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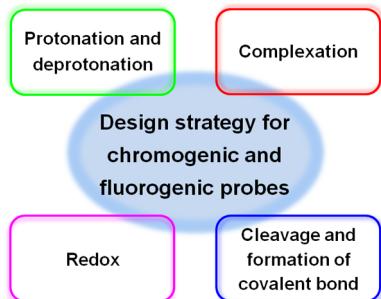
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Design Strategies for Water-Soluble Small Molecular Chromogenic and Fluorogenic Probes

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1. INTRODUCTION

Chromogenic and fluorogenic probes may be described as the reagents that can interact with analytes (targets) accompanied by the changes of their spectroscopic (chromogenic, or luminescent including chemiluminescent) properties; on the basis of such changes, the analytes can thus be determined.^{1,2} They have been extensively investigated and widely used in many fields because of their powerful ability to improve analytical sensitivity, and in particular to offer greater temporal and spatial sampling capability for *in vivo* imaging studies. Typically, chromogenic and fluorogenic probes are comprised of three different moieties: (1) spectroscopic or signaling moiety, whose properties should be changed upon reaction with the analyte of interest; (2) labeling or recognition moiety responsible for the selective reaction with analyte; and (3) a suitable spacer or linker that connects the two former moieties (sometimes the two moieties are integrated without any linker).^{1–4} So far, various small molecular chromogenic and fluorogenic probes with superior properties have been developed for different analytes.^{1,4–10} A close examination reveals that the analyte detection by the probes is usually achieved through one of the following reaction mechanisms: (1) protonation–deprotonation; (2) complexation (including

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direct complexation and competitive displacement complexation); (3) cleavage and formation of covalent bonds; and (4) redox reaction. In fact, these reactions have been extensively employed as design strategies for developing small molecular chromogenic and fluorogenic probes, which however have not been systematically summarized to our knowledge (although many reviews on small molecular chromogenic and fluorogenic probes have been reported, they mainly focus on either diverse fluorochromes^{11–13} or analytes^{5–9}). In this Review, we will make such an effort to comprehensively review the progress of these design strategies in developing a variety of water-soluble small molecular chromogenic and fluorogenic probes that are of good applicability to biosystems, and it is our wish that this Review may provide help to those who are interested in the continually growing research field.

Note that different terms such as indicators, sensors, chemodosimeters, chemosensors, and dyes can be seen on this topic in the literature, among which chemosensors and chemodosimeters feature a reversible and irreversible spectroscopic response, respectively. In this Review, these terms may be unified as probe(s) according to the above description. Moreover, it should be pointed out that the cited literature sources in this Review are mainly from the last 10 years. For detailed information about the early but still widely used probes, the reader is directed to some excellent books on the subject.^{1,14–18}

2. CHROMOGENIC AND FLUOROGENIC PROBES BASED ON PROTONATION–DEPROTONATION

Protonation–deprotonation reactions are usually used to develop chromogenic and fluorogenic pH probes. In general, pH probes provide one with accurate pH determinations over a range of only about two pH units, that is, $pK_a \pm 1$, which however can meet the requirement for normal biosystems because of their rather small pH variation. Among the existing pH probes, most of them are designed for near neutral pH measurements, and only a small number of them are for extreme pH values (e.g., acidic stomachic or basic intestinal environments) by using $-\text{OH}$, $-\text{COOH}$, or an amino group as a pH-sensitive unit. Moreover, pH probes with linear response over a wide pH range are also rare, although they are needed for some special applications. To broaden the response range of pH, an effective strategy is to design a probe with multiple H^+ binding sites by assembling several electronegative atoms (e.g., N and/or O) in the different positions of a conjugated molecule.¹⁹

On the other hand, the typically used pH-sensitive groups, such as $-\text{COOH}$, $-\text{OH}$, and an amino group, also have a high affinity for common metal ions, thus resulting in complexation and nonspecific response of the probe to H^+ . To overcome this problem and increase the selectivity, the arrangement of electronegative atoms in the designed pH probe should not provide a suitable cavity or a convenient formation of five- and six-membered ring complexes for metal ions.

Currently, a large number of pH probes have been reported in the literature (some are commercially available¹), and a literature survey reveals that traditional fluorochromes, such as xanthene,^{20–23} boron dipyrromethene difluoride (BODIPY),¹² and cyanine,²⁴ are still the first choice to design pH probes for various purposes.^{1,25,26}

2.1. Xanthene-Based pH Probes

Xanthene-based pH probes mainly include fluorescein, seminaphthorhodafluor, and rhodamine derivatives.^{1,25–27} For cellular studies, fluorescein is one of the earliest used pH probes, whose fluorescence intensity is closely related to the change of pH (Figure 1): below pH 5 fluorescein exists as a

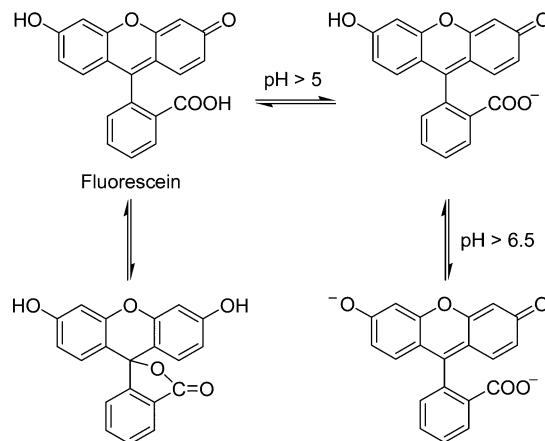
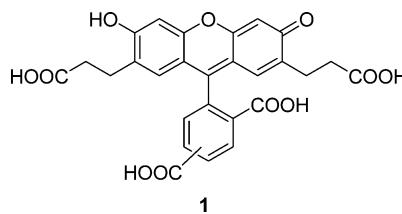


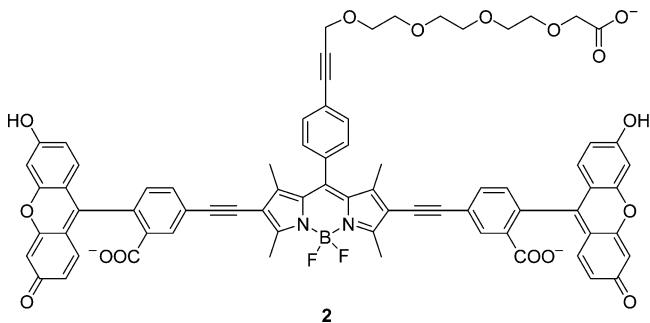
Figure 1. The protonation–deprotonation equilibria of fluorescein.

weakly fluorescent charge-neutral species and equilibrates with a nonfluorescent spirolactone isomer; between pH 5 and 6.5 its monoanionic (carboxylate) species with a quantum yield of 0.37 predominates; whereas at higher pH (>6.5) the equilibrium is shifted toward the highly emissive dianion with the quantum yield of 0.93.^{1,28,29} However, fluorescein suffers from the rapid leakage from cells.^{1,27} To solve this problem, in 1982 Tsien and co-workers developed a highly charged fluorescein derivative, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (**1**),³⁰ which is often used as a ratiometric fluorescent probe for measuring cytoplasmic pH because the excitation profile of the probe changes significantly with pH. Introduction of electron-withdrawing groups (e.g., Cl atom) into xanthenes lowers pK_a values,^{25–27} and the as-obtained chlorinated fluoresceins are suited for detection of lower pH values, such as acidic cells.^{31–33}

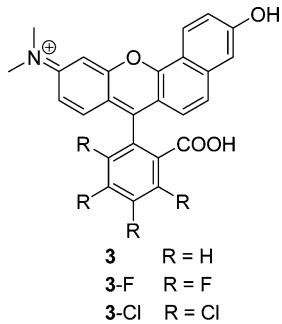


Several fluorescein derivatives containing a BODIPY core have also been designed as ratiometric pH probes. These probes work with a TBET (through-bond energy transfer) mechanism, resulting in a large Stokes shift.^{34–37} For instance, Burgess et al.³⁸ reported **2** as a pH probe, whose maximum emission is located at 600 nm in pH 4.0–6.5 and 525 nm in pH 7–8. Therefore, this probe can be used for ratiometric detection of pH, and such an application has been demonstrated by imaging pH change in living COS-7 cells.

Compound **3**, a seminaphthorhodafluor (SNARF) derivative, is also a very useful pH probe.^{1,27} In particular, its dual-emission properties allow a ratiometric pH measurement. However, **3** has a relatively high pK_a (~7.62), which limits its intracellular applications.²⁵ As in the case of fluorescein-based pH probes,



3's derivatives with fluorinated/chlorinated xanthene rings also have lower pK_a values.³⁹ For instance, the pK_a of 3-F is 7.38, which makes it more suitable for intracellular applications.



For rhodamine-based pH probes,^{40–44} their response mechanism is based on the structural change between spirolactam and open-ring forms. In near neutral media, rhodamine-based probes exist in a spirocyclic form, which is colorless and nonfluorescent; addition of H^+ leads to the spirolactam ring-opening, resulting in an appearance of pink color and fluorescence. These probes have pK_a values of about 4–6, and may be used for acidic organelles such as lysosomes and endosomes. Probe 4 is such an example (Figure 2),⁴⁴ which has been employed to quantitatively detect the chloroquine-induced increase in lysosomal pH and monitor changes in the acidity of lysosomes during apoptosis in live cells.

In a recent study, 9-aryl-9*H*-xanthen-9-ol has been reported as an interesting platform for the development of fluorescent pH probes 5–7.⁴⁵ These probes offer a number of advantages, such as high selectivity of spectroscopic response to pH, and facile adjustment of pK_a by introducing appropriate substituents (Figure 3). Furthermore, the conversion of neutral leuco-forms into xanthylum ions yields more than 1300-fold fluorescence enhancement. This large signal contrast is beneficial for sensitive monitoring of pH change. Among these probes, 7 with an apparent pK_a of 6.5 is suitable for biological applications, and its applicability has been demonstrated by imaging the change of pH in NIH-3T3 cells.

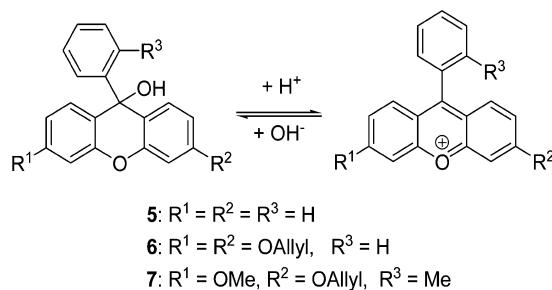


Figure 3. The protonation–deprotonation equilibria of 5–7.

2.2. BODIPY-Based pH Probes

The parent BODIPY has been adopted to make different pH probes.^{12,26,27} Actually, the high potential for BODIPY skeletons as building blocks in fluorescent probes was first demonstrated in pH probes,^{46–48} and has been followed by numerous examples of BODIPY-based probes for other analytes.¹² Up to now, many BODIPY derivatives^{49–55} bearing amino or hydroxyl groups (e.g., 8⁵⁵) have been reported as fluorescent probes for the detection of pH in aqueous and mixed aqueous–organic media. However, very few of them are suited for intracellular pH measurements. Compounds 9–12 are some exceptions, which are proposed as pH-activatable fluorescence probes for imaging acidic endosomes in cancer cells.⁵⁶ These probes are almost nonfluorescent in the nonprotonated form but become highly fluorescent when the aniline nitrogen is protonated. When the pH-triggerable fluorescent probes are conjugated to a cancer-targeting monoclonal antibody (trastuzumab) and incubated with cancer cells for 4 h, the probe–antibody conjugates accumulate in endosomes where the acidic pH makes them highly fluorescent. Therefore, probes 9–12 can potentially be used as a clinical tool for cancer detection and real-time monitoring of therapy.

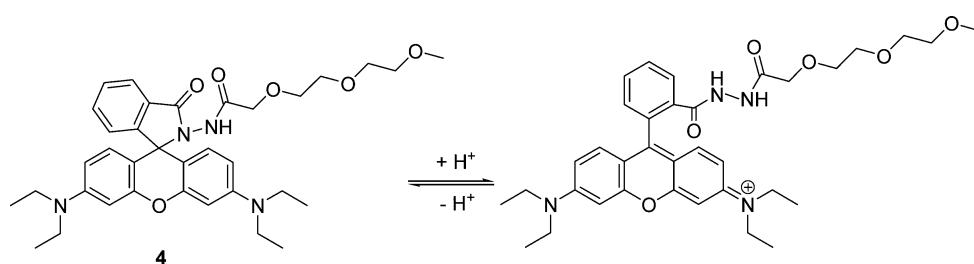
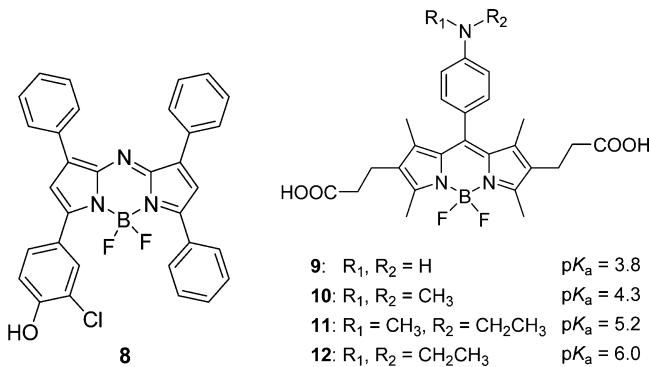
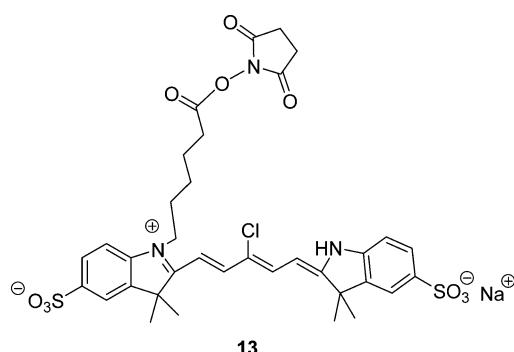


Figure 2. The protonation–deprotonation equilibrium of 4.

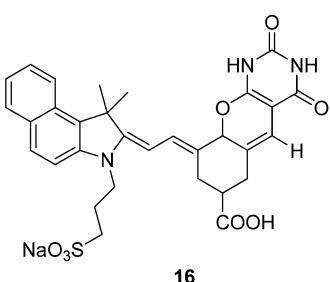
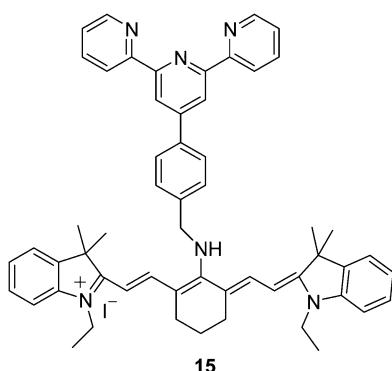
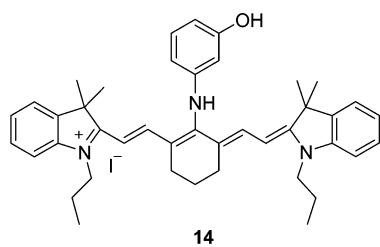
2.3. Cyanine-Based pH Probes

Cyanine dyes are frequently used as the scaffolds to develop near-infrared (NIR) fluorescent probes for pH measurement.



Some cyanine derivatives with non-*N*-alkylated indolium structures have been reported as pH-sensitive probes.^{57–61} These probes are almost nonfluorescent when the nitrogen atom is not protonated, but highly fluorescent upon protonation. Hilderbrand et al.⁶² labeled bacteriophage particles with 13 and Cy7 to prepare an NIR ratiometric fluorescent pH probe. The emission of Cy7 remains stable in the range of pH 5–9, while the emission signals of 13 ($pK_a = 6.2$) vary with pH. With the as-prepared probe, accurate measurements of intracellular pH have been made.

In recent years, some cyanine-based pH probes with *N*-alkylated indolium structures have been reported. Tang et al.^{63,64} synthesized 14



and 15 as pH-sensitive fluorescent probes. Probe 14 is a dual NIR fluorescent pH probe with a pK_a value of 5.14 under acidic conditions and 11.31 under basic conditions, and has been applied for monitoring intracellular pH in HepG2 cells. Probe 15 uses terpyridine as the H^+ -receptor, with a pK_a of ~7.1. With probe 15, real-time imaging of intracellular pH in living HepG2 and HL-7702 cells was performed. Probe 16 was prepared by an unusual barbiturate-mediated synthetic pathway.⁶⁵ The probe is highly fluorescent in the NIR region and possesses excellent spectral sensitivities to environmental pH changes, which makes it of potential use in biosystems.

By introduction of diamino moieties into cyanine, Nagano et al. synthesized 17–21 as pH-sensitive probes, in which a proton is delocalized at the two nitrogen atoms, as depicted in Figure 4 with 17 as an example.⁶⁶ Under acidic conditions,

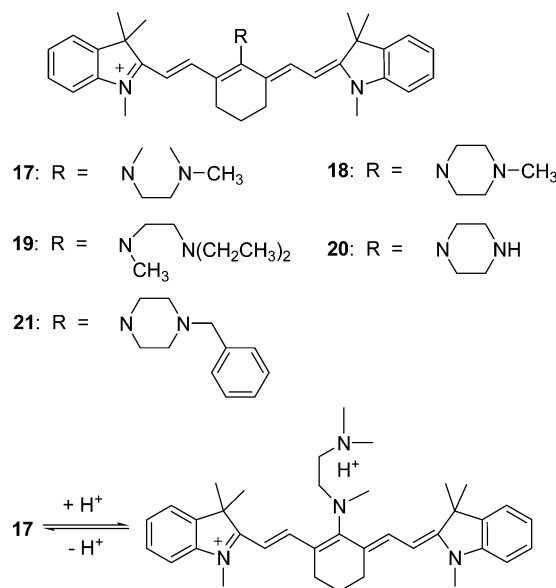


Figure 4. Structures of 17–21 and the protonation–deprotonation of 17.

these pH probes show a 46–83 nm red shift in the absorption maximum, which is reversible and is large enough to permit their use as ratiometric pH probes, whereas monoamine-substituted aminocyanines show irreversible changes because of their instability under acidic conditions. Furthermore, the pK_a values of such probes can be precisely tuned by referring to the calculated pK_a value of the diamine moiety. The application of these probes such as 17 has been demonstrated for monitoring intracellular pH changes.

Some hemicyanines have been reported as pH probes as well (Figure 5).^{67,68} Compound 22 is a red-emissive zwitterionic hemicyanine probe containing tetraphenylethene and *N*-alkylated indolium.⁶⁷ The multiple reactive sites of 22 toward OH^-/H^+ , together with the aggregation-induced emission feature of tetraphenylethene, enable the probe to sense pH in a broad range by showing different emission colors and intensities: strong to moderate red emission at pH 5–7, weak to no emission at pH 7–10, and no to strong blue emission at pH 10–14. The acid/base-switched red/blue emission transition is reversible, and, more importantly, a linear regression of the emission intensity to acidity was established in the physiological pH range of 5–7, revealing the great potential of this probe for pH sensing in biosystems.

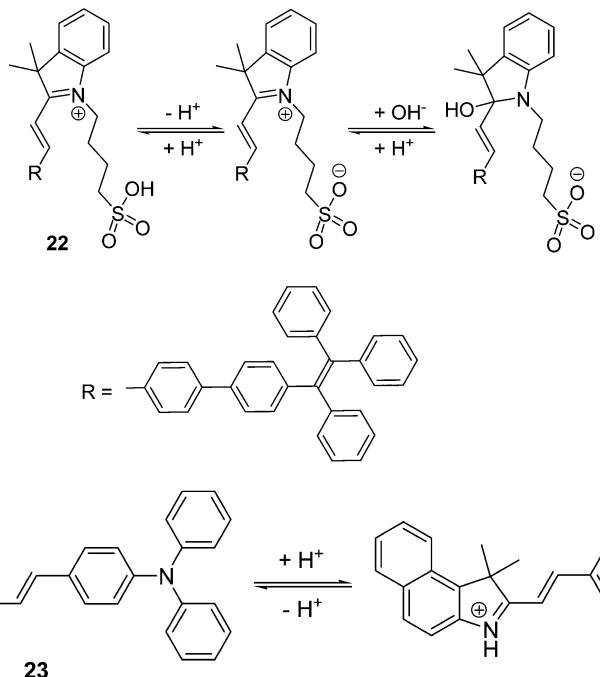


Figure 5. The protonation–deprotonation of 22 and 23.

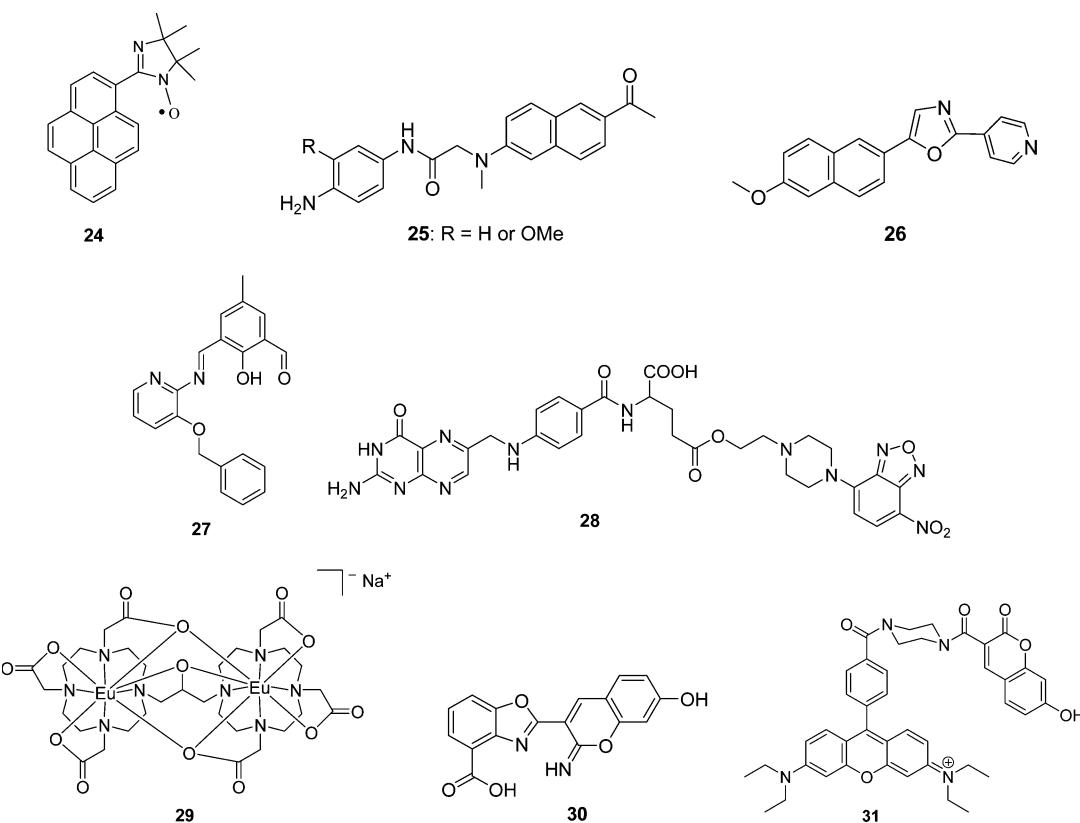


Figure 6. Structures of pH probes 24–31.

Compound 23, a styrylcyanine-based NIR pH probe with a pK_a of 4.40⁶⁸ is fluorescent in acidic media but nonfluorescent in neutral environments. Results from confocal fluorescence microscopy showed that the probe has the potential for imaging acidic organelles in live cells.

2.4. Other Fluorochrome-Based pH Probes

In addition to the pH probes described above, other fluorochromes,^{1,69–75} such as anthracene,^{76–78} pyrene,⁷⁹ and 1,8-naphthalimide,⁸⁰ have been employed in developing pH probes.

Compound 24, an imino nitroxide radical (Figure 6), has been characterized as a burst-type fluorescent pH probe.⁷⁹ The

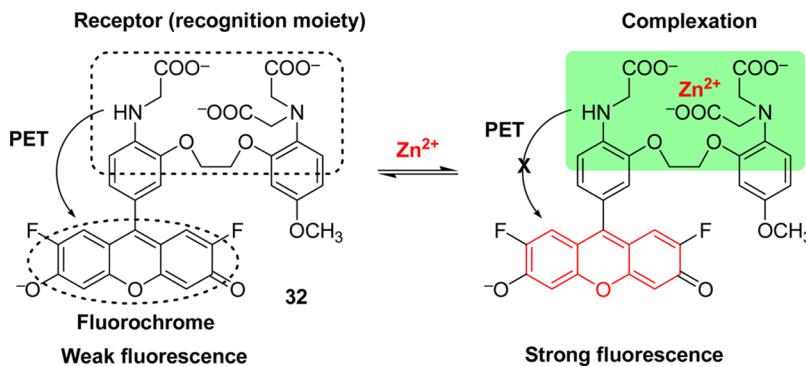


Figure 7. Response mechanism of a PET probe (32) to Zn^{2+} .

probe fluoresces weakly in the ethanol–water (85:15, v/v) media at pH ≥ 4.35 , but can produce a 4-fold burst increase in the fluorescence when the pH value is decreased from 4.35 to 4.31, which is reversible within this narrow pH range, indicating that 24 may serve as a fluorescent switch for monitoring the pH change around the point of 4.35. Compounds 25 and 26 were reported as two two-photon (TP) pH probes ($pK_a \approx 4.5$ or 5.0).^{81,82} With these probes, TP microscopy images were obtained for rat hippocampal tissue and human tissues (stomach and esophagus), respectively. Compound 27, a Schiff's base derivative of 2-aminopyridine, behaves as a highly selective fluorescent pH probe.⁸³ The probe shows a 250-fold increase in fluorescence intensity within the pH range of 4.2–8.3 with a pK_a value of 6.63, which may be useful for studying many of the biological organelles. Compound 28 is constructed as a pH probe by using 4-nitrobenzo[1,2,5]oxadiazole (NBD) as the fluorochrome and piperazine as the proton receptor ($pK_a = 5.70$),⁸⁴ which displayed about 30-fold fluorescence enhancement under acidic conditions. The probe is applicable to imaging pH in acidic organelles. Compound 29 is a Eu(III)-containing complex,⁸⁵ whose luminescence decay rate increases from pH 8 to pH 4 due to the protonation of a bridging alkoxide at lower pH values. 29 may be useful in the noninvasive imaging of *in vivo* pH. Kikuchi et al. have reported some fluorescent “off-on-off” pH probes,⁸⁶ in which the deprotonation of hydroxyl group, carboxylic acid, and benzoxazolium ion is accompanied by the alteration of absorption and fluorescence properties simultaneously. Of these probes, 30 showed the highest fluorescence quantum yield under physiological pH conditions, and was applied in live cell imaging studies. In addition, based on the coumarin–rhodamine platform, a dual-excitation ratiometric fluorescent pH probe 31 was developed,⁸⁷ and the probe was further transformed into a photocaged pH probe with the improved spatiotemporal resolution.

3. COMPLEXATION-BASED CHROMOGENIC AND FLUOROGENIC PROBES

There are two strategies for designing complexation-based chromogenic and fluorogenic probes: one is direct complexation, and the other is competitive displacement complexation. In the strategy of direct complexation, the recognition unit (receptor) and the signaling unit (reporter) are covalently linked, and the resulting probes can directly complex with analyte via the following ways: coordination, electrostatic interaction, or hydrogen bonding. This strategy, as shown below, is a major one in the development of complexation-based chromogenic and fluorogenic probes, and the probes

based on such a mechanism usually have rapid spectroscopic response and good reversibility, which are rather suited for monitoring the dynamic change in the analyte concentration. It should be pointed out that most of the molecular recognitions based on hydrogen bonding work well only in organic solvents, which are not included in this Review.

In the strategy of competitive displacement complexation, however, the receptor and the reporter are not covalently linked; instead, they form a complex (molecular ensemble), which serves as a probe.^{88,89} Upon addition of analyte into the solution of the receptor–reporter ensemble, the stronger complexation of analyte with the receptor leads to the release of the reporter, thereby causing the spectroscopic signal change of the system.

It should be kept in mind that complexation-based probes usually suffer from pH influence because different protonation states of the electronegative atoms in the ligands have different coordinating ability. This is why the effect of pH should be examined and a pH buffer is often required in the detection system. Moreover, the design of this kind of probes with high selectivity for some metal ions remains a great challenge, because many metal ions have similar reactivities and may interfere with each other (e.g., Mg^{2+} vs Ca^{2+} , Ag^+ vs Hg^{2+} , and Cd^{2+} vs Zn^{2+}).

Obviously, the key issue for designing complexation-based chromogenic and fluorogenic probes is how to construct a specific receptor for analyte. Toward this end, different novel receptors and thereby the corresponding probes for analytes have been developed by combining various photophysical processes and modern coordination reactions, which will be discussed in detail below.

3.1. Direct Complexation

3.1.1. Probes for Metal Ions. Direct complexation strategy is often used to develop chromogenic and fluorogenic probes for metal ions (Ca^{2+} , Zn^{2+} , Cu^{2+} , Hg^{2+} , etc.) by virtue of their strong coordinating abilities with electronegative heteroatoms (N, O, S, etc.).⁹⁰ Common measures available for this purpose are based on: (1) matchable ring/cavity size for a given ion, such as crown ethers with different ring sizes for alkali and alkaline earth metal ions; (2) suitable ligands for convenient formation of five- and six-membered ring complexes with metal ions, like the probes with EGTA (ethylene glycol tetraacetic acid) moiety for Ca^{2+} ; and (3) soft–hard acid–base principle, such as the soft sulfur-containing receptors with high affinity for soft metal ions (e.g., Ag^+ and Hg^{2+}).

On the other hand, in the design of such probes, a photophysical process like photoinduced electron transfer

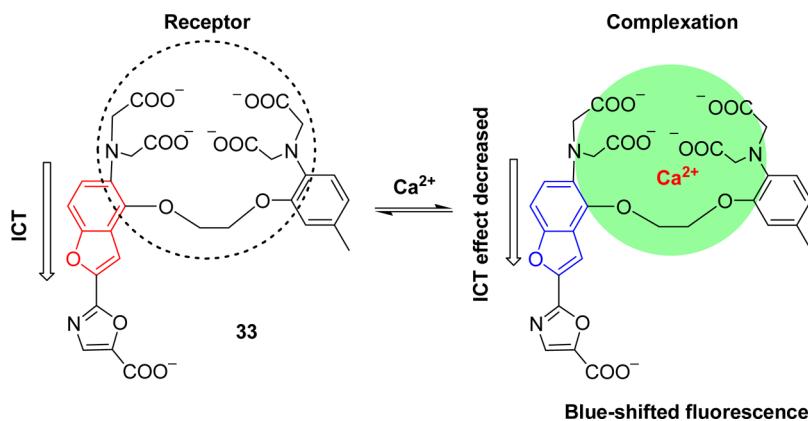


Figure 8. Response mechanism of an ICT probe (33) to Ca^{2+} .

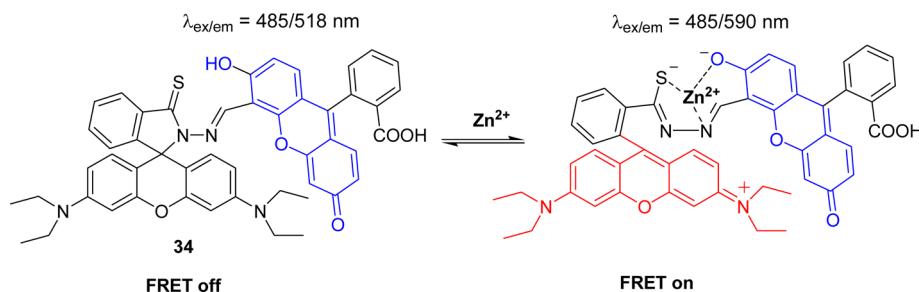


Figure 9. Response mechanism of a FRET probe (34) to Zn^{2+} .

(PET), internal charge transfer (ICT), or fluorescence resonance energy transfer (FRET) should be incorporated to translate analyte binding into a spectroscopic signal change.

In PET probes, a fluorochrome is usually connected via a spacer to a receptor, and the receptor containing electronegative atoms (e.g., nitrogen atom) has a relatively high-energy nonbonding electron pair, which can transfer an electron to the excited fluorochrome, leading to the quenching of fluorescence. However, upon coordination to a cation, the reduction potential of the receptor is increased so that the corresponding highest occupied molecular orbital (HOMO) becomes lower in energy than that of the fluorochrome; consequently, the PET process from the receptor to the fluorochrome is prohibited, and fluorescence intensity is enhanced.^{3,91,92} The PET mechanism is the most frequently used one in designing chromogenic and fluorogenic probes for metal ions. Figure 7 shows a typical example of PET probes, FluoZin-3 (32),^{1,93} whose fluorescence is rather weak due to the PET process from the receptor to the fluorochrome. However, the weak fluorescence can be greatly enhanced by inhibiting the PET process via complexation with Zn^{2+} of the tailor-made recognition moiety containing *N,N,N'*-triacetic acid. This probe has been used in the selective imaging of Zn^{2+} in living cells.^{93,94}

In ICT probes, an electron-donating group (often an amino group) in the receptor is directly linked to a fluorochrome; therefore, the nonbonding electron pair of the electron-donating group participates in the conjugation of the fluorochrome, which essentially differs from PET-based probes. In this case, the ICT process from the electron donor to the fluorochrome may occur upon excitation by light.^{95,96} However, coordination of metal ions with the receptor changes not only the energy gap between LUMO (lowest unoccupied molecular orbital) and HOMO but also the efficiency of ICT, causing

both absorption and fluorescence intensity changes as well as spectral shifts, which makes ratiometric measurements possible. Figure 8 depicts a typical example of ICT probes, Fura 2 (33),^{1,97,98} whose complexation with Ca^{2+} leads to a remarkable blue-shifted fluorescence. This enables intracellular Ca^{2+} to be determined in a ratiometric mode, which effectively overcomes the influence of several variants like concentration and optical path length.

