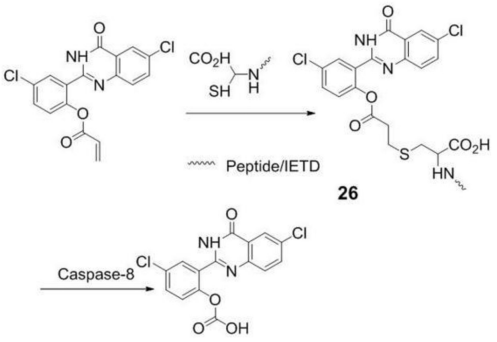

Fig. 26. The design and synthesis of probe **26** for caspases.

The sensitive detection of β-galactosidase is thus of great importance. Tong *et al.* reported a fluorescent probe **25** with light-up response to β-galactosidase (Fig. 25), which could be well retrained in living cells and emit strong fluorescence [56]. Probe **25** had a Stokes shift of 190 nm, while Pan *et al.* developed a probe combining a similar reaction with an ICT mechanism, which only exhibits a Stokes shift of around 30 nm [57]. The combination of hydroxyl protection strategy and ESIPT mechanism in constructing probes fully exemplifies their unique advantages.

4.5. Caspases

Caspases as aspartate-specific cysteine proteases, play vital roles in apoptosis and inflammation. Their inactivation inevitably causes some diseases including neurodegenerative diseases. Kuang *et al.* constructed a novel peptide probe **26** for peptidases through the Michael addition reaction between an acryloylated ESIPT fluorophore and a cysteine-appended peptide substrate [58]. The peptide bond in the probe was broken under the catalysis of intracellular peptidase, accordingly, the substrate peptide was released and the ESIPT fluorophore was recovered along with intramolecular cyclization (Fig. 26). The design strategy of probe **26** was generally applicable, which enabled the development of a variety of probes for peptidases. The performance parameters of the above probes were cited in detail and summarized in Table S1 (Supporting information).

5. Conclusion and outlook

The design and synthesis of probes based on regulating ESIPT through hydroxyl protection and deprotection are relatively simple and mature. Many related achievements have been made in developing fluorescent probes for RSS, ROS and enzymes in recent

years. Unfortunately, most of these probes belong to esters, amides or ethers, which are easy to hydrolyze and sensitive to pH. Moreover, the solubility of these probes is poor, and the detection is carried out in systems containing dimethylsulfoxide or acetonitrile. All of these limit the application of such probes in complex biological systems. Considering that ESIPT emission could be regulated by electronic effects and intermolecular hydrogen bonding, therefore, we put forward the following outlook: (1) Based the specific chemical reaction, ESIPT process is switched through analyte-induced electronic effect, realizing the detection of the analyte; (2) The ESIPT switch can also be regulated by the new-born intromolecular hydrogen bonding from the adduct of probe with analyte, thus possible for constructing multiple- sites probes.

Declaration of competing interest

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.

CRediT authorship contribution statement

Haixian Ren: Writing – original draft. **Yuting Du:** Software. **Xiaojing Yang:** Data curation. **Fangjun Huo:** Validation. **Le Zhang:** Formal analysis. **Caixia Yin:** Project administration.

Acknowledgments

We thank the National Natural Science Foundation of China (Nos. 22277104, 22325703, 22074084), the Natural Science Foundation of Shanxi Province (No. 202203021212184), Research Project supported by Shanxi Scholarship Council of China (No. 2022–002), the Basic Research Program of Shanxi Province (Free Exploration) (No. 202203021221009), 2022 Lvliang City science and technology plan project (Nos. 2022SHFZ51, 2022GXYF15) and Scientific Instrument Center of Shanxi University (No. 201512).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2024.109867.

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