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Small-molecule fluorescent probes based on covalent assembly strategy for chemoselective bioimaging

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Fluorescent probes have been widely studied and applied in environment and health analysis, where among them small molecular "covalent assembly" probes are a novel type of reaction probes with many advantages, including no background interference, remarkable colorimetric change, rapid response, high sensitivity, and strong fluorescent signal. During the past decade, significant contributions have been made globally to both the application and mechanism of covalent assembly probes. In this review, we summarize the recent development of covalent assembly probes, classifying them based on different analytes, such as anions, metal ions, small biological molecules, reactive oxidative spices (ROS), reactive nitrogen species (RNS), nerve agent mimics, and enzymes, and introduce their detection mechanism in detail. Furthermore, the perspective on the next generation of covalent-assembly probes toward biomolecules imaging is presented.

1. Introduction

Fluorescent probes are molecular agents that interact with a target analyte and show spectroscopical fluorescence changes simultaneously. Based on the specific stimulus–response interaction and resulting output change, the analyte substrate can be ascertained. Because small molecular fluorescent probes possess a well-defined structure and their spectral properties can be tuned based on their physical and optical characteristics, they have been widely applied in several fields such as chemical analysis, bioassay, and disease diagnosis.[1] The recognition principles of most probes are based on the cleavage or formation of covalent bonds or coordination bonds, which can achieve fluorescent signals "turn-on" or "turn-off". Due to their high sensitivity and detection ability in complicated systems, small molecular fluorescent probes have attracted increasing attention in several fields, especially in the biological and medical fields.

However, with the continuous development of social science and technology, the requirements for the performance of small molecule probes are also increasing. To achieve accurate and real-time detection results in a complex detection environment, the research route of improving molecular sensitivity and selectivity and reducing the interference from background signals through new action mechanisms is an important direction for the development of molecular probes.

In 2005, Anslyn *et al.* designed a specific palladium ion detection probe through the design idea of covalent assembly.[2] Through a palladium-catalysed Heck reaction, olefin compounds are coupled, an intramolecular cyclization reaction occurs, and then the fluorescence signal is generated, and the goal of detection is realized. This detection method is a new method for heavy metal analysis by catalytic signal amplification. The signal amplification scheme relies on exogenous inhibitors to deliberately inactivate the organometallic reaction that catalyzes the generation of fluorophores. However, the slow initiation of the Heck catalyst limits the sensitivity of the probe.

Subsequently, in 2014, Yang *et al.* first reported a novel mechanism based on the principle of "covalent assembly".[3] This mechanism is mainly triggered by the substrate to be tested. Through an intramolecular covalent cascade reaction, two fragments are combined to form fluorophores achieving the detection effect. In principle, the covalent assembly process results in a new electronic "push–pull" backbone and a rigidified bridge,[4,5] which can cause a significant change in the fluorescence emission of small molecular probes. In comparison with other types of probes, covalent assembly probes have many outstanding advantages, such as a more remarkable

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colorimetric change, more rapid response, higher sensitivity, and stronger fluorescent signal. Significantly, they have no background interference due to the novel mechanism of the "covalent assembly" reaction with specific analytes. Therefore, they have been widely investigated in the field of fluorescent probes.

2. Rational design of covalent assembly-based fluorescent probes

Over the past decade, many groups, especially Yang's group[4] and Romieu's group,[5] have made extraordinary contributions to the development of this novel mechanism and improving its sensitivity for *in vitro* and *in vivo* detection. These probes have been widely applied for the detection of various ions, small biological molecules, reactive oxygen species (ROS), reactive nitrogen species (RNS), and enzymes. Herein, this review focuses on the rational design and recent development of small molecular probes based on the covalent assembly principle for fluorescent detection and multi-functional bioimaging, as well as the potential challenges and future directions in this field.

2.1 Probes for anions

2.1.1 Probes for F^-. Characterized as being the smallest anion, the fluoride anion (F^-) shows extraordinary chemical properties, which plays essential roles in a series of physiological processes, such as the prevention of dental caries and treatment of osteoporosis. It can also be utilized as a chrome-cleaning agent, glass-etching agent, insecticide, and rodenticide. However, the excess intake of fluoride may also adversely influence human health such as gastric and kidney disorders, skeletal fluorosis, and urolithiasis. Consequently, it is deemed necessary to develop new selective and sensitive methods for the accurate determination of F^- in biological systems, the detection of which is challenging using traditional ion-selective electrodes.

Fluorescence resonance energy transfer (FRET) is a common luminescence phenomenon. It refers to the phenomenon that the excitation of a donor fluorescence molecule can induce the receptor molecule to emit fluorescence, and the fluorescence intensity of the donor fluorescence molecule itself decays. Nowadays, this type of luminescence phenomenon is widely used in the field of biosensors, including the detection of F^- *in vivo*. However, the conditions of this phenomenon are harsh, where the fluorescence spectrum of the donor molecule must overlap the excitation spectrum of the receptor molecule and the distance between the donor and receptor must be close.

In 2003, several fluorescent F^- probes were first designed by Swager *et al.*, where their response mechanism involved the formation of iminocoumarin derivatives through covalent-assembly (Fig. 1A).[6] Compared to most semiconductor polymer sensor schemes, which depend on the variation in emission intensity, this sensor system utilizes a new fluorescent signal. The direct electrical connection between the indicator and the band structure of polymers is a promising alternative to FRET. Subsequently, many groups reported various probes

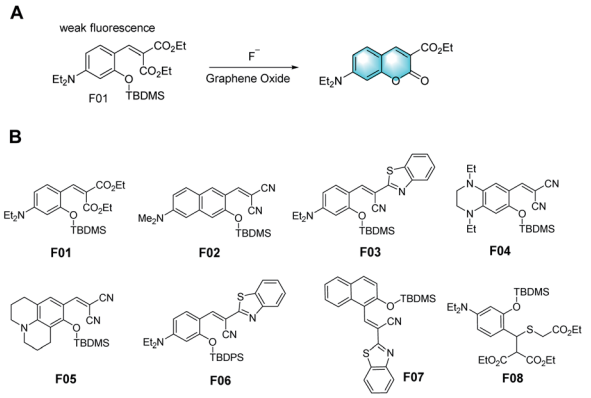

Fig. 1 (A) Response mechanism of a covalent assembly probe for F^-. (Adapted with permission from ref. 6 copyright 2003, Wiley). (B) Molecular structures of representative probes.

using *tert*-butyldimethylsilyl (TBDMS) or *tert*-butyldiphenylsilyl (TBDPS) as the recognition groups in accordance with the same mechanism, making full utilization of the high affinity of silicon for fluoride ions (Fig. 1B).[7–14] Besides, alkyl-amine groups or strong electron-donating groups (EDG) were introduced into the probes to achieve better fluorescence performances. Subsequently, some of them were modified, exhibiting a more remarkable Stokes shift, higher sensitivity, and better organelle targeting performance. Furthermore, these probes could even detect other ions simultaneously for application as molecular logic gates.[15]

As is known, the common phenomenon named aggregation concentration quenching (ACQ) is exhibited by most organic luminescent materials, which tends to weaken or even disappear completely in high-concentration solution or when these materials are in the aggregation state because of the formation of intermolecular aggregation. Different from the traditional ACQ principle, aggregation-induced luminescence (AIE) is a new luminescence mechanism reported by Tang's group. According to this mechanism, the intramolecular rotation of this type of molecule is limited and the non-radiative energy decays as a result of the limited space. The excited molecules can only return to the ground state by radiative transition, and consequently the fluorescence is significantly enhanced. This type of molecular mechanism overcomes the problem of fluorescence quenching and fluorescence concentration reduction in the aggregation state of traditional molecules and has good application prospect. Taking advantage of AIE, in 2019, a probe containing 4-(*tert*butyldimethylsilyloxy) benzyl as the recognition group with AIE activity was developed for the detection of fluoride ions (F^-) *via* the covalent assembly mechanism.[16] This fluorophore, which shows aggregation in water and emits strong yellow-orange fluorescence, exhibited significant AIE properties. Moreover, by improving the structure of the naphthalene ring, a fluorescent probe for the real-time monitoring of fluorine ions based on two-photon fluorescence covalent assembly was developed.[7] However, because of the prominent hydration effect of fluoride in aqueous environments, the performance of most of these probes is affected by the presence

of a large number of organic solvents.[1] Because of the inevitable hydration of fluorine ions and the limitation of low concentration for chemical dosimeters, the long response time of a large number of probes used for the detection of fluorine ions is unsatisfactory. In addition, the shortcomings of many organic fluorophores used for detection, such as poor water solubility, weak cell penetration, and poor photostability and chemical stability, are also urgent problems to be solved.

Thus, to overcome these problems, Wang *et al.* concluded that the interaction between the fluorescent chemical dosimeter and target analyte is essentially an organic chemical reaction.[9] Accordingly, improving the reaction efficiency will be beneficial to the performance of fluorescent chemical dosimeters. They attempted to offer a solution, where the self-assembly of fluorescent chemo-dosimeter molecules on the surface of graphene oxide (GO) can avoid the above-mentioned problems by taking advantage of the excellent chemical catalysis and nanocarrier functions of GO (Fig. 2). In the cell imaging experiment, the fluorescence response of the graphene oxide-loaded probe to fluorine ions was weaker than that of the non-loaded probe. Nevertheless, the loaded graphene oxide could give the probe the function of reducing its own background, short response time and response to more analytes. This was the first attempt to design a fluorescent probe by combining GO for sensing and bioimaging application. Thus, this simple design approach can help develop more effective fluorescent probes for other analytes through the combination of different nanomaterials.

2.1.2 Probes for CN^-. Currently, cyanide is a compound widely used in industry. It plays an important role in the synthesis of resins, drugs, insecticides, and chemical fertilizers and as a gold extraction reagent. However, due to the common use of cyanide, a large number of cyanide ions are exposed in the air, and thus pollute the environment. Moreover, its biological toxicity is extremely high, resulting in the inhibition of respiratory system of animals. Thus, due to the substantial toxicity of cyanide, researchers have devoted great attention to the sensitive detection of cyanide. Developing probes for cyanide characterized by high sensitivity and practical capacity is of great significance to environmental protection and health analysis.

Many sensors for toxic CN^- have been developed based on the strong nucleophilicity of cyanide to metal ions or carbonyl carbon atoms. These sensors showed an adequate limit of detection (LOD) for cyanide, but the LOD for analyte was reduced in the case of an excessive amount of cyanide. Thus, to solve this problem, Kim H. *et al.* reported a fluorescence probe, CN1, based on 2-hydroxy cinnamate for the detection of cyanide (CN^-).[17] They introduced an α,β-unsaturated carbonyl moiety in the latent fluorophore, where the 2-hydroxycinnamate group was employed as the fluorogenic unit and the α,β-unsaturated carbonyl group as the reaction unit with cyanide (Fig. 3A). Different from the previous work, cinnamate underwent a rapid cyanide-catalyzed reaction to form a stable coumarin product based on covalent assembly. Characterized by a limit of detection (LOD) of 1.3 μM, this probe also displayed a highly selective and sensitive fluorogenic response to cyanide in the presence of other anions with a micromolar limit for fluorometric detection (Fig. 3B). Kim's work presents an efficient way for developing probes capable of sensing cyanides at a catalytic level.

2.1.3 Probes for NO_2^-. Nitrite is a nitrogen-containing compound, which widely exists in the natural environment. As a storage form of biological NO, nitrite can be reduced through a variety of mechanisms and plays a key role in the nitrogen cycle. Nitrite is also widely used in daily life, such as in food additives, preservatives, agricultural fertilizers, industrial dyes and bleaches, and vasodilators. However, an excessive intake of nitrite is also harmful to human health, especially children and pregnant women. Excessive concentrations of nitrite can lead to methemoglobinemia and blue baby syndrome. Thus, it is essential to develop practical methods for the quantification of nitrite in traditional disciplines, including environmental monitoring, food safety, disease diagnosis and basic scientific research.

The Griess assay is a common method for the quantitative detection of nitrite ions.[18] It involves the mechanism of covalent assembly to cause *p*-amino benzenesulfonic acid to react with analytes to form diazonium ions in an acidic environment, and then produce azo dyes through intermolecular electrophilic substitution reaction with naphthylamine, and its absorption intensity is detected by UV-vis spectroscopy to achieve the detection effect. Nevertheless, due to the toxicity of the materials employed for detection using traditional methods, it is worth improving or developing new and effective methods for the quantitative detection of nitrite ions.