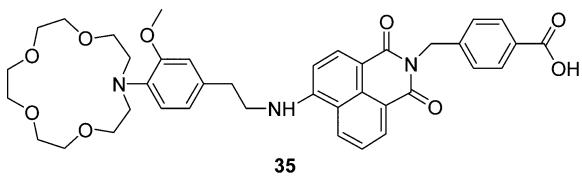
FRET usually arises from interaction between a pair of different fluorochromes, in which one acts as a fluorescence donor and the other as an acceptor. In the FRET process, the excited donor molecule passes its energy to the nearby acceptor at the ground state, and then the excited acceptor relaxes back to its ground state by emitting fluorescence. In some cases, the acceptor can be a quencher. FRET mainly depends on three factors: the distance between the donor and the acceptor, the extent of spectral overlap between the donor emission and acceptor absorption spectrum, and the relative orientation of the donor emission dipole moment and acceptor absorption moment.²⁷ Because of its distance-dependent efficiency, FRET has been widely used in the measurement of the distances between the two interacting fluorophores in macromolecules, especially biomacromolecules. On the other hand, when the acceptor is a fluorescent dye, FRET causes the relative change of fluorescence at two wavelengths, and this behavior can serve as an effective measure to design small molecular ratiometric probes by integrating the fluorescence donor and acceptor into one molecule. Figure 9 shows such a ratiometric probe 34, in which fluorescein and rhodamine function as fluorescence donor and acceptor, respectively, because the fluorescence spectrum of fluorescein matches well with the absorption spectrum of rhodamine.⁹⁹ In the absence of Zn^{2+} , the rhodamine moiety in 34 exists in a spirolactam form that has almost no absorption in the visible region, and thus cannot act as an

acceptor; when excited at 485 nm, probe **34** shows only green emission at 518 nm characteristic of fluorescein. However, upon addition of Zn^{2+} , complexation of **34** with Zn^{2+} results in the opening of spirolactam ring and the generation of an absorption peak at 560 nm, which leads to the occurrence of FRET and thus the change of fluorescence intensity ratio at 590/518 nm. It is noteworthy that the spiro-ring-opening of rhodamine has also been proved to be an efficient way for developing various spectroscopic off-on probes for metal ions.¹⁰⁰

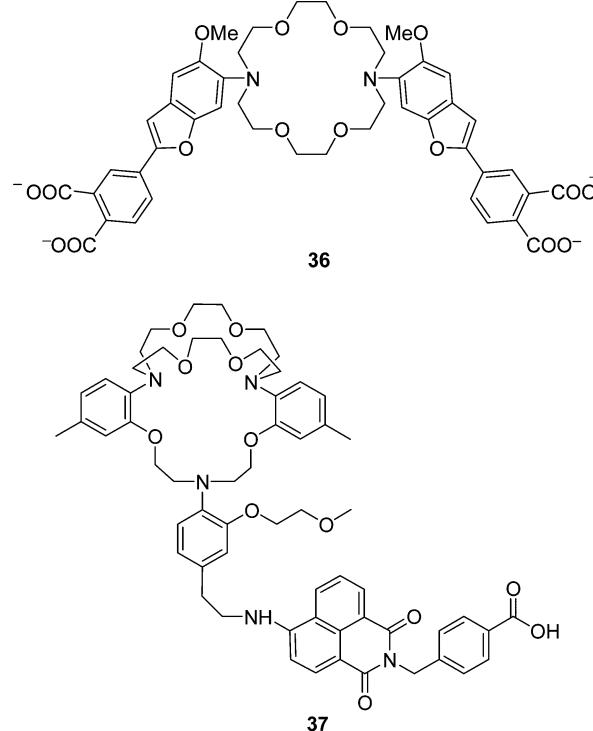
Until now, the above strategies have led to the appearance of a series of chromogenic and fluorogenic probes for metal ions, which will be reviewed in the order of first alkali and alkaline earth metal ions, then transition metal ions, and finally other metal ions.

3.1.1.1. Probes for Alkali and Alkaline Earth Metal Ions (Na^+ , K^+ , Mg^{2+} , and Ca^{2+}). The alkali and alkaline earth metal ions such as Na^+ , K^+ , Mg^{2+} , and Ca^{2+} are the major biological metal ions, whose concentrations in biosystems are rather high (usually over mM levels) except the intracellular Ca^{2+} concentration (about μM levels).¹⁰¹ Therefore, the design of chromogenic and fluorogenic probes with high selectivity and moderate complexation constants for these ions is of great importance for practical applications, but such excellent probes are still relatively rare.¹⁰²

Minta and Tsien developed the first fluorescence probes suitable for the measurement of intracellular Na^+ concentrations (ca. 30 mM) by using a crown ether, 1,7-diaza-4,10,13-trioxacyclo-pentadecane, as a recognition unit.¹⁰³ However, the low dissociation constants ($K_d < 50$ mM) of these probes preclude their use for detecting the high extracellular Na^+ concentration (ca. 140 mM). Later, de Silva et al. synthesized a PET probe with the same crown ether receptor, which is also only suitable for the detection of low Na^+ concentration (0.1–10 mM).¹⁰⁴ To meet the requirement for the assay of high extracellular Na^+ concentration, He et al. designed a PET probe (**35**)¹⁰⁵ by choosing *N*-(*o*-methoxyphenyl)aza-15-crown-5 as a receptor because of its sodium binding in the desired measuring range ($K_d \approx 80$ mM). Complexation of **35** with Na^+ causes a large increase in fluorescence at 540 nm, and the probe can be immobilized in a hydrophilic polymer layer for practical measurement of sodium in serum and whole blood samples.



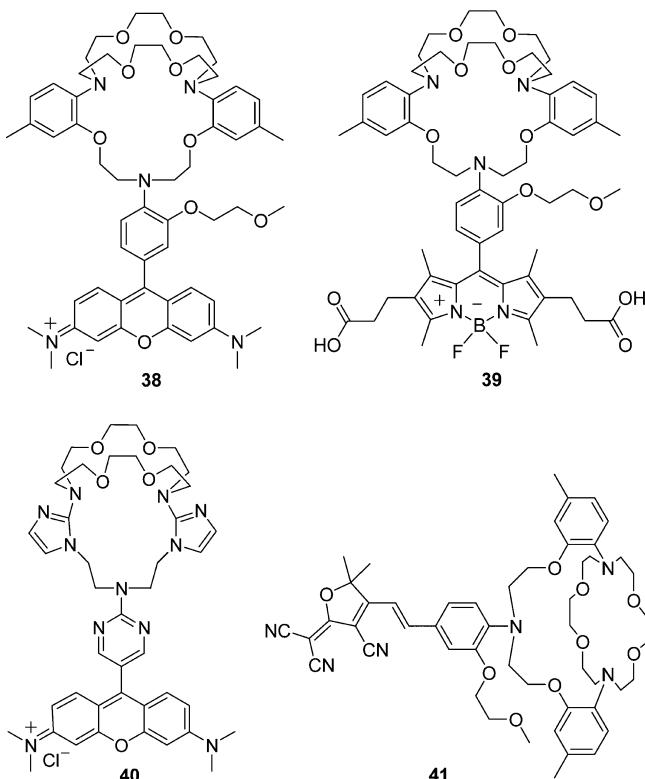
By using a larger crown ether, 1,10-diaza-4,7,13,16-tetraoxacyclooctadecane, as a recognition unit and benzofuran skeleton as a fluorochrome, a K^+ -selective probe (**36**) was obtained.¹⁰³ However, the $K^+:Na^+$ selectivity of this probe is only modest, and the sensitivity for extracellular K^+ is not ideal due to its low complexation constant. To increase selectivity and sensitivity, He et al. developed a PET probe **37** with triazacryptand as a recognition unit for K^+ .¹⁰⁶ Complexation of **37** with K^+ increases greatly the fluorescence at 540 nm, and this response is highly selective for K^+ over Na^+ . The probe can be used to monitor the change of extracellular K^+ concentration (e.g., K^+ in serum and whole blood samples), and no interference from cellular Ca^{2+} or pH is observed.



By using triazacryptand as the recognition unit, more fluorescent K^+ probes have been developed by Verkman's group.^{107–110} Compound **38** was first reported as a fluorescent K^+ probe, whose fluorescence (7 μM probe) increased up to 14-fold in the presence of 0–50 mM K^+ in 5 mM pH 7.04 HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] with K^+ -to- Na^+ selectivity >30. The probe was used to follow K^+ waves in the extracellular space in brain.¹⁰⁷ However, this probe can significantly partition into many cell types, limiting its extracellular utility. Later, they synthesized **39** with BODIPY skeleton as a fluorochrome,¹⁰⁸ which has been conjugated to dextran to afford a membrane-impermeable probe for detecting extracellular K^+ efflux. This K^+ transport assay may replace methods requiring radioactive rubidium and is suitable for high-throughput identification of K^+ transport modulators. Moreover, they coupled both **39** and a tetramethylrhodamine reference fluorochrome to dextran for ratiometric fluorescence measurement of extracellular K^+ in airway surface liquid (the thin fluid layer lining airway surface epithelial cells).¹⁰⁹ This was the first noninvasive measurement of K^+ in the airway surface liquid, and their results revealed the involvement of apical and basolateral membrane ion transporters in maintaining a high airway surface liquid K^+ . Recently, they also reported an efficient route to synthesizing a structurally simplified fluorescent probe **40** for extracellular K^+ with similar performance.¹¹⁰

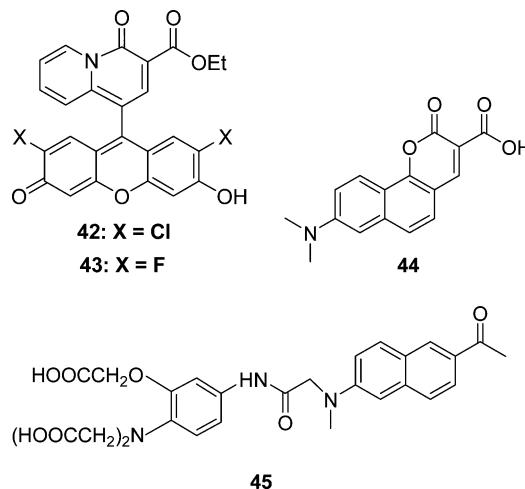
Zhou et al. synthesized an ICT probe **41** for K^+ by using triazacryptand as the recognition unit and multiple cyano groups as strong electron-withdrawing unit.¹¹¹ The as-designed probe **41**, with a K_d of ~88 mM, can selectively respond to K^+ up to 1.6 M. This is the first fluorescent K^+ probe suitable for sensing intracellular K^+ over a broad and high concentration range, and confocal fluorescence microscopy has established its utility for live-cell K^+ detection.

A number of fluorescent Mg^{2+} probes such as Mag-fura-2 and Mg Green have been proposed and commercialized.¹ However, the commercialized probes suffer from Ca^{2+} interference

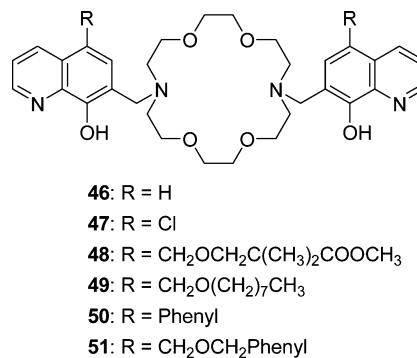


because of the similarity of their recognition unit, *o*-amino-phenol-*N,N,O*-triacetic acid, to that of Ca^{2+} probes. To overcome this problem, a series of Mg^{2+} -selective probes with β -diketone as recognition unit were reported by Suzuki et al.^{112–114} These probes exhibited high selectivity for Mg^{2+} over the other ions at their physiological concentrations. Probes 42 and 43 are two representative examples of β -diketone-based probes, which have PET response characteristics upon binding with Mg^{2+} .¹¹³ The two probes possess suitable dissociation constants ($K_d = 2 \text{ mM}$) for normal intracellular Mg^{2+} concentrations (0.1–6 mM). Reaction of 5 μM of 42 or 43 with 100 mM of Mg^{2+} produces about 10-fold fluorescence enhancement, which enables intracellular Mg^{2+} to be measured in many types of cells.^{115–117} Compounds 44 and 45 were reported as TP fluorescent Mg^{2+} probes,^{118,119} in which β -keto acid and *o*-aminophenol-*N,N,O*-triacetic acid are employed as the Mg^{2+} -selective binding site, respectively. Both of the probes have a large TP cross section and are capable of imaging Mg^{2+} in live cells and tissues by employing the TP microscopy. The dissociation constants ($K_{d,\text{Mg}}$) of 44 and 45 are 2.5 and 1.6 mM, respectively. Moreover, the $K_{d,\text{Mg}}/K_{d,\text{Ca}}$ ratio of 44 is 0.36, which is comparable to those of the above 42 and 43 but much smaller than those ($K_{d,\text{Mg}}/K_{d,\text{Ca}} = 76$ and 167, respectively) of Mag-fura-2 and Mg Green, suggesting that the selectivity of β -diketone-based probes (42–44) for Mg^{2+} over Ca^{2+} is much higher than that of Mag-fura-2 and Mg Green.

On the other hand, diaza-18-crown-6 is linked to 8-hydroxyquinoline (8-HQ) to develop fluorescent Mg^{2+} probes (46 and 47).¹²⁰ 8-HQ itself is weakly fluorescent. In its excited state, the intramolecular photoinduced proton transfer from hydroxyl group to the nitrogen atom provides a route to nonradiative relaxation; however, complexation of the diaza-18-crown-6 unit with Mg^{2+} can decrease the pK_a of the hydroxyl group in 8-HQ, thus facilitating the dissociation of the hydroxyl group. The leaving of this proton makes quinoline exist in a highly fluorescent form.^{121,122} Consequently, the probes



containing 8-HQ usually provide an off-on sensing response upon complexation with metal ions. Indeed, reactions of 46 and 47 with Mg^{2+} lead to a dramatic fluorescence enhancement due to the inhibition of both PET and photoinduced proton transfer. Probes 46 and 47, with K_d of 44 and 73 μM , respectively, can quantitatively assess total intracellular Mg^{2+} by simple fluorescence assay. Moreover, the fluorescence response of these probes is highly selective for Mg^{2+} , and alkali metal ions at 100 mM do not interfere with Mg^{2+} detection. A limitation of 46 and 47 is that they can be excited only in the UV region, and 47 shows an incomplete intracellular retention. To circumvent these problems, new compounds (48–51) were synthesized, and the potential of these new fluorescent probes as effective tools for total intracellular Mg^{2+} distribution and homeostasis has been demonstrated.¹²³ However, the above 8-HQ-based probes suffer from the severe influence of pH, which means that the detection can only be performed under the strict control of pH of reaction solutions.



Fluorescent Ca^{2+} probes have been the focus of biological and chemical researches for a long time due to the vital role of Ca^{2+} in various cellular processes. As mentioned above, the concentration of intracellular Ca^{2+} is usually at or even below micromolar levels, whereas that of intracellular Mg^{2+} is at millimolar levels; that is, the concentration difference between the intracellular Ca^{2+} and Mg^{2+} is as large as 3 orders of magnitude. Therefore, the designed intracellular Ca^{2+} probes should have not only high selectivity but also high sensitivity. Tsien's group has contributed a lot in this regard. By developing 1,2-bis(*o*-amino-phenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) with the EGTA structural feature as a novel recognition moiety for Ca^{2+} , Tsien et al. synthesized various Ca^{2+} probes, including the UV light-excited probes

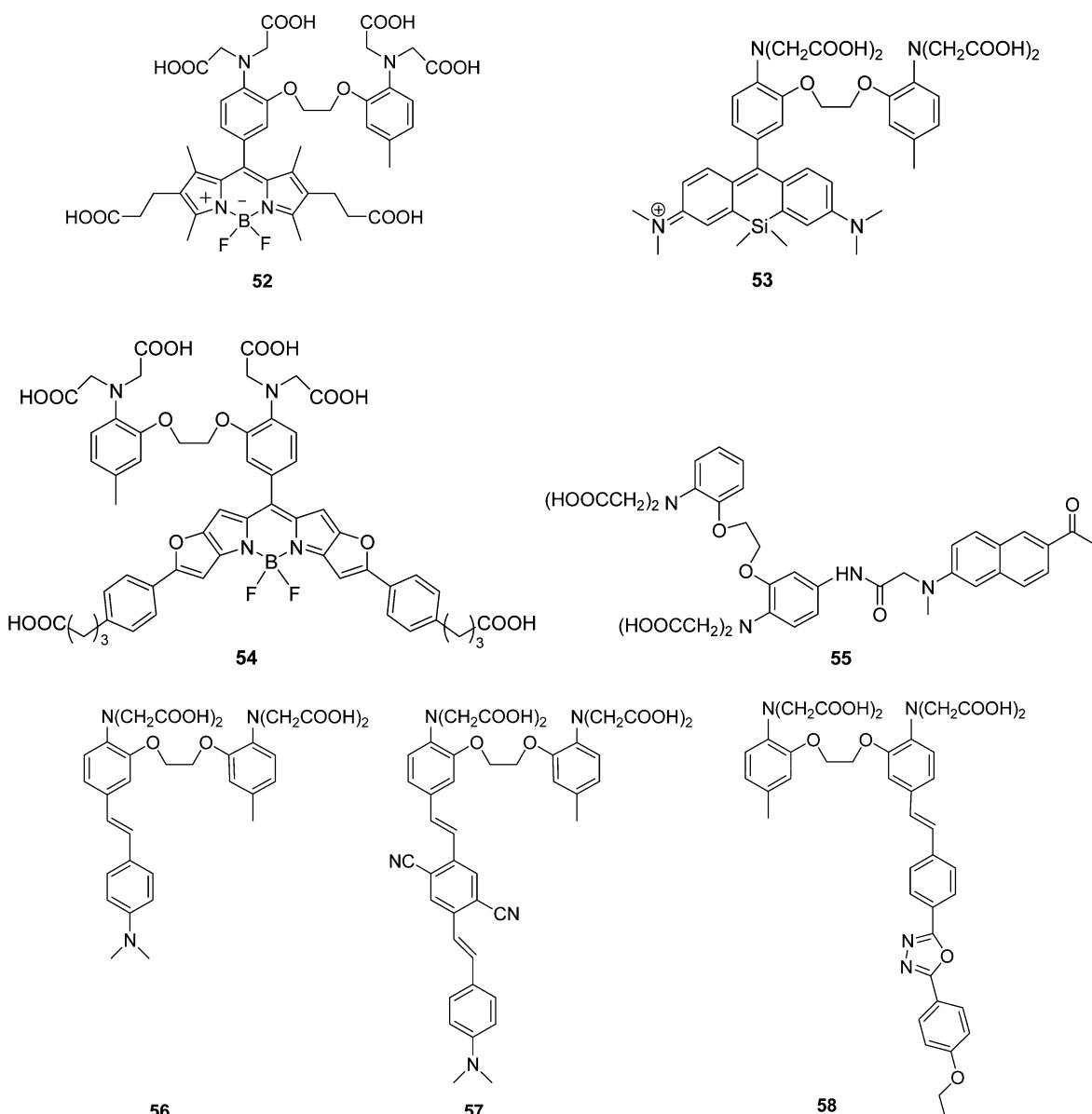


Figure 10. Structures of Ca^{2+} probes 52–58.

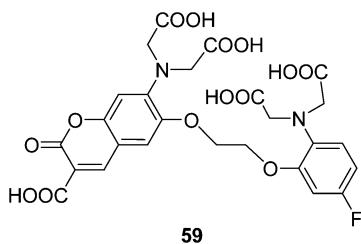
(Indo-1, Quin-2, and Fura-2)⁹⁷ and visible light-excited probes (Fluo-, Rhod-, and Ca Green-series).¹²⁴ The dissociation constants of these probes are close to micromolar levels (e.g., the K_d values of Fura-2 and Ca Green-1 are 145 and 190 nM, respectively), indicating that these probes are suitable for the detection of intracellular Ca^{2+} . Moreover, because of their good reversibility, these Ca^{2+} probes have been widely used to monitor the change of Ca^{2+} concentration in various cells, which greatly improves the understanding of Ca^{2+} function in biology and physiology.^{125–129}

In the past decade, more new fluorescent Ca^{2+} probes have been reported,^{130–136} and some of them have been applied to *in vivo* imaging of Ca^{2+} . For example, compound **52** (Figure 10), prepared by connecting BAPTA to BODIPY at the mesoposition,¹³¹ reacts with Ca^{2+} resulting in about 250-fold fluorescence increase through the inhibition of PET process. The high sensitivity of **52** ($K_d = 0.3 \mu\text{M}$) makes it a powerful tool for tracing the intracellular Ca^{2+} change with high spatiotemporal resolution. Probe **53** was reported as a Ca^{2+} probe by Nagano's group by using Si-rhodamine as the

fluorochrome and BAPTA as the Ca^{2+} chelator.¹³² The probe shows over 1000-fold fluorescence increase upon complexation with Ca^{2+} ($39 \mu\text{M}$) through the efficient switch-off of a PET process ($K_d = 0.58 \mu\text{M}$). The NIR fluorescence characteristic and high signal-to-background ratio of **53** are the most important advantages over other existing Ca^{2+} probes, which makes it suitable for fluorescence imaging of Ca^{2+} in neuronal systems. Compound **54** is a BODIPY-based NIR fluorescent Ca^{2+} probe,¹³³ which also shows good spectroscopic properties such as intense NIR fluorescence emission ($\Phi = 0.24$) at 670 nm and high signal-to-background ratio (120) upon complexation with $39 \mu\text{M} \text{ Ca}^{2+}$ ($K_d = 0.5 \mu\text{M}$). The probe has been applied to real-time imaging of intracellular Ca^{2+} . Compound **55** is a TP Ca^{2+} probe.¹³⁴ Complexation of **55** with Ca^{2+} ($39 \mu\text{M}$) leads to a 40-fold fluorescence enhancement via the blocking of a PET process ($K_d = 0.25 \mu\text{M}$). **55** can be used for detecting the dynamic change of intracellular free Ca^{2+} .^{134,135} Compounds **56** and **57** are also TP Ca^{2+} probes. The two probes were constructed on the basis of the ICT process, in which part of the chelating domain (BAPTA) also belongs to

the fluorescence domain. Binding of **56** or **57** to Ca^{2+} resulted in a decrease of fluorescence intensity. In addition, due to the small K_d values (51 nM for **56** and 39 nM for **57**), these two probes are unsuitable for measuring the Ca^{2+} wave in living cells.¹³⁶ Very recently, a 1,3,4-oxadiazole derivative **58** (Figure 10) has been proposed as a ratiometric fluorescent Ca^{2+} probe, which exhibits a large Stokes shift of 202 nm and a highly selective ratiometric emission response (490/582 nm) to Ca^{2+} via an ICT mechanism. Probe **58**, with a K_d of 0.56 μM , has been successfully used to reveal the change of Ca^{2+} concentration in human umbilical vein endothelial cells.¹³⁷ Despite the great progress made to date, the Ca^{2+} probes (e.g., Fura 2 and Indo 1) developed by Tsien's group are still the most widely used, and often serve as the gold standard in Ca^{2+} detection due to their superior properties and commercial availability.^{1,97,98}

A coumarin derivative **59** was prepared as a multianalyte (Ca^{2+} , Mg^{2+}) probe based on an ICT mechanism.¹³⁸ The probe was constructed by introducing two recognition units of BAPTA (for Ca^{2+}) and β -diketone (for Mg^{2+}) into a single molecule. Upon complexation of BAPTA (electron donor) with Ca^{2+} , **59** shows a 45-nm blue shift in absorbance and a 5-nm blue shift in fluorescence. On the contrary, the binding of Mg^{2+} to β -diketone (electron acceptor) induces a 21-nm red shift in absorbance and a 5-nm red shift in fluorescence. The probe has been successfully applied for the simultaneous imaging of intracellular Ca^{2+} and Mg^{2+} in PC12 cells.



3.1.1.2. Probes for Transition Metal Ions (Ag^+ , Cd^{2+} , Co^{2+} , Cr^{3+} , Cu^{2+} , Cu^+ , Fe^{3+} , Hg^{2+} , Ni^{2+} , Pt^{2+} , Zn^{2+}). The concentrations of transition metal ions, including biologically important Cu^{2+} , Fe^{3+} , and Zn^{2+} , are extremely low (usually $<10^{-7}$ M) in biosystems.¹⁰¹ Thus, the designed chromogenic and fluorogenic probes should be of high sensitivity for practical applications, but regrettably most of the current probes are unable to detect the basal levels of transition metal ions in biosystems. In the following section, we will present the probes for the transition metal ions in order of Ag^+ , Cd^{2+} , Co^{2+} , Cr^{3+} , Cu^{2+} , Fe^{3+} , Hg^{2+} , Ni^{2+} , Pt^{2+} , and Zn^{2+} .

There are relatively few chromogenic and fluorogenic probes for Ag^+ ,^{90,139} which are often designed by utilizing the receptors containing sulfur atom because this kind of receptors has high affinity for soft transition metal ions including Ag^+ . The small molecular chromogenic and fluorogenic Ag^+ probes can be mainly classified as the following types: probes with cyclic thiaoxaaza-crown[N,S,O] receptors,^{140–142} probes bearing acyclic receptors,^{143–148} and excimer-based probes.^{149–152} However, the water solubilities of most of the current Ag^+ probes are not ideal,^{139,153} and a large amount of organic solvents has to be used in the system, which would disturb the normal functions of biomolecules and thus is not very suitable for biological studies.

Compound **60** (Figure 11) was reported as a fluorescent Ag^+ probe.¹⁴⁰ Reaction of **60** (10 μM) with Ag^+ (30 μM) shows a 14-fold fluorescence enhancement in $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ (50:50, v/v) media of pH 7.4. The probe can be used to detect Ag^+ down to 1.0×10^{-8} M ($K_d = 8.06 \mu\text{M}$). Probe **61** exhibits negligible fluorescence in the absence of Ag^+ , whereas a ca. 35-fold increase in fluorescence is produced upon complexation with Ag^+ (30 μM) in 50 mM HEPES buffer of pH 7.2, which is ascribed to the inhibition of a PET process.¹⁴⁵ The probe is selective for Ag^+ , except that Cu^+ and Cu^{2+} cause slight fluorescence enhancement. The K_d of **61** for Ag^+ is 2.0 μM , suggesting that the probe is also sensitive. Probes **62** and **63** both show large fluorescence enhancement upon binding to

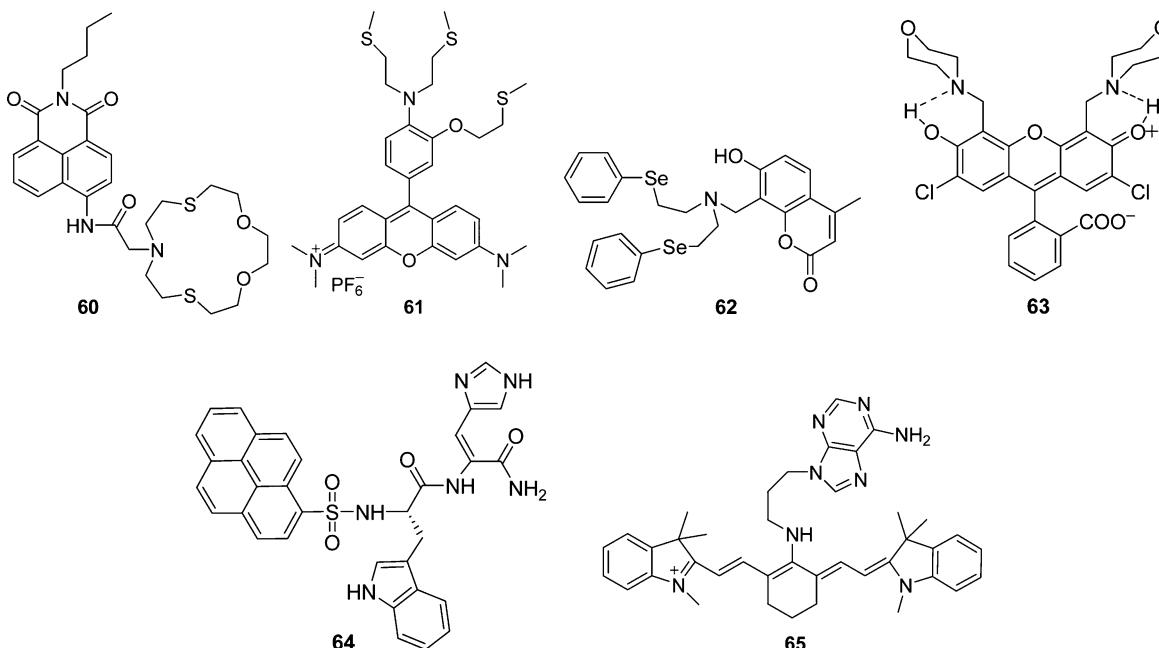


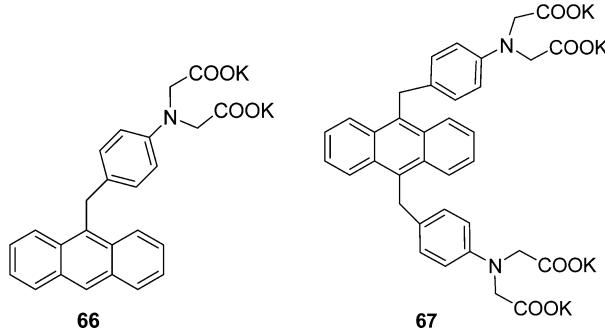
Figure 11. Structures of Ag^+ probes **60**–**65**.

Ag^+ and can be applied to selectively detect Ag^+ in the organic-aqueous solutions.^{147,148}

Pyrene derivatives have been proposed as Ag^+ probes, whose fluorescence responses are based on excimer formation.^{149–152} Typically, these probes show emission bands at around 378 and 395 nm characteristic of pyrene. Upon addition of Ag^+ , a decrease of the pyrene monomer emissions and the appearance of pyrene excimer fluorescence at about 480 nm are observed, which is ascribed to the formation of a 1:2 Ag^+ –probe complex. Probe **64** (Figure 11) is such an example, which not only showed a ratiometric fluorescence response to 1 equiv of Ag^+ or Hg^{2+} (30 μM), but also good solubility in aqueous solution at physiological pH (pH 7.4 HEPES buffer solution containing 1% DMF).¹⁵² The K_d of **64** for Ag^+ and Hg^{2+} is 1.1×10^{-13} and $4.4 \times 10^{-13} \text{ M}^2$, respectively. Interestingly, complexation of **64** with Ag^+ exhibited a strong excimer emission at 480 nm with a small decrease of monomer emission bands, whereas that with Hg^{2+} showed a weak excimer emission with a considerable decrease of the monomer emission bands. This behavior may be used to differentiate Ag^+ and Hg^{2+} . Another ratiometric Ag^+ probe is **65**,¹⁵⁴ whose fluorescent response is based on the cyanine aggregation induced by the specific interaction between adenine and Ag^+ in aqueous solution. The binding of adenine to Ag^+ induced cyanine aggregation, causing a distinct decrease in the 731 nm fluorescence and an increase in the 546 nm fluorescence. The emission ratio ($I_{546 \text{ nm}}/I_{731 \text{ nm}}$) of **65** (20 μM) increases with the increasing Ag^+ concentrations from 0–10 μM , thus allowing Ag^+ to be determined ratiometrically with a detection limit of 34 nM.

There exist many fluorescent Cd^{2+} probes. However, most of the probes can respond to Zn^{2+} as well because the two metal ions often exhibit similar coordination properties.^{155–162} Therefore, the significant challenge in the design of fluorescent Cd^{2+} probes is to discriminate Cd^{2+} from Zn^{2+} . Toward this end, various novel receptors for Cd^{2+} have been designed.^{163–168}

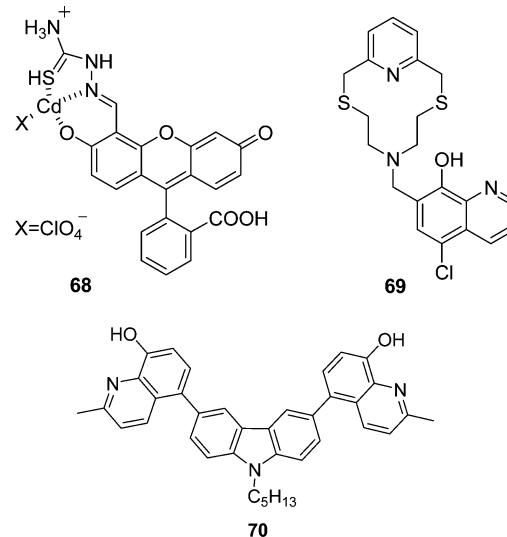
The anthracene derivatives **66** and **67** with either one or two iminodiacetate receptors have been reported as fluorescent Cd^{2+} probes, whose K_d values are 158 and 63.1 μM , respectively.¹⁶³ Both **66** and **67** have good water solubility and are pH-independent in the physiological pH range. Upon addition of Cd^{2+} , the formation of charge-transfer complexes induced fluorescent enhancement at $\lambda_{\text{max}} = 506$ and 500 nm for **66** and **67**, respectively, whereas addition of Zn^{2+} only causes the increase of anthracene emission of **67** and a red-shifted emission of **66** ($\lambda_{\text{max}} = 468$ nm). Thus, both **66** and **67** demonstrated sufficient Cd^{2+} selectivity over Zn^{2+} under physiological conditions.



Compound **68** is a water-soluble fluorescent probe for detecting Cd^{2+} .¹⁶⁴ The probe, with a K_d of 0.123 μM , shows

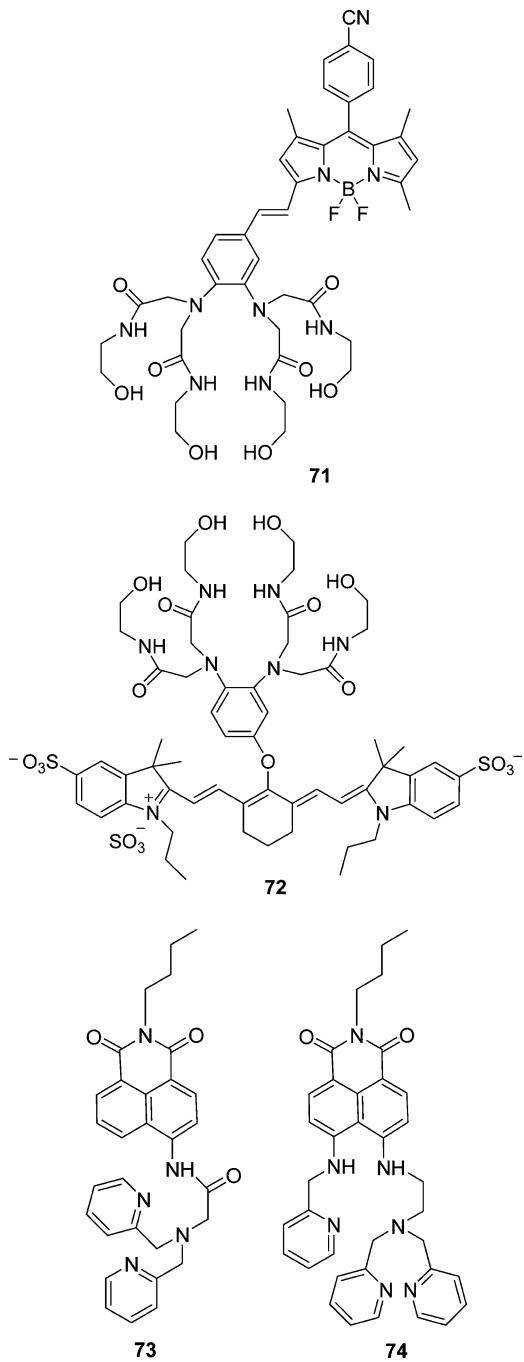
selectively enhanced fluorescence for Cd^{2+} over other metal ions in HEPES buffer solution, which is ascribed to the complexation-induced prevention of the rotation of $\text{C}=\text{N}$ bond as confirmed by NMR and mass analysis.

With 8-HQ as the fluorochrome and 2,8-dithia-5-aza-2,6-pyridinophane as the receptor, a fluorescent Cd^{2+} probe **69** was synthesized.¹⁶⁵ **69** can form a 1:1 complex with Cd^{2+} ($K_d = 0.62 \mu\text{M}$) and exhibits a significant fluorescence increase toward Cd^{2+} in aqueous solution. The probe has been applied for selective imaging of Cd^{2+} in living cells. In addition, a ratiometric fluorescent probe **70** containing the 8-HQ skeleton has been prepared, which can distinguish Cd^{2+} from Zn^{2+} by both fluorescence intensity and emission shift based on different coordination actions.¹⁶⁶ Similar to the above 8-HQ-based probes for Mg^{2+} , these Cd^{2+} probes also have the narrow pH working range, which may hinder their application.



By using polyamides as the receptor, several fluorescent Cd^{2+} probes have been synthesized.^{168–170} Reactions of the probes (e.g., **71**¹⁶⁸ and **72**¹⁷⁰) with Cd^{2+} all caused significant fluorescence increases due to the inhibition of PET. Continuous variation method (Job's plot) revealed that two Cd^{2+} ions coordinated to one probe molecule, and the K_d values of **71** and **72** are 1.07×10^{-9} and $6.0 \times 10^{-10} \text{ M}^2$, respectively. More importantly, introduction of Zn^{2+} into the solution of the probes did not cause any noticeable fluorescence change, indicating that they can well distinguish Cd^{2+} from Zn^{2+} . Living cell experiments revealed the potential of these probes for Cd^{2+} imaging.

On the other hand, the di-2-picolyamine (DPA) moiety, widely employed as Zn^{2+} -selective receptors, and its derivatives can also serve as the construction of Cd^{2+} -selective receptors if it is combined with proper auxiliary groups such as N-containing heterocycles.^{5,91,171,172} Yoon et al. synthesized a naphthalimide-based fluorescent probe **73** for ratiometric Cd^{2+} and Zn^{2+} detection by designing an amide-DPA receptor.¹⁷³ Complexation of both Cd^{2+} and Zn^{2+} with **73** caused large fluorescence enhancement. Job's plots indicated that **73**– Cd^{2+} and **73**– Zn^{2+} complexes all have 1:1 stoichiometry with a K_d of 48.5 and 5.7 nM, respectively. Interestingly, Cd^{2+} induced a blue shift in fluorescence, while Zn^{2+} caused a red shift via Zn^{2+} -triggered amide tautomerization. Therefore, Cd^{2+} and Zn^{2+} can be differentiated by **73**. Qian et al.¹⁷⁴ developed another naphthalimide-based probe **74** for discrimination of this cation



pair on the basis of two reverse ICT processes for Cd^{2+} and Zn^{2+} . The probe can also form a 1:1 complex with Cd^{2+} or Zn^{2+} , with a K_d of 1.74 and 6.06 μM , respectively.

Jiang et al. synthesized a series of quinoline-based fluorescent probes with DPA as the receptor for detecting Cd^{2+} as well as Zn^{2+} .^{175–177} For example, probe 75 can distinguish Cd^{2+} from Zn^{2+} via two different sensing mechanisms (PET for Cd^{2+} ; ICT for Zn^{2+}).¹⁷⁶ As shown in Figure 12, these differences result from the different coordination modes of $75-\text{Cd}^{2+}$ and $75-\text{Zn}^{2+}$, which were also confirmed by ^1H NMR studies. Moreover, the central ion of $75-\text{Zn}^{2+}$ can be displaced by Cd^{2+} with stronger affinity, resulting in another ratiometric sensing signal output. Thus, 75 can be used as a dual mode Cd^{2+} -selective probe via the chelation-enhanced fluorescence mechanism and ratiometric displacement approach, and the detection limit for Cd^{2+} was $2.38 \times 10^{-6} \text{ M}$.

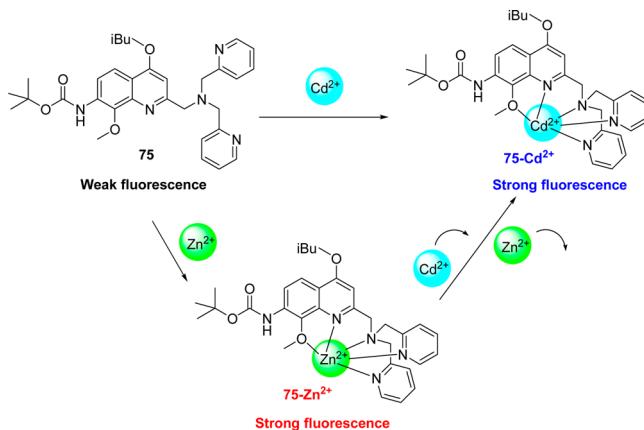
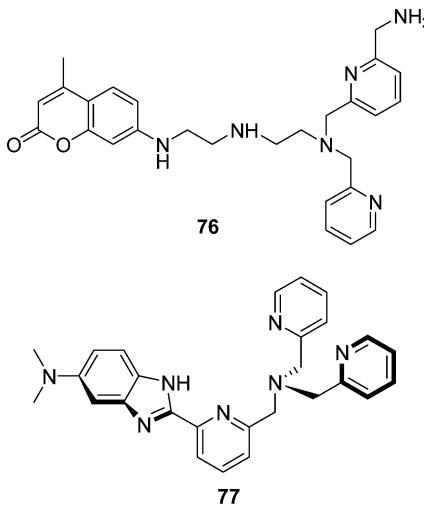


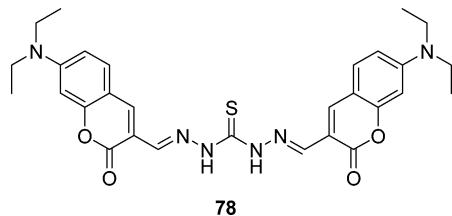
Figure 12. Proposed complexation modes for 75 with Cd^{2+} and Zn^{2+} . Reprinted with permission from ref 176. Copyright 2009 American Chemical Society.

Taki et al. developed a ratiometric fluorescent Cd^{2+} probe 76, whose fluorescence ratio is only slightly affected by Zn^{2+} .¹⁷⁸ The probe has very high affinity for Cd^{2+} ($K_d = 0.16 \text{ nM}$) at pH 7.2, and nonlinear fitting analysis revealed that 76 is suitable to determine free Cd^{2+} between 40 and 660 pM. In addition, the ratio-imaging experiments demonstrated that 76 may be a useful tool for detecting changes in Cd^{2+} concentrations in living mammalian cells. He et al. also designed a ratiometric fluorescent probe (77) with extremely high affinity for Cd^{2+} ($K_d = 25 \text{ pM}$) in aqueous media.¹⁷⁹ Probe 77 features a large Cd^{2+} -induced red-shifted fluorescence. Moreover, this probe is able to discriminate Cd^{2+} from Zn^{2+} and has been used for ratio-imaging Cd^{2+} in living cells.

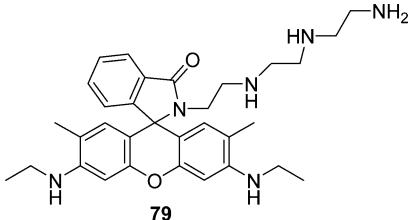


For Co^{2+} , the design of complexation-based probes with fluorescence enhancement features is rather difficult due to its paramagnetic nature.¹⁸⁰ However, there are several colorimetric Co^{2+} probes, although their water solubility and selectivity are relatively poor.^{181,182} To improve selectivity, thiocarbanohydrazone was linked to coumarin,¹⁸³ and the resulting probe 78 can selectively complex with Co^{2+} in 2:1 stoichiometry ($K_d = 1.12 \times 10^{-8} \text{ M}^2$), concomitant with the color change from light yellow to deep pink. 78 has been used to develop practically viable colorimetric kits and to stain Co^{2+} in microorganisms.

Similarly, it is a challenging task to design complexation-based fluorescence off-on probes for the paramagnetic metal

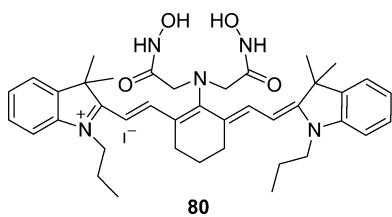


ions of Cr^{3+} , Cu^{2+} , and Fe^{3+} due to their inherent quenching ability.^{184,185} In this context, the above-mentioned rhodamine framework, due to the reversible opening reaction of spiro-ring, may provide a good choice to construct such probes.^{11,100} For Cr^{3+} , water-soluble fluorescent probes are still rare, but the rhodamine-based probe **79** is an exception, which can work in aqueous solution.¹⁸⁶ The probe itself is colorless and nonfluorescent because of its five-membered spirolactam structure. Complexation of the probe with Cr^{3+} leads to the formation of a 1:1 complex ($K_d = 24 \mu\text{M}$), resulting in the spirolactam ring-opening and strong fluorescence. In addition, other rhodamine-based fluorescent Cr^{3+} probes that function in the mixed media of organic solvent and water have been reported,^{187,188} as summarized in a recent review.¹¹



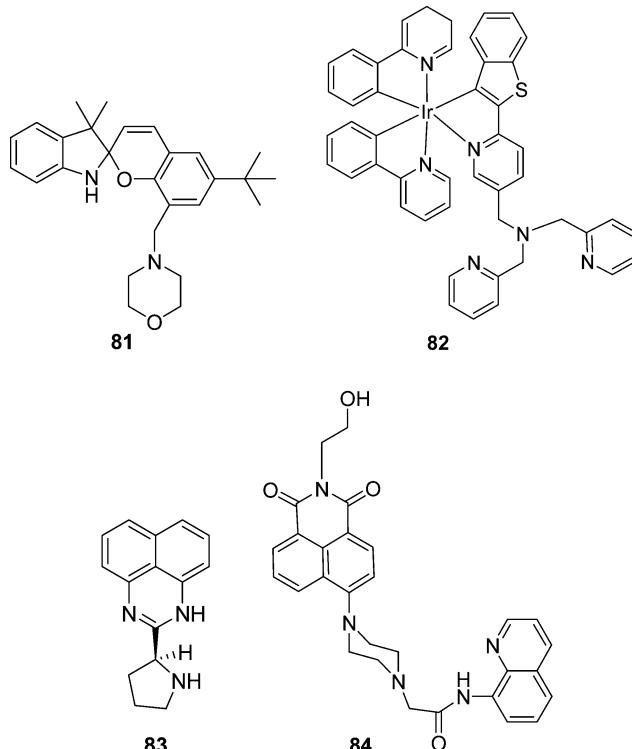
For Cu^{2+} , many probes that show reversible color and fluorescence off-on response upon complexation are designed on the basis of rhodamines.^{189–191} This has been systematically summarized in some reviews.^{11,100,192}

In addition, compound **80** has been reported as a selective NIR fluorescent probe for Cu^{2+} with a K_d of $2.7 \mu\text{M}$.¹⁹³ Probe **80** itself displayed weak fluorescence in 10 mM HEPES (pH 6.85, 1% DMSO as cosolvent) by the PET quenching mechanism. Upon addition of Cu^{2+} , more than 10-fold fluorescence enhancement was produced due to complexation-induced suppression of the PET process. With the usage of $5 \mu\text{M}$ of **80**, a detection limit of $5.0 \times 10^{-8} \text{ M}$ was obtained for Cu^{2+} . The probe can be employed to image Cu^{2+} in living cells and tissues.



Some ratiometric fluorescent Cu^{2+} probes have also been reported. For example, Yang et al. developed **81** for Cu^{2+} ($K_d = 120 \mu\text{M}$) on the basis of the metal-coordination tunable photochromism of a spirobenzopyran.¹⁹⁴ Upon addition of Cu^{2+} to the 50% ethanol–water solution of **81**, the fluorescence intensity at 475 nm decreased with the concomitant formation of a red-shifted emission centered at 640 nm. The dual fluorescence of **81** modulated by Cu^{2+} allows Cu^{2+} to be determined ratiometrically. Lippard's group reported a multi-chromophoric iridium(III) complex **82** as a phosphorescent

Cu^{2+} probe, in which the two different ligands result in two emission bands.¹⁹⁵ Probe **82** exhibits a reversible and selective complexation reaction with Cu^{2+} ($K_d = 16 \mu\text{M}$), accompanying the multimodal change in both phosphorescence intensity ratio and lifetime. **82** can be used for detecting intracellular copper ratiometrically. In another study, probe **83** was synthesized, which shows high selectivity for Cu^{2+} .¹⁹⁶ Upon complexation with Cu^{2+} ($K_d = 38.8 \mu\text{M}$), **83** also displayed a remarkable color change (from colorless to purple and then to blue) and a large red shift in emission (from 540 to 650 nm) due to the enhanced ICT effect. Very recently, a new ratiometric fluorescent probe for Cu^{2+} , **84**, has been developed via integrating a 1,8-naphthalimide fluorochrome with 8-aminoquinoline.¹⁹⁷ Probe **84** exhibits a highly selective ratiometric response to Cu^{2+} over other transition metal ions in aqueous media by formation of a 1:1 complex with Cu^{2+} ($K_d = 34.5 \mu\text{M}$). The probe has been applied for imaging intracellular Cu^{2+} in MCF-7 cells.



Other ratiometric fluorescent Cu^{2+} probes have been reported,^{198–200} which however need to use a high percentage of organic solvents due to their poor water solubility. In addition, ratiometric Cu^{2+} detections can also be achieved through replacing the central metal ion (e.g., Cd^{2+} or Zn^{2+}) in a complex by Cu^{2+} .^{201,202}

Fahrni et al. synthesized **85** (Figure 13) as a highly selective fluorescent probe for Cu^{+} ,²⁰³ which may exist in a stable state within the reducing environment of the cytosol. Reaction of **85** ($5 \mu\text{M}$) with equimolar Cu^{+} caused a 4.6-fold fluorescence enhancement via a 1:1 complex formation ($K_d = 39.8 \text{ nM}$). The probe has been used in elucidating the subcellular localization of labile intracellular copper pool, which however requires ultraviolet excitation.

Chang's group also designed a series of fluorescent probes for Cu^{+} . They first synthesized **86** (Figure 13) as a Cu^{+} probe based on the PET mechanism by combining BODIPY with a thioether-rich receptor bis{2-[2-(2-ethylthio)ethylthio]ethyl}.

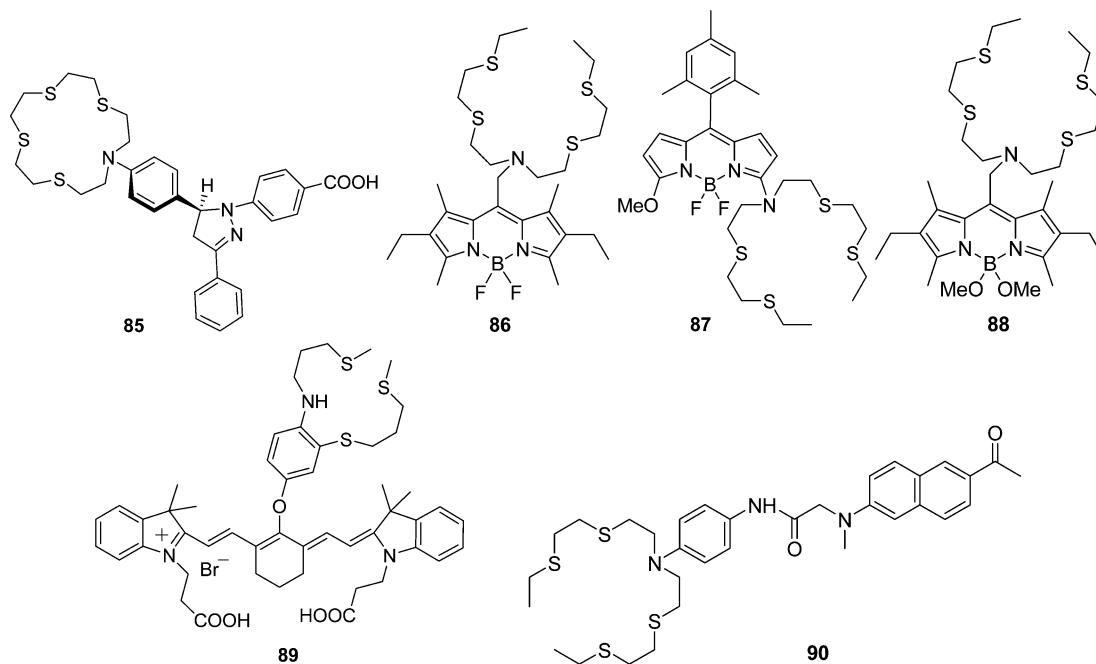


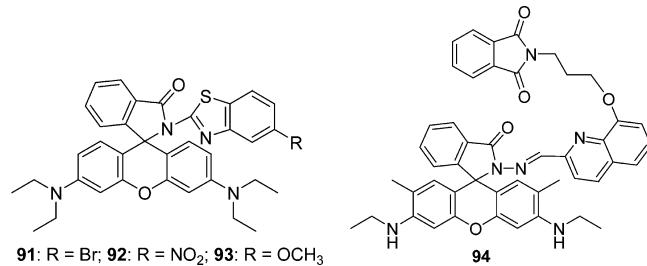
Figure 13. Structures of Cu^+ probes 85–90.

amine (BETA).^{204,205} Probe 86 features an excellent selectivity for Cu^+ over other biologically relevant metal ions (including Cu^{2+}) in water. Complexation of the probe ($2 \mu\text{M}$) with equimolar Cu^+ resulted in a 10-fold fluorescence increase. The probe has a K_{d} of 3.6 pM for Cu^+ and may be used to detect Cu^+ in living cells.²⁰⁶ Later, they proposed a ratiometric Cu^+ -specific fluorescent probe 87 by employing the same receptor but based on an ICT mechanism (Figure 13).²⁰⁷ 87 has a K_{d} of $4.0 \times 10^{-11} \text{ M}$ for Cu^+ and can detect changes in intracellular Cu^+ concentration upon exogenous copper addition. Moreover, incorporation of a triphenylphosphonium group into 87 afforded a mitochondrial-targeting probe for imaging exchangeable mitochondrial copper pools in living cells.²⁰⁸ By structural modification of 86, another Cu^+ probe (88; Figure 13) that showed much improved off-on response and quantum yield was obtained.²⁰⁹ Reaction of 88 ($4 \mu\text{M}$) with equimolar Cu^+ produced a 75-fold fluorescence increase. The probe has a K_{d} of $8.9 \times 10^{-14} \text{ M}$ and can be used to monitor labile copper pools in living cells under basal and copper-depleted conditions. Very recently, they reported an NIR fluorescent Cu^+ probe 89 (Figure 13) with a K_{d} of $3.0 \times 10^{-11} \text{ M}$.²¹⁰ In the presence of equimolar Cu^+ , the probe ($2 \mu\text{M}$) shows a 15-fold fluorescence enhancement, and this response is highly selective for Cu^+ . The probe has been used to detect exchangeable copper stores in living mice under basal, copper-overload, or copper-deficient conditions.

Cho et al. presented the first TP probe 90 for Cu^+ by using 2-methylamino-6-acetylnaphthalene as the reporter and BETA as the Cu^+ -selective receptor (Figure 13). Binding of 90 with equimolar Cu^+ (0.36 nM) gave rise to a 4-fold enhancement in fluorescence intensity. The probe has a K_{d} of 20 pM for Cu^+ and may be used to detect intracellular Cu^+ in live cells and tissues at depths more than 90 mm for long periods of time without mistargeting and photobleaching problems.²¹¹

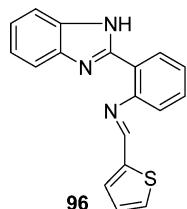
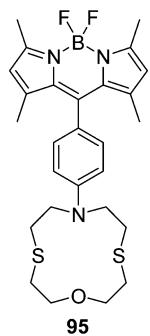
Similar to the above situation of Cr^{3+} , most of the fluorescent off-on probes for Fe^{3+} are also based on the rhodamine scaffold.^{1,192,212–221} For example, Li et al. reported three rhodamine-based probes (91–93) for selective detection of

Fe^{3+} , which all showed linear fluorescence increase with the increase of Fe^{3+} concentrations in the range of $5\text{--}20 \mu\text{M}$ with a detection limit of $5 \mu\text{M}$. Among the probes, 93 has a K_{d} of $2.21 \mu\text{M}$, and can be used to monitor Fe^{3+} in living cells.²²¹ 94 is another rhodamine-based Fe^{3+} probe, which displays a reversible fluorescence off-on response to Fe^{3+} via a 1:1 binding mode ($K_{\text{d}} = 0.91 \mu\text{M}$) with high selectivity.²²² Bioimaging studies demonstrated that 94 could also act as a fluorescent Fe^{3+} probe in living cells.



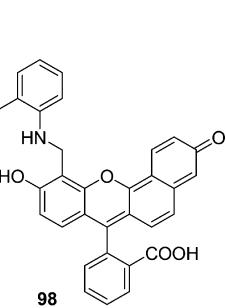
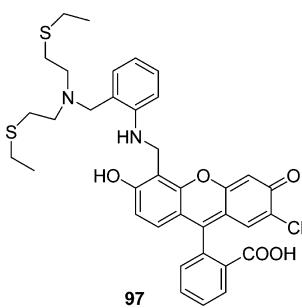
Interestingly, Bricks et al. introduced a size-restricted dithia-aza-oxa macrocycle into the BODIPY skeleton via a phenyl linker and obtained 95 as a ratiometric fluorescent probe for Fe^{3+} .²²³ In 3-(N-morpholino)propanesulfonic acid buffer (0.01 M) of pH 5.1, the probe shows a selective fluorescence response to Fe^{3+} over the other metal ions tested, but its sensitivity is not very high ($K_{\text{d}} = 100 \mu\text{M}$).

Compound 96 has been reported as a highly selective ratiometric fluorescent probe for Fe^{2+} at pH 4.0–5.0 and Fe^{3+} at pH 6.5–8.0 in acetonitrile–HEPES buffer (1:4, v/v) media.²²⁴ The probe displays a decrease in fluorescence at 412 nm but increase in fluorescence at 472 nm for Fe^{2+} and 482 nm for Fe^{3+} , which is ascribed to the formation of 1:1 ($96:\text{Fe}^{2+}$) and 2:1 ($96:\text{Fe}^{3+}$) complexes with a K_{d} of $2.58 \mu\text{M}$ for Fe^{2+} and $4.76 \times 10^{-2} \text{ M}^2$ for Fe^{3+} , respectively. In addition, the probe can discriminate Fe^{2+} from Fe^{3+} in acetonitrile–water (1:4, v/v) media by the naked eye.



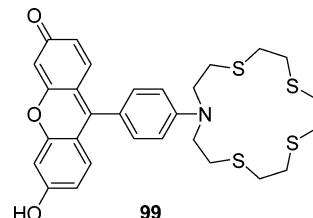
The development of chromogenic and fluorogenic Hg^{2+} probes has aroused great interest because of its high toxicity to organisms.^{6,167} Until now, most of the complexation-based reversible fluorescent probes for Hg^{2+} are designed on the basis of the PET mechanism, although some rhodamine-based probes that show reversible response to Hg^{2+} in aqueous solution were reported as well.^{6,11,225–231}

Lippard et al. reported a fluorescein-based probe 97 for the selective detection of Hg^{2+} by appending a thioether-rich receptor to the “top” xanthone moiety of a fluorescein platform.²³² The probe is almost nonfluorescent due to PET from the aniline moiety to fluorescein. Coordination of 97 to Hg^{2+} results in enhancements of both quantum yield and molar absorptivity. 97 can detect 2 ppb Hg^{2+} in buffered aqueous solution. However, both Cu^{2+} and Cu^+ interfere with the Hg^{2+} -induced fluorescence response.²³³ Later, other “top ring” substituted fluoresceins were presented, which can give fluorescence enhancement upon reacting with Hg^{2+} .⁶ However, Cu^{2+} and Ni^{2+} interfere with the detection of Hg^{2+} due to the removal of sulfur atom from the above receptor. Further, they developed ratiometric fluorescent Hg^{2+} probes based on the seminaphthofluorescein platform.^{234,235} 98 is an example of these ratiometric probes, whose selectivity for Hg^{2+} is similar to that of 97.²³⁵ Analyses of natural water samples spiked with mercuric salts indicated that 98 can rapidly complex with Hg^{2+} , demonstrating its potential utility in the field.

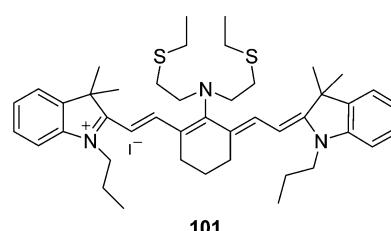
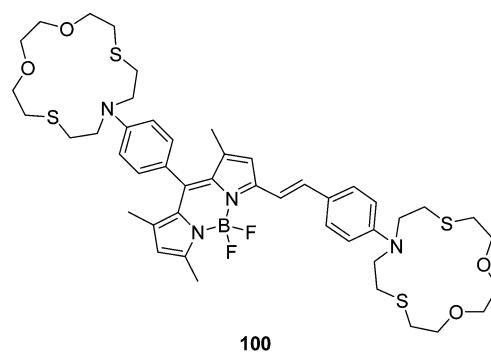


Installation of a receptor on the “bottom” ring of fluorescein or a fluorescein derivative offers another route to constructing water-soluble Hg^{2+} probes. Chang’s group synthesized such a probe 99, which contains a thioether-rich NS₄ macrocycle.²³⁶ The known preference of the thioether-rich macrocycle for Hg^{2+} over Cu^{2+} overcomes the Cu^{2+} interference observed for

many Hg^{2+} probes. Therefore, 99 exhibits excellent selectivity for Hg^{2+} over environmentally relevant metal ions. Complexation of 99 with Hg^{2+} (2 equiv, 2 μM) affords >170-fold fluorescence enhancement. The probe has a wide linear range (0.1–8 ppm) for Hg^{2+} assay with a detection limit of 60 nM, and can be employed to detect Hg^{2+} extracted from fish samples that were digested with microwave irradiation.

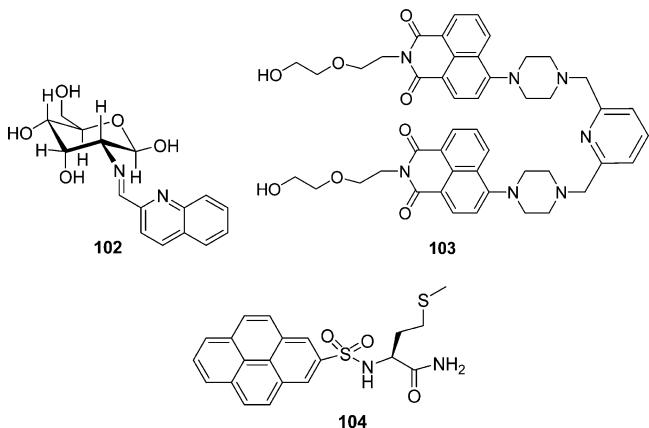


Other water-soluble fluorescent Hg^{2+} probes have also been reported.^{237–239} For instance, a BODIPY-based probe (100) with two dithiadioxaaza macrocycles was designed for the ratiometric detection of Hg^{2+} in aqueous solution by both fluorescence and color changes.²³⁸ Job’s plots indicated formation of a 1:2 100– Hg^{2+} complex ($K_d = 7.82 \times 10^{-10} \text{ M}^2$), where each Hg^{2+} binds per macrocycle, and both PET and ICT were invoked to rationalize the fluorescence response of 100 to Hg^{2+} . In another case, an NIR fluorescent Hg^{2+} probe 101 was synthesized.²³⁹ Probe 101 exhibits fluorescence increase upon binding of Hg^{2+} based on the inhibition of the PET quenching mechanism, and its applicability is demonstrated by monitoring the real-time uptake of Hg^{2+} within HepG2 cells and 5-day-old zebrafish.

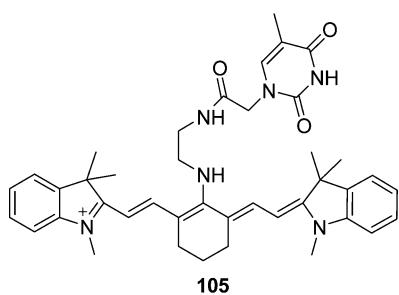


Incorporation of water-soluble units (e.g., sugar, polyamide) into a fluorochrome is an effective measure to afford water-soluble fluorescent probes.^{240–243} For example, the introduction of D-glucosamine into quinoline via the formation of Schiff base yields 102, which can complex with Hg^{2+} in a 1:1 mode in aqueous solution, resulting in fluorescence enhancement.²⁴⁰ By combining two 2-hydroxyethoxy groups with two aminonaphthalimides via 2,6-bis(aminomethyl)pyridine, the obtained compound 103 shows high selectivity for Hg^{2+} in aqueous Tris-HCl [tris(hydroxymethyl)aminomethane hydrochloride] buffer of pH 7 containing 10% (v/v) ethanol.²⁴¹ In addition, the usage

of amino acids as the receptors usually gives probes with high water solubility.^{244–246} For instance, a pyrene derivative containing methionine (**104**) has been reported as a fluorescent Hg²⁺ probe in aqueous solution.²⁴⁶ Upon Hg²⁺ binding ($K_d = 1.28 \times 10^{-13} \text{ M}^2$), **104** exhibits a considerable excimer emission at 480 nm along with a decrease of monomer emission at 383 nm, which allows selective and sensitive ratiometric detection of Hg²⁺ without any interference from other metal ions.

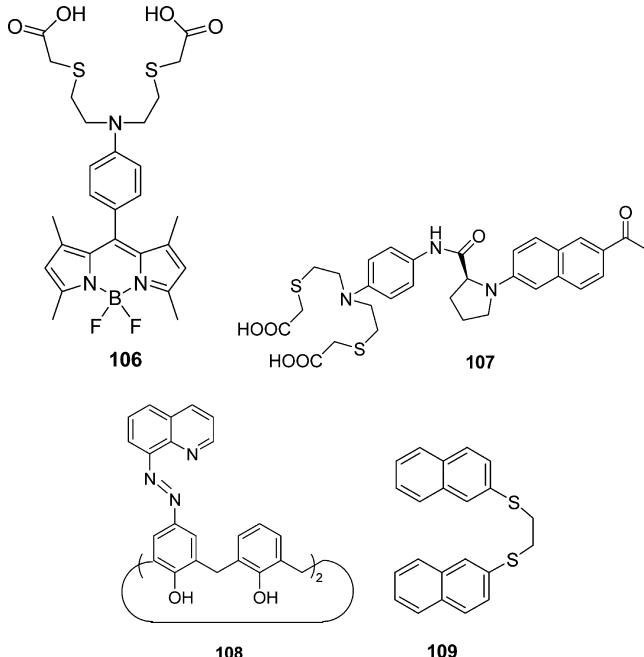


On the basis of the thymine–Hg²⁺–thymine binding mode,²⁴⁷ a ratiometric fluorescent Hg²⁺ probe **105** was developed with heptamethine cyanine as a fluorochrome.²⁴⁸ The binding of thymine to Hg²⁺ induced cyanine aggregation in aqueous solution, causing a distinct increase in the emission ratio ($I_{537 \text{ nm}}/I_{714 \text{ nm}}$), thus allowing Hg²⁺ to be determined ratiometrically with a detection limit of 4.8 nM.

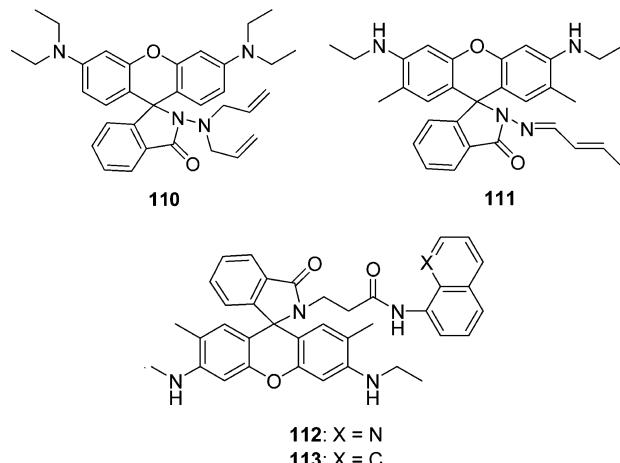


Chromogenic and fluorogenic probes for Ni²⁺, Pd²⁺, and Pt²⁺ have been reported occasionally. Chang's group synthesized **106** as a selective, water-soluble fluorescent off-on probe for Ni²⁺ by using BODIPY as the fluorochrome and *N,N*-bis[2-(carboxymethyl)thioethyl]amine (CTEA) as the Ni²⁺ chelator.²⁴⁹ **106** features visible wavelength spectral profiles and a ca. 25-fold fluorescence increase upon Ni²⁺ binding ($K_d = 193 \mu\text{M}$). Confocal microscopy experiments showed that this probe can reliably monitor changes in Ni²⁺ levels within living cells. Kim et al. linked CTEA to 2-acetyl-6-dialkyl-aminonaphthalene and synthesized a TP fluorescent probe (**107**) for Ni²⁺.²⁵⁰ **107** shows 26-fold TP-excited fluorescence enhancement in response to Ni²⁺ with a dissociation constant of $K_d = 89 \mu\text{M}$, and can selectively detect Ni²⁺ in live cells and fish organs at depths of 80–150 μm by TP microscopy. Ma's group developed a calix[4]arene-based colorimetric Ni²⁺ probe **108**, in which the quinolylazo group serves as a Ni²⁺ receptor, and 8-aminoquinoline as a chromogenic moiety.²⁵¹ Reaction of **108** with Ni²⁺ forms a 1:1 complex ($K_d = 3.45 \times 10^{-7} \text{ M}$), accompanying a large bathochromic shift in the absorption peak from 400 to 580 nm ($\Delta\lambda = 180 \text{ nm}$). **108** can be used to

detect Ni²⁺ selectively in aqueous solution without interference from common transition metal ions such as Ag⁺, Cd²⁺, Cu²⁺, Fe³⁺, Hg²⁺, and Zn²⁺. In addition, a ratiometric fluorescent probe (**109**) for Ni²⁺ was reported on the basis of the Ni²⁺-induced intramolecular excimer formation of a naphthalene fluorochrome in DMSO–water (1:1, v/v) medium,²⁵² and the probe has been utilized for the detection of Ni²⁺ in plant cells.