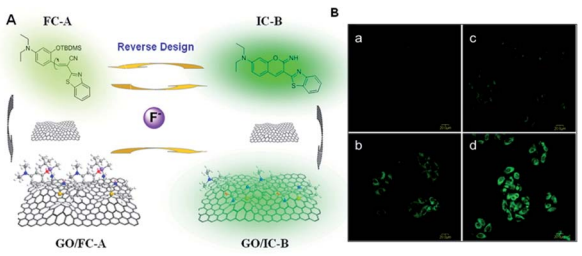

Fig. 2 (A) Response process design of fluorescent chemodosimeter FC-A and GO-based fluorescent nanodosimeter GO/FC-A to fluorine ions. (B) Fluorescent confocal images of the graphene oxide-loaded probe to fluorine ions in living cells. (Adapted with permission from ref. 9 copyright 2014, the American Chemical Society).

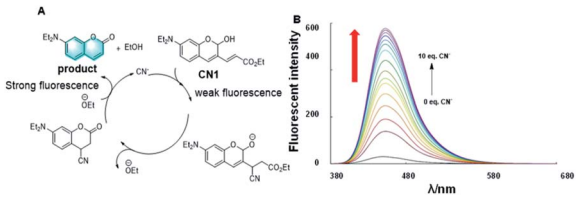

Fig. 3 (A) Response mechanism of a covalent assembly probe for CN^-. (B) Fluorescence responses of **CN1** toward CN^- in different eq. CN^-. (Adapted with permission from ref. 17 copyright 2016, Elsevier).

Based on this, Unob *et al.* proposed a new strategy to optimize the Griess analysis.[19] In this strategy, cellulose thread was modified by *p*-aminobenzoyl, and chromotropic acid was used to replace the toxic aromatic amine *n*-(1-naphthyl) ethylenediamine (NED), and thus in an acidic environment, the thread acts as a reaction platform for nitrite ions, *p*-aminobenzoyl and NED. Diazotization reaction and covalent assembly were carried out successively to form azo dyes to form color bands. The effect of the quantitative analysis of nitrite ion concentration was realized according to the dyed length of the thread. This method is convenient and intuitive. Furthermore, the concentration of each reagent and the sample volume were optimized. The proposed method allowed the detection of nitrite in the concentration range of 50–1000 μM. Unob's work further optimized the Griess assay, which realized the accurate quantitative detection of nitrite in a more intuitive and convenient way, exhibiting potential as a detection method.

For detection methods for disease diagnosis, high sensitivity, short response time and efficient and accurate detection effects are the standards to measure their quality. In recent years, fluorescent probes have been developed for the detection of nitrite ions.[20–26] However, most of these probes have limited have been developed practical application due to their low sensitivity, high background fluorescence and long response time. In contrast, a novel fluorescent sensor for the quantification of nitrite was designed based on the "covalent assembly" principle by Yu in 2021.[27] The response mechanism is actually an intramolecular azo coupling mechanism. In strong acidic medium (pH = 0–1), as is shown in Fig. 4, once the diazotization between nitrite and the aromatic amine part of the probe **AAC** is completed, the newly formed aryldiazo and the coumarin part of **AAC** undergo electrophilic aromatic substitution reaction, intramolecular cyclization and protonation, finally forming dye molecules with strong fluorescence. It is worth mentioning that this mechanism utilizes the strategy of covalent assembly. The experimental data showed that the quantum yield of the probe **AAC** in acidified medium is zero, which shows the characteristics of zero fluorescence background. In addition, its emission spectrum reaches the near-infrared window, which also indicates that it is suitable for practical detection. In addition, this probe also showed some other excellent properties, such as excellent sensitivity and high selectivity. It is worth mentioning that the detection limit is 6.7 nM, which means that the probe is also suitable for low-concentration nitrite environmental monitoring. Consequently, Yu's experiments revealed that their probe is a potential detection tool for practical disease detection.

2.2 Probes for metal ions

2.2.1 Probes for Au^{3+}. Owing to their excellent biocompatibility and unique chemical properties, gold ions have always been a focus in biochemistry. However, it was reported that some gold ions, especially Au^{3+}, can lead to cytotoxicity in living organisms once bound to specific enzyme. Thereby, developing gold ion probes to evaluate the amount of gold ions in the environment is of significant importance.

Accordingly, the question arises, what group can specially respond to gold ions? In 2004, Richard *et al.* reported the process of the gold-catalysed activation of a C–C multiple bond in alkyne.[28] Impressively, in the presence of Au^{3+}, the alkyne was coordinated by the Au^{3+} in a gold–alkyne complex to realize hydro-arylation, and then underwent intramolecular electrophilic aromatic substitution with a Michael receptor, which indicates that the alkyne group can be used in the design of probes that respond to Au^{3+}.

In 2010, Yoon *et al.* reported a probe for Au^{3+} based on the mechanism of gold-catalyzed hydro-arylation, followed by intramolecular electrophilic aromatic substitution with a Michael acceptor (Fig. 5A).[29] Through the special response of Au^{3+}, the probe completed the hydro-arylation reaction to afford a coumarin structure, presenting strong fluorescence by covalent-assembly. It is a highly selective and sensitive fluorescence "turn-on" probe for Au^{3+} ions in protic solvents, which could even realize the bioimaging of Au^{3+} in live cells.

Subsequently, many probes based on the cascade reaction between gold and alkynyl have been developed.[30–41] Their

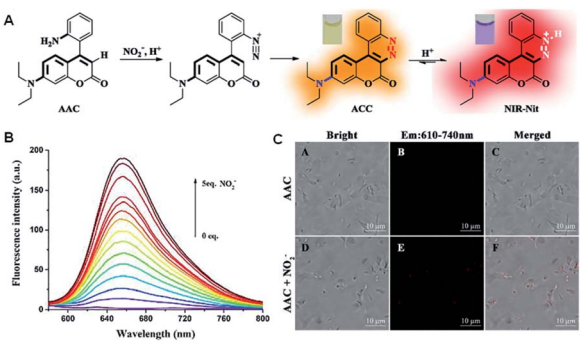

Fig. 4 (A) Response mechanism of the covalent assembly probe AAC for NO_2^-. (B) Fluorescence responses of AAC toward NO_2^-. (C) Fluorescent confocal microscopy images of *E. coli* stained with 10 μM **AAC** for 10 min in the presence of nitrite (5 mM). (Adapted with permission from ref. 27 copyright 2021, Elsevier).

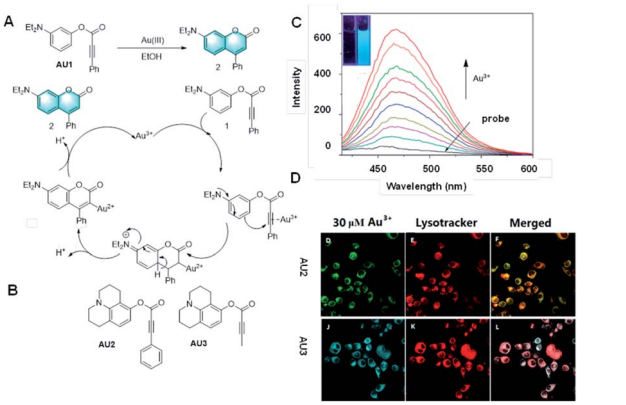

Fig. 5 (A) Response mechanism of a covalent assembly probe for Au^{3+}. (B) Structures of covalent assembly probes to Au^{3+}. (C) Response of the probe **Au2** towards different concentrations of Au^{3+}. (D) Fluorescent images of Au^{3+} in HaCaT cells. (Adapted with permission from ref. 42 copyright 2016, Elsevier).

luminescence mechanisms are rich and diverse and have excellent performance, but they cannot be strictly classes as the covalent assembly mechanism. It is worth mentioning that two new colorimetric and fluorescent probes were developed for the rapid and highly selective detection of Au^{3+} (Fig. 3B).[42] Acetylene compounds were utilized as probes to rearrange potential fluorophores into fluorescent coumarin derivatives, which could realize the detection and recognition of Au^{3+} *via* a gold ion-mediated hydro-arylation reaction. The experimental results showed that these two probes have good selectivity and sensitivity for the detection of gold ions, and their detection limit was as low as 0.1 nM. More importantly, due to their good biocompatibility and cell membrane permeability, these two probes could also be applied for the fluorescence imaging of MCF-cells. They are detection tools with great practical application potential and are expected to bring some practical application value to practical clinical detection. The application of potential fluorophores in the screening of effective catalysts is also an ongoing research topic.

2.2.2 Probes for Cu^{2+}/Cu^{+}. Playing a critical role as information messengers and enzyme cofactors, copper has an indispensable role in living systems. However, excessive unbound copper ions will also cause oxidative damage and protein misfolding, leading to many neurodegenerative diseases and deleterious effects on human health. Therefore, the development of fluorescent probes that specifically respond to Cu^{2+} for its detection in aqueous solutions and under physiologically relevant conditions is still necessary.

In 2004, Anslyn *et al.* first reported a highly sensitive probe *via* thew Heck reaction, which showed high selectivity for Cu^{2+}.[43] The recognition mechanism also involved covalent assembly. However, although this probe demonstrated a good detection limit of 30 nM, it also suffered from the drawback that its detection time is nearly 1.5 h.

In recent years, through the same mechanism of N=N isomerization, many probes for Cu^{2+} detection have been reported (Fig. 6C).[44–54] These probes demonstrated better water solubility, better selectivity, better biocompatibility, higher sensitivity through structural modification, and were applied in cell imaging or paper-based detection devices.

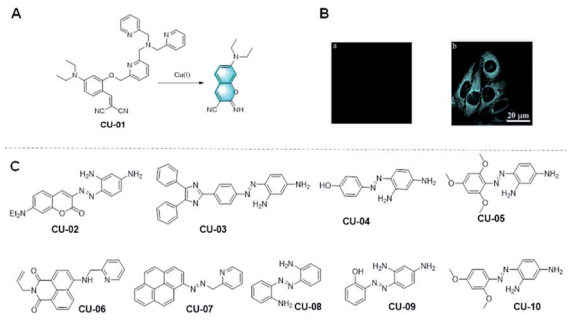

Fig. 6 (A) Response mechanism of a covalent assembly probe for Cu^{2+} and (B) fluorescent images of Cu^{+} in HepG2 cells. (Adapted with permission from ref. 55 copyright 2016, Elsevier). (C) Structures of the probes for Cu^{2+} detection based on the mechanism of N=N isomerization.

As the dominant oxidation state in the physiological reducing conditions, recently, Cu^{+} has been a focus, and thus several synthetic probes have been developed for realizing its detection in biological systems. Among them, many probes contain tris[(2-pyridyl)-methyl] amine (TPA) as the recognition group for Cu^{+} detection because of the selective promotion of oxidative cleavage of the benzylic ether bond (C–O) upon binding Cu^{+}.

However, the previous designs of TPA-based probes for Cu^{+} are limited by their dependence on fluorophore scaffolds containing phenolic groups. It is worth mentioning that Hu *et al.* found that the use of TPA in probes can also be extended to the fluorophore precursors containing hydroxyl groups. According to their strategy, TPA was used in a probe and connected with the phenylphenol group of the fluorophore precursor (Fig. 6A).[55] Through the complexation of the analyte cuprous ion, the carbon–oxygen bond is broken, and the fluorophore precursor is covalently assembled through Pinner cyclization to form a fluorophore with strong fluorescence signal. The probe **CU-01** made according to this strategy not only showed excellent solubility in aqueous solution and living cells, but also had a low fluorescence background and rapid response to analytes in living cells, which are worthy of recognition. In conclusion, Hu's work solved the limitation of using the TPA group as a cuprous ion recognition group, broadened the detection method of cuprous ions, and provided a good idea for the further preparation of fluorescent probes for the detection for Cu^{+} with more sensitive performances and more significant detection effects.

2.2.3 Probes for Hg^{2+}. Mercury, which is considered one of the most common toxic metals in the environment, can easily penetrate biofilms such as respiratory and gastrointestinal tissues and skin. Once it passes through the human body, it will cause damage to the central nervous system and endocrine system. Moreover, it is reported that both elemental mercury and mercury ions can be transformed into methylmercury by bacteria in the environment, and then heavy metal mercury can be bioaccumulated through the biological chain, finally resulting in mercury poisoning in organisms. Therefore, the imaging of Hg^{2+} in living cells is the key to clarifying its biological effect.

A class of probes for the detection of Hg^{2+} based on vinyl ethers as the reaction unit was reported.[56–59] The mechanism of these probes is that the Hg^{2+}-promoted hydrolysis of the vinyl ether group results in a hydroxy-ester intermediate, which undergoes a fast cyclization reaction to afford a fluorescent coumarin structure. Extending coumarin derivatives with electron-donating substituents, such as amino and dimethylamino groups, can improve their photophysical properties. For example, as shown in Fig. 7C–E, upon the addition of Hg^{2+} in pure aqueous medium, the Hg^{2+}-mediated hydrolysis of vinyl ether and subsequent cyclization reactions converted the probe HGb2 into the corresponding iminocoumarin dye, which is strongly fluorescent and can also be applied for imaging Hg^{2+} in living cells. The probe HGb4 has a remarkable fluorescence enhancement at 625 nm, a large Stokes shift and low limit of detection (7.1 nM) when 1,4-diethyl-1,2,3,4-tetrahydroquinoxaline is present instead of a benzene ring in

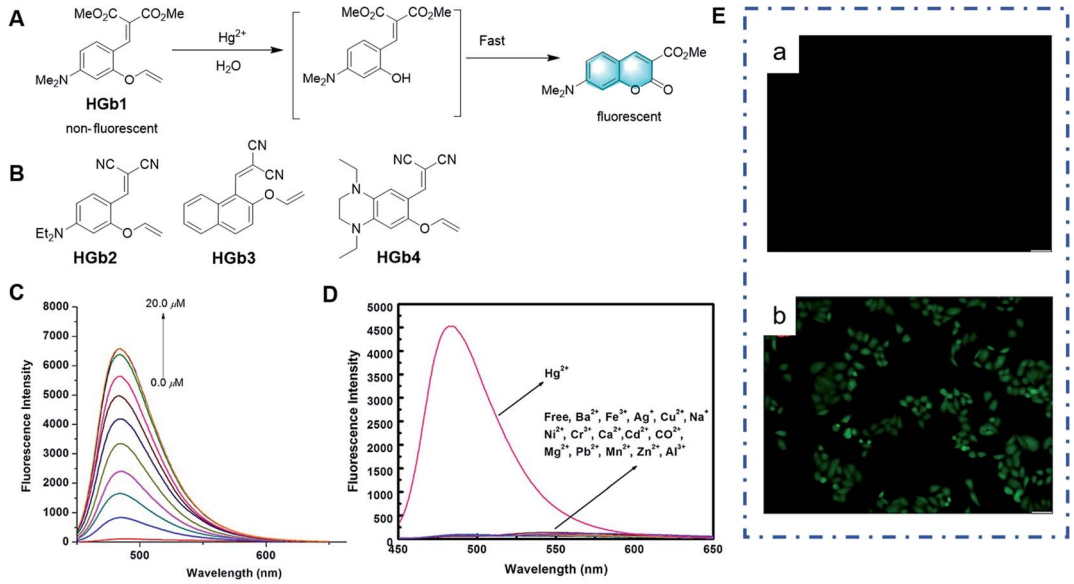

Fig. 7 (A) Response mechanism of the probe **HGb1** to Hg^{2+}. (B) Structures of probes for Hg^{2+} based on the same mechanism. (C) Fluorescence responses of **HGb2** toward different concentrations of Hg^{2+} and (D) different metal ions. (E) Images of HeLa cells: (a) fluorescent images of HeLa cells incubated with **HGb2** (10 μm) and (b) fluorescent images of HeLa cells treated with **HGb2** (10 μm) for 30 min, and then incubated with 30 μm Hg^{2+} for another 30 min. (Adapted with permission from ref. 57 copyright 2014, Wiley).

its structure. This probe exhibited a good performance for the detection of Hg^{2+} *in vivo* and *in vitro*.

The effects of mercury exposure on the nervous system are mainly attributed to the organic form of mercury, methylmercury ($MeHg^{+}$), which acts physiologically by binding with the sulfhydryl groups in proteins or cysteine, forming water-soluble complexes in tissues, accumulating in the food chain and crossing the human brain blood–brain barrier. However, it is a laborious task to develop molecular probes for the selective detection of $MeHg^{+}$ in the presence of Hg^{2+}. In 2014, Yang *et al.* reported Hg^{2+}-promoted desulfurization, with ring-opening of 1,3-dithiolane, and subsequent covalent-assembly with the amino group on the benzene ring owing to the strong nucleophilicity of the amino group.[60] As shown in Fig. 8A and B, in the probe HGc1, the conjugated structure of the product generated by detection was expanded, which greatly enhanced the fluorescence. Based on this mechanism, other probes, HGc2 and HGc3, also used 1,3-dithiolane as the reaction unit to achieve the same covalent assembly strategy.[61,62] Among them, it is worth mentioning that the probe HGc3 exhibited high sensitivity and selectivity towards Hg^{2+} and $MeHg^{+}$, which emitted strong fluorescence. Moreover, it was successfully applied for the quantitative detection of Hg^{2+} in real environmental samples and imaging of Hg^{2+} in HeLa cells.

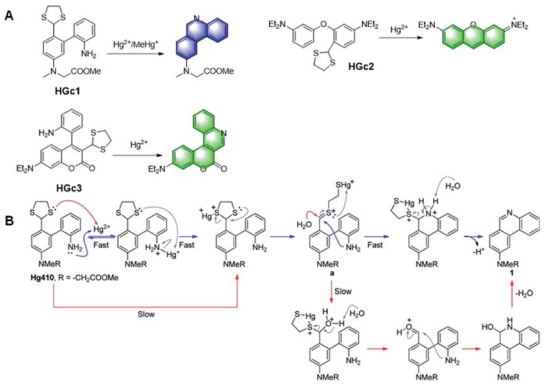

Fig. 8 (A) Structures of probes **HGc1**, **HGc2**, and **HGc3** and their response to Hg^{2+}. (B) Proposed mechanism of the probe **HGc1** to Hg^{2+}. (Adapted with permission from ref. 60 copyright 2014, the American Chemical Society).

2.2.4 Probes for Pd^{2+}. Acting as a catalyst for the synthesis of various molecules, including drugs, palladium plays a crucial role in chemical conversions. However, palladium ions may also cause a series of cellular processes upon binding to proteins, DNA, thiol-containing amino acids, other macromolecules, *etc.*, and thereby have a negative impact on human life and the environment. Consequently, the development of efficient methods to detect the presence of palladium species is desirable.

Over the last few years, considerable efforts have been focused on the exploration of novel palladium ion fluorescent probes based on the coordination action of Pd^{2+} with heteroatomic ligands, ring-opening reactions, oxidative cyclization reactions, as well as demercuration and deallylation reactions catalyzed by palladium. However, many of them still have limitations, such as working only in organic solvents and/or water organic cosolvents, requiring additional reagents, laborious synthetic processes using expensive chemicals, interference from other coexisting metal ions, and long response time. In addition, among the few available Pd^{2+} chemical dosimeters reported, most of them use the pH-sensitive fluorescein or rhodamine as fluorophores, and their pH dependence may

cause detection errors. Hence, for practical applications, it is necessary to develop new simple and specific fluorochemical dosimeters to detect Pd^{2+}.

In 2005, Anslyn *et al.* designed a specific palladium ion detection probe *via* the 'covalent assembly principle'.[2] Through palladium-catalysed Heck reaction, an intramolecular cyclization reaction occurs, which can turn the fluorescence signal on and realize the detection. However, the slow initiation of the Heck catalyst limits the sensitivity of this probe (Fig. 9A).

It has been reported that through a palladium-catalyzed hydrolysis reaction, the terminal propargyl ether can be cleaved to form the corresponding free hydroxyl group.[63–65] Based on the Tsuji Trost reaction, the palladium ion is reduced to palladium(0) by triphenylphosphine in the corresponding process, and then the allyl group in the molecular probe is oxidized with palladium(0) to release a hydroxyl intermediate, which spontaneously forms coumarin derivatives with high fluorescence intensity. The strong fluorescence change before and after the response is also an intuitive manifestation of the effective and sensitive detection of palladium ions. In 2016, Han *et al.* reported the probe **PD2** based on this mechanism (Fig. 9A).[66] The free hydroxyl group combined with the cyano-group can form a coumarin structure, which significantly changes its fluorescence. It was successfully utilized for the visualization of Pd^{2+} *in vitro* and in living cells (Fig. 9C and D). Later, Yang *et al.* reported another probe **PD3** for the detection of Pd^{2+} with a simple structure based on the terminal propargyl ether, which was easy to prepare and had a lower detection limit of 1.65 nM in an aqueous solution.[67] Because of the non-radiative energy consumption caused by intramolecular rotation, the probe did not show fluorescence in solution. As an effective tool for detecting Pd^{2+}, it can effectively recognize Pd^{2+}, and thereby emit an obvious fluorescent "turn-on" response. Moreover, it exhibited outstanding properties, such as rapid response (within 3 min), good linearity ($R^2 = 0.9945$), specific selectivity, and superior recovery, demonstrating its extraordinary potential for the analysis of real samples.

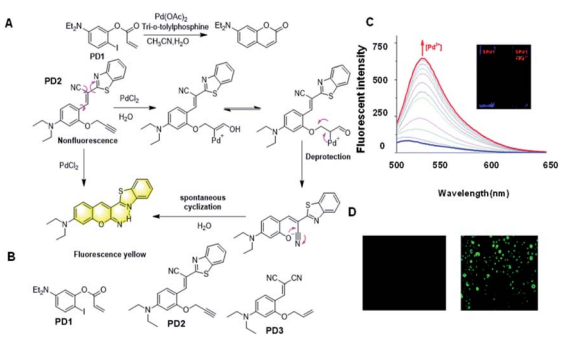

Fig. 9 (A) Response mechanism of the probes **PD1** and **PD2** for Pd^{2+} detection. (B) Structures of probes for Pd^{3+}. (C) Fluorescence responses of **PD2** toward Pd^{2+} and (D) The fluorescent images of Pd^{2+} in living cells. (Adapted with permission from ref. 80. Copyright 2016, Elsevier).

2.3 Probes for small molecules

2.3.1 Probes for biothiols. Biothiols, such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), play as crucial roles in mediating a series of physiological and pathological processes in mammalian systems. It has been reported that an abnormal level of these biothiols is closely related to many diseases. Therefore, to better explore the different physiological mechanisms of various biothiols on diseases, it is crucial to develop efficient methods for the selective and sensitive detection of biothiols, particularly for those that can distinguish the above-mentioned three different species.

In 2015, Phillips and the co-workers used 2,4-dinitrobenzesulfonyl (nosyl) as the reaction unit for detecting thiols in a polymeric monomer.[68] As shown in Fig. 10, the nosyl group of the probe **BT1** cleaved selectively in response to the added thiol and the free phenol cyclized into a thioester, releasing one equivalent of ethanethiol, while forming an equivalent of fluorescent 7-alkoxycoumarin by the covalent assembly strategy simultaneously. Phillips' work described a responsive polymer membrane realizing the trace level detection of specific molecular signals (thiol), and subsequently triggering a self-propagating reaction in the material to transform the non-fluorescent film into a global fluorescent film. More importantly, according to these findings, it is obvious that the self-propagating reaction in synthetic materials is a promising strategy. In the process of synthesizing materials, due to the self-propagating reaction, the materials can realize self-transformation in response to a trace level of a specific application signal in an autonomous and complete way, realizing the new biomimetic amplification reaction.