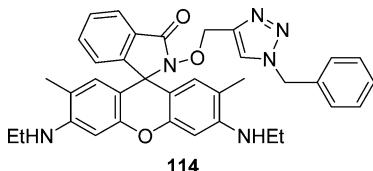


For Pd²⁺, several rhodamine-based probes (**110**–**113**) have been reported, which are designed by utilizing the specific complexation of Pd²⁺ with the probes as well as the reversible ring-opening of spirolactam.^{11,253–255}



There is only one reversible rhodamine-based probe **114** for Pt²⁺ in the literature.²⁵⁶ The probe, containing a dual binding unit (a hydroxamate and a triazole), shows a high selectivity and sensitivity toward Pt²⁺ over a wide range of other metal ions in water.

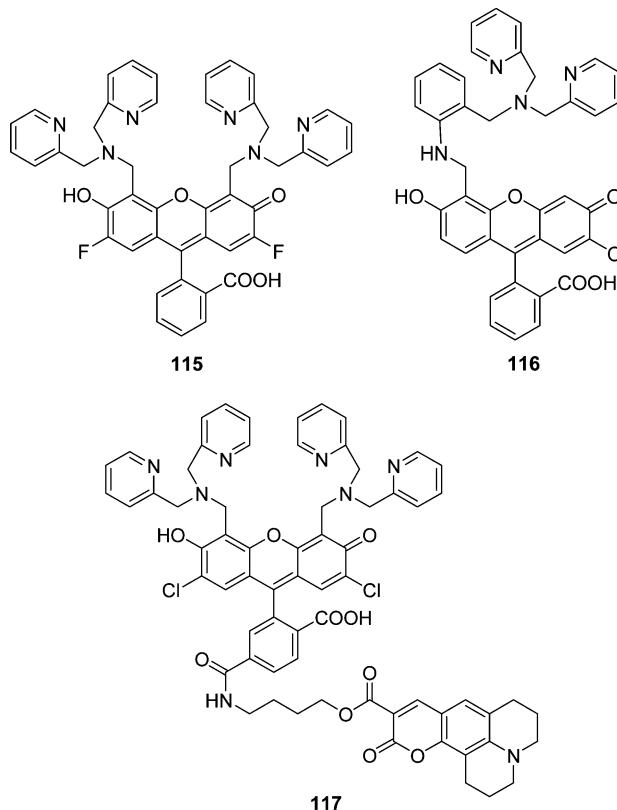
In the past decade, significant advances have been made in the design of fluorescent probes for Zn²⁺,^{5,91,171,192,257–259} because this metal ion is of great interest in the field of neurobiology.^{260–262} As mentioned above, DPA is one of the most commonly used receptors for Zn²⁺. Other N-containing ligands, such as quinoline, iminodiacetic acid, acyclic and cyclic



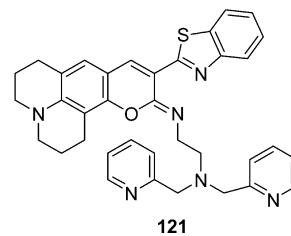
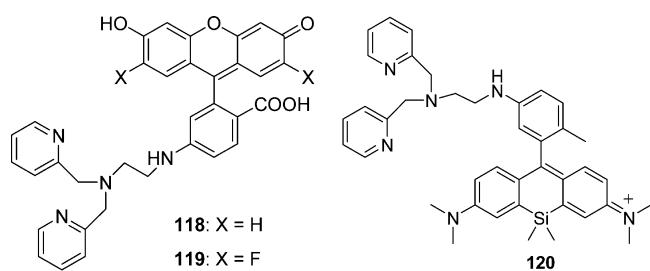
polyamines, bipyridine, or Schiff bases, have also been adopted as Zn^{2+} -receptors.¹⁶⁷ In the following section, the fluorescent Zn^{2+} probes are reviewed according to the type of receptors.

DPA and its derivatives have been proved to be highly selective for Zn^{2+} over alkali and alkaline-earth metal ions (e.g., Na^+ , K^+ , Ca^{2+} , and Mg^{2+}) that exist at much higher concentrations in biological samples; therefore, they are widely employed as receptors to develop Zn^{2+} fluorescent probes.^{157,263–268} For example, with DPA as the receptor, Lippard's group designed a series of fluorescein-based Zn^{2+} probes, which work via the PET mechanism.^{91,192,269–275} Two representatives of these probes are **115** and **116**, which are of high affinity for Zn^{2+} . **115** is synthesized by incorporating two fluorine atoms into fluorescein, and the introduction of the F atoms reduces the pK_a to 6.8 as well as the fluorescence quantum yield to $\Phi = 0.15$.²⁷¹ Zn^{2+} binding ($K_d = 0.8 \text{ nM}$) causes a 6-fold increase in fluorescence ($\Phi = 0.92$). Moreover, probe **115** is cell-permeable and capable of imaging endogenous Zn^{2+} pools in brain systems. The incorporation of an aromatic amine into the probe structure (**116**) can also reduce the background fluorescence ($\Phi = 0.06$).²⁷⁵ Complexation of **116** with Zn^{2+} ($K_d = 0.65 \text{ nM}$) leads to fluorescence increases by ca. 6-fold ($\Phi = 0.34$). Probe **116** has been used to selectively image Zn^{2+} -damaged neurons in tissue slices.²⁷⁶ Replacing one of the pyridine arms with a pyrazine in the chlorine-substituted fluorescein yields a probe exhibiting higher signal/background ratio, and such a probe has been applied to monitor changes in mobile zinc from prostate during progression of prostate cancer in mouse.^{277–279} Lippard's group also synthesized some ratiometric Zn^{2+} probes with the DPA receptor.^{280,281} For example, probe **117** was prepared by incorporating a Zn^{2+} -insensitive coumarin moiety into a Zn^{2+} -sensitive fluorescein moiety that bears a DPA receptor.^{270,280} **117** is membrane-permeable and essentially nonfluorescent ($\Phi = 0.04$). However, treatment of **117** with porcine liver esterase in vitro leads to hydrolysis of the ester linkage, yielding a coumarin moiety and a fluorescein moiety with the DPA receptor. The former shows a fluorescence at $\lambda_{\text{ex/em}} = 445/488 \text{ nm}$, which is not sensitive to Zn^{2+} , whereas the latter binds with Zn^{2+} ($K_d = 0.25 \text{ nM}$) causing a large change in its fluorescence at $\lambda_{\text{ex/em}} = 505/534 \text{ nm}$. As a result, the fluorescence intensity ratio of $I_{534 \text{ nm}}/I_{488 \text{ nm}}$ changes from 0.5 in the absence of Zn^{2+} to 4.0 in the presence of a 2-fold excess of Zn^{2+} , which permits Zn^{2+} to be imaged ratiometrically in live cells.

By introducing DPA to the bottom aromatic ring, Nagano et al. also developed a series of fluorescein-based Zn^{2+} probes.^{157,282,283} **118** and **119** are two representatives of these probes.²⁸² Because of the PET effect, **118** displays low background fluorescence ($\Phi = 0.02$) and large fluorescence enhancement (51-fold) upon complexation with Zn^{2+} ($K_d = 2.7 \times 10^{-9} \text{ M}$). Fluorinated product (**119**) of **118** has an improved signal/background ratio (60-fold; $K_d = 5.5 \text{ nM}$).²⁸³ The diacetyl version of **119** is membrane-permeable and can image labile Zn^{2+} pools in acute rat hippocampal slices.²⁸³ The authors also developed **120** as a far-red to NIR Zn^{2+} probe with a K_d of 1.4 nM,²⁸⁴ which shows a 15-fold fluorescence enhancement in the



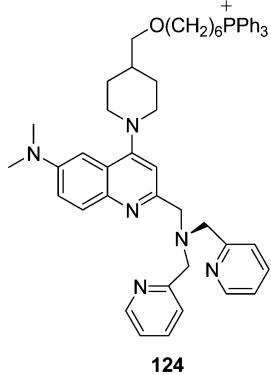
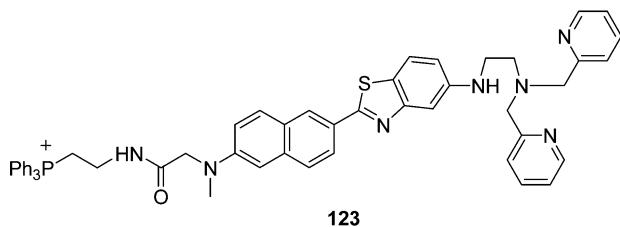
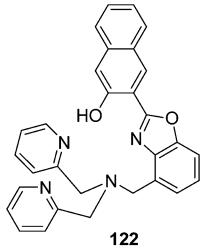
presence of Zn^{2+} via a PET mechanism. The probe has been employed for imaging Zn^{2+} in living cells. In addition, Nagano et al. have reported some ratiometric Zn^{2+} probes as well.^{96,159} For instance, the iminocoumarin-based probe **121** can function as a single-excitation, dual-emission ratiometric probe upon complexing with Zn^{2+} in aqueous media via an ICT mechanism.⁹⁶ The ratio of emission intensities ($I_{558 \text{ nm}}/I_{543 \text{ nm}}$) with $\lambda_{\text{ex}} = 513 \text{ nm}$ varies from 0.8 in the absence of Zn^{2+} to 1.9 in the presence of equimolar Zn^{2+} (5 μM). The probe is highly sensitive to Zn^{2+} ($K_d = 1.3 \text{ pM}$) and has been used to image endogenous Zn^{2+} in living HEK293 cells and rat hippocampal slices.



Other Zn^{2+} probes featuring off-on or ratiometric fluorescence response with DPA or its derivatives as receptors have also been reported.^{285–289} For example, Kwon et al. synthesized **122** as a Zn^{2+} probe by connecting 2-(2'-hydroxy-

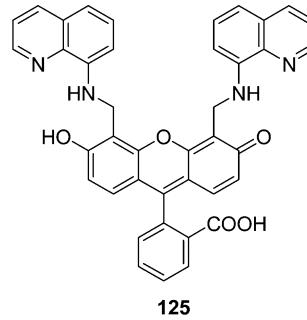
3'-naphthyl)benzoxazole to the DPA receptor through a methylene bridge.²⁸⁵ In pH 7.0 buffer, **122** (10 μM) displays a 44-fold fluorescence enhancement for Zn^{2+} ($K_{\text{d}} = 12 \text{ pM}$), which is ascribed to the suppression of PET. Moreover, the fluorescence response of **122** to Zn^{2+} is independent of pH over a broad range. The probe has been utilized to visualize endogenous Zn^{2+} during apoptosis in live cells.

Incorporating triphenylphosphonium salt (an effective mitochondrial targeting group) into naphthalene skeleton affords a TP Zn^{2+} probe **123** with a K_{d} of 3.1 nM, which can be used to detect mitochondrial Zn^{2+} in a rat hippocampal slice at a depth of 100–200 μm without interference from other metal ions.²⁹⁰ Another mitochondrial targeting probe **124** was proposed by Jiang's group.²⁹¹ The probe can bind with Zn^{2+} in aqueous media with a K_{d} of 0.45 nM and has been used for ratiometric imaging of mitochondrial Zn^{2+} in NIH-3T3 cells.

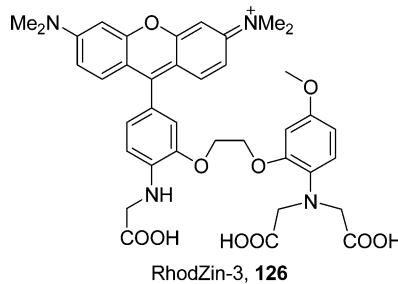


Quinoline can also be utilized as a Zn^{2+} receptor.^{292–298} For example, Lippard's group reported a fluorescein derivative **125**, which contains two 8-aminoquinoline units and can serve as a Zn^{2+} probe.²⁹⁵ The combination of quinoline and fluorescein provides probe **125** with a lower Zn^{2+} affinity ($K_{\text{d}} = 41 \mu\text{M}$) than the DPA-based probe ($K_{\text{d}} = 2.7 \text{ nM}$), which means that **125** can detect higher concentrations of mobile Zn^{2+} reversibly without probe saturation.

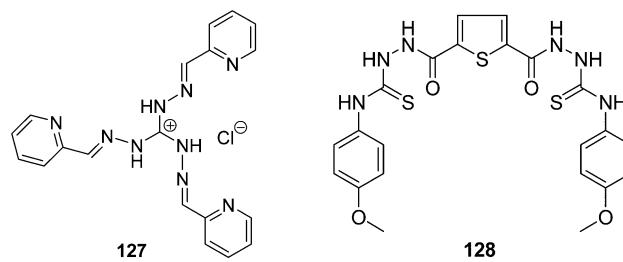
Several Zn^{2+} probes, such as the FluoZin and RhodZin, are constructed on the basis of the scaffold of Ca^{2+} probes.^{1,33,299} For instance, the rhodamine-based probe RhodZin-3 (**126**) has one acetic acid removed from the BAPTA structure, resulting in greatly reduced affinity for Ca^{2+} but increased affinity for Zn^{2+} ($K_{\text{d}} = 65 \text{ nM}$).³⁰⁰ As compared to the above fluorescein-based



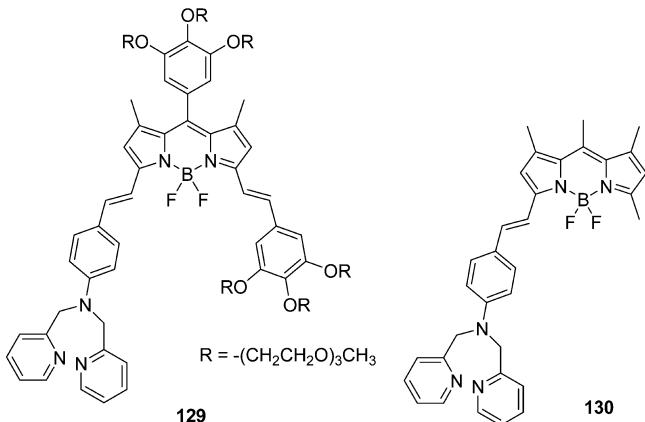
counterpart (FluoZin-3, Figure 7), **126** allows Zn^{2+} detection at longer wavelengths with a 75-fold fluorescence increase.



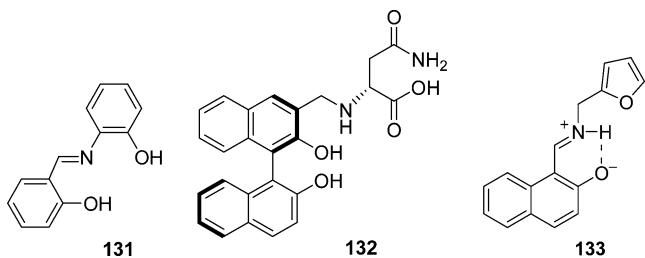
In addition to the above receptors, acyclic and cyclic polyamines,^{301–307} bipyridines,^{308,309} Schiff bases,^{310,311} and other ligands^{99,160,161,312–319} have been exploited as alternative receptors in the development of Zn^{2+} probes as well. For example, Zhang et al. presented a C_3 -symmetric Schiff base (**127**) as a highly selective Zn^{2+} probe.³¹⁶ **127** features a large Zn^{2+} -induced red emission shift and a distinguishable color change under physiological conditions when Zn^{2+} was present. With the usage of 50 μM **127**, a detection limit of $2.5 \times 10^{-6} \text{ M}$ was obtained for Zn^{2+} . The probe has been successfully applied to the detection of intracellular Zn^{2+} . In a recent study, *N*-amidothioureas containing electron-donating methoxy moieties, such as **128**, have been demonstrated for recognition of Zn^{2+} with high selectivity and sensitivity in $\text{DMSO}/\text{H}_2\text{O}$ (1:99, v/v).³¹⁷ In addition, Lippard's group developed some fluorescent Zn^{2+} probes by designing other novel receptors, which have been summarized in a recent account.⁹¹



It should be pointed out that many Zn^{2+} probes also show fluorescence response to Cd^{2+} . However, Cd^{2+} would have little influence on Zn^{2+} detection *in vivo* because it exists at very low concentrations.³²⁰ Interestingly, changing the solution composition could make the probes with very similar structures and the same receptor display distinct selectivities for Zn^{2+} and Cd^{2+} . For example, probe **129** is selective for Zn^{2+} in the mixed solution of ethanol–water (5:95, v/v; 0.1 M HEPES, pH 7.2),³²¹ whereas probe **130** is selective for Cd^{2+} in the mixed solution of acetone–water (9:1, v/v; 0.01 M Tris-HCl, pH 7.4).¹⁶²

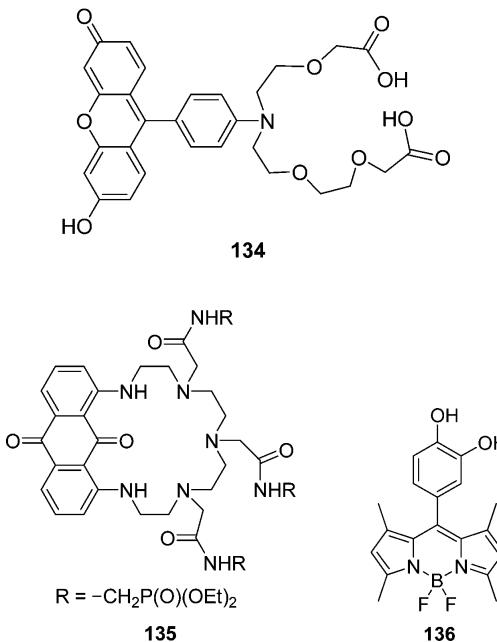


3.1.1.3. Probes for Other Metal Ions (Al^{3+} and Pb^{2+}). There are very few chromogenic and fluorogenic Al^{3+} probes.^{322–324} Unfortunately, they all need organic solvents as a cosolvent. For example, Kim et al. developed a salicylimine-based probe (**131**) for Al^{3+} .³²⁴ In methanol–water (1:1, v/v) solution, Al^{3+} could selectively complex with **131**, resulting in color change and fluorescence enhancement at 510 nm. The probe can be applied to image the intracellular distribution of Al^{3+} . **132** is another fluorescent Al^{3+} probe,³²⁵ which shows a selective 11.8-fold fluorescent enhancement toward Al^{3+} in 99% aqueous solution (1% CH₃OH, pH 5.0). **133** exhibits a ca. 11-fold fluorescence enhancement toward equimolar concentration of Al^{3+} in HEPES buffer (1% DMSO, pH 7.4),³²⁶ which may be ascribed to the complexation-induced restriction of ICT and rotation in the molecule. **133** has been used for detection of intracellular Al^{3+} by fluorescence microscopy.



Several complexation-based probes for Pb^{2+} have been reported.^{327–329} **134** was designed by combining fluorescein with the dicarboxylate pseudocrown receptor.³²⁸ The probe showed selective fluorescence turn-on response toward Pb^{2+} ($K_d = 23 \mu M$) in aqueous solution and has been used to trace the change in Pb^{2+} levels within living cells. A 1,8-diaminoanthraquinone derivative **135** was reported as a colorimetric probe for visual detection of Pb^{2+} in water at neutral pH.³²⁹ Addition of Pb^{2+} to the solution of **135** induced a dramatic color change with the maximum absorption wavelength blue-shifted by 47 nm, suggesting a strong complexation of Pb^{2+} with **135**. Another colorimetric Pb^{2+} probe **136** was synthesized by incorporating catechol into the BODIPY skeleton,³³⁰ which displays a selective and sensitive colorimetric response (from yellow to pink) to Pb^{2+} in aqueous media.

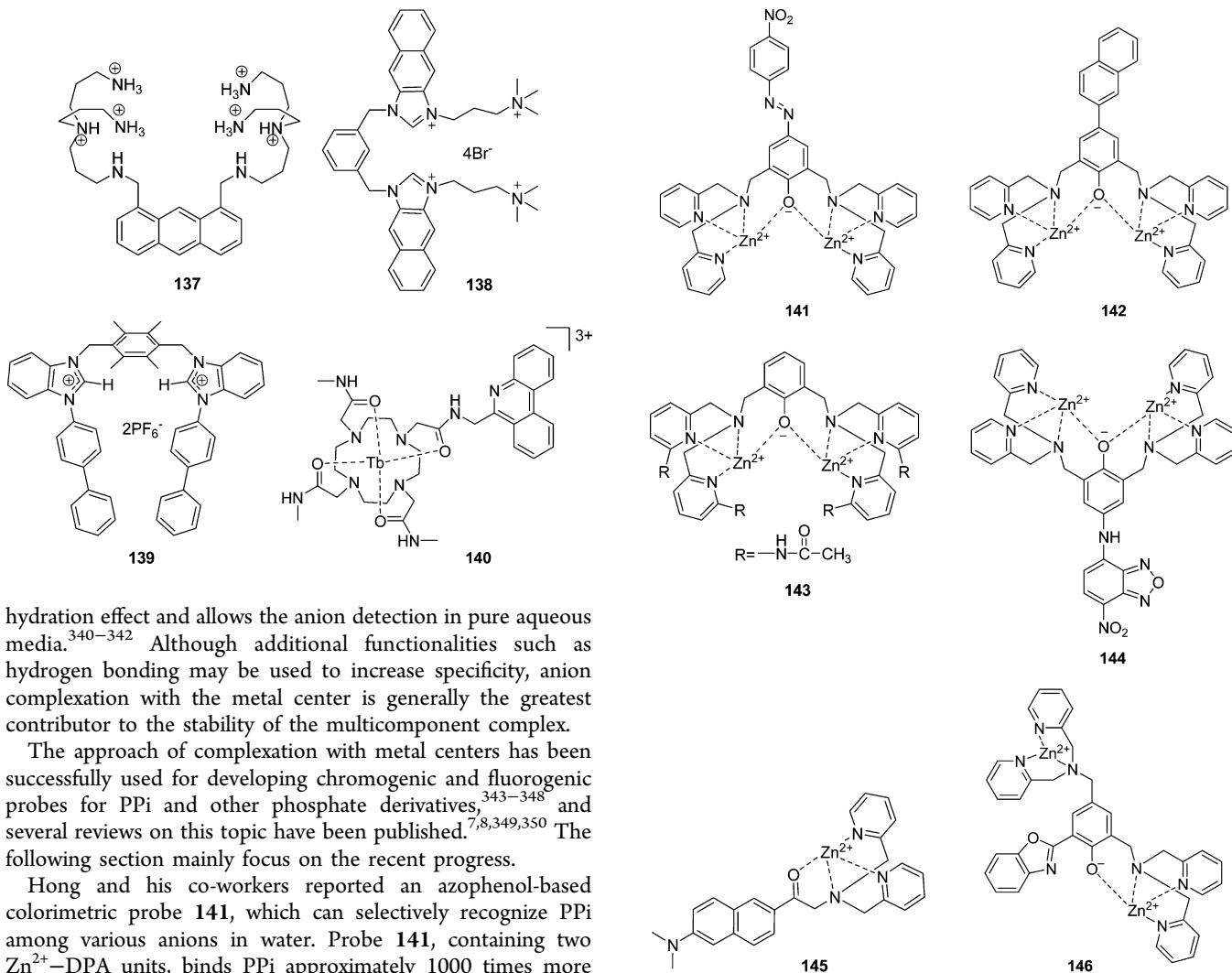
3.1.2. Probes for Anions. The design of chromogenic and fluorogenic probes with high sensitivity and selectivity for anions is a challenging project because of the strong hydration effects of anions in an aqueous system. Toward this end, different interactions (mainly including electrostatic interac-



tions and complexation with metal centers) between receptors and analytes have been exploited.

Electrostatic interactions between anions and positively charged receptors such as polyamines and imidazolium groups are often used to construct chromogenic and fluorogenic probes.^{331–336} Polyamines can be made in a variety of shapes (open chain, cyclic, branched, etc.) and thus have been widely employed as anion receptors. Furthermore, the good water solubility of polyamines makes the resulting probes suitable for aqueous environments.⁸⁹ Compound **137** is a typical fluorescent probe containing polyamine receptor, which complexes with pyrophosphate (PPi) in 1:1 mode in aqueous media causing fluorescence enhancement. Importantly, **137** can distinguish PPi from phosphate on the basis of electrostatic interactions and matchable size.³³² Compound **138** was constructed by combining two naphthoimidazolium groups with two quaternary ammonium groups, which displays a selective fluorescence enhancement for ATP (adenosine triphosphate) in pure aqueous solution, although GTP (guanosine triphosphate) could quench the fluorescence.³³⁵ Compound **139** has been reported as a highly selective fluorescent probe for perchlorate.³³⁶ The probe exhibits fluorescence quenching toward perchlorate among a number of other anions, and can be used for the detection of ClO₄⁻ in drinking water. It should be pointed out that probes based on electrostatic interactions usually suffer from poor selectivity and moderate sensitivity for a particular anion because the binding affinity of receptors for analytes and other competitive species is similar and relatively low.³³⁷ Nevertheless, in some cases, the selectivity of anion recognition can be improved via the combination of electrostatic interactions and π -stacking effects, as exemplified by the luminescent probe **140**, which has been used for selective detection of adenosine nucleotides.³³⁸

Complexation with metal centers is an important approach for designing chromogenic and fluorogenic probes for anions, in which a metal ion complex is utilized as both a receptor and a reporter. The strong binding affinity of the metal ion for a negatively charged anion leads to the formation of a multicomponent complex, concomitant with the change of spectroscopic signal.³³⁹ This strategy overcomes the competing



hydration effect and allows the anion detection in pure aqueous media.^{340–342} Although additional functionalities such as hydrogen bonding may be used to increase specificity, anion complexation with the metal center is generally the greatest contributor to the stability of the multicomponent complex.

The approach of complexation with metal centers has been successfully used for developing chromogenic and fluorogenic probes for PPi and other phosphate derivatives,^{343–348} and several reviews on this topic have been published.^{7,8,349,350} The following section mainly focus on the recent progress.

Hong and his co-workers reported an azophenol-based colorimetric probe 141, which can selectively recognize PPi among various anions in water. Probe 141, containing two Zn²⁺-DPA units, binds PPi approximately 1000 times more tightly than phosphate in a wide pH range of 6.5–8.3.³⁵¹ The same group later extended this system to a fluorescent probe 142 for the detection of PPi, which exhibits a selective fluorescence increase upon complexation with PPi instead of ATP.³⁵² This is the first example of a metal complex that can discriminate PPi from ATP in aqueous solution. In addition, Hong et al. also examined the synergistic effect of metal coordination and hydrogen bonding by synthesizing 143 as a colorimetric probe for PPi detection in water.³⁵³ The formation of four hydrogen bonds between the amide groups and PPi in 143 results in a greatly improved binding affinity ($K_d = 20 \text{ pM}$) as compared to probe 141 ($K_d = 15 \text{ nM}$).

On the basis of Zn²⁺-DPA complexes, other fluorescent probes for PPi recognition were also developed. For example, an NBD-phenoxy-bridged dinuclear Zn²⁺ complex (144) is found to be an effective colorimetric probe for PPi in aqueous solution over a wide pH range.³⁵⁴ The probe shows high binding affinity (association constant $K_a \approx 3 \times 10^8 \text{ M}^{-1}$) and high selectivity for PPi. The mononuclear Zn²⁺-DPA complexes (145) with an auxiliary ligand can discriminate PPi over ATP with high selectivity, which is ascribed to the synergistic effect of metal coordination and π - π stacking interactions.³⁵⁵

Ratiometric fluorescent PPi probes with two Zn²⁺-DPA units have also been reported.^{356,357} For example, complexation of probe 146 with PPi ($K_a = 9.2 \times 10^7 \text{ M}^{-1}$) in 10 mM HEPES buffer of pH 7.4 shows a large bathochromic shift in

fluorescence from 420 to 518 nm via excited-state intramolecular proton transfer.³⁵⁸ The probe has been applied to detect PPi released from a PCR experiment.

Complex 147 is a spiropyran derivative, which bears a Zn²⁺-DPA unit and can serve as fluorescent probe for PPi (Figure 14).³⁵⁹ Interactions between 147 and PPi lead to the full

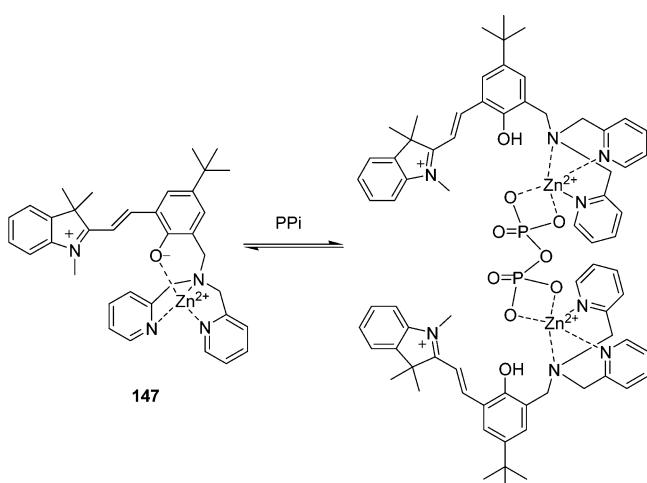


Figure 14. Complexation of 147 with PPi.

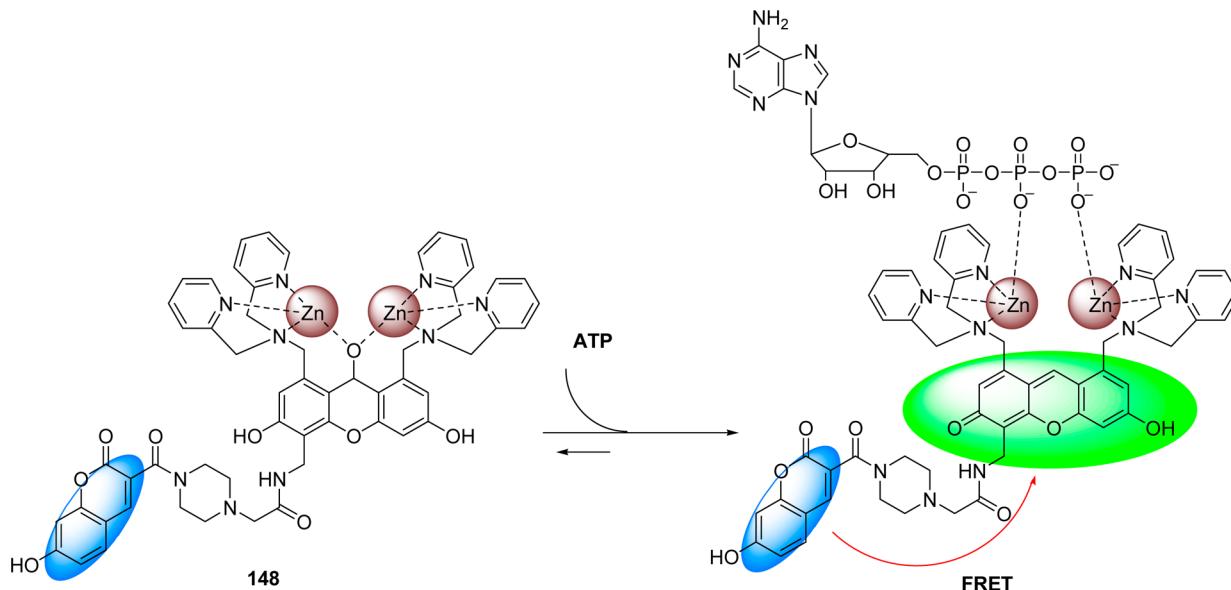
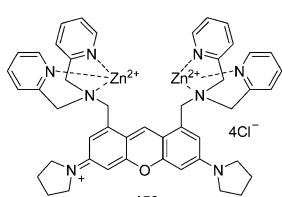
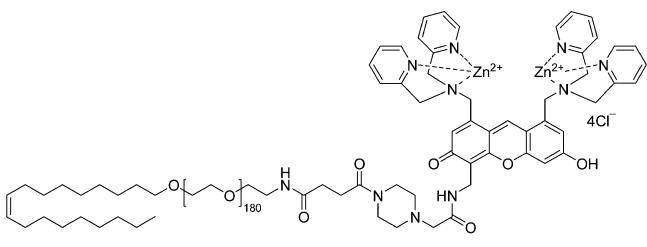


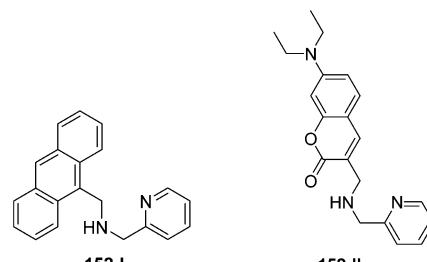
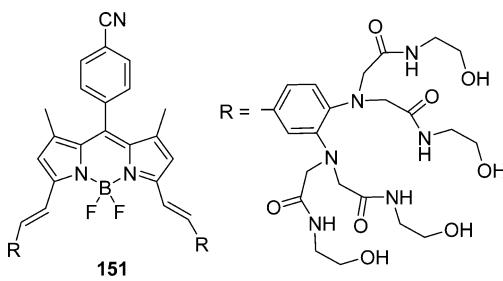
Figure 15. Complexation of ATP with **148**. Reprinted with permission from ref 362. Copyright 2010 American Chemical Society.

quenching of fluorescence at 620 nm but the recovery of fluorescence at 560 nm. The fluorescence intensity ratio, I_{560}/I_{620} , is proportional to the PPi concentration in the range from 1.0×10^{-6} to 1.0×10^{-4} M with a detection limit of 4.0×10^{-7} M, which makes the probe capable of quantitatively determining PPi in urine.

Fluorescent probes with high selectivity for polyphosphates such as ATP have also been proposed on the basis of the Zn^{2+} -DPA unit.^{347,360–362} For instance, **148** with two Zn^{2+} -DPA units was synthesized as a ratiometric polyphosphate probe based on binding-induced modulation of FRET between coumarin and xanthene skeleton (Figure 15).³⁶² Upon binding to polyphosphates in aqueous solution, **148** exhibits a clear dual-mission signal change at 454/525 nm, while no detectable emission change is observed with monophosphates and phosphodiester species as well as various other anions. The probe has been used for real-time fluorescence monitoring of enzyme reactions and ratiometric visualization of ATP in living cells. Recently, two more fluorescent probes (**149** and **150**) with the same recognition unit were reported for the detection of nucleoside polyphosphates on plasma membrane surfaces (**149**) and in mitochondria (**150**), respectively.³⁶³



Aside from the Zn^{2+} -DPA system, other ligands such as polyammonium macrocycles and quinoline have been employed for the construction of Zn^{2+} complexes in selective sensing of phosphate derivatives.^{342,348,364–367} In addition, the complexes from Cu^{2+} and Cd^{2+} have been utilized as well for developing chromogenic and fluorogenic probes for phosphate derivatives.^{356,368–372} For example, the complex of **151** with Cd^{2+} can be used to detect PPi selectively and sensitively in water.³⁷² The ternary complex (**151-I/151-II/Cu²⁺**) can serve as a selective ratiometric fluorescent probe for ATP in aqueous solution.³⁷³



3.2. Competitive Displacement Complexation

In the strategy of competitive displacement complexation, as mentioned above, a receptor-reporter ensemble is used as a probe, and the receptor binds an analyte more strongly than the reporter, thus causing the release of the reporter and an irreversible change of spectroscopic signal.^{7,88,340,374} In fact, this strategy resembles the classical compleximetric titration in analytical chemistry, where EDTA is often used as a

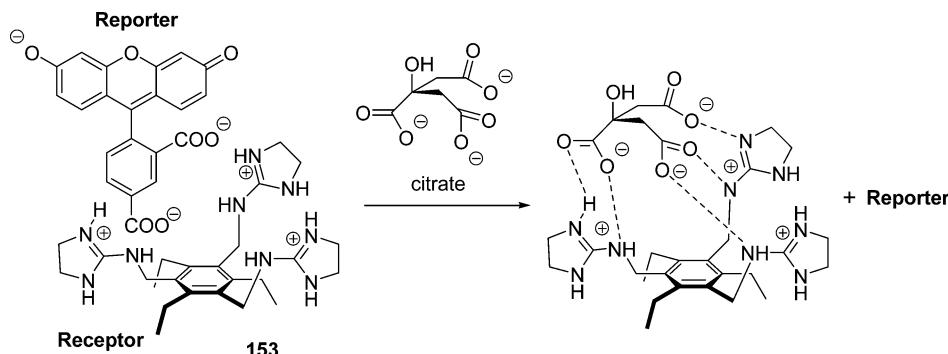
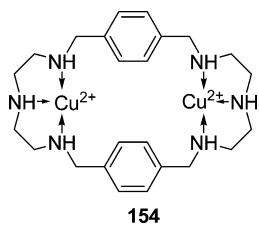


Figure 16. The chemosensing ensemble for the detection of citrate.

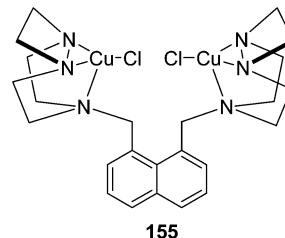
titrant.^{375,376} For example, the dye Eriochrome Black T binds Mg^{2+} forming a red complex at pH 10, and at the end point EDTA can seize Mg^{2+} in the complex, releasing Eriochrome Black T and thus producing a blue color.³⁷⁷ Competitive displacement complexation has led to the development of various chemosensing ensembles for analytes.^{88,374}

Ansyn et al. proposed the first colorimetric probe for detecting citrate in aqueous medium.³⁷⁸ The probe was constructed by assembling the receptor containing three guanidinium groups (**153**) and reporter (5-carboxyfluorescein), as depicted in Figure 16. Binding between the receptor and reporter lowers the pK_a of the phenol moiety of the reporter, causing its deprotonation. Upon reaction of the probe with citrate, the reporter is released as a phenol-protonated species via competitive displacement complexation, and a decrease in the reporter's fluorescence is observed, which allows the quantitative detection of citrate. The probe is selective for citrate over simple dicarboxylic and monocarboxylic acids in water and has been used to determine citrate concentration in commercial beverages. Later, they reported other chemosensing ensembles for detecting various organic or inorganic substances, such as tartrate,³⁷⁹ phosphate,³⁸⁰ amino acids,^{381,382} and carboxylates.³⁸³

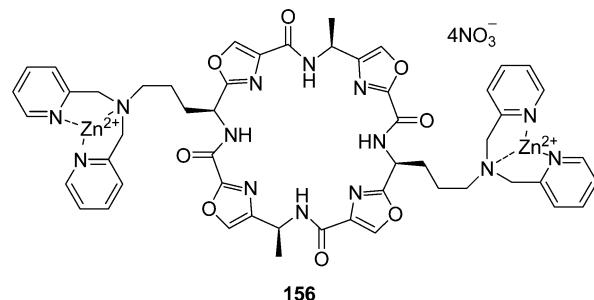
Fabbrizzi and his co-workers further exploited the application of metal complexes in the field of chemosensing ensembles.³⁸⁴ They utilized the Cu^{2+} complex **154** as the receptor and fluorescein derivatives as reporters to prepare chemosensing ensembles for PPi assays in neutral aqueous solution. The formation of the ensembles ($K_a = 1.0 \times 10^7 M^{-1}$) completely quenched the fluorescence of fluorescein derivatives. However, reaction of the ensembles with PPi ($K_a = 1.0 \times 10^8 M^{-1}$) restored the fluorescence, and no interference was observed from other anions including phosphate. Similar strategies have been employed by the same group for developing other chemosensing ensembles for amino acids,³⁸⁵ dicarboxylates,³⁸⁶ and guanosine monophosphate.³⁸⁷



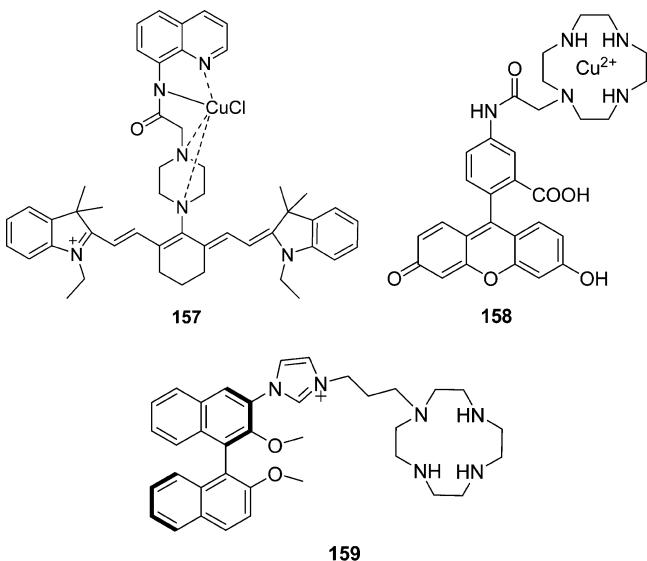
Another dinuclear Cu^{2+} compound **155** can complex with eosine Y yielding an ensemble ($K_a = 5.6 \times 10^4 M^{-1}$), which has been used for the sensitive and selective fluorescence detection of oxalate ($K_a = 1.3 \times 10^5 M^{-1}$) in neutral aqueous solution.³⁸⁸



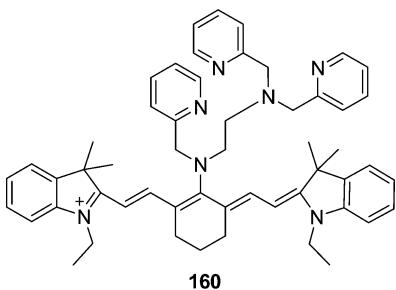
Zn^{2+} -DPA units have also been used to construct the corresponding ensembles for PPi assay.^{7,8,349,350} For example, introduction of two Zn^{2+} -DPA units into a cyclic peptide produces **156**,³⁸⁹ and **156** complexes with 6,7-dihydroxy-4-(sulfomethyl)-coumarin forming an ensemble ($K_a = 1 \times 10^5 M^{-1}$), which can be used to detect PPi ($K_a = 1 \times 10^8 M^{-1}$) in water with high selectivity.



Spectroscopic responses in the following examples are based on anion-induced demetalation. Compound **157**, a Cu^{2+} complex, was designed for detection of sulfide.³⁹⁰ Its response mechanism is based on the fact that the fluorescence of the tricarbocyanine skeleton can be almost completely quenched by Cu^{2+} , and sulfide can complex with Cu^{2+} to form CuS precipitate (solubility product constant, $K_{SP} = 1.27 \times 10^{-36}$), releasing the tricarbocyanine. As a result, **157** showed a significant fluorescence increase in the presence of sulfide in aqueous media. Compound **158** was reported as a fluorescent probe for H_2S .³⁹¹ When H_2S binds to Cu^{2+} in **158**, the fluorescein moiety would be released resulting in fluorescence enhancement. High concentrations of reduced glutathione (GSH) do not interfere with the detection of H_2S , and the probe has been applied for fluorescence imaging of H_2S in live cells. **159** is a water-soluble fluorescent probe, which can form a 2:1 (**159**: Cu^{2+}) complex with Cu^{2+} in HEPES buffer solution (pH 7.4)³⁹² and may have a potential for detecting sulfide based on the same anion-induced demetalation. Similarly, other Cu^{2+} complexes have been employed for developing fluorescent probes for sulfide.^{393–395}



The high affinity of CN⁻ for Cu²⁺ has been used to design chromogenic and fluorogenic probes for CN⁻ detection.^{396–398} Binding of Cu²⁺ to 160 forms a complex, which can further react with CN⁻ causing the removal of Cu²⁺ from the 160–Cu²⁺ complex and the NIR fluorescence enhancement.³⁹⁷ This system has been used to determine CN⁻ produced by *P. aeruginosa* in *C. elegans*.



In addition, on the basis of the anion-induced demetalation, other metal complexes have been used as chemosensing ensembles for the detection of cysteine (Cys),^{399,400} I⁻,^{401,402} F⁻,⁴⁰³ PPi,^{404–406} and amino acids.⁴⁰⁷

The displacement of simple ligands (e.g., H₂O and Cl⁻) in some of the metal ion complexes by an analyte could also produce the change of spectroscopic signal.^{366,408,409} Wolfbeis and co-workers creatively developed such a system based on a europium complex,⁴¹⁰ in which H₂O can be replaced by H₂O₂ to form a strongly luminescent complex without the occurrence of a redox reaction. This system can be used to detect H₂O₂ produced in oxidase-catalyzed reactions. Similarly, 161 can serve as a colorimetric probe for CN⁻ via the substitution of H₂O.⁴¹¹

Lippard et al. reported several NO probes, which are designed on the basis of iron cyclam,⁴¹² ruthenium porphyrin,⁴¹³ and dirhodium tetracarboxylate complexes.⁴¹⁴ In these probes, the fluorescence of a fluorochrome is quenched by a paramagnetic transition metal ion, and reaction of the probes with NO forms a metal–NO adduct, causing the fluorochrome release and thus fluorescence enhancement (e.g., 162⁴¹³ in Figure 17). However, these NO probes are not ideal for in vivo applications because of their low stability or poor water solubility.^{415,416}

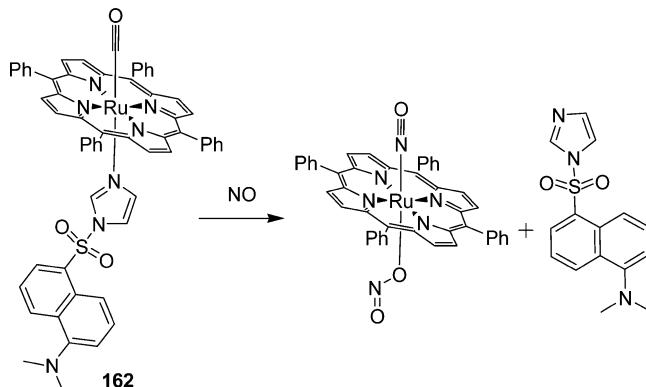
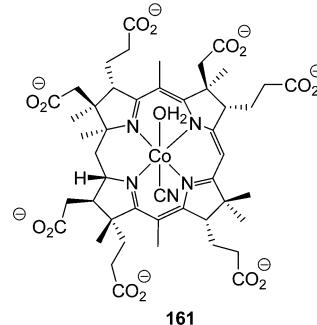


Figure 17. Reaction of 162 with NO.

On the basis of competitive displacement complexations, Anslyn et al. developed an array assay for small peptides and their phosphorylated analogues.⁴¹⁷ In this approach, five different receptors containing two tripeptide arms, three metal cations (Ni²⁺, Co²⁺, and Cu²⁺), and three indicators (pyrocatechol violet, celestine blue, and galloxyanine) were used to create an array of 45 metal/receptor/indicator combinations that was used for pattern recognition of Pro-Ser-Glu, Ser-Glu-Glu, Pro-pSer-Glu, and pSer-Glu-Glu tripeptides.

4. CHROMOGENIC AND FLUOROGENIC PROBES BASED ON THE CLEAVAGE AND FORMATION OF COVALENT BONDS

4.1. Chromogenic and Fluorogenic Probes Based on the Cleavage of Covalent Bonds

In the past decade, chromogenic and fluorogenic probes based on the cleavage of covalent bonds by analytes have received great attention. This type of probe is usually constructed by employing the following strategy: a fluorochrome is attached to a quencher via a cleavable active bond by virtue of various chemical reactions such as protection–deprotection. The as-obtained probes often have no or weak fluorescence; upon addition of an analyte, the active bond in the probe is cleaved and the fluorochrome is released, accompanying the retrieval of fluorescence. This type of probes usually has an irreversible spectroscopic off–on response, which is favorable for sensitive detection but is unsuitable for studying the dynamic change of analyte concentrations. Moreover, most of these probes show higher selectivity for analytes than complexation-based chromogenic and fluorogenic probes, which provides an alternate route to the specific determination of many analytes (e.g., enzyme activity, metal ions, reactive oxygen species, and thiols). Ma's group has summarized the progress of this type of

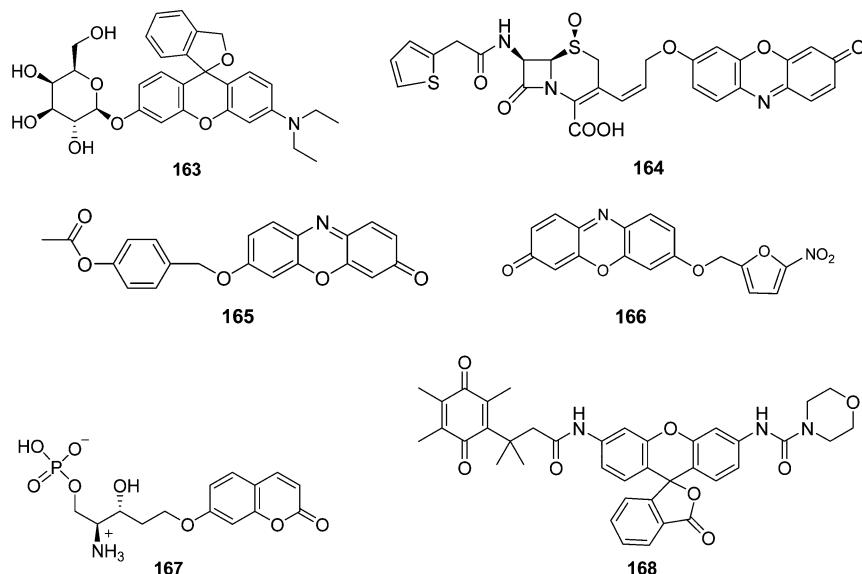


Figure 18. Structures of fluorescent probes **163–168** for detecting enzyme activity.

probes in a recent review.² Herein, we will give a more comprehensive review on this topic.

4.1.1. Cleavage of Covalent Bonds by Enzymes.

Measurement of enzyme activity, especially in living systems, has seen great progress in recent years, and in this respect chromogenic and fluorogenic probes with enzyme-triggerable off-on responses are playing an important role due to their high signal/background ratio.^{2,4,418–420} In earlier studies, *p*-nitrophenol, *p*-nitroaniline, 7-amino-, and 7-hydroxyl-coumarin were mainly employed as the skeletons to develop chromogenic and fluorogenic probes with cleavable active bonds for enzyme activity detection;¹ in the last 10 years, fluorochromes with long-wavelength emission, such as fluoresceins, rhodamines, and resorufins, are more often used. In these probes, phenolic O-alkylation or anilinic N-amidation of the fluorochromes with an enzyme-specific substrate results in the quenching of their spectroscopic signals. Upon reaction with the enzyme, the fluorochrome will be released producing spectroscopic off-on response. Representative structures of the fluorescent probes for enzyme activity are summarized in Figure 18.

Compound **163** (Figure 18), a hybrid structure of fluorescein and rhodamine, has been reported as a fluorescent probe for β -galactosidase assay by Nagano's group.⁴²¹ The probe, prepared by the phenolic O-alkylation of the hybrid with β -galactopyranoside, shows weak fluorescence due to the favored existence of the spirocyclic form. Reaction of **163** with β -galactosidase leads to the removal of β -galactopyranoside and thereby fluorescence increase. The probe has been employed to visualize β -galactosidase activity in living cells and in *Drosophila melanogaster* tissue. Nagano's group also reported other fluorescent probes for enzyme detection (e.g., nucleotide pyrophosphatase) activity.^{422–425}

Other researchers developed fluorescent probes for monitoring the activity of proteases,^{426–429} α -glucosidase,⁴³⁰ β -lactamase,⁴³¹ glutathione transferase,⁴³² monoamine oxidase,⁴³³ etc. Rao et al. reported **164** (Figure 18) as a fluorescent probe for β -lactamase.⁴³⁴ The probe was synthesized by introducing the substrate moiety of β -lactamase to the 7-hydroxy of resorufin. This alkylation quenches the fluorescence of resorufin, but reaction of **164** with β -lactamase causes the release of resorufin and thus the recovery of fluorescent signals.

The acetoxyethyl (AM) ester of **164** is cell membrane permeable, and the probe has been used to image the β -lactamase activity in β -lactamase stably transfected C6 glioma cells.

On the other hand, enzyme-initiated domino cleavage reactions have also led to the successful design of many chromogenic and fluorogenic probes, in which a self-immolative spacer (e.g., *p*-hydroxymethyl phenol) is usually incorporated between the enzyme substrate moiety and the fluorochrome. For instance, Ma et al. reported **165** (Figure 18) as a carboxylesterase probe.⁴³⁵ Probe **165** shows very low background signal, but its reaction with carboxylesterase results in the cleavage of the carboxylic ester bond, followed by 1,6-rearrangement-elimination. This domino cleavage reaction leads to the release of resorufin and thus the recovery of both color and fluorescence signal. On the basis of this behavior, a simple and sensitive fluorescence method has been established for assaying carboxylesterase activity with a detection limit of 8.6×10^{-5} U/mL, which is much lower than those of the existing fluorescence approaches (5.2×10^{-3} to 0.01 U/mL). The probe is cell membrane permeable and has been applied for monitoring carboxylesterase activity in HeLa cells. Recently, Ma's group reported a resorufin-based probe (**166**) for monitoring nitroreductase activity, which also follows a 1,6-rearrangement-elimination reaction mechanism. The probe (5 μ M) shows a linear fluorescence response to nitroreductase in the concentration range of 15–300 ng/mL with a rather low detection limit of 0.27 ng/mL. The ready reduction of the nitro group in **166** by intracellular nitroreductase under hypoxic conditions leads to the establishment of a simple fluorescence method for imaging the hypoxic status of tumor cells. With this method, HeLa and A549 cells under normoxic and hypoxic conditions (even for different extents of hypoxia) can be differentiated successfully.⁴³⁶ On the basis of the similar 1,6-rearrangement-elimination reaction, a chemiluminescence (CL) probe for the in vitro detection of caspase-3 was synthesized.⁴³⁷ The CL probe shows a highly sensitive response to caspase-3 with a detection limit of about 1 pmol; this high sensitivity may result from the fact that CL-based detection usually has an extremely low background signal because no excitation light source is required (instead, CL

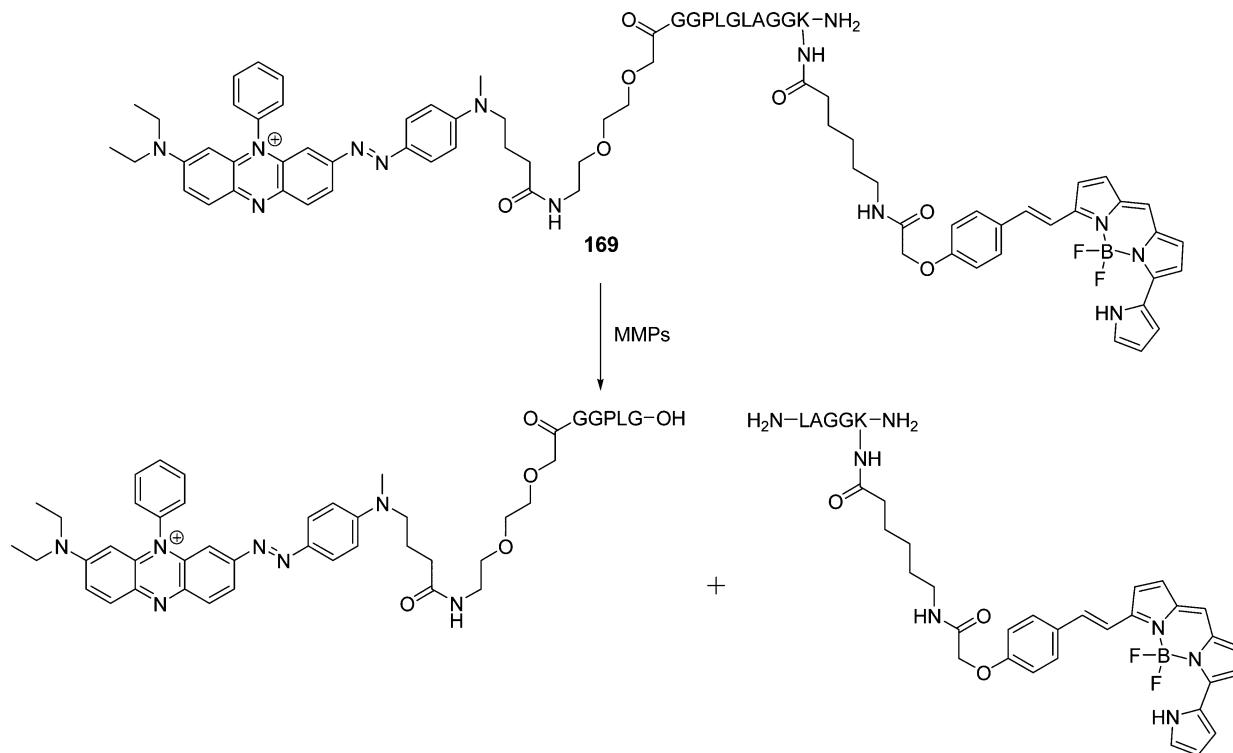


Figure 19. Reaction of **169** with MMPs.

probes are excited via chemical reaction). Moreover, other fluorescent probes with the same response mechanism have been reported for monitoring the activity of enzymes such as penicillin G acylase,⁴³⁸ reductases,^{439,440} sulfatase,⁴⁴¹ and β -galactosidase.⁴⁴²

Besides the 1,6-rearrangement-elimination reaction, the release of fluorochromes containing phenolic or anilinic group may also be achieved via β -elimination, and this mechanism has been used to design the fluorescent probes for sphingosine-1-phosphate lyase (**167**, Figure 18),⁴⁴³ β -glucuronidase,⁴⁴⁴ monoamine oxidase,^{445–448} and esterase.⁴⁴⁹

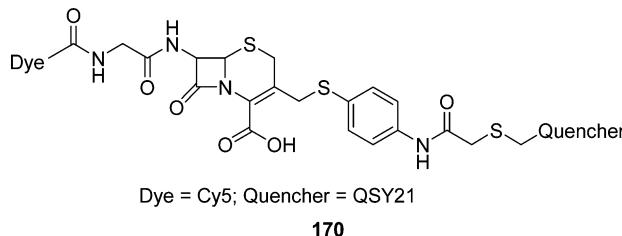
Intramolecular cyclization can also be employed in enzyme-initiated domino cleavage reactions, as exemplified by the fluorescent probes for alkaline phosphatase,^{450,451} penicillin amidase,⁴⁵² reductase (**168**, Figure 18),^{453–455} and esterase.^{456,457}

Alternatively, fluorescence off-on probes for the assay of enzyme activity can be obtained by constructing a FRET system with a fluorochrome as the donor and a quencher as the acceptor, which are connected via an enzyme cleavable linker (i.e., a peptide sequence or the specific substrate of an enzyme). Upon reaction with the target enzyme, the linker is cleaved, and fluorescence of the donor will be recovered.

One can select various peptide sequences to satisfy different requirements.^{458–462} For example, compound **169** (Figure 19) was constructed by linking a BODIPY moiety as a FRET donor to the dark quencher as a FRET acceptor via a peptide substrate of matrix metalloproteinases (MMPs).⁴⁶⁰ Reactions of **169** with MMPs cleave the peptide, leading to fluorescence increase. **169** has been used to visualize the activity of MMPs inside living cells and in a mouse xenograft tumor model. Besides, several FRET-based probes have been developed for caspase-3 detection by using its core peptide sequence, AGVA.^{463–465} The core peptide sequences of proteolytically active prostate-specific antigen and MMP-2 are HSSKLQ⁴⁶⁶ and PLGVR,⁴⁶⁷

respectively, which may serve as cleavable linkers to construct corresponding FRET systems for the assay of these enzymes.⁴⁶⁸

Cleavable linkers in FRET systems are not limited to peptide sequences. Instead, nonpeptide enzyme substrates can also be used as cleavable linkers. For instance, probe **170** is constructed by using Cy5 (donor) and QSY21 (quencher) as the FRET pair, and the substrate structure of β -lactamase as a cleavable linker.⁴⁶⁹ The probe can be used to image tuberculosis in live mice. Similarly, Xing et al.⁴⁷⁰ reported another FRET system for β -lactamase, which may also be useful for antibiotic selection.

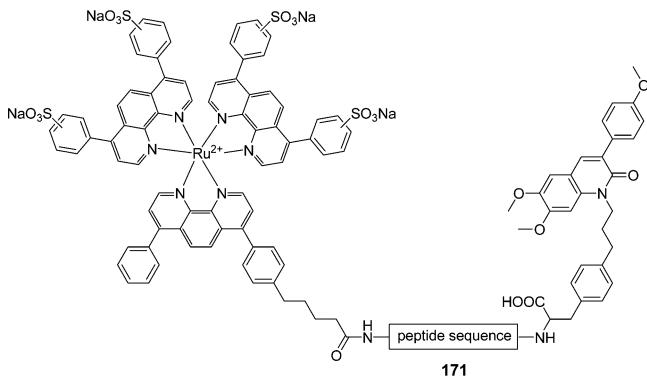


Dye = Cy5; Quencher = QSY21

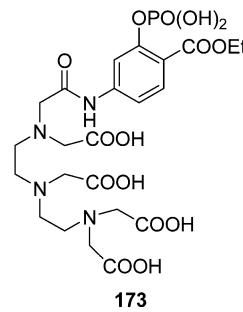
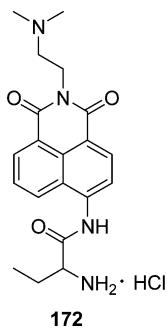
170

In addition to the above probes with fluorescence off-on responses, ratiometric fluorescent probes have also been reported for the assay of enzyme activity. One frequently used approach for designing such probes is to construct a FRET system by using one fluorochrome as the donor and the other as the acceptor, which can also be connected through an enzymatically cleavable linker.^{2,420,471–477} For example, compound **171** is a ratiometric probe, which consists of a carbostyryl moiety as the FRET donor and a ruthenium(II) bathophenanthroline complex as the FRET acceptor.⁴⁷² Reaction of **171** with thrombin leads to the cleavage of the peptide sequence and thus the change of the fluorescence intensity ratio of the donor and the acceptor at 420/618 nm. In addition, with ester bond, phosphodiester bond, or the

substrate structure of β -lactamase as a cleavable linker, FRET-based ratiometric probes have been reported for monitoring the activity of esterase,⁴⁷³ phosphodiesterase,^{474,475} and β -lactamase.⁴⁷⁷



Ratiometric fluorescent probes can also be designed by linking a substrate unit of an enzyme to a specific fluorochrome that could produce spectral shift upon removal of the enzyme substrate. Compound 172 is such a probe, whose reaction with aminopeptidase N leads to the cleavage of the amide bond and the removal of 2-aminobutanoic acid, resulting in the fluorescence wavelength change from 460 to 550 nm.⁴⁷⁸ The probe was applied for ratiometric imaging of aminopeptidase N in cells. The complex of 173 with Tb³⁺ has been reported as a ratiometric luminescent probe for monitoring alkaline phosphatase activity.⁴⁷⁹ In this complex, salicylic acid was used as the antenna, whose excitation spectrum would be changed upon removal of the phosphate group by alkaline phosphatase, allowing ratiometric detection by excitation at two wavelengths.



4.1.2. Cleavage of Covalent Bonds by Metal Ions.

Various metal-driven chemical cleavage reactions, such as metal-promoted hydrolysis, metal-induced desulfurization followed by hydrolysis, and metal-induced deprotection, have been utilized for the development of chromogenic and fluorogenic probes for metal ions.^{2,480}

4.1.2.1. Probes for Ag⁺. On the basis of the fact that selenium has a strong affinity for silver, Ma's group has designed rhodamine B selenolactone (174; Figure 20) as a Ag⁺ fluorescent probe by incorporating Se atom into the spirocyclic structure of rhodamine.⁴⁸¹ The probe produces a rapid and specific fluorescence off-on response to Ag⁺ or Hg²⁺ and is capable of reacting with Ag⁺ in the presence of high concentration of Cl⁻ because Se has a stronger binding ability toward Ag⁺ than does Cl⁻. Furthermore, the probe has been used to compare the imaging behaviors of Hg²⁺ and Ag⁺ in HeLa cells for better understanding their different toxicities. It was observed that the reaction of Ag⁺ with the probe inside the

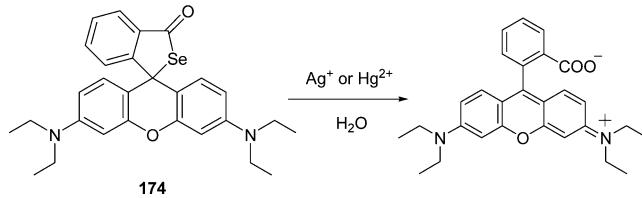


Figure 20. Reaction of 174 with Ag⁺ or Hg²⁺.

cells occurs much slower than that of Hg²⁺, which is ascribed to the high concentration of cellular chloride ions (Cl⁻ barrier) inhibiting the formation of sufficient free Ag⁺. This observation is helpful to get an insight into the different interaction mechanism of Hg²⁺ and Ag⁺ with cells.

By virtue of the AgI formation and the subsequent irreversible tandem ring-opening and formation processes, Ahn et al. developed a rhodamine-based probe 175 for Ag⁺ in 20% ethanolic water solution (Figure 21).⁴⁸² The probe shows

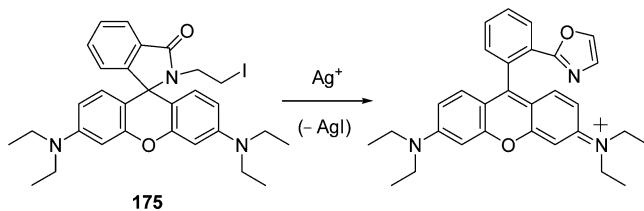


Figure 21. Reaction of 175 with Ag⁺.

a linear fluorescence response to Ag⁺ in the range of 0.1–50 μ M with a detection limit of 1.4×10^{-8} M. Moreover, taking advantage of the oxidation of silver nanoparticles to Ag⁺ by hydrogen peroxide, the probe can also be applied to the quantification of silver nanoparticles in consumer products.

4.1.2.2. Probes for Au³⁺/Au⁺. Compound 176 (Figure 22) was proposed as a chromogenic and fluorogenic probe for Au³⁺/Au⁺,^{483,484} based on Au³⁺/Au⁺-induced ring-opening of the spirolactam followed by intramolecular cyclization to form oxazolecarbaldehyde.^{483–485} The formyloxazole compound was found to be a major product in aqueous medium, which is

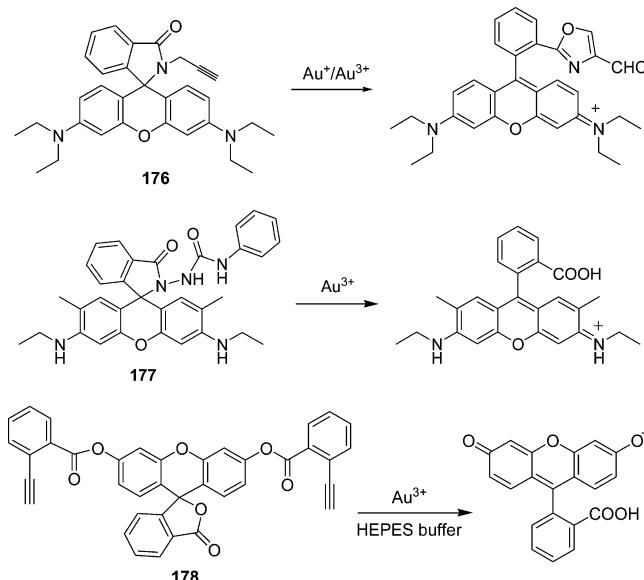


Figure 22. Reactions of 176, 177, and 178 with Au⁺/Au³⁺.

presumably formed via a vinylgold intermediate. Compound **177** was also reported as a Au^{3+} probe based on Au^{3+} -mediated spirolactam ring-opening and hydrolysis of acylsemicarbazide to carboxylic acid (Figure 22).⁴⁸⁶ Reaction of **177** with Au^{3+} generates an intense fluorescence emission at 549 nm, and this fluorescence increase is linear to the concentrations of Au^{3+} from 0.5 to 70 μM with a detection limit of 290 nM. The probe can be used for the detection of Au^{3+} in living cells. To alleviate side reactions identified in an *N*-propargyl-rhodamine lactam sensing system, Ahn et al. synthesized a fluorescein (2-ethynyl)benzoate (**178**) that can undergo Au^{3+} -promoted ester hydrolysis selectively over other metal ions with high sensitivity,⁴⁸⁷ resulting in a fluorescence off-on response in HEPES buffer of pH 7.4 (Figure 22).

Compound **179**, a nonfluorescent I-BODIPY derivative, was reported as a probe for the detection of Au^{3+} (Figure 23).⁴⁸⁸

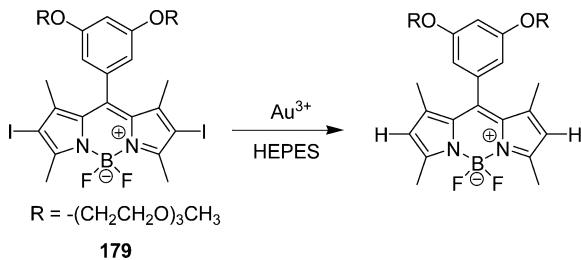


Figure 23. Reaction of **179** with Au^{3+} .

whose fluorescent response is based on the following two reactions: first, HEPES can reduce Au^{3+} to Au forming HEPES-capped gold nanoparticles at ambient temperature and neutral pH; second, the formed gold nanoparticles can effectively trigger C–I bond scission in **179**, yielding the highly fluorescent H-BODIPY. The probe showed remarkable selectivity for Au^{3+} over other metal ions.