For a long time, due to the similarity of GSH, Hcy and Cys, it was a challenge to develop a specific probe for the recognition of Cys in the presence of GSH, Hcy and Cys. Thus, to solve this

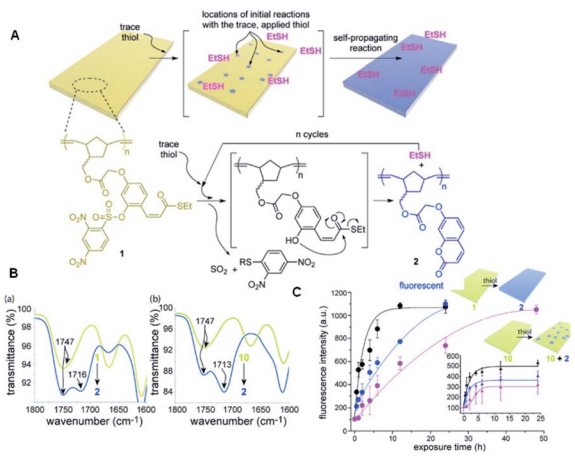

Fig. 10 (A) Illustration of the polymeric film response to thiol. (B) Change in IR signature in the carbonyl region when film 1 (a) or 10 (b) is exposed to 10 mM thiol. (C) Fluorescence kinetics when 0.1 mM (pink data), 1 mM (blue data), and 10 mM (black data) solutions of L-cysteine were exposed to films of polymers 1 and 10 at 23 °C. (Adapted with permission from ref. 82 copyright 2015, the American Chemical Society).

problem, in 2016, Song *et al.* designed a probe **BT2** using acrylate as the recognition group for the detection of cysteine (Fig. 11).[69] In the presence of Cys, GSH and Hcy, probe **BT2** formed thioether due to a nucleophilic addition reaction. The difference is that after cysteine treatment, the thioether formed by the probe can further undergo intramolecular cyclization to form an imino coumarin dye and emit strong red fluorescence. However, the addition reaction of Hcy and GSH with the probe could not further cyclize to form imino coumarin dyes, and thus the specific recognition effect of Cys in the presence of biothiols with similar structures was achieved. Consequently, this probe had an excellent performance, displaying a low detection limit of 6.6 nM and a pronounced Stokes shift of 148 nm. It could also be applied for cell imaging because of its permeability and biocompatibility.

Using the same structure, Churchill *et al.* applied the probe Reals-C in HaCaT human keratinocytes and mice liver.[70] Thus, a new preeminent fluorescent probe featuring a red emission, large Stokes shift of cysteine and water-based detection was prepared. The probe detected analytes through the complex interaction established by its leaving group to distinguish and form a red emission analyte sensing platform ($\lambda_{ex} = 471$ nm and $\lambda_{em} = 637$ nm) *via* a chemical cascade reaction. Furthermore, the responsive performance of the probe was proven by *in vitro* and *in vivo* experiments. Compared with a sensitive commercial thiol probe, this probe could successfully detect endogenous cysteine in human keratinocytes. Reals-C showed preeminent *in vivo* cysteine detection in a drug-induced liver injury model. The considerable results suggest that this type of Cys-recognizing fluorescent probe can be extended in the future to serve as a promising tool for exploring microbiological systems, such as intracellular organelles related to Cys.

2.3.2 Probes for H_2S/H_2S_n. Recognized as the third gas transmitter after carbon monoxide and nitric oxide, hydrogen sulfide plays a key role in biological processes. Hydrogen sulfide participates in a series of important biological reaction processes and disease-related redox regulation to achieve intracellular homeostasis and inhibit cell oxidative stress. According to many reports, the expression level of hydrogen sulfide can display the degree of oxidative stress in organisms, where an abnormal expression of hydrogen sulfide is closely connected to diabetes, arterial disease, Down's syndrome, pulmonary hypertension, Alzheimer's disease and other diseases. In addition, hydrogen polysulfides (H_2S_n, $n > 2$ or $n = 2$) can be produced by endogenous hydrogen sulfide or other enzymatic pathways. Compared with hydrogen sulfide, H_2S_n can activate some ion channels and transcription factors. However, its exact biological function still needs to be further studied. Accordingly, efficient and practical detection methods are important for better understanding the biological roles of H_2S/H_2S_n.

The use of the benzyl azide group as a recognition unit for H_2S has been widely reported.[71–73] The mechanism is that benzylazide group can be reduced to a benzylamine intermediate in the presence of H_2S. The resulting benzylamine fraction is unstable, and thus subsequently self-eliminated by 1,6-disulfide to release a hydroxyl group. Then it will undergo intramolecular cyclization to produce imino coumarin molecules. As shown in Fig. 12A, different probes based on this reaction mechanism *via* structure modification have been developed. It is gratifying that most of these probes showed superior performances in biological imaging, such as high sensitivity, rapid response time, and good water solubility.

In 2019, Zhao *et al.* reported an outstanding probe, **PZC-S_n**, based on a phenothiazine coumarin dye for the detection of H_2S_n *via* covalent assembly (Fig. 12B).[74] H_2S_n can react with the recognition group (2-fluoro-5-nitrobenzoate moiety) by replacing the F atom to produce an intermediate with an –SSH group and induce cyclization between the ester group and –SSH group. Remarkably, this probe emitted red emission with a massive Stokes shift and showed high sensitivity to H_2S_n with a fluorescence turn-on response and low cytotoxicity to cells. The bioimaging analysis of exogenous and endogenous H_2S_n in living RAW264.7 cells and zebrafish was performed, which showed superior results without autofluorescence.

2.3.3 Probes for thiophenol. Thiophenols, which are widely utilized in the preparation of medicines, pesticides and materials, are highly toxic substances. It has been reported that thiophenols can produce reactive oxygen species (such as superoxide radical and hydrogen peroxide) in the process of autoxidation in biological systems, causing serious oxidative damage to cells. Therefore, exposure to thiophenol can lead to many diseases, such as increased respiration, central nervous system injury, muscle weakness, hind limb paralysis, coma, and even death. Consequently, the establishment of sensitive and selective platforms for the detection of thiophenols in environmental and biological samples is of great importance.

In recent years, many small molecule fluorescent probes have been developed for the detection of thiophenol, but most of them have long response times, relatively small fluorescence enhancement ratio and high detection limit. Thereby, it is challenging to prepare sensitive, rapid response and low detection limit probes for practical biological detection. Based

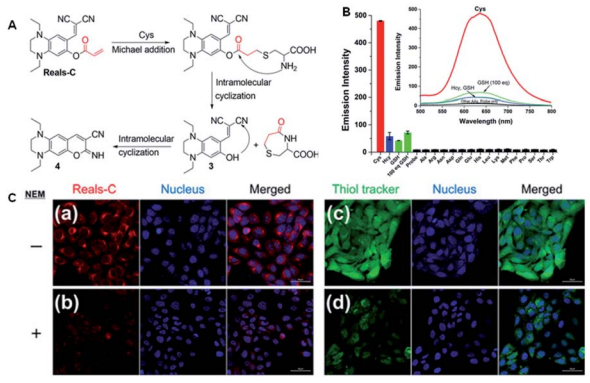

Fig. 11 (A) Proposed mechanism of **Reals-C** with Cys. (B) Emission intensity of **Reals-C** (10 μm) with 10 equiv. of canonical amino acids and biothiols. (C) Confocal fluorescence images of HaCaT cells incubated with **Reals-C** (a) and 20 μm thiol tracker (c) for 30 min. Cells were pre-treated with NEM (1 mm) for 30 min, followed by incubation with 10 μm of **Reals-C** (b) and 20 μm thiol tracker (d) for 30 min. (Adapted with permission from ref. 69 copyright 2016, Elsevier).

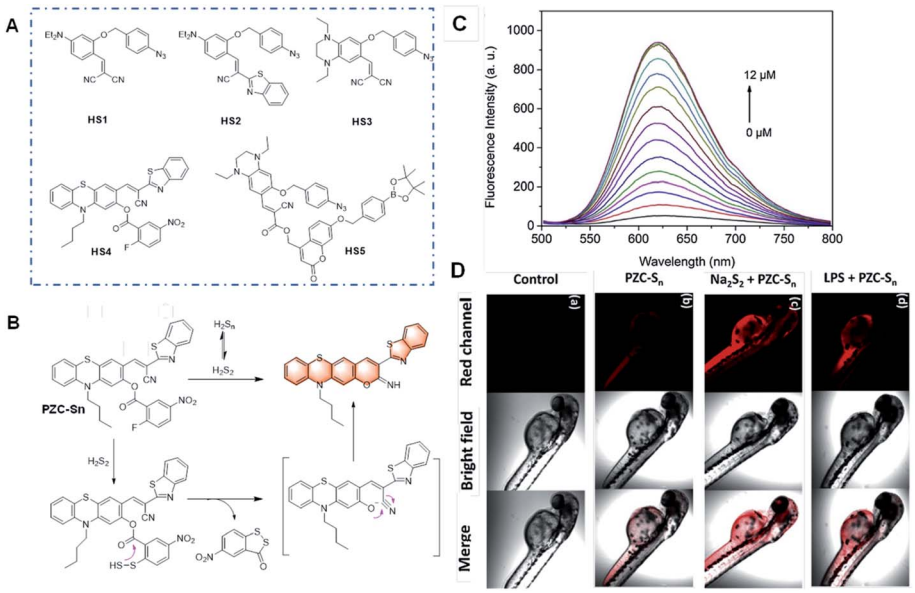

Fig. 12 (A) Structures of the probes for H_2S_n based on the benzyl azide group as the recognition unit. (B) Structure and response mechanism of **PZC-S$_n$** to H_2S_n. (C) Fluorescence responses and (D) images of **PZC-S** *in vitro* and in zebrafish. (a) Zebrafish without any treatment; (b) zebrafish stained with **PZC-S$_n$**; (c) zebrafish treated with 12 μM Na_2S_2 followed by the staining with **PZC-S$_n$**; and (d) zebrafish treated with LPS for 12 h followed by the staining with **PZC-S$_n$**. (Adapted with permission from ref. 74 copyright 2010, The Royal Society of Chemistry).

on the mechanism of thiophenol-induced cleavage of the 2,4-dinitrophenolate moiety and a subsequent cyclization reaction, some novel probes have been reported for the detection of thiophenol.[75–77] For example, Song *et al.* developed a probe for the detection of thiophenol.[76] As shown in Fig. 13, **TP1** was essentially nonfluorescent due to the mechanism of the photo-induced electron transfer (PET) process. In the presence of thiophenol, the 2,4-dinitrophenolate moiety in TP1 would be selectively cleaved and a subsequent cyclization reaction would produce an imino-coumarin dye. The probe **TP1** displayed a rapid response (within seconds) and a massive Stokes shift (129 nm). Furthermore, this probe emitted in the red or near infrared (NIR) regions, and thus it can penetrate the cell membrane and allow the visualization of intracellular thiophenol in living cells.

2.3.4 Probes for nerve-agents. Nervous agents, such as sarin, soman and tabun, are a class of organophosphate derivatives. Because of their strong electrophilic ability, these nerve-agents can easily interact with the central nervous enzyme acetylcholinesterase, destroying nerve impulse conduction and resulting in acute toxicity.[78–87]

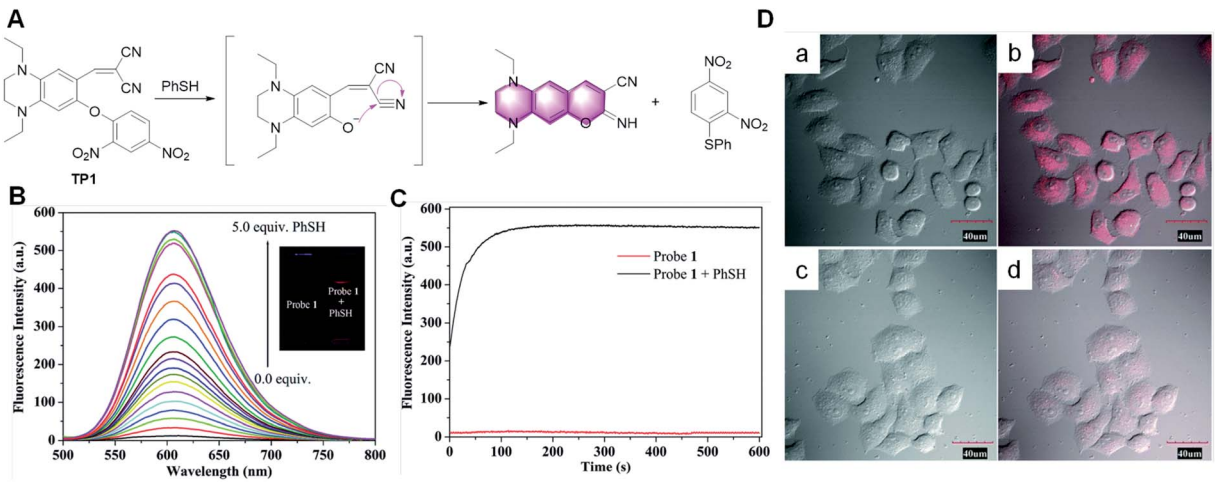

Fig. 13 (A) Proposed sensing process of probe **TP1** for thiophenol. (B) Fluorescence spectra of **TP1** (10.0 μM) upon the addition of thiophenol. (C) Kinetics of the fluorescence enhancement in the absence (red line) and the presence (black line) of thiophenol. (D) Fluorescence (left), bright field (middle) and merged (right) images of living HeLa cells. Top row: cells pre-treated with thiophenol (50.0 μM), and then incubated with **TP1** (10.0 μM) for 30 min at 37 °C (a and b). Bottom row: cells incubated with **TP1** (10.0 μM) for 30 min at 37 °C (c and d). (Adapted with permission from ref. 76 copyright 2010, The Royal Society of Chemistry).