4.1.2.3. Probe for Cd^{2+} . Until now, only one fluorescent probe (**180**) for Cd^{2+} has been reported on the basis of the cleavage of covalent bonds,⁴⁸⁹ that is, deprotection of the aldehyde group (Figure 24). Because of the thiophilicity of

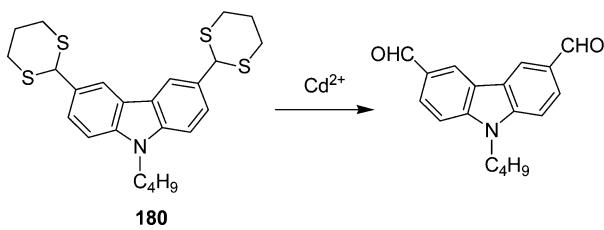
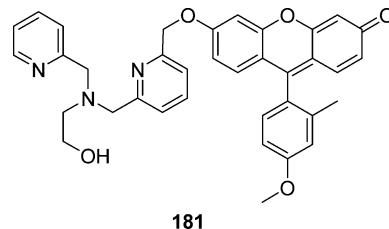


Figure 24. Reaction of **180** with Cd^{2+} .

Cd^{2+} , the electron-rich 1,3-dithiane protective group in **180** can be removed by Cd^{2+} , forming the fluorescent precursor that contains two electron-withdrawing aldehyde groups. As a result, an ICT process from the electron-donating *N*-butylcarbazole moiety to the aldehyde groups occurs, leading to a significant bathochromic shift (>50 nm) in the emission spectrum and showing a ratiometric fluorescent response to Cd^{2+} .

4.1.2.4. Probe for Co^{2+} . Chang et al. prepared a Co^{2+} probe (**181**) by connecting a tetradeятate ligand to a xanthene fluorochrome through a cleavable ether linker.⁴⁹⁰ Alkylation of the phenol moiety with the ligand results in weak fluorescence. However, reaction of **181** with Co^{2+} in the presence of O_2 can

break the C–O benzyl ether bond and release the fluorochrome. The probe is highly selective for Co^{2+} over a variety of biologically relevant s- and d-block metal ions, and can be used to monitor the change of Co^{2+} concentrations added in living cells.



4.1.2.5. Probes for Cu^{2+} and Cu^+ . On the basis of Cu^{2+} -promoted hydrolysis of hydrazides,⁴⁹¹ Ma and Chen have characterized spiro form fluorescein hydrazide (**182**; Figure 25)

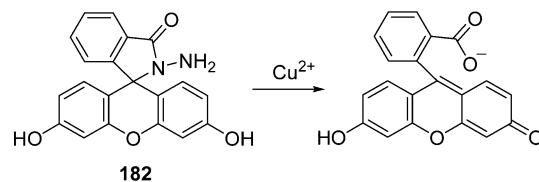
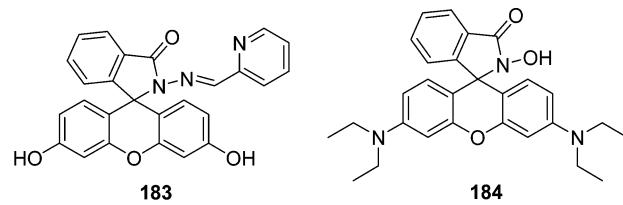


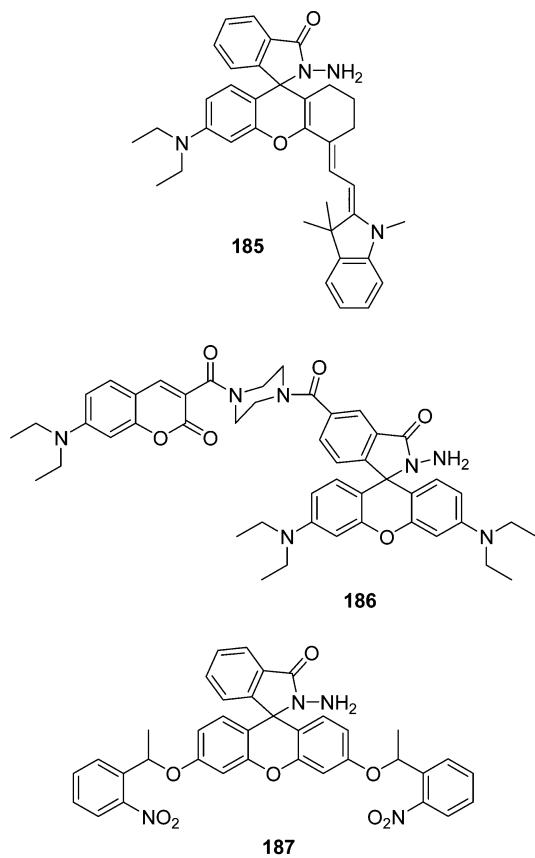
Figure 25. Reaction of **182** with Cu^{2+} .

as a highly selective and sensitive fluorescence probe for Cu^{2+} .⁴⁹² In 0.01 M Tris-HCl buffer (pH 7.2), the probe displays a highly selective fluorescence off-on response to Cu^{2+} only, instead of other common metal ions. The reaction mechanism has been further confirmed as that the hydrazide group of **182** recognizes and binds Cu^{2+} , and the subsequent complexation of Cu^{2+} promotes hydrolytic cleavage of the amide bond, causing the release of fluorescein and thereby the retrieval of fluorescence. The probe shows a linear fluorescence response to Cu^{2+} in the concentration range of 0.1–10 μM with a detection limit of 64 nM, also showing a highly sensitive feature. **182** has been used for the determination of trace Cu^{2+} in real biological fluids such as human serum and cerebrospinal fluid. Compounds **183** and **184** have also been proposed as highly selective and sensitive fluorescence probes for Cu^{2+} based on the Cu^{2+} -promoted spirolactam ring-opening of **493,494**



Lin's group introduced a unique strategy to design a NIR fluorochrome with the carboxylic acid-controlled fluorescence on-off switching mechanism by spirocyclization.⁴⁹⁵ On the basis of this platform, they synthesized **185** as an NIR fluorescent probe for Cu^{2+} . The probe exhibited a sensitive fluorescence off-on response (>500-fold) to Cu^{2+} .⁴⁹⁵ They also designed a FRET probe **186** based on the coumarin-rhodamine scaffold.⁴⁹⁶ Cu^{2+} -promoted hydrolysis of the rhodamine B hydrazide moiety in **186** leads to the occurrence of FRET between coumarin and rhodamine. The ratio (I_{581}/I_{473}) of fluorescence intensities at 581 and 473 nm exhibits a

linear change with Cu^{2+} concentration in the range of 0.08–30 μM with a detection limit of 13 nM. The probe has been applied to ratiometrically imaging Cu^{2+} in living cells. Moreover, they reported **187** as a photocontrollable Cu^{2+} -responsive fluorescent probe.⁴⁹⁷ Upon photolysis, the photolabile nitrobenzyl groups are removed to release the Cu^{2+} -responsive fluorescein hydrazide, which can be used to detect Cu^{2+} in solutions as well as in living cells.



By inserting a nitrogen atom in the spiro-ring of rhodamine B spiro thiohydrazide (a known Hg^{2+} probe⁴⁹⁸), the resulting derivative (**188**)⁴⁹⁹ with a six-membered spiro-ring prefers recognizing Cu^{2+} to Hg^{2+} (Figure 26). Addition of Cu^{2+} to the

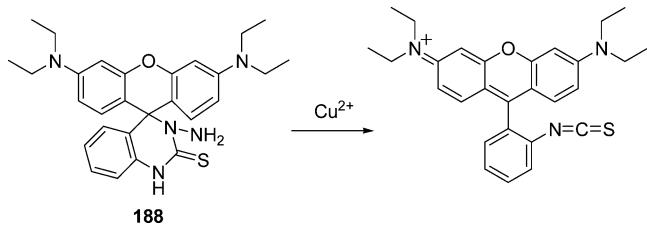


Figure 26. Reaction of **188** with Cu^{2+} .

probe solution produces strong fluorescence, and this fluorescence increases linearly with increasing the concentration of Cu^{2+} from 0.1 to 10 μM . Reaction mechanism studies revealed that **188** opened its spiro-ring by a Cu^{2+} -induced transformation of the cyclic thiosemicarbazide moiety to an isothiocyanate group.

In addition, Cu^{2+} can induce the hydrolysis of activated esters, Schiff bases, and hydrazone^{500–504}, which provides alternative ways for designing Cu^{2+} probes, as exemplified by

compounds **189–191**. These probes show fluorescence off-on response to Cu^{2+} via Cu^{2+} -promoted hydrolysis of the ester bond, Schiff bases, or hydrazone (Figure 27).^{502–504} However, most of the hydrolysis reactions can only proceed in the presence of a high content of organic solvents.

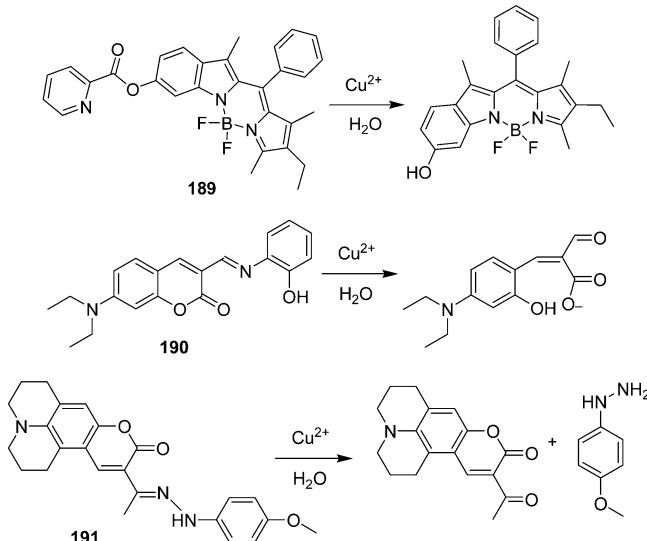
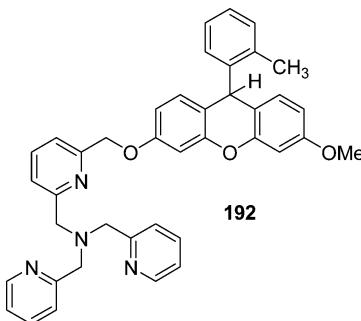


Figure 27. Reactions of **189–191** with Cu^{2+} .

By connecting a tetradeinate ligand, tris[(2-pyridyl)-methyl]amine (TPA), to the reduced form of a xanthene skeleton through a benzyl ether linkage, Taki and co-workers prepared **192** as a turn-on fluorescent probe for Cu^{+} .⁵⁰⁵ Complexation of the TPA moiety with Cu^{+} leads to the cleavage of the benzyl ether bond, accompanied by fluorescence generation. The probe is selective for Cu^{+} over other transition and heavy metal ions, and can be used to detect intracellular Cu^{+} under physiological conditions.



4.1.2.6. Probes for Fe^{3+} . It is known that Fe^{3+} can promote Schiff-base hydrolysis in acidic media, and this reaction was used for the design of the fluorescence off-on probe **193** (Figure 28).⁵⁰⁶ Probe **193**, prepared by incorporating 2-hydroxy-5-nitrobenzaldehyde into a rhodamine core via a hydrolyzable imine linkage, shows significant fluorescence enhancement upon reaction with Fe^{3+} , which is due to the Fe^{3+} -induced Schiff-base hydrolysis and the spirolactam ring-opening reaction. The probe has been used for imaging free Fe^{3+} in HepG2 cells without any detrimental effect on Fe^{3+} -based enzymes. On the basis of the Fe^{3+} -mediated hydroxylamine oxidation and the subsequent hydrolysis, compound **194** has also been synthesized as a turn-on fluorescent probe for detecting Fe^{3+} in living cells (Figure 28).⁵⁰⁷

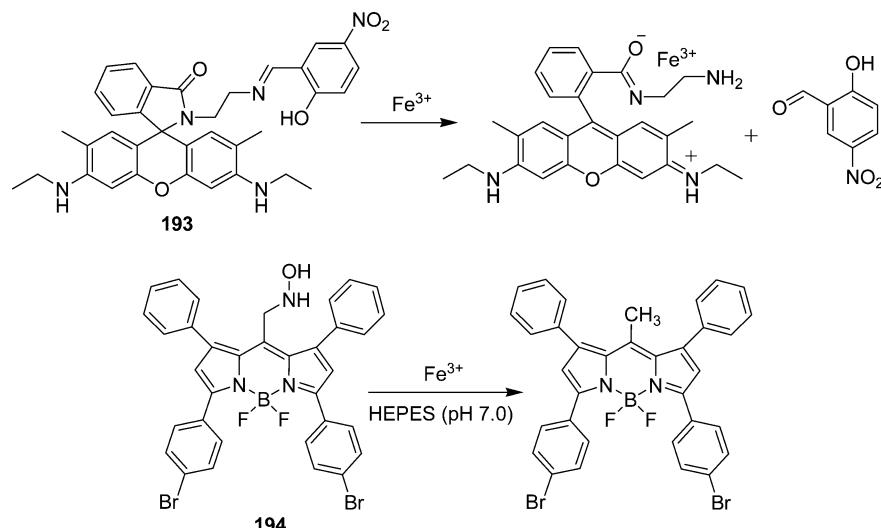


Figure 28. Reactions of **193** and **194** with Fe^{3+} .

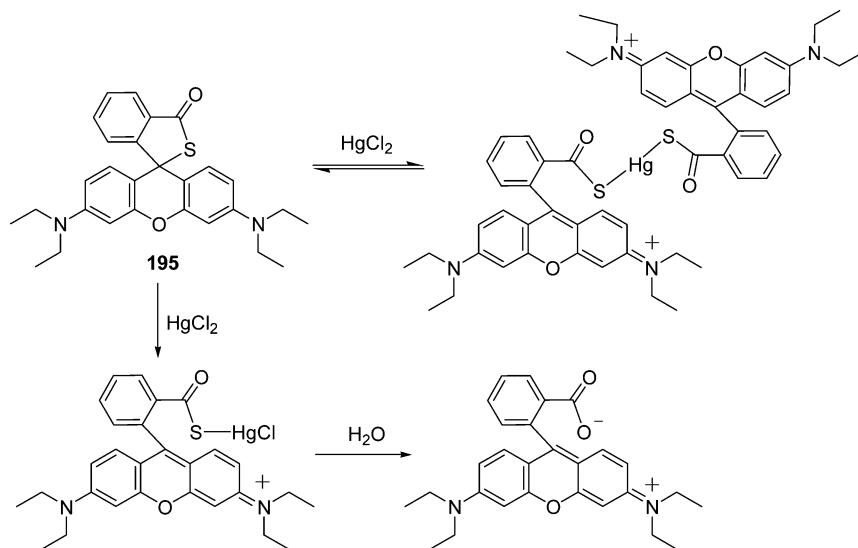


Figure 29. Proposed reaction mechanism of **195** with Hg^{2+} .

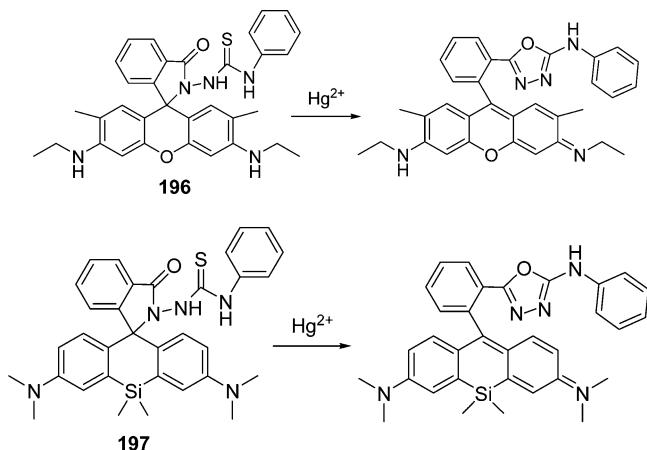
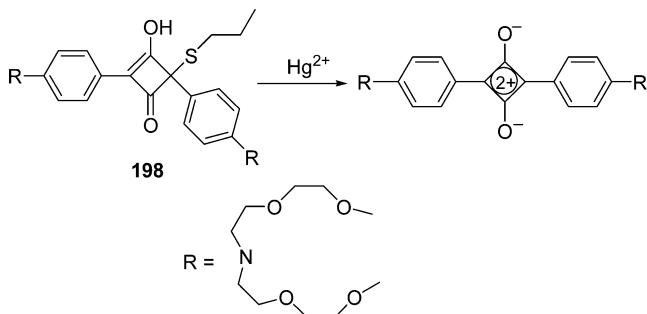
4.1.2.7. Probes for Hg^{2+} . The strong affinity of Hg^{2+} for S or Se atom, combined with various cleavage reactions, has been extensively used to design chromogenic and fluorogenic Hg^{2+} probes. The commonly used cleavage reactions include Hg^{2+} -induced ring-opening of spirocyclic systems^{508–515} and desulfurization.^{6,167,516,517}

Ma and Shi⁵⁰⁹ and Xu et al.⁵¹⁰ independently designed rhodamine B thiolactone (**195**) as a color and fluorescence off-on probe for Hg^{2+} . The probe undergoes ring-opening triggered by complexation with Hg^{2+} , and the introduction of KI to the system can reverse the reaction only in the presence of no more than 0.5 equiv of Hg^{2+} . Combined with ESI-MS analytical data, Ma et al. further proposed that the reaction proceeds following the route depicted in Figure 29. The 1:2 complex of Hg^{2+} with **195** is relatively stable in the solution; however, the 1:1 complex can be further degraded to rhodamine B. With the usage of 5 μM **195**, the fluorescence increase is proportional to the concentration of Hg^{2+} in the range of 0.5–5 μM with a detection limit of 20 nM. The probe is highly selective for Hg^{2+} over other common species and has been used to image Hg^{2+} in *Arabidopsis thaliana*.⁵¹⁸ In addition,

an analogue of **195**, rhodamine 6G thiolactone, was also proposed for Hg^{2+} detection.⁵¹⁹

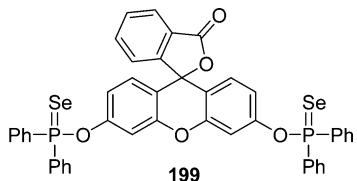
Compound **196** was also reported as a Hg^{2+} probe,^{520,521} which can undergo desulfurization in the presence of Hg^{2+} at pH 7.4, followed by the formation of 1,3,4-oxadiazole and the ring-opening of the spirocyclic structure (Figure 30). Reaction of **196** with Hg^{2+} results in a 30-fold fluorescence enhancement as well as the color change from colorless to bright pink. The probe is cell membrane-permeable and has been utilized to image accumulated Hg^{2+} in zebra fish organs.⁵²² Recently, an NIR Hg^{2+} probe **197** was proposed on the basis of the spirolactonized Si-rhodamine (Figure 30).⁵²³ Similar to **196**, reaction of **197** with Hg^{2+} leads to strong fluorescence at around 680 nm due to the Hg^{2+} -promoted ring-opening via desulfurization and cyclization. **197** is capable of imaging Hg^{2+} in living cells.

On the basis of Hg^{2+} -triggered desulfurization, a regenerative squaraine-based probe (**198**, Figure 31) has been synthesized for sensing Hg^{2+} in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (1:4, v/v, pH 9.6) media.⁵²⁴ Probe **198** is colorless in solution. Introduction of Hg^{2+} removes propanethiol, releasing the π -conjugated squaraine

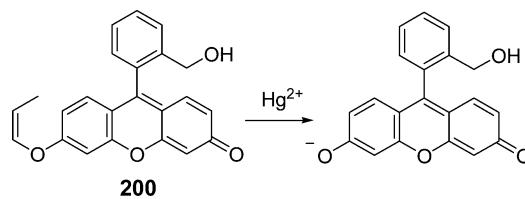
Figure 30. Reactions of 196 and 197 with Hg^{2+} .Figure 31. Reaction of 198 with Hg^{2+} .

and thus producing fluorescence enhancement and color change from colorless to blue. The probe is selective for Hg^{2+} , and Hg^{2+} down to 2 ng/mL can be fluorimetrically detected with a 100 nM probe.

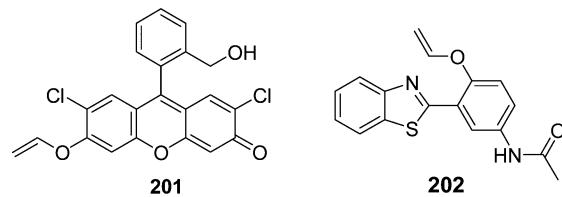
The selenophilic character of Hg^{2+} can also induce the ring-opening of the spirocyclic structure of the above 174.⁴⁸¹ By using 5 μM of 174, a linear response in fluorescence intensity was found in the concentration range of 0.1–5 μM Hg^{2+} with a detection limit of 23 nM. 174 can be used to detect Hg^{2+} and methylmercury species in cells and zebrafish.^{481,525} In addition, compound 199 can undergo deselenation reaction with Hg^{2+} , releasing fluorescein.⁵²⁶ The selectivity of 199 for Hg^{2+} over other relevant metal ions is very high. With the usage of 2.5 μM 199, a good linearity between the fluorescence increase and the concentration of Hg^{2+} in the range of 0.07–3.4 μM was obtained. The detection limit of 199 for Hg^{2+} is 1.0 nM. 199 has been applied to the imaging of Hg^{2+} in RAW 264.7 cells.



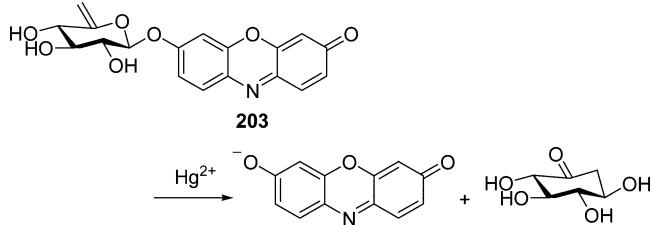
On the basis of the π -electrophilicity of Hg^{2+} , Koide and co-workers developed several probes for sensing Hg^{2+} and CH_3HgCl species.^{527,528} For example, compound 200 can be hydrolyzed through the vinyl ether oxymercuration, accompanied by the generation of strong fluorescence (Figure 32). 200 can be used to detect 1 ppb of Hg^{2+} in water. Compound 201 was reported by Ahn and co-workers as a selective

Figure 32. Reaction of 200 with Hg^{2+} .

fluorescent probe for Hg^{2+} ,⁵²⁹ whose response mechanism also follows the vinyl ether oxymercuration. With this probe (5 μM), a linear relationship between the fluorescence intensity and the concentration of Hg^{2+} was obtained in a wide concentration range (1.0×10^{-9} to 1.0×10^{-5} M). The probe has been used to image methylmercury in zebrafish. The vinyl ether derivative (202) of 2-(benzothiazol-2-yl)phenol was proposed as a ratiometric fluorescent probe for mercury species based on the same mechanism.⁵³⁰

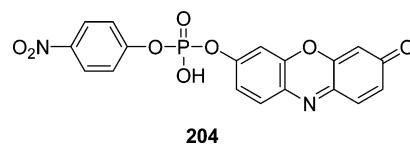


In addition, 203 was prepared as a fluorescent Hg^{2+} probe.⁵³¹ Reaction of the probe with Hg^{2+} causes the removal of the carbohydrate moiety and thus the fluorescence recovery of resorufin (Figure 33). With the usage of 25 μM probe, the

Figure 33. Reaction of 203 with Hg^{2+} .

fluorescence intensity increased about 25-fold in the presence of 1 μM Hg^{2+} , and the detection limit was calculated to be 0.15 μM . The probe also shows high selectivity for Hg^{2+} and has been applied to the imaging of Hg^{2+} in A549 cells and zebrafish.

4.1.2.8. Probe for Pb^{2+} . Pb^{2+} can efficiently cleave phosphate ester bonds, and this behavior has been used to design a fluorescent probe (204) for the specific detection of Pb^{2+} by Ma's group.⁵³² The probe is nonfluorescent. Upon reaction with Pb^{2+} , the phosphate ester bonds in the probe were cleaved, and the fluorescent resorufin was released. The fluorescence increase is proportional to the concentration of Pb^{2+} in the range of 50–125 nM with a detection limit of 22 nM. 204 may have potential use for specific detection of Pb^{2+} because of its excellent selectivity for Pb^{2+} over a wide range of alkaline earth and other transition metal ions, such as Ca^{2+} , Cd^{2+} , Cu^{2+} , Fe^{2+} , Hg^{2+} , Mg^{2+} , and Ni^{2+} .



4.1.2.9. Probe for Palladium and Platinum Species. Koide and co-workers have demonstrated that Tsuji–Trost⁵³³ and Claisen⁵³⁴ reactions of metal–allyl complexes are capable of sensing palladium and platinum species (e.g., **205** in Figure 34).

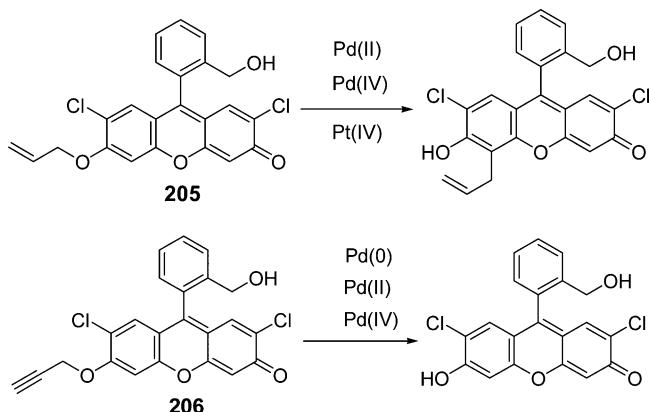
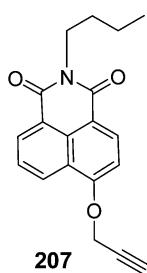


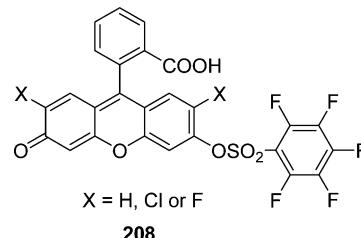
Figure 34. Reactions of **205** and **206** with palladium and platinum species.

Reaction of **205** with the metal species proceeds through hydrolysis of an allyl ether. pH adjustment, ligand addition, and other experimental condition changes can promote specificity for palladium or platinum and even for their oxidation states. The detection limits of **205** for Pd(II) and Pt(IV) are 3.9 μM and 0.54 nM, respectively. Ahn's group synthesized an *O*-propargylated fluorescein derivative (**206**, Figure 34), which can be used for monitoring palladium in a variety of oxidation states (Pd(0), Pd(II), and Pd(IV)) and for imaging palladium in zebrafish.⁵³⁵ On the basis of the same reaction mechanism, Zhang and co-workers have prepared a ratiometric naphthalimide-based probe (**207**) for these metal species.⁵³⁶ The probe has a detection limit of 0.07 μM for Pd^{2+} and has been used to image Pd^{2+} in live RAW 264.7 macrophage cells.



4.1.3. Cleavage of Covalent Bonds by Reactive Oxygen Species. Reactive oxygen species (ROS), that is, the highly reactive oxygen-containing species [e.g., hydrogen peroxide (H_2O_2), superoxide ($\text{O}_2^{\bullet-}$), hydroxyl radical ($\cdot\text{OH}$), singlet oxygen (${}^1\text{O}_2$), peroxy nitrite (ONOO^-), hypochlorite (OCl^-), and nitric oxide (NO)], have been studied extensively because they are important mediators for the pathological conditions of various diseases.^{2,537–539} However, the lifetimes of most ROS are extremely short, and their basal levels in biosystems are usually rather low. Moreover, some ROS have similar strong oxidability. Therefore, the development of fast, sensitive, and selective methods for ROS assay is a great challenge. In this respect, the specific cleavage of some chemical bonds by ROS has led to the successful design of various chromogenic and fluorogenic probes with good performance for ROS detection.^{416,480,530–542}

4.1.3.1. Probes for H_2O_2 . H_2O_2 is one of the most extensively studied ROS, and many chromogenic and fluorogenic probes for H_2O_2 have been developed. For instance, compound **208** was prepared as a fluorescent H_2O_2 probe based on hydrolyzable sulfonic ester bonds.^{543,544} The nonfluorescent **208** reacts with H_2O_2 leading to the cleavage of the sulfonic ester bond and the release of the fluorescein moiety. However, the probes with sulfonic ester bonds are also reactive to other nucleophiles such as superoxide anions and thiols, which preclude their use in biosystems.^{545–547}



By utilizing the unique chemical reactivity of benzil with H_2O_2 , **209** was synthesized as a fluorescent probe for the detection of H_2O_2 .⁵⁴⁸ The weakly fluorescent probe can react with H_2O_2 at pH 7.4, producing the highly fluorescent 5-carboxyfluorescein (Figure 35). Probe **209** is sensitive and selective for H_2O_2 and has been applied to imaging H_2O_2 generation in live RAW 264.7 macrophages as well as in A431 human epidermoid carcinoma cells.

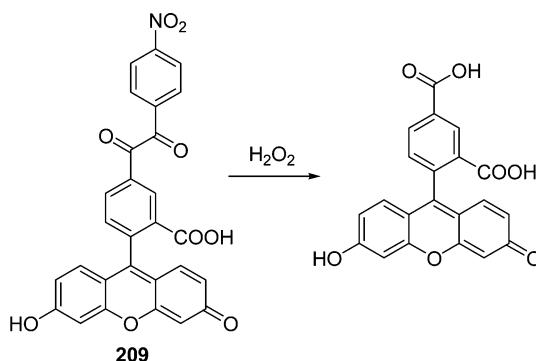
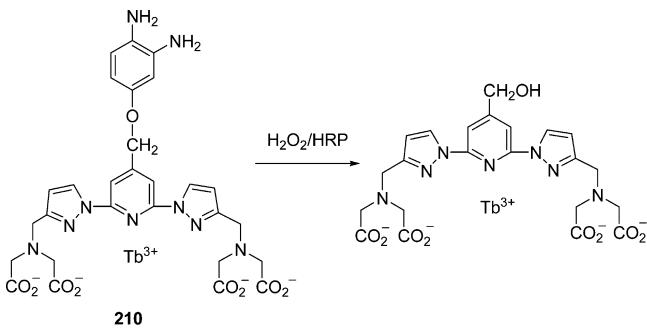
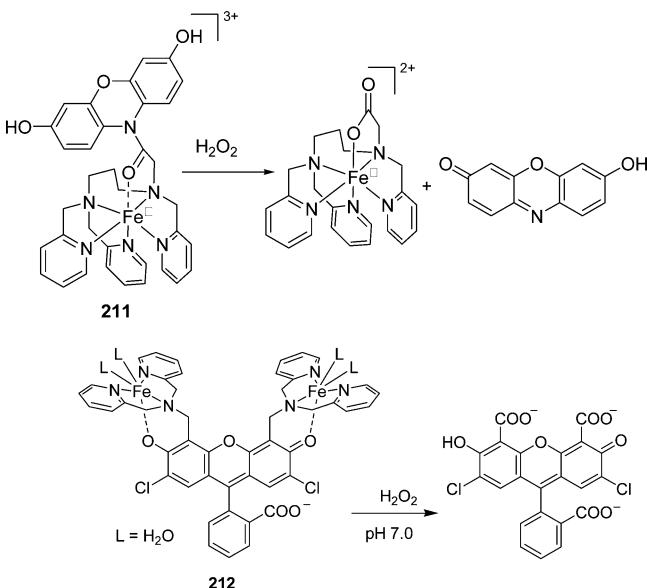


Figure 35. Reaction of **209** with H_2O_2 .

By incorporating a diaminophenyl moiety into a Tb^{3+} complex, Yuan et al. developed a luminescent probe, **210**, for the detection of H_2O_2 .⁵⁴⁹ The probe was also designed on the basis of the PET mechanism from the diaminophenyl moiety to the Tb^{3+} complex. Reaction of the probe with H_2O_2 in the presence of peroxidase removes the diaminophenyl moiety, yielding a highly luminescent product with a long luminescence lifetime (Figure 36). The luminescence enhancement showed a good linearity against the H_2O_2 concentration in the range of 10^{-8} – 10^{-6} M, with a slope of 0.756 and a detection limit of 3.7 nM H_2O_2 . **210** has been applied for time-resolved luminescence imaging of the H_2O_2 generation in living plant tissues.

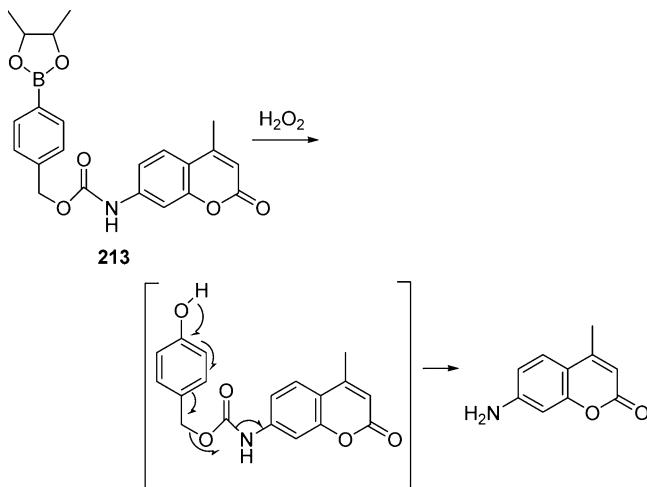
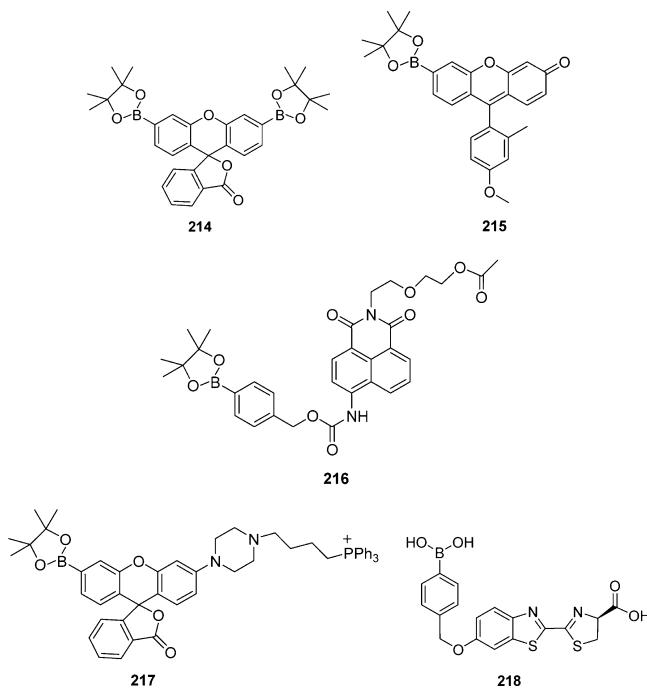
Compound **211** was reported as a fluorescent probe for H_2O_2 assay (detection limit, 3.2 μM).⁵⁵⁰ This probe has an iron complex as a reaction site for H_2O_2 and a 3,7-dihydroxyphenoxazine moiety as a fluorescent reporter unit. Reaction of **211** with H_2O_2 leads to the release of fluorescent resorufin (Figure 37). As compared to many of other H_2O_2 probes, the **211**-based reaction is selective for H_2O_2 and can eliminate the

Figure 36. Reaction of **210** with H_2O_2 .Figure 37. Reactions of **211** and **212** with H_2O_2 .

interference from phenol derivatives. Similarly, the Fe^{3+} complex **212** has been proposed as a fluorescent H_2O_2 probe (detection limit, 29 μM H_2O_2),⁵⁵¹ whose sensing mechanism involves H_2O_2 -induced oxidative *N*-dealkylation of the Fe^{3+} -ligand unit to liberate the fluorescein moiety (Figure 37). The unique reaction affords a high selectivity for H_2O_2 over other ROS, and the probe can be used to detect intracellular H_2O_2 in lysosomes.

Arylboronates can be selectively hydrolyzed to the corresponding phenols by H_2O_2 , and this reaction has been exploited for developing H_2O_2 fluorescent probes. The first example of such probes is compound **213**, in which aminocoumarin is masked by a *p*-dihydroxyborylbenzyloxycarbonyl group.⁵⁵² The intermediate formed by H_2O_2 -promoted hydrolysis of the arylboronate group in **213** undergoes spontaneous 1,6-elimination to generate fluorescent aminocoumarin (Figure 38). In vitro experiments showed that a linear correlation exists between the fluorescence response and the concentration of H_2O_2 in the range of 0.1–5.0 μM .

Chang's group synthesized a series of H_2O_2 probes based on the diboronate- and monoboronate-masked xanthene, resorufin, and other structures. Figure 39 shows some of the typical structures (**214**–**218**).^{553–560} Among them, **216** is a ratiometric probe, and can be used to visualize the localized increase of H_2O_2 in the phagosomes of PMA-stimulated macrophages.⁵⁵⁸ Probe **217** with a lipophilic phosphonium cation can be employed to target mitochondrial H_2O_2 in living

Figure 38. Reaction of **213** with H_2O_2 .Figure 39. Boronate-based probes (**214**–**218**) for H_2O_2 detection.

HeLa cells,⁵⁵⁹ while **218** can function as a bioluminescent probe for imaging H_2O_2 in living animals.⁵⁶⁰

The above oxidative hydrolysis of boronates has also led to the design of a cyanine-based H_2O_2 probe **219** (Figure 40).⁵⁶¹ Upon removal of the boronate triggered by H_2O_2 , the cyanine fluorochromes with strong NIR fluorescence are released via the rearrangement of the π -electron system. The probe has been used to image endogenous H_2O_2 produced in an acute inflammation model in mice.

Lin et al. developed a novel approach for the construction of an NIR fluorescent probe (**220**) for H_2O_2 .⁵⁶² The probe is almost nonfluorescent in the absence of H_2O_2 when excited at around 690 nm; however, upon addition of increasing concentrations of H_2O_2 (0–250 μM), the probe shows an up to 180-fold fluorescence increase at 708 nm. Furthermore, the probe is capable of imaging endogenously produced H_2O_2 not only in living cells but also in living mice, demonstrating the great potential of **220** in bioanalysis. By using boronate as the

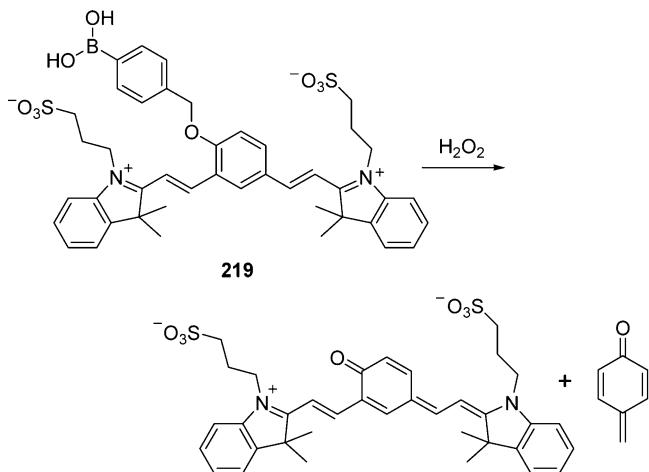
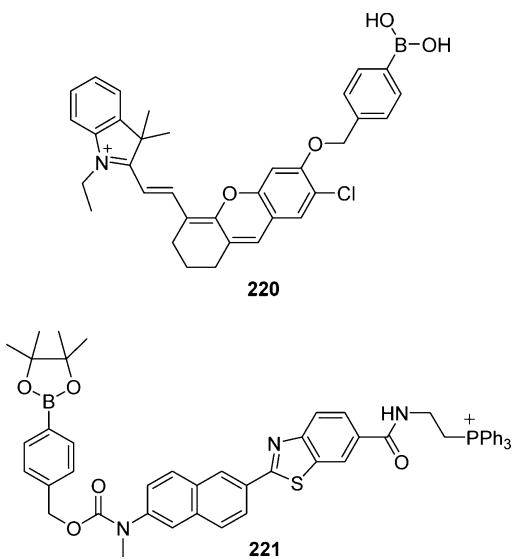
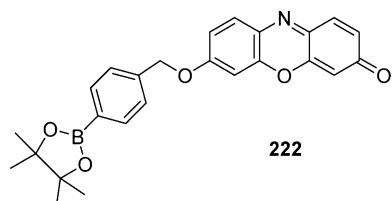


Figure 40. Reaction of 219 with H_2O_2 .

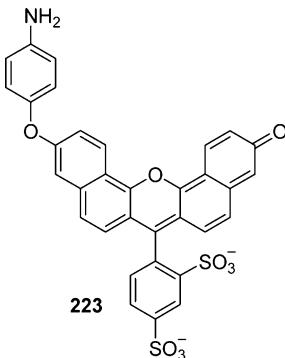
reaction moiety, some TP fluorescent H_2O_2 probes have been developed.^{563,564} Compound 221 is such a probe, which has a detection limit of $4.6 \mu\text{M}$ H_2O_2 , and can ratiometrically detect mitochondrial H_2O_2 in intact tissues at $>100 \mu\text{m}$ depth through the use of TP microscopy.⁵⁶⁴



4.1.3.2. Probe for Benzoyl Peroxide (BPO). Because of the similar reactivity of BPO to H_2O_2 , arylboronate may also serve as a recognition unit for BPO. This has been confirmed with compound 222. Reaction of 222 with BPO unmasks resorufin via deboronation, concomitant with the recovery of fluorescence at 585 nm. The fluorescence enhancement is directly proportional to the concentration of BPO in the range of $0.5\text{--}26 \mu\text{M}$ with a detection limit of 23nM , showing high sensitivity. The probe has been applied to detecting BPO in some real samples such as wheat flour and antimicrobial agent.



4.1.3.3. Probes for Hypochlorite (OCl^-). Taking advantage of the strong oxidative reactivity of OCl^- , many fluorescent OCl^- probes have been designed. For instance, the OCl^- -mediated oxidative bond-cleavage was used by Libby and co-workers in designing 223 as a fluorescent probe for OCl^- detection.⁵⁶⁶ Reaction of the probe with OCl^- results in the removal of the 4-aminophenyl moiety and the generation of strong fluorescence, while other ROS cause only a slight increase in fluorescence. In vitro experiments showed that OCl^- as low as $2 \mu\text{M}$ can still be detected, which enables 223 to be used for monitoring the production of OCl^- and myeloperoxidase in human atherosclerotic arteries.



Nagano and co-workers developed a fluorescence off-on probe 224 for OCl^- .⁵⁶⁷ Reaction of the probe with OCl^- at pH 7.4 results in the oxidation of spirothioether to sulfonic group, accompanied by the spiro-ring-opening of rhodamine (Figure 41) and thus the fluorescence increase. The probe has been

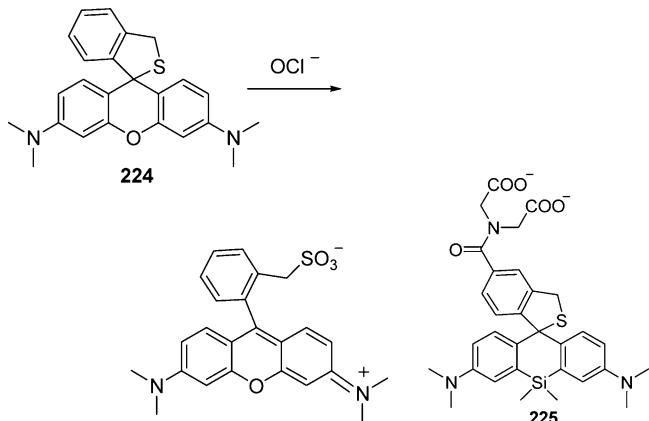


Figure 41. Reaction of 224 with OCl^- and structure of 225.

used to real-time monitoring of the production of hypochlorous acid inside phagosomes of living neutrophils. By replacing the oxygen atom in the tetramethylrhodamine with a silicon atom, an NIR fluorescent probe 225 was prepared,⁵⁶⁸ which has excellent properties such as pH-independence and tolerance to both autoxidation and photobleaching. The probe shows a sensitive and selective fluorescence response to HOCl and has been used for real-time fluorescence imaging of phagocytosis.

Ma's group first proposed dibenzoylhydrazine as an OCl^- recognition moiety, with which *N*-benzoyl rhodamine B hydrazide (226) has been synthesized and characterized as a new fluorescent probe for OCl^- .⁵⁶⁹ At pH 12, reaction of 226 with OCl^- causes a largely enhanced fluorescence, and the fluorescence increase is highly sensitive and selective for OCl^- .

over other common ions and oxidants. A good linear equation of $\Delta F = 52.69 \times [\text{OCl}^-] (\mu\text{M}) - 29.83$ was obtained between the fluorescence increase (ΔF) and the OCl^- concentration in the range of 1–10 μM , with a detection limit of 27 nM OCl^- . The reaction mechanism has been proposed as that OCl^- selectively oxidizes the hydrazone group in the probe, forming the analogue of dibenzoyl diimide, which in turn hydrolyzes releasing the fluorochrome (Figure 42). Further, on the basis of

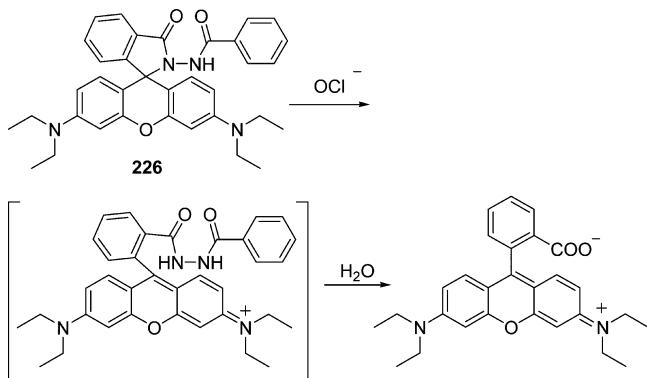
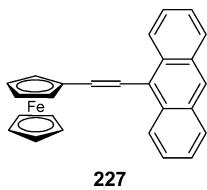


Figure 42. Reaction of 226 with OCl^- .

the specific HOCl-mediated oxidative cleavage of the *N*-acylsulfonohydrazide linker, a FRET probe has been synthesized by our group. The probe has a detection limit of 80 nM HOCl and has been applied to cell imaging of HOCl.⁵⁷⁰ It should be pointed out that, at about pH 7, some of the rhodamine-based probes with the reactive dibenzoylhydrazine moiety can also respond to Cu^{2+} .⁵⁷¹

In another study, Ma et al. prepared 227 as a fluorescent probe for the selective detection of OCl^- .⁵⁷² The probe is nonfluorescent due to electron transfer from the electron-rich ferrocene group to the anthracene moiety. Upon reaction with OCl^- , the double bond between anthracene and ferrocene is cleaved, leading to a more than 100-fold increase in fluorescence. The fluorescence increase is directly proportional to the OCl^- concentration from 1 to 10 μM with a detection limit of 0.3 μM . In aqueous buffer (pH 7.4), 227 also shows a high selectivity for OCl^- over other ROS, and its applicability has been demonstrated for fluorescence imaging of HOCl in HeLa cells.



On the basis of the hypochlorite-induced oxidative desulfurization ($-\text{SCl}$), several sulfur-containing Hg^{2+} probes have also been proposed for hypochlorite detection. For example, the above Hg^{2+} probe, rhodamine B thiolactone (195),^{509,510} has been demonstrated with the ability of sensing HOCl at pH 7.4 (detection limit, 0.3 μM HOCl).⁵⁷³ Similarly, rhodamine 6G thiolactone⁵⁷⁴ as well as compounds 228–230 can also be used for the detection of HOCl,^{495,575} among which 228 features an NIR fluorescence response (Figure 43),⁴⁹⁵ and 230 is a FRET probe.⁵⁷⁵

Protection–deprotection of an aldehyde has been used to design OCl^- probes, such as 231.⁵⁷⁶ In the presence of OCl^- ,

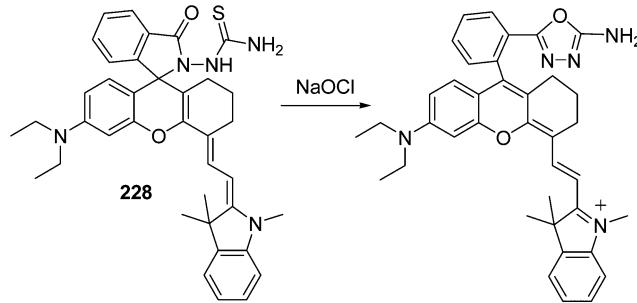
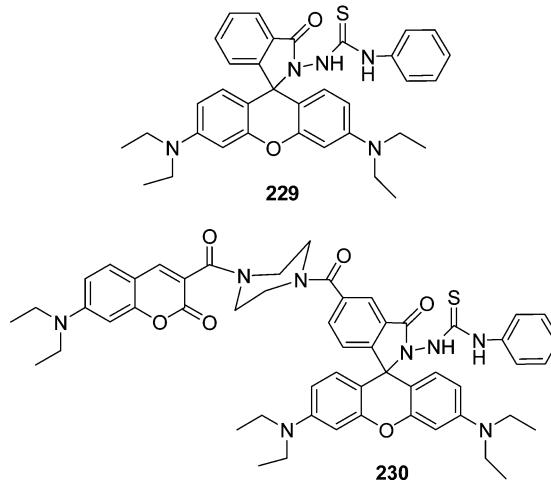


Figure 43. Reaction of 228 with OCl^- .



the oxime group in 231 is cleaved to produce an electron-withdrawing aldehyde group (Figure 44). This change results in

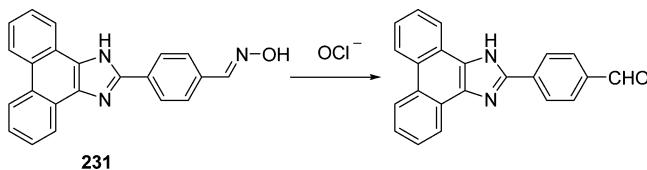
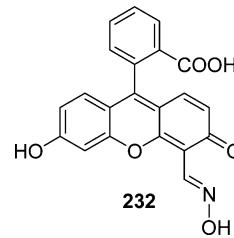
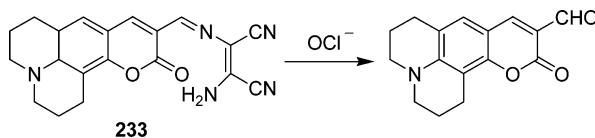


Figure 44. Reaction of 231 with OCl^- .

a red-shift in fluorescence that arises from ICT. In aqueous buffer (pH 9.0, 25% DMF), this probe displays a ratiometric fluorescent response to OCl^- with the emission intensity ratio (I_{509}/I_{439}) increasing from 0.28 to 2.74. Moreover, probe 231 can respond to OCl^- down to micromolar levels and is highly selective for OCl^- over other oxidants, such as H_2O_2 , NO_2^- , and ClO_3^- . On the basis of the same reaction mechanism, another fluorescent turn-on probe (232) has been synthesized, which can react with OCl^- at pH 7.⁵⁷⁷

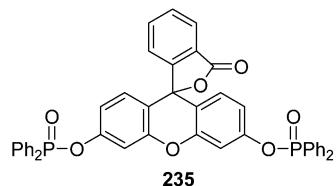
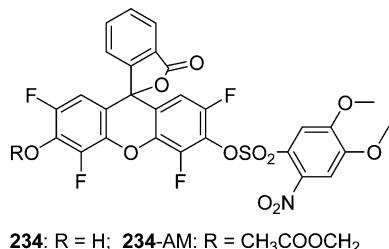


Utilizing the OCl^- -induced dediazoniumaleonitrile reaction, compound 233 was designed as a ratiometric fluorescent probe for OCl^- (Figure 45).⁵⁷⁸ 233 displays a fluorescence maximum

Figure 45. Reaction of 233 with OCl^- .

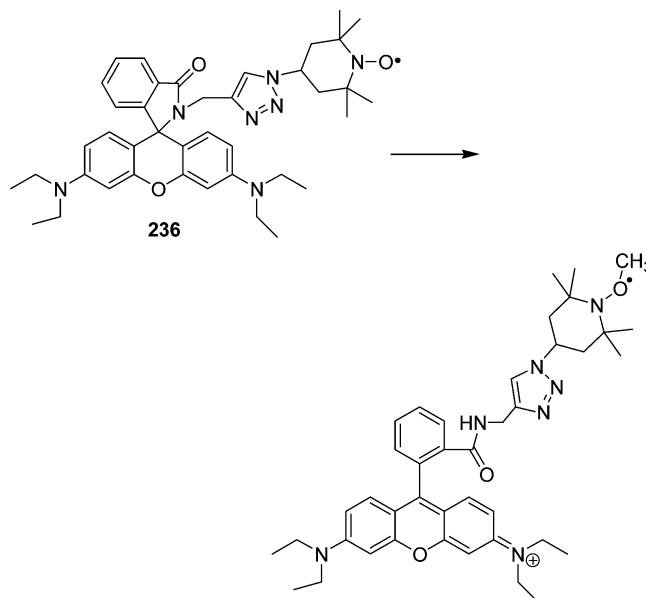
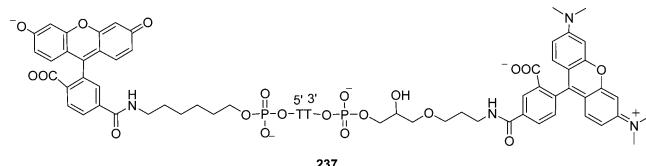
at 585 nm. Upon addition of OCl^- , the emission intensity at 585 nm decreased gradually with the simultaneous appearance of a new blue-shifted emission band centered at 505 nm, affording the ratiometric detection. Probe 233 has a detection limit of 0.3 μM and has been used for ratiometric imaging of OCl^- in live MCF-7 cells.

4.1.3.4. Probes for Superoxide Radical ($\text{O}_2^{\bullet-}$). Protection-deprotection reactions of sulfonic or phosphinate ester bonds are useful in designing fluorescent $\text{O}_2^{\bullet-}$ probes.^{579–581} For example, compound 234 was prepared as a sensitive and selective $\text{O}_2^{\bullet-}$ probe based on the deprotection of benzene-sulfonyl fluorescein.⁵⁷⁹ As compared to bis(2,4-dinitro-)tetrafluorofluorescein,⁵⁴⁵ probe 234 exhibits greater specificity toward $\text{O}_2^{\bullet-}$ over Fe^{2+} , reductases, diaphorase, GSH, and other ROS such as H_2O_2 , NaOCl , $t\text{-BuOOH}$, ${}^1\text{O}_2$, and ONOO^- . This feature allows 234 and its AM-ester to serve as fluorescent probes for the detection of extra- and intracellularly generated $\text{O}_2^{\bullet-}$. Probe 235 was designed on the basis of the $\text{O}_2^{\bullet-}$ -mediated deprotection of phosphinated fluorescein.⁵⁸⁰ Upon reaction with $\text{O}_2^{\bullet-}$, 235 exhibits a strong fluorescence with high selectivity for $\text{O}_2^{\bullet-}$ over other ROS and some biological compounds. The probe is cell-permeable and can detect the change of $\text{O}_2^{\bullet-}$ concentration at micromolar levels in living cells by using confocal microscopy.

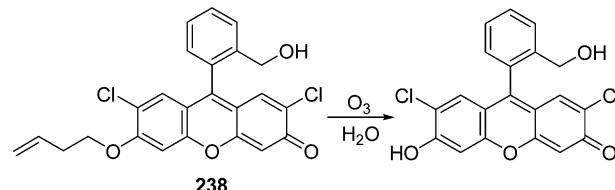


4.1.3.5. Probes for $\cdot\text{OH}$. The reaction of nitroxides with $\cdot\text{OH}$ in the presence of DMSO has been utilized to develop fluorescent probes for monitoring hydroxyl radical.^{582–584} Yapici et al. prepared a series of such fluorescent probes (e.g., 236) based on rhodamines.⁵⁸⁴ These probes are highly selective for $\cdot\text{OH}$ in aqueous solution, and can avoid interferences from other ROS, which makes them suitable for diverse applications in biological imaging (Figure 46).

Compound 237 was designed as a FRET probe with 6-carboxyfluorescein and 5-carboxytetramethylrhodamine as the respective donor and acceptor, which were linked by a cleavable deoxythymidine dimer.⁵⁸⁵ Reaction of 237 with $\cdot\text{OH}$ cleaves the linker and leads to the cancellation of the FRET effect. The probe shows a good selectivity for $\cdot\text{OH}$ over other ROS.

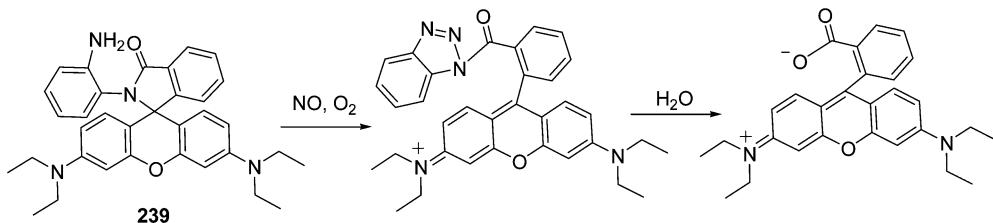
Figure 46. Reaction of 236 with $\cdot\text{OH}$.

4.1.3.6. Probe for O_3 . Koide and co-workers developed 238 as a fluorescence off-on probe for ozone based on a tandem ozonolysis- β -elimination mechanism (Figure 47).⁵⁸⁶ The

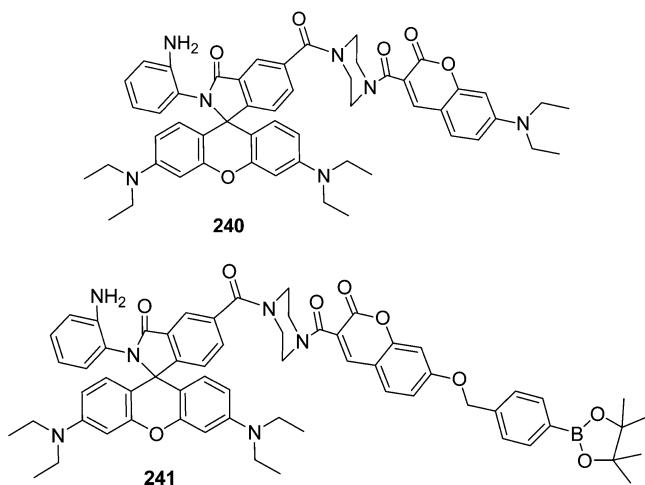
Figure 47. Reaction of 238 with O_3 .

probe showed a selective fluorescence response to ozone instead of other ROS or biological antioxidants. Reaction of 238 with ozone in the concentration range from 50 nM to 12.5 μM gave an increase of signal-to-background ratio from 2.6 to 310. Later they found that pyrrolidine in pH 9 or azetidine in pH 6 buffer could accelerate the elimination, which makes the fluorescence generation rapid enough to monitor ozone in real time.⁵⁸⁷

4.1.3.7. Probes for NO. Compound 239 was reported by Xu and co-workers, which contains a NO-reactive group of *o*-phenylenediamine.⁵⁸⁸ Reaction of 239 with NO in the presence of oxygen forms a triazole structure, which undergoes further hydrolysis to generate rhodamine B and thus strong fluorescence (Figure 48). Experiments showed that addition of 1.0 equiv of NOC13 [1-hydroxy-2-oxo-3-(3-aminopropyl)-3-methyl-1-triazene, a NO donor] to the solution of 239 (10 μM) caused a ca. 2400-fold fluorescence increase at 574 nm, and the detection limit was determined to be 3.0 nM NOC13. Therefore, 239 may be used as a fluorescent probe for NO detection.

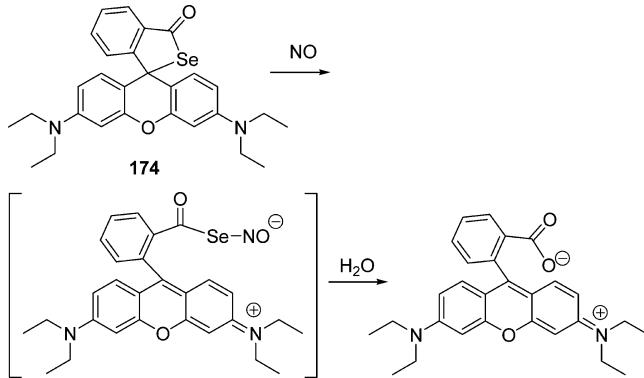
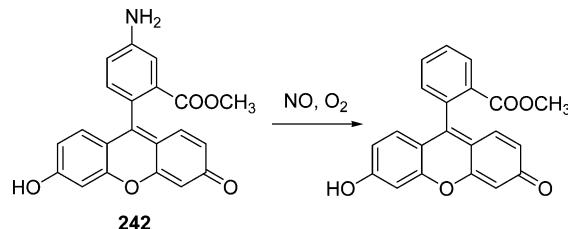
**Figure 48.** Reaction of **239** with NO.

On the basis of the same reaction, Lin's group developed a ratiometric fluorescent probe (**240**) for NO detection.⁵⁸⁹ The probe exhibits a large variation (up to 420-fold) in the fluorescence ratio (I_{583}/I_{473}) with a detection limit of 30 nM, and is capable of imaging NO in macrophage cells. Later, by using the 7-hydroxycoumarin–rhodamine platform, they further developed a single fluorescent probe (**241**), which can report H_2O_2 , NO, and $\text{H}_2\text{O}_2/\text{NO}$ with different fluorescence signal patterns.⁵⁹⁰ Significantly, **241** is capable of simultaneously detecting H_2O_2 and NO in living cells by dual-color fluorescence imaging. The probe is promising to report the production and dynamics of H_2O_2 and NO in the complex interaction networks of the signal transduction and oxidative pathways.



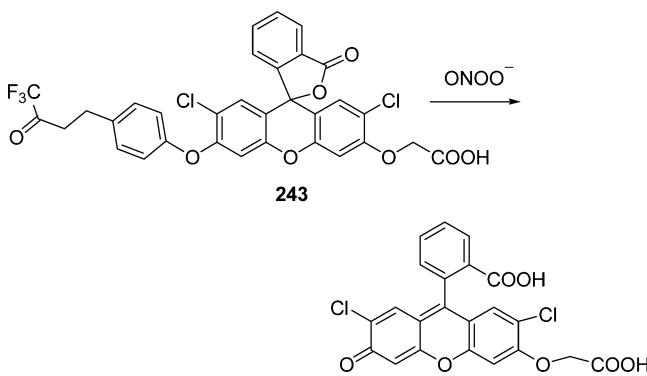
Enlightened by the interaction between NO and the SeH group of selenoproteins in biosystems,⁵⁹¹ Ma et al. used the above rhodamine B selenolactone (**174**) as a probe to develop a new approach for NO assay.⁵⁹² Reaction of **174** with NO forms a Se–NO bond, which causes the ring-opening of rhodamine B selenolactone and the subsequent hydrolytic release of rhodamine B (Figure 49). A good linearity between the fluorescence increase and the NO concentration in the range of 2.5–30 μM was observed, with a regression equation of $\Delta F = 48.5 \times [\text{NO}] (\mu\text{M}) - 9.5$, and the detection limit was determined to be 38 nM. Moreover, probe **174** is highly selective for NO over other ROS and common inorganic salts. The applicability of the probe to biosystems has been demonstrated in fluorescence imaging of NO in HeLa cells. The unique detection strategy may provide a perspective to design new selenide probes for NO sensing.

Compound **242** as a fluorescent NO probe was designed on the basis of reductive deamination of an aromatic primary monoamine (Figure 50).⁵⁹³ The probe itself is weakly fluorescent due to PET, and its reaction with NO results in significant fluorescence enhancement with a detection limit of

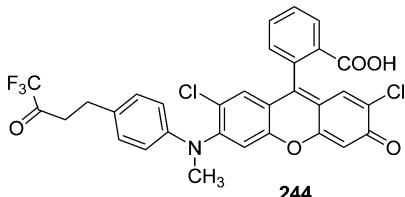
**Figure 49.** Reaction of rhodamine B selenolactone with NO.**Figure 50.** Reaction of **242** with NO.

44 nM NO. Furthermore, the good aqueous solubility of both **242** and its deaminated product as well as their high fluorescence stability over a wide range of pH ensures potential application of the probe for NO bioimaging, and this capability has been demonstrated with Raw 264.7 murine macrophages.

4.1.3.8. Probes for ONOO^- . Yang et al. found that ONOO^- can react with activated ketones to form dioxiranes,⁵⁹⁴ and such a reaction did not occur in the case of the other ROS. On the basis of this, they designed a fluorescent ONOO^- probe **243** by linking a ketone unit to a dichlorofluorescein moiety through an aryl ether linkage (Figure 51). Reaction of **243** (20 μM)

**Figure 51.** Reaction of **243** with peroxy nitrite.

with 15 equiv of ONOO^- resulted in ca. 8-fold fluorescence enhancement.⁵⁹⁵ To increase sensitivity, they further prepared a rhodol-based fluorescent probe **244**.⁵⁹⁶ This probe ($5 \mu\text{M}$) reacting with 1 equiv of ONOO^- produced 140-fold fluorescence increase, and the detection limit for ONOO^- was estimated to be 50 nM. Probe **244** has been used for cellular imaging of peroxynitrite.⁵⁹⁶



4.1.4. Cleavage of Covalent Bonds by Anions and Small Molecules. Nucleophilic reaction of some anions (e.g., F^- and SO_3^{2-}) and small molecules (e.g., thiols and hydrazine) can lead to the cleavage of the covalent bond between a removable group (usually electron-deficient groups) and a fluorochrome. As a result, the fluorochrome is released with fluorescence recovery. This behavior has been utilized to design fluorescent probes for some anions and small molecules.

4.1.4.1. Probes for F^- . By virtue of the high affinity of silicon for F^- , Swager et al. developed several fluorescent F^- probes, whose response mechanism is based on F^- -triggered Si–O bond cleavage.⁵⁹⁷ Later, more fluorescent off–on or ratiometric F^- probes were reported on the basis of this cleavage reaction.^{598–606} However, most of the probes perform well merely in the presence of large amount of organic solvents because of the strong hydration effect of F^- in aqueous environments, and only a few of them can work in aqueous solution. For instance, Hong et al.⁵⁹⁹ reported **245** as a fluorescent off–on probe with appropriate water solubility. Reaction of **245** with 1 mM NaF for 3 h in aqueous phosphate buffer results in more than 4-fold enhancement of fluorescence intensity. The applicability of **245** has been demonstrated for the detection of NaF in A549 human epithelial lung cancer cells (Figure 52). Compound **246** is a ratiometric fluorescent F^-

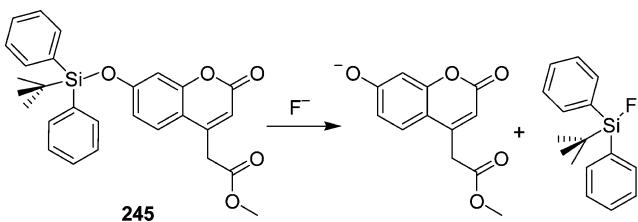


Figure 52. Reaction of **245** with fluoride.

probe,⁶⁰⁴ which reacts with F^- in 20 mM phosphate buffer of pH 7.4 containing 30% (v/v) ethanol producing a significant emission red-shift from 500 to 558 nm. Additionally, there was a good linearity between the fluorescence intensity ratio (I_{500}/I_{558}) and the concentration of F^- in the range of 0.5–28 mM with a detection limit of 0.08 mM. The probe has been applied to ratiometric fluorescence imaging of F^- in living cells.

4.1.4.2. Probes for SO_3^{2-} . By utilizing the sulfite-selective deprotection of levulinic, a few chromogenic and fluorogenic probes for sulfite have been developed,^{607,608} such as **247** and **248**. The reaction of **247** with SO_3^{2-} at pH 7.0 produces pink color and strong red fluorescence characteristic of resorufin (Figure 53), while that of **248** generates not only a fluorescence

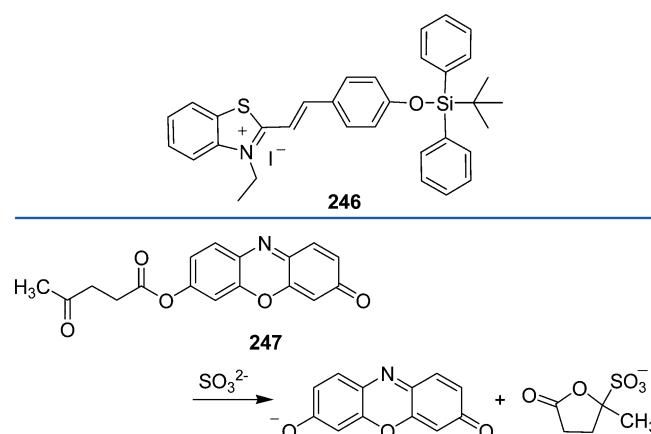
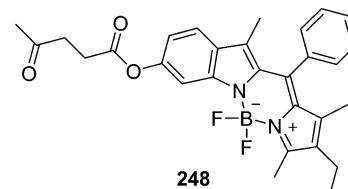


Figure 53. Reaction of **247** with sulfite.

ratiometric change but also a dramatic color change from orange to blue, which is favorable for applications in environmental analysis.