In 2014, Yang *et al.* developed a novel probe, **NA1**, to detect sarin *via* a Vilsmeier–Haack reaction employing the covalent assembly strategy (Fig. 14A).[3] The highly electron deficient phosphorus centers of the nerve agents, their mimics, and precursors were capable of activating the 4-diethylaminobenzaldehyde in **NA1** toward nucleophilic attack. After the reaction, the probe emitted strong yellow-orange fluorescence, thus realizing the detection of the sarin. Overall, this is the first case of using the covalent assembly strategy to prepare a small molecule fluorescent probe. Yang's work combined the advantages of the proposed covalent-assembly principle and existing chemistry for the recognition of a nerve agent. Subsequently, the application of the covalent assembly fluorescence response strategy in the sensing field was further expanded. In 2016, Goswami *et al.* reported a probe, **NA2**, which could detect F^- and the nerve-agent mimic diethyl cyano-phosphonate in mixed aqueous media (Fig. 14A).[86] This probe could discriminate diethyl cyanophosphonate (DCNP) and diethyl chlorophosphate (DCP) *via* cyclization-induced fluorescence enhancement. After the addition of fluoride, the probe **NA2** formed a green fluorescent intermediate, **NA2F**, through a specific intramolecular cyclization reaction triggered by the affinity of fluoride to silicon. Subsequently, in the presence of DCNP, the lone pair electrons on the imino group of **NA2F** first attacks DCNP to release CN^-, which attacks the double bond of the imino-coumarin moiety. Accordingly, the six-membered ring product is formed by the resonance of the imino-coumarin to the benzothiazole nitrogen through the ICT mechanism, which also has strong fluorescent on a different channel to distinguish F^- and DNCP. In contrast, in the presence of DCP, the lone pair electron attack of the imino nitrogen of the intermediate **NA2F** caused a slight fluorescence enhancement, thus achieving the effect of distinguishing DCNP from DCP. This probe was fabricated on test strips, making the test more rapid and convenient. Moreover, the unique "off–on–on" fluorescence response mechanism also provides a reference for the specific detection of fluoride based on the covalent assembly mechanism.

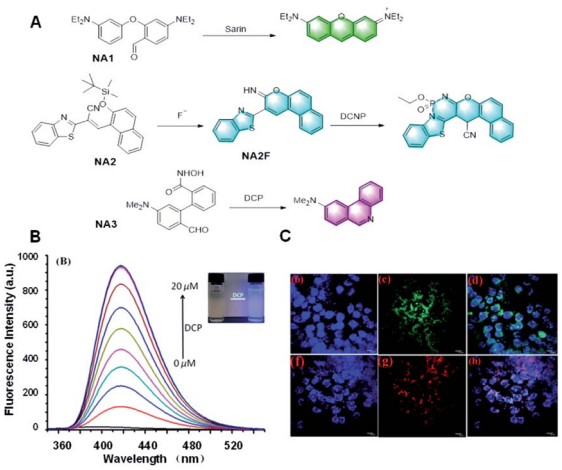

Fig. 14 (A) Response mechanism of the probes NA1/2/3 to nerve-agents. (B) Fluorescence responses of probe NA3 toward DCP and (C) fluorescent images of DCP in live cells. (Adapted with permission from ref. 87 copyright 2019, the American Chemical Society).

In addition, Yang *et al.* reported a probe, **NA3**, for the detection of DCP based on the Lossen rearrangement (Fig. 14A).[87] The hydroxamic acid group in probe **NA3** was combined with DCP, and the newly formed phosphoryl intermediate was rapidly rearranged to provide the corresponding isocyanate, which tended to further react with water to release an NH_2 group. This probe had many advantages, such as low detection limit (10.4 nM), sensitive response (100 s), and outstanding linearity ($R^2 = 0.9993$) in the range of 2 to 16 μM. More importantly, the successful detection of DCP in the gas state in this paper also proved the practicability of the probe. It is believed that this probe will be a useful tool for sensing nerve agents.

2.4 Probes for ROS/RNS

2.4.1 Probe for HOCl. Hypochlorous acid (HOCl) is an essential biological reactive oxygen species. It plays a bactericidal role in the immune system. However, the uncontrolled production of OCl^- can lead to some undesirable effects, such as neuronal degeneration, arthritis, and cancer. Therefore, it is necessary to quantitatively detect OCl^- in biological samples and allow the imaging of OCl^- through simple pretreatment and cheap instruments *in vivo*.

The "light-up" probe **CL1** for the detection of ClO^- based on 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) oxidation was reported in 2019 for the first time (Fig. 15A).[88] During the reaction process, the primary alcohols in the probe **CL1** are first oxidized to aldehydes by the active intermediates generated by ClO^- and TEMPO. The new generated formyl groups are captured swiftly by the adjacent NH_2 groups initially, resulting in the spontaneous formation of phenanthridine compounds with strong fluorescence emission. Further research showed that this probe had excellent performance, such as fast response (within 2 min), low detection limit (63.7 nM), and good linearity ($R^2 = 0.9863$). It was also applied for the analysis of everyday samples, such as swimming pool water and tap water. In general, it is a useful strategy for the special detection of ClO^-.

2.4.2 Probes for H_2O_2. Playing critical roles in several physiological and pathological processes, hydrogen peroxide (H_2O_2) is one of the most important reactive oxygen species (ROS). Thus, the qualitative and quantitative detection of H_2O_2 is significant because abnormal levels of H_2O_2 are closely related to many diseases, such as disorders, neurodegenerative diseases, cancers, aging, and diabetes.

In recent years, numerous fluorescent probes have been reported for the detection of H_2O_2. The use of an aromatic boronic ester as a detecting unit for H_2O_2 has been widely studied for a long time.[89] In 2012, Phillips *et al.* designed several fluorescent H_2O_2 probes, in which the response mechanism was based on covalent assembly to form imino-coumarin derivatives.[90] The initial results demonstrated that appropriate reagent design enabled rapid down-selection before quantitative fluorescence assays were conducted. This strategy should facilitate the time-consuming process of performing quantitative assays in resource-limited environments.

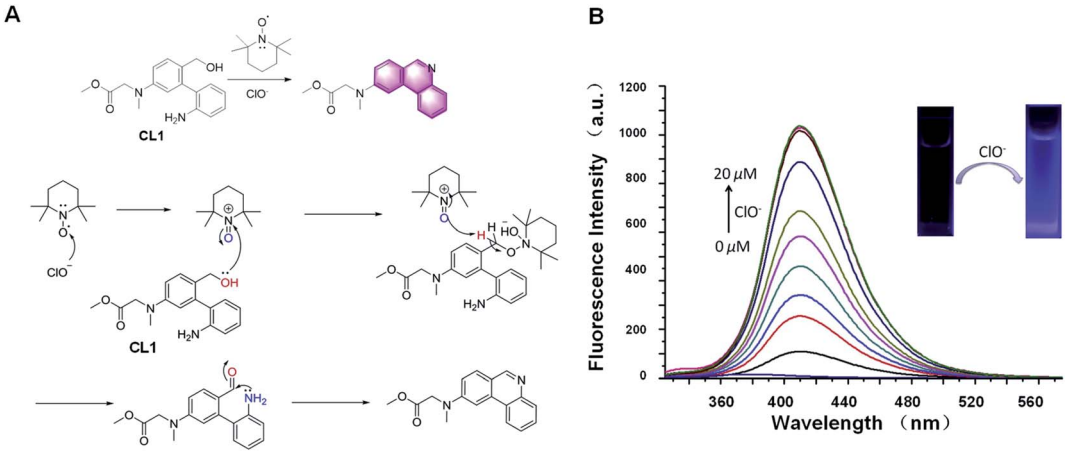

Fig. 15 (A) Response mechanism of the probe **CL1** to HOCl. (B) Proposed mechanism for the reaction of **CL1** with HOCl. (B) Fluorescence responses of probe **CL1** toward HOCl (adapted with permission from ref. 88 copyright 2017, The Royal Society of Chemistry).

Aromatic boronic esters are the preferred scaffolds for designing fluorescent probes with fluorophores characterized by the red/near-infrared emission and large Stokes shift. This is because long wavelength light can penetrate deep into tissues under low background interference, while a large Stokes shift can minimize self-absorption to improve the detection sensitivity. However, to date, fluorescent probes capable of H_2O_2 detection with both a red/near-infrared emission and a large Stokes shifts are rare. To better visualize H_2O_2 detection for bioimaging, in 2018, Song *et al.* showed a highly sensitive and selective fluorescence probe for the detection of H_2O_2 based on the same mechanism (Fig. 16).[91] During the detection process, Probe **1** was non-fluorescent initially. Once treated with H_2O_2, H_2O_2 caused the cleavage of the boronic ester moiety and the quinone derivative would leave quickly, subsequently resulting in an interference ability in aqueous media, which can also be applied to detect intracellular H_2O_2 in HeLa cells.

2.4.3 Probes for peroxynitrite ($OONO^-$). Peroxynitrite ($OONO^-$) is produced by the near diffusion control combination of nitric oxide (NO) and the superoxide anion radical. It mainly mediates the obvious cytotoxicity of NO, and can also induce lipid peroxidation and DNA damage. Thus, methods to detect cellular $OONO^-$ levels are of practical significance for both pathophysiology and disease diagnosis.

In 2012, Yang *et al.* first reported a peroxynitrite probe based on a green-emitting coumarin derivative (Fig. 17A).[92] The amino

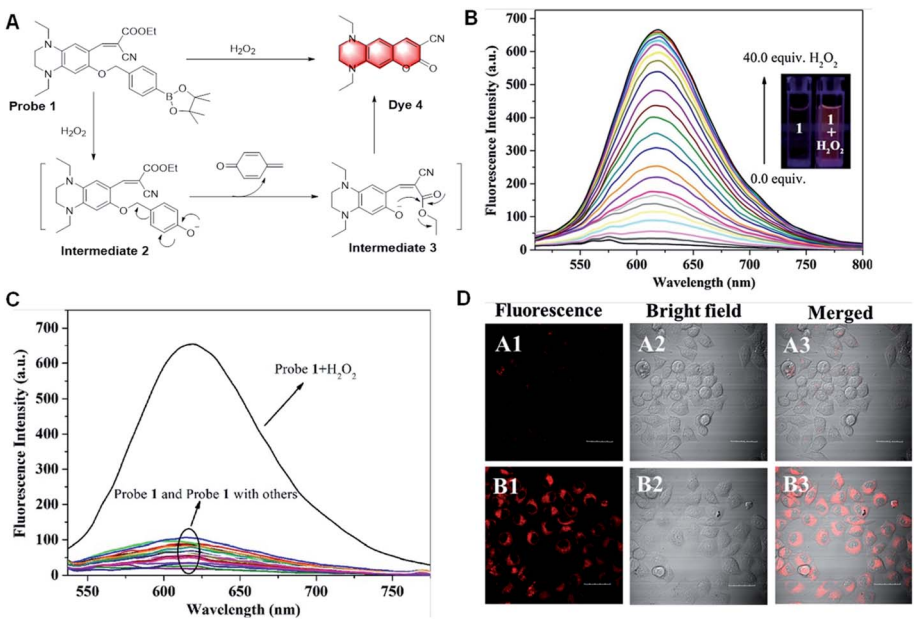

Fig. 16 (A) Proposed response mechanism and (B) fluorescence responses of probe **1** toward H_2O_2. (C) Fluorescence response of probe 1 toward relevant testing species. (D) Images of HeLa cell. A1–A3: cells incubated with probe **1**. B1–B3: cells pre-treated with H_2O_2, and subsequently incubated with probe **1**. (Adapted with permission from ref. 91 copyright 2018, Elsevier).

group on the coumarin moiety was oxidized by peroxynitrite, which transformed the molecule into a red-emitting resorufin derivative *via* an orange-emitting intermediate by expanding the conjugated structure. This probe showed three-channel signals, which could distinguish peroxynitrite from other ROS and RNS. Moreover, because of its membrane permeability and low cytotoxicity, it could also be utilized in a cell imaging (Fig. 17B) intramolecular cyclization reaction to form a dye emitting intense fluorescence. The probe had high selectivity and anti-interference ability in aqueous media, which could also be applied to detect intracellular H_2O_2 in HeLa cells.

Compared with H_2O_2, aromatic boronic esters react with peroxynitrite nearly a million times faster.[93] Consequently, a series of probes containing aromatic boronic esters have been developed to detect peroxynitrite in recent years.[94–98] The hydroxyl group obtained by the cleavage of aromatic boronic esters reacts with the cyano group. Finally, these probes form imino-coumarin or coumarin derivatives with strong fluorescence by cyclization (Fig. 17C). These probes have many advantages, such as fast response, excellent selectivity, and sensitivity, and they were used to detect peroxynitrite in cell imaging. However, all these probes displayed low hydrophilicity and the test solvent contained 10% or more organic solvent, such as ethanol, acetonitrile, or DMSO. Thus, there are still some limitations in the application of this type of probe, and consequently more research is needed to solve these problems for its further application.

2.4.4 Probes for NO. Nitric oxide (NO) is a small molecule uncharged free radical, which plays a critical role in cardiovascular, neurological, immune and other physiological and pathological processes. Thus, several methods have been developed for the detection of NO to investigate its multi-roles in the fields of physiology and pathophysiology *in vivo*.

In the early years, Nagano *et al.* first reported a diaminofluorescein as an NO indicator.[99] Different form the traditional structure of fluorescein, amino fluorescein shows quenched fluorescence due to the strong electron donor group of amino. Upon interaction with NO, the amino group in the probe is transformed into a relatively weak amide electron donor group, its electronic structure changes and the fluorescence recovers, thus realizing the detection of NO. It is worth mentioning that the triazole product has strong green fluorescence, with the fluorescence quantum yield increasing by more than 100 times. It also had a low detection limit of 5 nM. Many groups followed this mechanism for the design of other probes by changing the fluorophore to achieve better optical performances or modifying the structure to optimize the performance.[100–107] However, this type of probe still has a limitation, that is, the probe will be disturbed by endogenous *o*-dicarbonyl compounds such as deoxyascorbic acid and methylglyoxal, which will distort the probe signal.

In 2010, Anslyn *et al.* reported an NO fluorescent probe, **NO1**, based on the formation of a diazo ring (Fig. 18A).[108] The electrophilic aromatic substitution on the electron-deficient nitrosamine yielded a hydroxyhydrazine derivative. Elimination of a molecule of water led to the formation of a diazo ring by covalent assembly. The expanded conjugation of the molecule led to a substantial increase in fluorescence. It also possessed many advantages, such as rapid and linear response, excellent selectivity, and low pH dependence.

Subsequently, they developed a series of probes based on the same mechanism. In 2020, Yang *et al.* summarized the probes

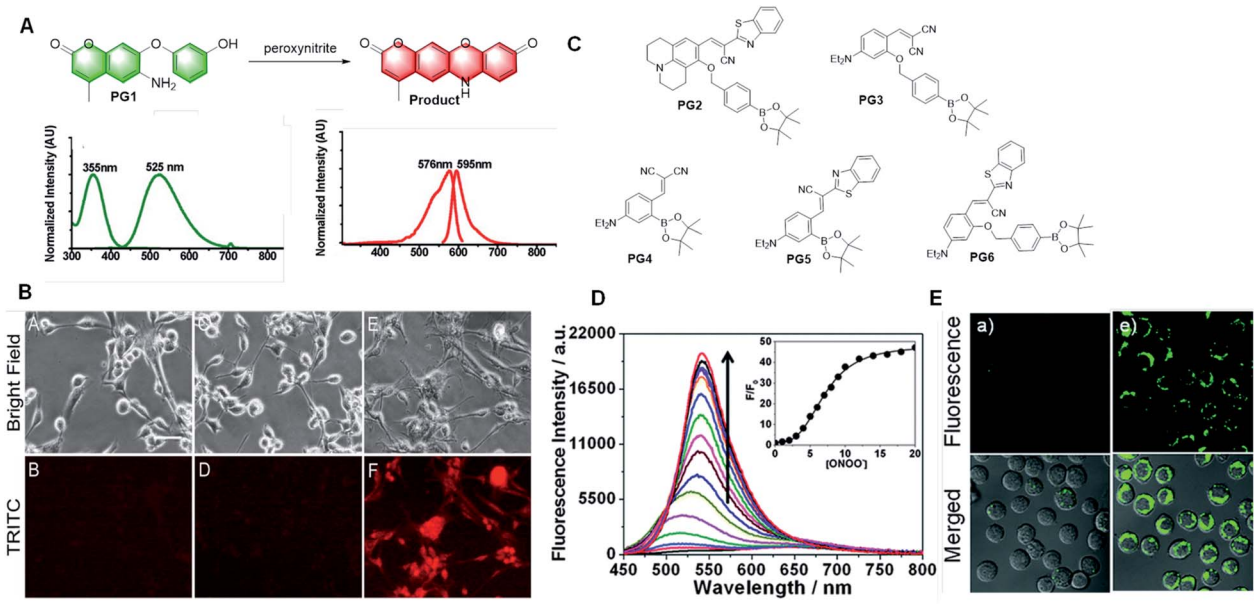

Fig. 17 (A) Response and absorption and emission spectra of peroxynitrite probe **PG1** based on a green-emitting coumarin derivative to peroxynitrite. (B) Fluorescent images of this probe for peroxynitrite imaging in living cells. (C) Structures of the probes based on aromatic boronic ester to peroxynitrite. (D) Fluorescence response of **PG2** toward relevant testing species. (E) Fluorescent images of the probe **PG2** for peroxynitrite imaging in living cells. (Adapted with permission from ref. 92 copyright 2018, Elsevier).

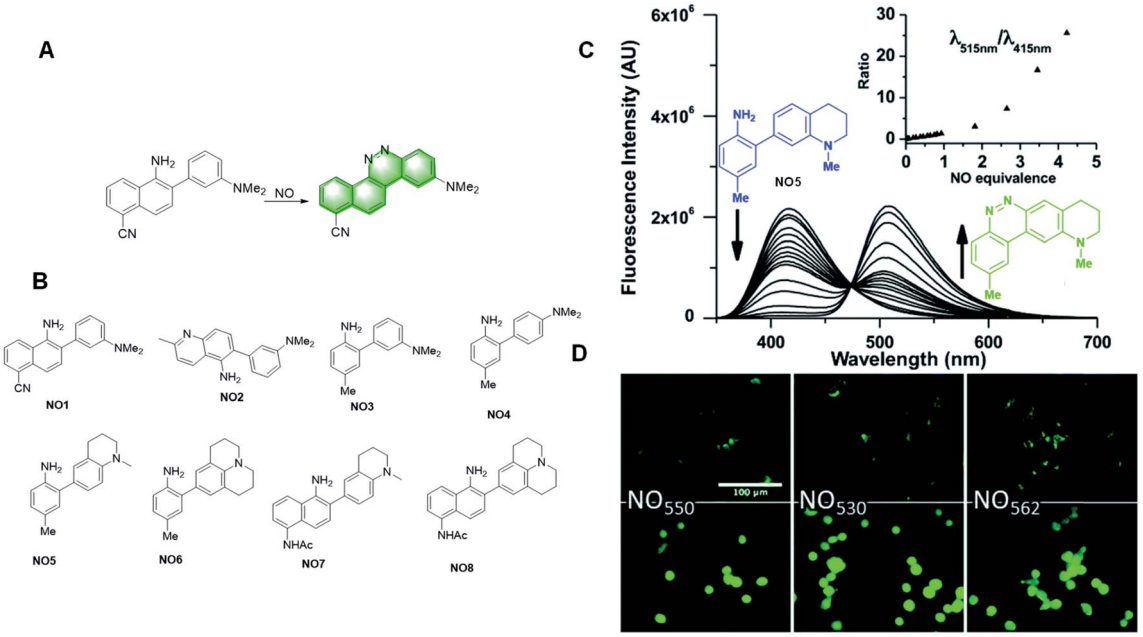

Fig. 18 (A) Response mechanism of probe **NO1** to NO. (B) Structures of the covalent assembly probes reported for NO detection. (C) Fluorescence responses of probe **NO5**. (D) 40× magnification, pseudo-colored FITC-filtered images of NIH-3T3 cells without NO dosing (B), and with 320 mM NO added (C) for **NO5 (NO550)**, **NO7 (NO530)**, and **NO8 (NO562)**. (Adapted with permission from ref. 109 copyright 2018, Elsevier).

they studied for the quantitative detection of NO. They developed probes with various structures based on the design idea of covalent assembly (Fig. 18B) and selected the probes more suitable for clinical detection by comparing their performances (Fig. 18B).[109] Among the probes, **NO7** acts as a promising material for live cell imaging. Due to its greater response to stimuli, higher selectivity for NO, and the ability to image both the reacted and unreacted probe, **NO7** provides an attractive option as a nitric oxide probe and a potentially valuable alternative to the commercial dye DAF-FM in various applications.

After analysing the capacity of the probes they studied, they found that attaching electron-donating groups, primarily dialkyl amines, on the nucleophilic aryl group, and avoiding the conjugation of electron-withdrawing groups to the 2-amino group, render the probes sufficiently nucleophilic to readily scavenge NO in cellular media. Furthermore, fusion of the aminoalkyl groups to the nucleophilic aryl group increased both the fluorescence wavelength and the fluorescence quantum yield of the cinnoline product.

2.4.5 Probe for oxidative radicals. Because of their high corrosiveness, radicals are utilized by the immune system as a host defense mechanism. However, radical species may cause unwanted damage to host cells, and further research has revealed that the radicals under control may also affect the development of various degenerative diseases in a negative way, such as Alzheimer's diseases, autoimmune diseases, coronary heart disease, and cancers. Thus, it is necessary to develop sensitive and selective probes for radicals.

In 2017, Yang *et al.* reported a probe, **OR570**, based on the covalent assembly principle, which could detect oxidative radicals (Fig. 19).[110] The methine linked with secondary benzylic is the hydrogen atom donor. The presence of the ethoxy group keeps the free radicals stable, thus enhancing the performance of the probe. The main advantage of the assembled probe is the zero-background fluorescence signal, which makes this probe capable of detecting analytes at a trace level with high sensitivity. However, due to its low hydrophilicity, it can only be used in organic solvents.

Moreover, the main structure of the probe reported by Yang Y. in this paper is the same as that of the probe previously reported for neurotransmitters and mercury ions, and the reaction mechanism is similar. Both of them follow the mechanism of covalent assembly. Once they are activated with the participation of the analytical substrate, intramolecular electrophilic aromatic substitution occurs, and then eliminated to obtain the xanthene dye with strong fluorescence. This means that the main structure of the probe has a good prospect, and it has unlimited possibilities in the detection of other specific substances.

2.5 Probes for enzymes

2.5.1 Probes for alkaline phosphatase (ALP). Alkaline phosphatase (ALP), a dimeric metalloprotein containing zinc, plays a vital role in the hydrolysis and transphosphorylation of a wide variety of monophosphate esters.[111,112] It is a type of enzyme that exists in human tissues and organs. The level of ALP is linked with many diseases such as biliary obstruction, leukemia response, osteoblastic bone tumors, osteomalacia, and diabetes. Phosphate has been used as the recognition group in small molecular probes to detect ALP for a long time.[113]

However, due to the high background fluorescence and detection error of water-soluble hydrolysates secreted by cells, the performance of these probes is restricted. At present, the commercial ELF-97 probe produces water-insoluble hydrolysate

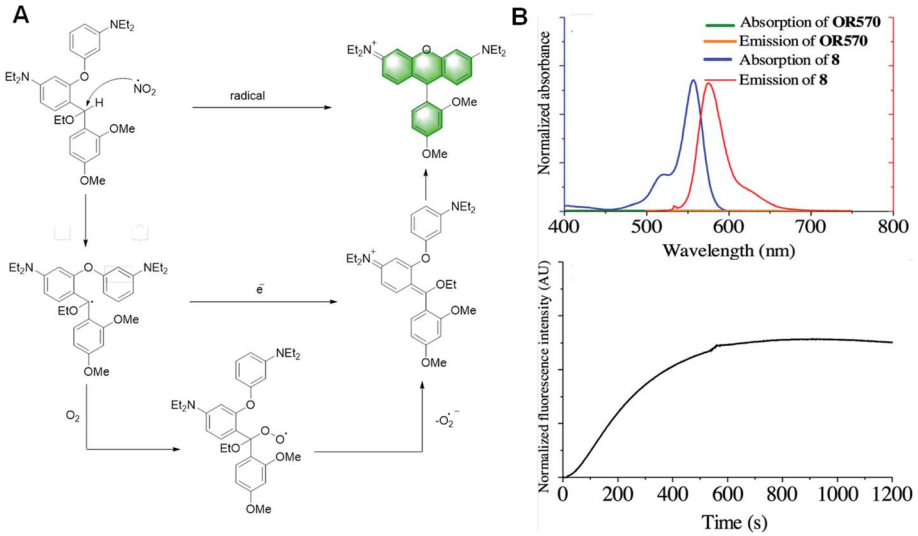

Fig. 19 (A) Tentative detection mechanism of **OR570** toward highly oxidative radicals. (B) (Top) absorption (black) and emission (red) spectra of **OR570** and compound 8. (Bottom) kinetic curve of the formation of **OR570** upon the addition of oxidative radicals. (Adapted with permission from ref. 110 copyright 2019, The Royal Society of Chemistry).

after enzymatic hydrolysis. However, under the irradiation of a 488 nm long wavelength Ar ion laser, the excitation effect of the probe is not good, which is the excitation wavelength commonly used by confocal laser scanning microscopes and flow cytometry. Therefore, new fluorescent probes are needed for the detection of ALP, which must be compatible with physiological conditions, have a high signal-to-noise ratio and can be excited on the 488 nm line of the AR laser.

Early on, Kim *et al.* reported a strategy for the detection of ALP using covalent assembly probes containing an imino-coumarin benzothiazole structure (Fig. 20).[114] The probes **ALP-1** and **ALP-2** reacted with ALP to make the phosphate leave and generate hydroxyl. Combined with the cyano group, the free hydroxyl group can form an imino-coumarin structure with strong fluorescence. They were also applied for imaging endogenous ALP activity in living cells.

Two years later, they introduced a benzyl group between the fluorophore and the phosphate to improve the probes, resulting in **ALP-3** with a faster response, and achieved real-time quantitative analysis (Fig. 20).[115] In addition, the potential utility of the probe in the screening of ALP inhibitors was demonstrated with levamisole. This work demonstrated the utility of self-immolated linkers in the design of fluorogenic substrates for enzymes possessing comparatively difficult access to the active catalytic site, which opens the possibility to improve existing fluorogenic substrates for various enzymes.

2.5.2 Probes for monoamine oxidase (MAOs). Monoamine oxidase (MAOs) is a flavin adenine dinucleotide-dependent

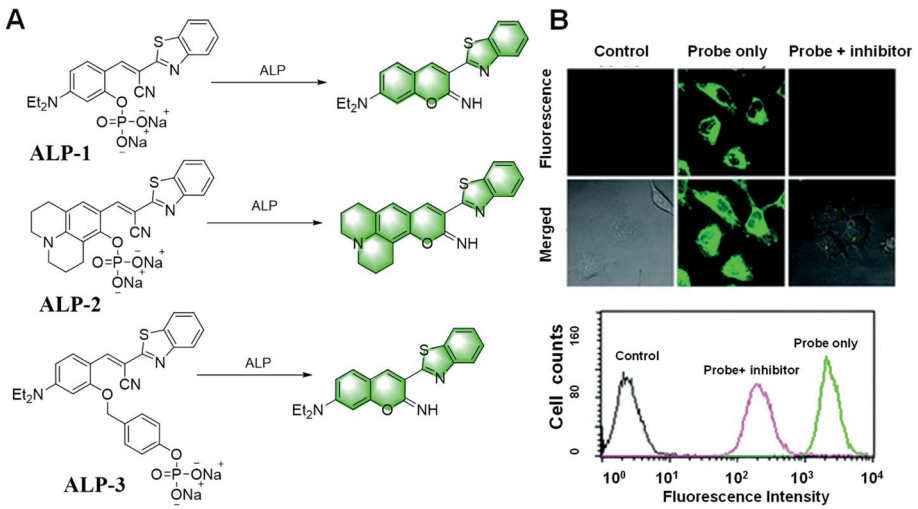

Fig. 20 (A) Illustration of the iminocoumarin-benzothiazole-based probes **ALP-1/2/3** for the detection of ALP. (B) Representative confocal fluorescence images of HeLa cells incubated with probe **ALP-1** for 2 min. (Adapted with permission from ref. 114 copyright 2011, The Royal Society of Chemistry).

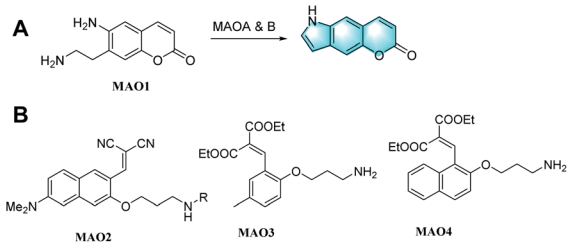

Fig. 21 Structures of the representative probes for the detection of MAOs.

enzyme distributed on the outer membrane of mitochondria. It has two subtypes of MAO-A and MAO-B. Its working mechanism is mainly through catalytic amine substrate, aerobic oxidation to corresponding imines, and then hydrolysis to aldehyde products. MAO plays a key role in the central nervous system. It is usually used to regulate the steady-state environment of neurotransmitters such as dopamine and serotonin. The concentration level of MAOs is closely related to a variety of diseases, such as Parkinson's disease, depression, and Alzheimer's disease.

In the early years, Sames *et al.* reported a probe, **MAO1**, based on intramolecular cyclization to generate an indole derivative by covalent assembly.[116] As shown in Fig. 21, enzymatic oxidation of the ethyl-amino group in probe **MAO1** affords an aldehyde intermediate, which subsequently undergoes spontaneous intramolecular condensation with the aniline amino group, furnishing an indole moiety in an irreversible fashion. This overall chemical process results in a profound electronic change in the system, which in turn should alter its fluorescence profile. In 2012, Kim *et al.* reported a two-photon absorption probe, **MAO2**, based on imino-coumarin derivatives (Fig. 21).[117] The aminopropyl and *N*-methylaminopropyl as the reactive groups could transform into iminium ions by catalysis with MAOs. The probe following the reaction of hydrolysis and b-elimination generated a hydroxyl group, which could condense with one of the nitrile groups to produce the final product with two-photon fluorescence. In 2019, Li *et al.* reported novel probes containing amino-propyl as the recognition group, which can detect MAO-B.[118] They chose coumarin as the dye group, while decorating the 3-aminopropoxy group for the detection of MAO-B. Once the enzymatic oxidation by MAO-B was triggered, intramolecular cyclization occurred and the fluorescence signal of coumarin was turned on. To date, these two probes, **MAO3** and **MAO4**, not only have excellent selectivity, which can distinguish MAO-B from MAO-A concisely (Fig. 22B), but also have a low detection limit and can be applied in human astrocyte (U87) imaging. Consequently, MAO-B can be selectively detected by the tandem reaction probe at a reasonable pH value (pH = 7.4) and mild temperature (37 °C). The signal enhancement in these two probes was obvious, showing the selectivity between MAO-B and MAO-A. Importantly, the novel design strategy of establishing fluorophores by targeting enzymes in the reaction process is significant in the future.

2.5.3 Probes for nitroreductase (NTR). Nitroreductase (NTR) is a flavin or flavin adenine dinucleotide-containing enzyme working with the co-enzyme nicotinamide adenine dinucleotide (NADPH), which can reduce nitro-aromatic or nitro-heterocyclic derivatives to the corresponding amines. The excessive expression of NTR is related to cellular reductive stress by hypoxia. Inducing reductive stress is a necessary feature of many diseases, such as inflammatory diseases, cardiac ischemia, and solid tumors. In a solid tumor, the NTR level is directly linked to the degree of hypoxia. Due to their high sensitivity, non-invasiveness, low cytotoxicity and the ability for real-time spatial imaging, optical imaging of fluorescent probes

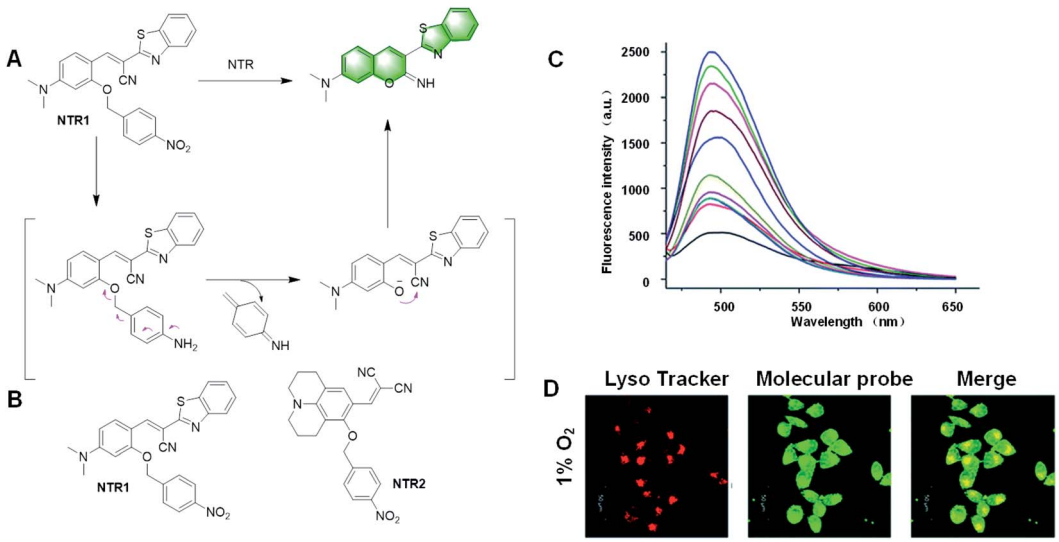

Fig. 22 (A) Response mechanism of the probe **NTR1** to NTR. (B) Structures of the covalent assembly probes reported for the detection of NTR. (C) Fluorescence response of **NTR1** toward different concentrations of NTR. (D) Confocal fluorescence images of MGC-803 cells treated with the probe under 1% O_2 conditions for 12 h. (Adapted with permission from ref. 119 copyright 2018, Elsevier).

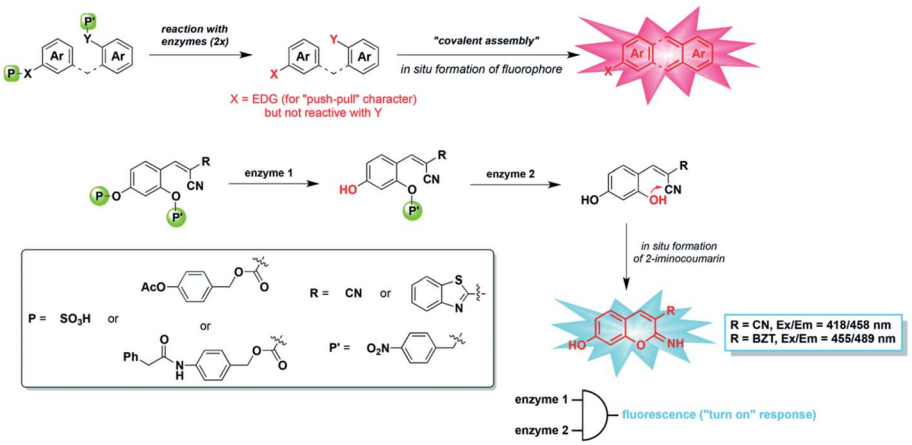

Fig. 23 Dual-enzyme sensing strategy explored in this work: (top) "covalent assembly" principle applied to the simultaneous detection of two distinct enzymes (X = NH or O, EDG = electron-donating group) and (bottom) *in situ* synthesis of 3-substituted-7-hydroxy-2-iminocoumarin scaffolds from a non-fluorescent caged precursor and through domino reactions triggered by the two different enzymes. (Adapted with permission from ref. 125 copyright 2015, The Royal Society of Chemistry).

based on NTR-triggered nitro-reduction reactions have been exploited and made great contribution to understanding the biological roles of NTRs in oncology.

In 2018, Gu *et al.* described a probe, **NTR1**, based on iminocoumarin derivatives, which could detect NTR in the presence of NADH.[119] The 4-nitrobenzyl is the reactive group that can leave by NTR catalysis (Fig. 22A). The intramolecular nucleophilic attack of the phenolic oxygen to the nitrile group resulted in the cyclized imino-coumarin-benzothiazole. This probe had a fast fluorescence response in the presence of NADH within only 20 min. **NTR1** showed high sensitivity and good selectivity to NTR and a LOD of 11 ng mL^{-1}. It could be applied in the imaging of endogenous NTR in HepG2 cells and tumor tissues under hypoxia conditions. Thus, **NTR1** has great potential to monitor NTR in the biological system (Fig. 22C and D).

In recent years, many fluorescent probes for detecting NTR have been developed, but most of them can only achieve single photon excitation, causing great damage to cells and poor permeability. In 2019, Liu *et al.* realized a two-photon fluorescent probe, **NTR2**, for the detection of NTR under hypoxic condition.[120] The julolidine structure, instead of the iminocoumarin derivative, had two-photon activity (Fig. 23A). The reaction mechanism is as follows: under anoxic condition, nitrating enzyme reduces the nitro group to the corresponding amino group, and then releases iminocoumarin. The NTR detection limit of the probe was 48 ng mL^{-1}. This probe could be applied to monitor the hypoxic status of MGC-803 cells, MCF-7 cells, and 3D tumour spheres by detecting endogenous NTR.

2.5.4 Probes for penicillin G acylase (PGA). As a non-specific acylase, penicillin G acylase (PGA) is an intracellular enzyme with low strict requirements for substrate transfer, which only preferentially hydrolyzes penicillin G. The hydrolysis conditions of PGA are mild, and the reaction can be completed without the harsh conditions of high temperature and high pressure or the addition of toxic reagents. Therefore, PGA is widely used in the preparation of semi-synthetic penicillin antibiotic intermediates and is popular in the field of pharmacy. Furthermore, as a catalyst capable of catalyzing penicillin G potassium (PG) to 6-aminopenicillanic acid (6-APA), PGA plays an essential role in the pharmaceutical field. Hence, PGA has always been a focus.

In recent years, characterized by the simple structure and low cost of PGA, a series of probes was reported with the development of the design principle of the "covalent assembly" based on PGA probe examples.[121–124] Recently, it was found that protease (*i.e.*, PGA and ALP) can initiate biocompatible cyclization/aromatization reaction from the mixed cage precursor of diarylether and construct anthracene-based fluorophores. The principal mechanisms of activation are based on the pyronin assembly triggered by the enzyme itself.

However, from a kinetic point of view, there is a problem that needs to be overcome, where the formation of pyronin takes over 10 h, which is too slow to achieve a significant fluorescence level for considering the implementation of this "covalent assembly" probe in diagnostic bioassays or *in vivo* fluorescence imaging of disease-related enzymes.

To realize a more suitable probe capable of detecting PGA, Romieu *et al.* reported a dual-reactive probe for the detection of PGA or pig liver esterase (PLE) as well as NTR simultaneously by covalent assembly (Fig. 23).[125] The probe was activated *via* the hydrolysis of an amide (or ester) bond and reduction of a nitro group, followed by domino 1,6-elimination/cyclization to produce 7-hydroxy-(2-imino) coumarin with strong fluorescence. Combined with the reaction/quenching groups of two different analytes, the dual active coumarin precursor in this work can be easily decorated to form a "double-lock" structure, where a dramatic fluorescence signal only occurs when the two analytes exist simultaneously. It is well known that the overproduction of a specific analyte may be more than one pathological feature, and there may be false-positive signals in healthy tissues. Thus, the concept of 'double-gating' structure enables the analysis of pathology more accurately by detecting

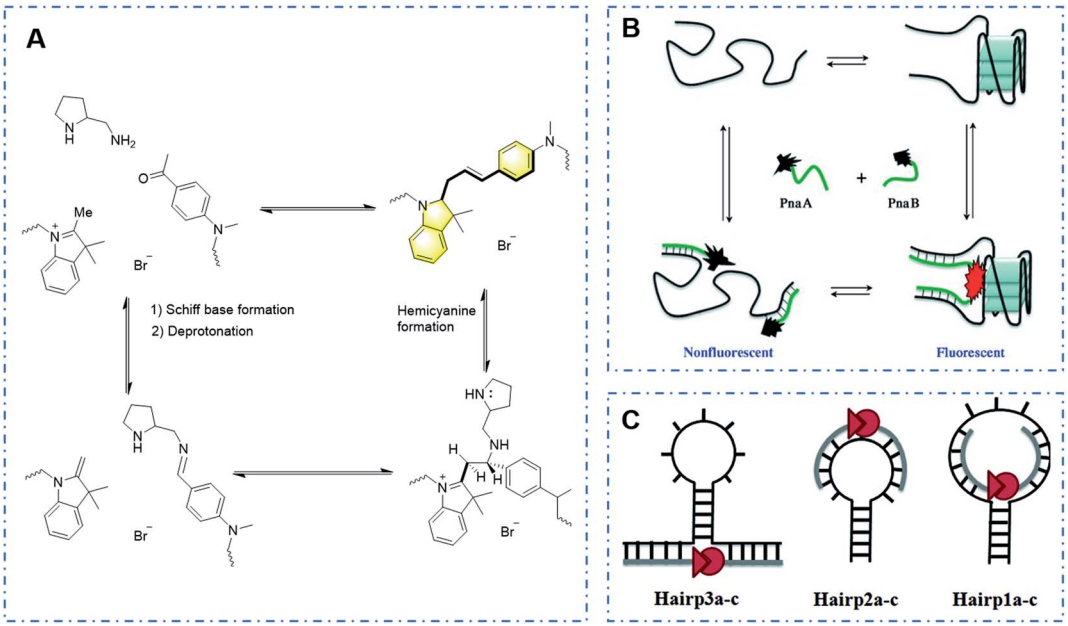

Fig. 24 (A) Response mechanism of a covalent assembly probe to DNA. (B) Novel strategy to examine the G-quadruplex structure *via* covalent assembly and (C) different ways of combination with hairpin structures to realize the detection of hairpin structures. (Adapted with permission from ref. 129 and 130 copyright 2010, the American Chemical Society, copyright 2010, Wiley).

another characteristic analyte.[126] Romieu's work provided a good strategy for the preparation of high-precision probes, especially those related to effectively distinguishing molecules with similar chemical reactivity (such as biothiols).

2.6 Probes for DNA

Nucleic acids play an important role in physiological genetics, and thus are a research hotspot. The traditional DNA double helix structure is the most abundant DNA structure in the human body. However, with the discovery from research and the progress of physiological regulation, DNA also contains other secondary structures. Among them, single chain hairpin structure, G-quadruplex structure, double chain cross structure and bimolecular G-quadruplex structure all play an important role in transcriptional regulation mechanism, but their specific physiological mechanism still needs to be studied. Therefore, it is necessary to further understand DNA sequence detection and the physiological mechanism of the DNA structure.

In recent years, with the development of small molecule fluorescent probes, many DNA fluorescent probes have been developed. Among them, a new covalent assembly method is attractive. This method uses DNA/RNA template strand recognition to shorten the distance with the substrate and induce the covalent assembly of the substrate to form dyes, effectively improving the effective concentration and reaction rate and realizing the detection of specific sequence of DNA/RNA fragments.

Initially recognized by Huang *et al.* in 2008 for its application, the DNA-template reaction of trimethylindole catalyzed by diamine with benzaldehyde was developed to finally produce hemicyanine-like dyes *via* the formation of a Schiff base, methylene indoline, proton transfer and carbon–carbon bond coupling.[127] As shown in Fig. 24A, through the DNA-template reaction, probe **DNA1** was studied and its spectroscopic performance (λ_{ex} = 550 nm and λ_{em} = 590 nm) showed its potential application in the detection of DNA.

Subsequently, Ladame's research group used the template chain reaction strategy to develop a series of high-performance probes for detecting the DNA sequence and DNA secondary structures.[128–130] This strategy makes use of the characteristics of "DNA sequence + structure". Through sequence combination, the DNA chain structure changes, and thus two parts of the dye structures connected at the head and end can be covalently assembled to form a hemicyanine dye due to structural changes, achieving the detection effect (Fig. 24B and C). Unlike other conventional DNA template reactions, its system has the following significant advantages: (i) its emission spectrum reaches the near-infrared window, and its excellent performance of strong permeability and high resolution makes it suitable for *in vivo* imaging and (ii) the template reaction does not require a heavy metal catalyst and has good biocompatibility.

It should be noted that this method is similar to the covalent assembly strategy mentioned above. Also, it is believed that this novel strategy can inspire us in the development of small molecular probes to overcome the problems shown in the previous works and design more prominent probes for application in daily life.

3. Conclusion

This review summarized the small molecular fluorescent probes based on covalent assembly for detecting and imaging various

ions (anions and cations), small biological molecules, reactive oxygen species (ROS), reactive nitrogen species (RNS), nerve agent mimics, enzymes and DNA. Compared with the traditional small molecular probes, the detection product exhibits a strong signal with pronounced red-shift of the absorption and emission bands; simultaneously, it also has the same advantages as the small molecular fluorescent probe, such as easy preparation and tunability. These probes generally contain common fluorophores such as coumarin, fluorescein, and naphthalene imide, which have the advantages of low cost, easy structural tuning, and excellent fluorescence performance. However, this approach also faces some limitations, such as a relatively narrow range of applicable substrates, which need to be further studied to meet the actual requirement.

Briefly, covalently assembled reactive probes are a type of probe materials that change the molecular structure of dyes to respond and produce changes in fluorescence signals. They induce the dye to assemble *in situ* into dye groups with strong fluorescence through the substrate. The specific methods involve: (1) decorating a push–pull electron group, (2) changing the conjugation degree of the push–pull electron backbone, and (3) changing the rigidity degree of the main chain.

Actually, since the concept of covalent assembly was proposed by Yang *et al.*, only a few covalent assembly reactive probes have been reported. Different from the general mechanism, *i.e.*, photo-induced electron transfer (PET) and fluorescence resonance energy transfer (FRET), covalent assembly probes involve the assembly of two dye fragments originally fixed by a linker into the corresponding dyes *in situ* in response to an analyte, ensuring that the fluorescence background of this type of reaction probe is very low, even up to zero background, while the other luminescence mechanisms mentioned above are to inhibit the self-fluorescence of the molecular probe through the modified group, and then react with the analyte to cause the departure of the group or increase the molecular spacing, and finally maintain the "on" state of the probe dye.

In recent years, many probes based on covalent assembly have been developed for better performance with two-photon fluorescence emission and near-infrared emission, which are more suitable for *in vivo* imaging with low background, high signal-to-noise ratio, and deep penetration depth. These probes tend to focus on detecting small biological molecules and enzymes, which are related to many diseases and physiological/pathological processes. Notably, increasing attention has been paid to explore novel mechanisms with the features of improved sensitivity, selectivity, low toxicity, water solubility, and biocompatibility. Thus, it is a bright future to develop and improve the performance of these probes in various systems.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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