4.1.4.3. Probes for Thiols. It is well-known that sulfonethers and sulfonamides can be readily cleaved by thiolate anions. By virtue of these reactions, various fluorescent probes with an electron-withdrawing group of 2,4-dinitrobenzenesulfonyl group (DNBS) have been developed for the detection of thiols.^{609–619}

Compound **249** is a fluorescence off–on probe for thiol assay.⁶⁰⁹ Reaction of the probe with thiols cleaves the sulfonether bond and releases fluorescein, thus generating greatly enhanced fluorescence (Figure 54). **249** may also be

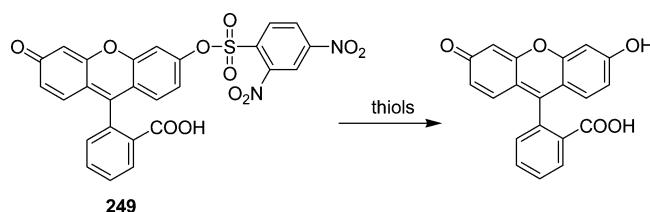
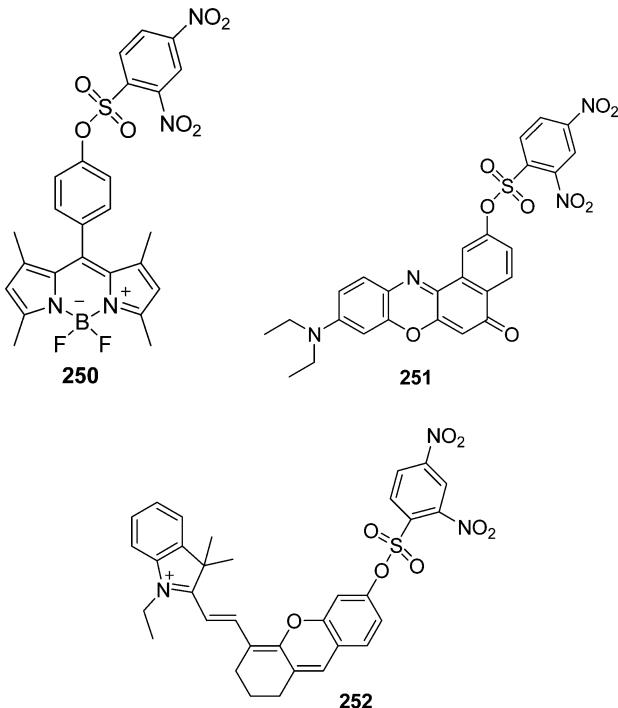


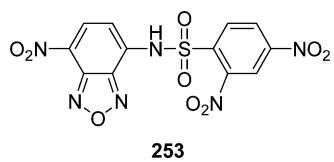
Figure 54. Reaction of **249** with thiols.

used for the screening of acetylcholinesterase inhibitors. **250** was synthesized as a fluorescent probe for Cys detection by incorporating the DNBS group into the BODIPY skeleton.⁶¹⁰ The probe itself is weakly fluorescent due to PET from the excited BODIPY fluorochrome to the electron-deficient DNBS moiety. The nucleophilic substitution reaction of Cys with **250** cleaves the sulfonether bond, causing the removal of the DNBS moiety and thereby the release of the highly fluorescent phenolic BODIPY. In addition, the fluorescence enhancement of the probe ($1 \mu\text{M}$) is directly proportional to the concentration of cysteine in the range of 2–12 μM , with a detection limit of 30 nM ($S/N = 3$). The probe has been successfully used for the determination of nonprotein Cys in human serum as well as for imaging of intracellular thiols.⁶¹¹

Compound **251**, constructed by incorporating DNBS into Nile Red, features a good water solubility and long-wavelength emission of about 650 nm.⁶¹² Reaction of **251** (10 μ M) with varied concentration of GSH in the range of 0.5–6 μ M shows linear fluorescence increase. The detection limit of **251** for GSH was determined to be 43 nM, and its applicability has been demonstrated on fluorescence imaging of reduced GSH in HeLa cells. Very recently, Lin's group synthesized an NIR thiol probe **252** based on a new NIR fluorochrome.⁵⁶² **252** also displayed a large fluorescence turn-on response to thiols. Furthermore, the probe could visualize endogenous thiols not only in living cells, but also in living mice.

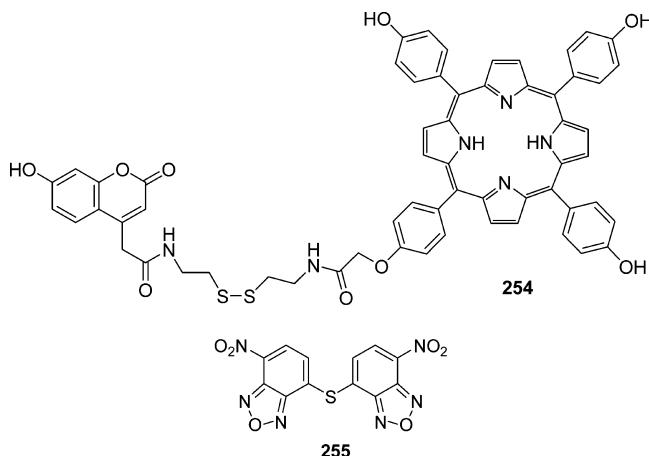


Fluorescent probes for thiols have also been developed on the basis of the thiol-induced sulfonamide cleavage. Compound **253**, reported by Jiang et al.,⁶¹⁹ can be used for aromatic thiol assay. Reaction of **253** with thiophenol in an aqueous medium of pH 7.3 causes a dramatic fluorescence enhancement, which is ascribed to the cleavage of the sulfonamide bond and removal of the DNBS moiety from the nonfluorescent **253**. Interestingly, the reaction is highly selective for thiophenols over aliphatic thiols and other nucleophiles, which enables **253** to be useful for the detection and quantification of highly toxic thiophenols in environment. On the basis of the similar reaction mechanisms, other fluorescent probes for the detection of aromatic thiols have been developed.^{620–623}



The cleavage reaction based on thiol/disulfide exchange is also frequently employed to construct fluorescent probes for thiols. Compound **254** is such a probe,⁶²⁴ which was synthesized by integrating porphyrin with coumarin. Reaction of **254** with a thiol such as Cys cleaves the disulfide bond, thereby switching off FRET between the two fluorochromes. As

a result, the probe exhibits a ratiometric fluorescent response to Cys, and can be employed for ratiometric fluorescence imaging of thiols in living cells. Compound **255** is not fluorescent, but can form a strong fluorescent adduct after reacting with a thiol through a sulfide–thiol exchange.⁶²⁵ This property can be used to image and quantify thiol changes in live cells through fluorescence microscopy.



In addition, the thiol-induced cleavage of a disulfide-based carbamate protecting group is often employed to construct fluorescent probes for thiols.^{626–628} By using triphenylphosphonium salt as a mitochondrial-targeting group, Kim et al. prepared **256** as a ratiometric TP probe for mitochondrial thiols.⁶²⁹ The reaction between **256** and a thiol cleaves the disulfide-based carbamate protecting group, resulting in a ratiometric fluorescence response, which is ascribed to the pronounced ICT effect. The large TP cross section of **256** enables the probe to visualize mitochondrial thiol levels in live cells as well as in living tissues at depths of 90–190 μ m. With galactose as a hepatocyte-targeting group, Kang et al. synthesized a thiol probe **257**,⁶³⁰ in which the galactose subunit serves to guide the probe to hepatocytes, while the cleavable disulfide is responsible for inducing the change of spectroscopic signal of naphthalimide. Reaction of the probe with thiols gives rise to an enhanced fluorescence emission. The potential utility of **257** in indicating pathogenic states and as a possible screening tool for antioxidants has been demonstrated with HepG2 cells.

Taking advantage of the selective cleavage of Se–N bond, some fluorescent thiol probes have been developed as well.^{631–633} Compound **258** is a rhodamine-based thiol probe, which contains a cleavable Se–N bond (Figure S5).⁶³¹ The probe exhibits larger fluorescence response to GSH than to other nonprotein thiols, and has been applied to imaging the difference in thiol concentrations between HL-7702 and HepG2 cells. Similarly, a tetraselenide derivative of rhodamine 110 (**259**)⁶³² and two NIR fluorescent probes (**260** and **261**)⁶³³ with better performance were synthesized for imaging thiols in live cells.

It was reported that the cleavage of diselenide bonds by thiols is much faster than that of disulfide bonds.⁶³⁴ On the basis of this behavior, a diselenide-containing fluorescent probe (**262**) for the rapid detection of thiols has been developed (Figure S6).⁶³⁵ The probe can also be used for monitoring the redox changes mediated by thiols and ROS in living cells.

Thiols can add to quinones in the Michael addition fashion, forming reduced hydroquinone adducts, and this chemistry was

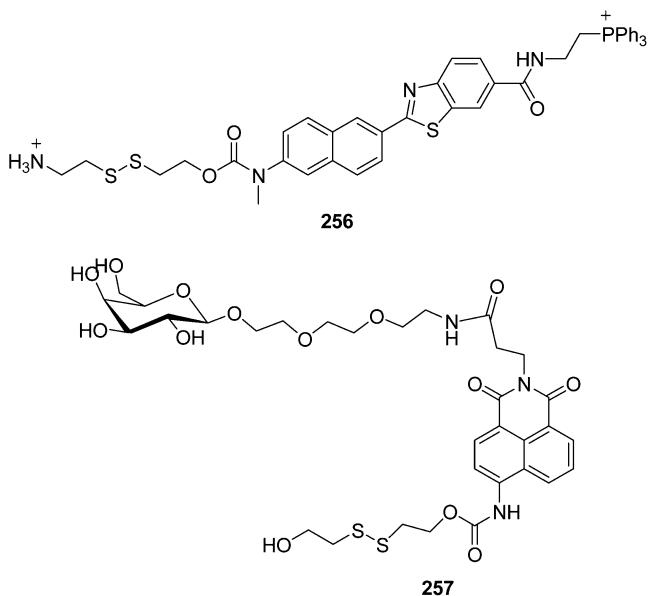


Figure 55. Reaction of 258 with thiols (RSH).

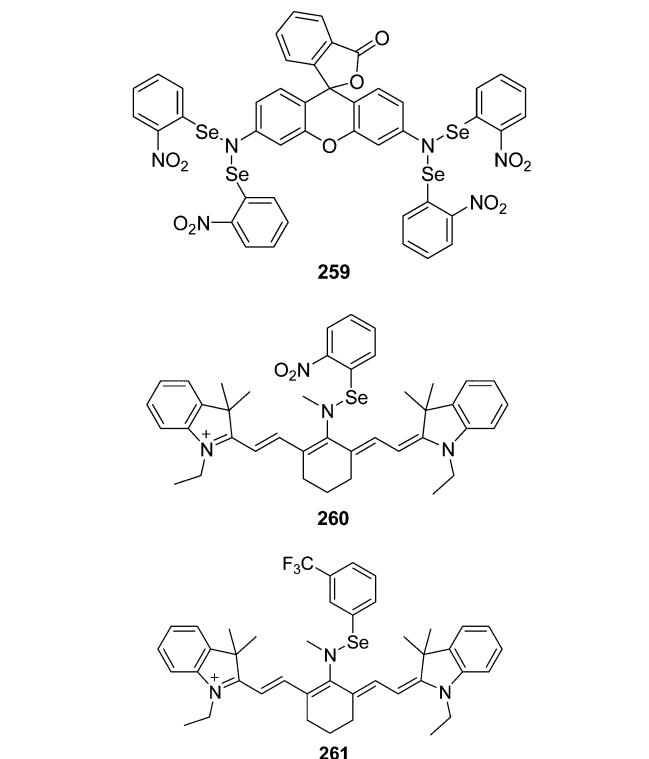


Figure 56. Reaction of 262 with thiols (R'SH).

used to develop a turn-on fluorescent probe (263) for thiols.⁶³⁶ Reaction of 263 with thiols leads to the cleavage of the ether bond and thus the release of the fluorescent coumarin moiety (Figure 57).

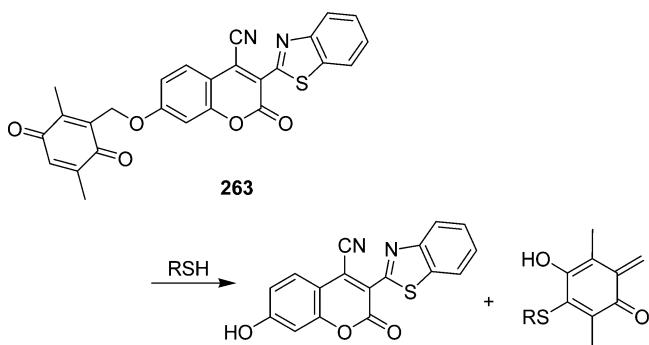


Figure 57. Reaction of 263 with thiols (RSH).

The addition reaction of Cys or homocysteine (Hcy) to acrylate generates a thioether, which can undergo further intramolecular cyclization. Taking advantage of this reaction, Strongin et al. developed a fluorescent thiol probe (264) by masking the hydroxy group in the fluorochrome of 2-(2'-hydroxy-3'-methoxyphenyl)benzothiazole with an α,β -unsaturated carbonyl moiety.⁶³⁷ Reaction of the probe with Cys or Hcy leads to the cleavage of the ester bond and the release of the fluorochrome as a result of the intramolecular cyclization (Figure 58). Importantly, the probe shows different reaction rates with Cys and Hcy, which permits them to be detected selectively. The probe has been used in the detection of Cys in

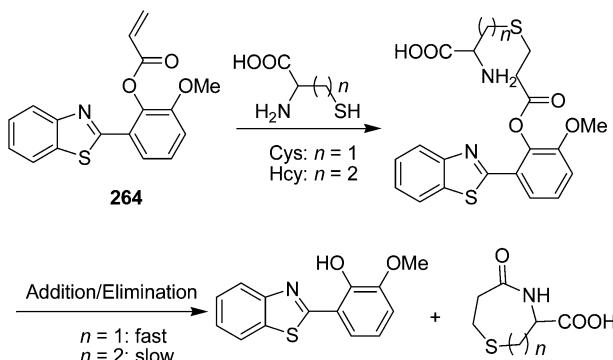
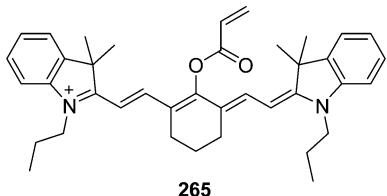


Figure 58. Reaction mechanism of 264 with Cys or Hcy.

diluted (10%) deproteinized human plasma. Similarly, on the basis of the different rates of intramolecular cyclizations between Cys and Hcy, other fluorescent Cys probes have been prepared,^{638–640} such as **265**, which shows a highly selective ratiometric response to Cys.⁶³⁹ The probe has been successfully applied for imaging of Cys in cancer cells.



Chemical ligation reaction between a peptide- α -thioester and an N-terminal Cys-peptide, initially used in peptide synthesis, has been used to prepare **266** as a FRET-type fluorescent probe for thiols.⁶⁴¹ Reaction of **266** with Cys caused the separation of rhodamine from BODIPY and thus the change of fluorescence intensity at 590/510 nm (Figure 59). The probe has been applied for ratiometric detection of thiols in biological fluids and also for imaging of thiols in living cells.

4.1.4.4. Probes for H_2S . Development of fluorogenic probes for the selective detection and imaging of H_2S has been a hot research topic in recent years.⁶⁴² Xian et al. designed a disulfide-containing probe **267** for selective detection of H_2S (Figure 60).⁶⁴³ The probe adopted a closed lactone conformation and exhibited no absorption in the visible region. Reaction of **267** with H_2S results in the formation of benzodithiolone with the release of the fluorochrome. Probe **267** is selective for H_2S , and its application has been demonstrated for detecting H_2S in plasma as well as in cells. Another turn-on fluorescent H_2S probe (**268**)⁶⁴⁴ utilized the H_2S -specific Michael addition-cyclization reaction to release the fluorochrome. Probe **268** also shows good sensitivity and selectivity for H_2S , and has been used for imaging of H_2S in NaHS-treated cells (Figure 60). On the basis of the similar reaction mechanism, a ratiometric fluorescence probe has also been reported for fluorescence imaging of H_2S in living cells.⁶⁴⁵

Compound **269** is a coumarin derivative, whose *o*-(azidomethyl) benzoyl moiety can be efficiently deblocked via the H_2S -triggered tandem reactions of the azido reduction and intramolecular amidation of the in situ generated amine group, followed by the release of highly fluorescent 7-hydroxy-4-

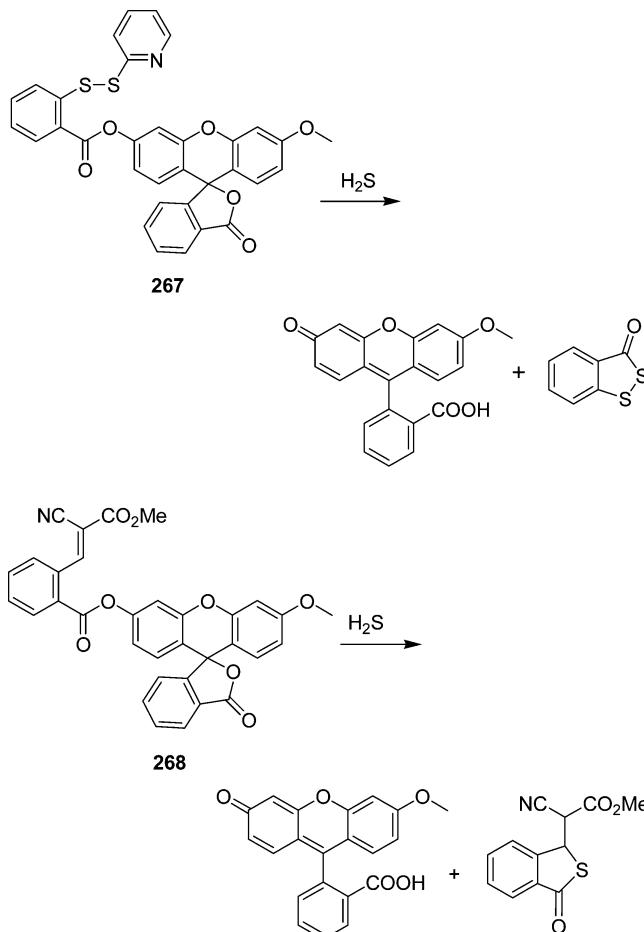


Figure 60. Reactions of **267** and **268** with H_2S .

methylcoumarin (Figure 61).⁶⁴⁶ **269** is highly selective for H_2S and is suitable for the study of H_2S -mediated biomedical events.

Lin et al. have developed an NIR fluorescent H_2S probe (**270**) based on the thiolysis of the dinitrophenyl ether by H_2S (Figure 62).⁶⁴⁷ Probe **270** has a high selectivity for H_2S over the other biological species tested, including GSH and Cys at the biologically relevant concentrations, and is thus suitable for fluorescent imaging of H_2S in living cells.

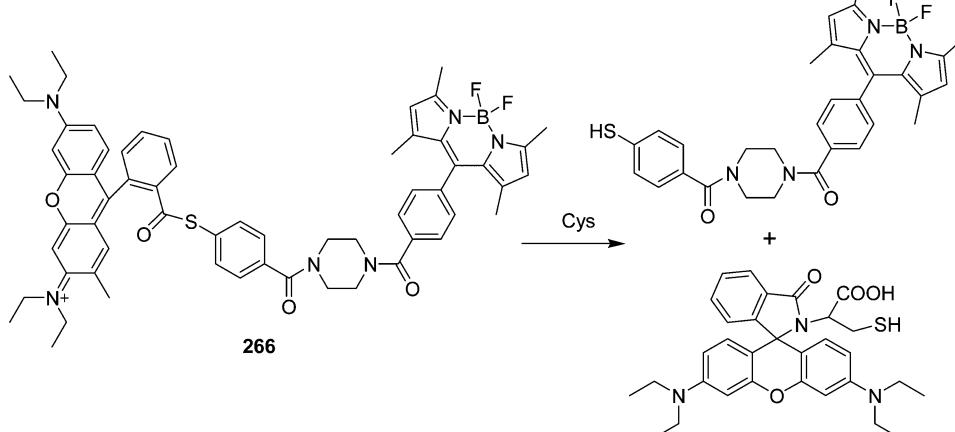


Figure 59. Reaction of **266** with Cys.

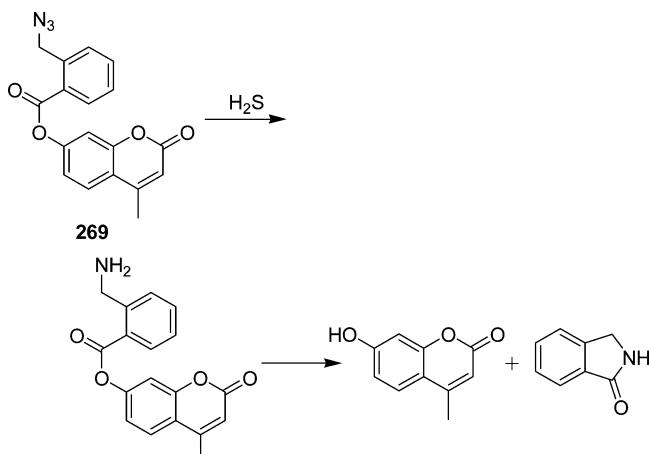


Figure 61. Reaction of **269** with H₂S.

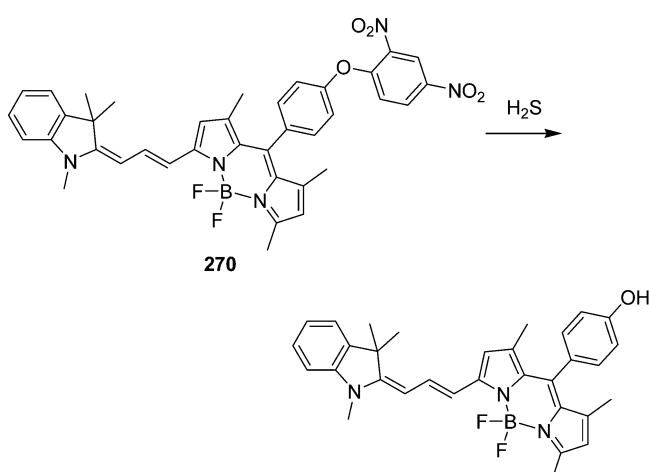
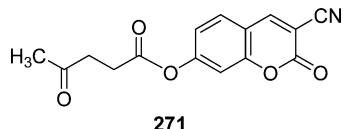


Figure 62. Reaction of **270** with H₂S.

4.1.4.5. Probe for Hydrazine. Hydrazine can remove the protective group of levulinic acid under acidic conditions, and this reaction has led to the development of a coumarin-based fluorescent off-on probe (**271**) for hydrazine.⁶⁴⁸

4.2. Chromogenic and Fluorogenic Probes Based on the Formation of Covalent Bonds

The formation of covalent bonds has long been used to develop chromogenic and fluorogenic probes for various analytes. In particular, such probes have been extensively used in chromatographic derivatization and biomolecular labeling via a variety of chemical reactions (e.g., acylation of amines, alkylation of thiols by maleimides, dansylation of alcohols,



esterification of carboxylic acids, Schiff base formation of carbonyl compounds, etc.). Traditional chromogenic and fluorogenic probes for derivatization and labeling purposes have been systematically documented in books.^{1,649} In this Review, we are concerned with the recent development of chromogenic and fluorogenic probes that are based on the formation of covalent bonds for the selective detection of analytes without chromatographic separation. This strategy

usually takes advantage of the nucleophilic addition reactions between fluorochromes and analytes (e.g., amines, phenols, thiols, sulfide, cyanide), or metal-ion-catalyzed/promoted ligation reactions (e.g., Cu^{+} -catalyzed click reaction between azide and alkyne, and Hg^{2+} -promoted intramolecular cyclic guanylation of thiourea derivatives), which will be reviewed below.

4.2.1. Probes for Small Molecules. 4.2.1.1. *Probes for*

Amines. Several chromogenic and fluorogenic probes have been reported for the assay of amines including amino acids in recent years.⁶⁵⁰ Ma et al. developed 272 as a fluorescent probe for in situ labeling of highly volatile methylamine.⁶⁵¹ The probe itself is weakly fluorescent due to fluorescence quenching by the electron-deficient 1,3,5-triazinyl group. Upon reaction with the electron-donating methylamine, the fluorescence quenching is greatly suppressed, and a large fluorescence enhancement is produced at $\lambda_{\text{ex/em}} = 340/405 \text{ nm}$, which enables 272 to serve as a probe for in situ labeling of highly volatile methylamine (Figure 63). The applicability of the probe has been demonstrated by measuring methylamine released during hydrolysis of *N*-methylcarbamates such as ethiofencarb.

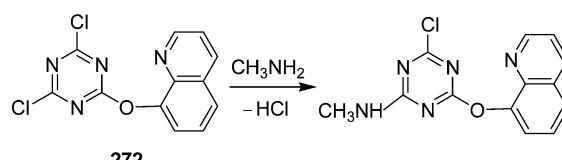


Figure 63. Reaction of 272 with CH_3NH_2 .

Wolfbeis et al. designed selective chromogenic and fluorogenic probes for primary aliphatic amines based on pyrylium dyes.^{652,653} Compound 273, a representative of these probes, exhibits weak fluorescence at $\lambda_{\text{ex/em}} = 611/665 \text{ nm}$. Upon exposure to primary aliphatic amines, the probe can be easily converted into a pyridinium derivative, accompanied by a distinct color change from blue to red and a greatly enhanced fluorescence at $\lambda_{\text{ex/em}} = 503/602 \text{ nm}$ (Figure 64). The probe

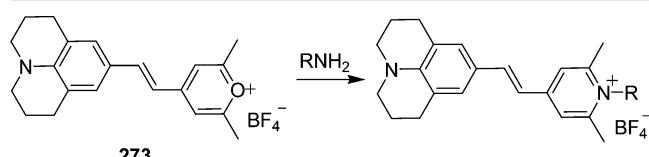


Figure 64. Reaction of **273** with an amine (RNH_2).

can be used for detection and labeling of amine-containing biomolecules. In addition, they also proposed a cyanine-based NIR probe for labeling of biogenic amines.⁶⁵⁴ The NIR probe, bearing an active chlorine, reacts readily with primary amines, producing not only a color change from green to blue but also a great increase in fluorescence.

Aldehyde-modified coumarin derivatives were reported as fluorogenic probes for the assay of amines via the formation of imines.⁶⁵⁵⁻⁶⁵⁷ Compound 274 is such a probe (Figure 65), which operates efficiently in high salt, neutral solution with excitation and emission profiles similar to those of BODIPY. Furthermore, intramolecular hydrogen bonding can increase fluorescence. The aldehyde-substituted distyrylbenzene, 275, can react with amines in water to form imines or cyclic aminals depending on the structure of the employed amine,⁶⁵⁸ accompanied by an off-on and blue-shifted fluorescence.⁶⁵⁸

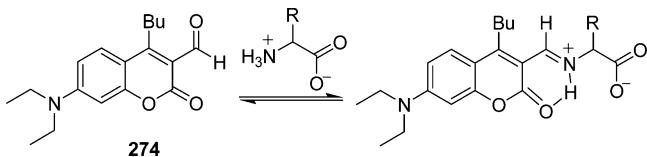
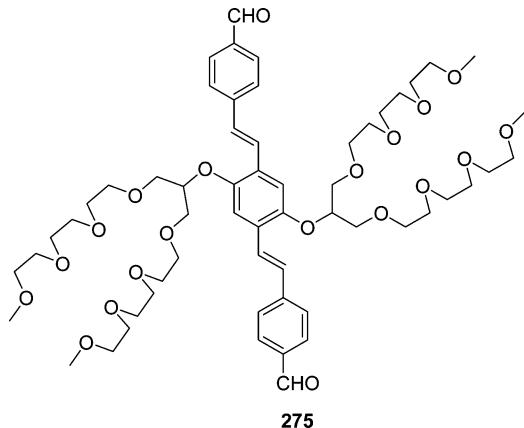
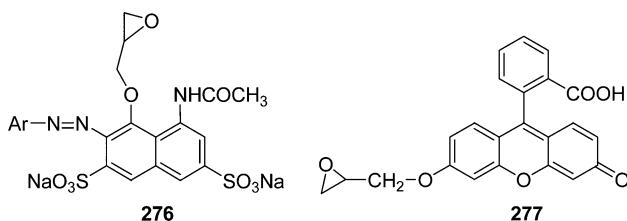


Figure 65. Reaction of 274 with an amino acid.

This property enables 275 to discern different amines, as demonstrated by photography.



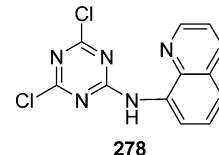
Addition reaction of the secondary amino group in imidazole with an active epoxy group has led to the successful design of two fluorescent probes, 276 and 277, for the selective analysis of histidine.^{659,660} In alkaline media, both of the probes react selectively with histidine rather than the other amino acids. Reaction of histidine with 276 causes a large decrease in fluorescence intensity, but that with 277 shows a great fluorescence increase. The two probes have been applied in the determination of histidine in human serum, and the results were in good agreement with those obtained by using histidine–nickel complex adsorptive voltammetry.



Oguri and co-workers found that 2,3-naphthalenedicarboxyaldehyde could act as a selective chromogenic probe for histamine in a weakly acidic buffer solution.⁶⁶¹ The reaction of histamine with 2,3-naphthalenedicarboxyaldehyde at pH 6.0 caused an increase in absorbance at 552 nm within 10 min. No similar color formation was observed for other amines except histidine and serotonin. On the basis of this reaction, a simple and convenient method for visual detection of histamine on a short silica–gel column cartridge was developed, and its applicability has been demonstrated on the analyses of fresh tuna meat.

4.2.1.2. Probes for Phenols. Compound 278 was proposed as a fluorescent probe for labeling of phenol.⁶⁶² Reaction of 278 with phenol in alkaline media caused the excitation and emission peak shifts of the probe from 334 and 443 nm to 361 and 485 nm, respectively, which provides the basis for establishing a simple method for the fluorimetric determination of phenol with a detection limit of 11 ng/mL. The method has

been applied to determining phenols in industrial wastewater, and the results obtained were in good agreement with those obtained by using 4-aminoantipyrine colorimetry.



Tanaka et al. proposed the imines derived from aniline derivatives as potential fluorogenic probes for phenols in aqueous buffer based on Mannich-type reactions.⁶⁶³ These probes are nonfluorescent or very weakly fluorescent. However, addition products of the imines to phenols show more than 100-fold higher fluorescence than the imines and the precursor amines. These fluorogenic imines might be useful for the detection of phenol-bearing molecules. Figure 66 illustrates the reaction mechanism of such a probe (279) with a phenol.

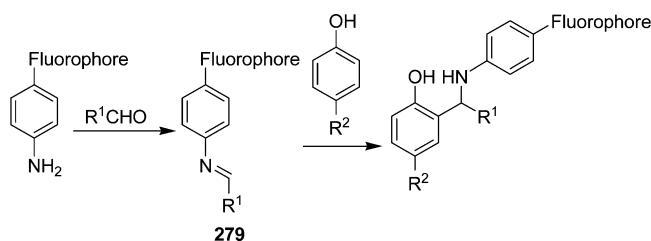


Figure 66. Mannich-type reaction of 279 with a phenol.

4.2.1.3. Probes for Thiols. A large number of chromogenic and fluorogenic probes for thiols have been reported on the basis of the formation of covalent bonds,^{480,547,664,665} among which Michael additions and cyclization reactions of aldehydes with Cys/Hcy have attracted much attention.

Some excellent fluorescent thiol probes have been developed by utilizing the Michael additions of thiols with maleimides,^{666–670} squaraines,^{671,672} quinones,^{673–675} α,β -unsaturated carbonyl moieties,^{676–683} α,β -unsaturated malonitrile,⁶⁸⁴ nitroolefin,⁶⁸⁵ maleate,⁶⁸⁶ and so on.^{480,547,664}

Incorporation of the maleimide moiety into fluorochromes such as BODIPY leads to weak fluorescence of the resulting probes due to the $n-\pi^*$ transition or PET.^{480,547} However, nucleophilic addition of thiol to the maleimide moiety in such probes eliminates the quenching processes and therefore results in fluorescence off-on response. Compound 280 is such a probe, whose reaction with thiol produces a 350-fold fluorescence enhancement via the inhibition of the PET process (Figure 67).⁶⁶⁸ Among the *ortho*-, *meta*-, and *para*-substituted maleimide derivatives of BODIPY, only the *ortho* one (i.e., 280) is fluorescently quenched through PET, which is

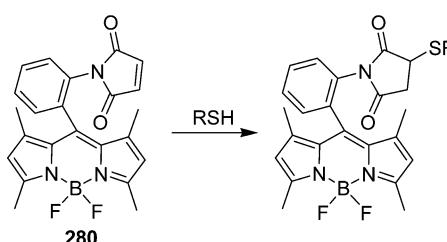


Figure 67. Reaction of 280 with a thiol.

ascribed to the closest distance between the electron donor (BODIPY) and acceptor (maleimide). Probe **280** has been used for detecting extremely low concentrations of bovine serum albumin in the gel after sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Nucleophilic addition of thiols to the electron-deficient central four-membered ring of squaraine has been employed to prepare **281** as a ratiometric NIR fluorescent probe.⁶⁷² Reaction of the squaric acid skeleton with a thiol breaks the large π -conjugated system of **281** (Figure 68), causing a blue

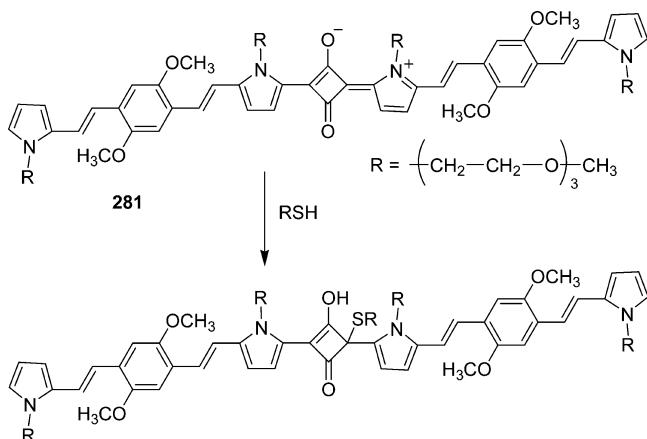


Figure 68. Reaction of **281** with a thiol.

shift in both absorption and fluorescence maxima. Therefore, the thiol concentration can be quantified ratiometrically by measuring the fluorescence ratio at 595/806 nm. The probe has been used for detecting aminothiols in human blood plasma and evaluating the smoking effect on the increased level of aminothiols in blood.

A number of thiol probes have been prepared by virtue of the Michael addition of thiols with α,β -unsaturated carbonyl moieties, most of which are based on the coumarin skeleton.^{676–683} Compound **282** is such a probe (Figure 69),

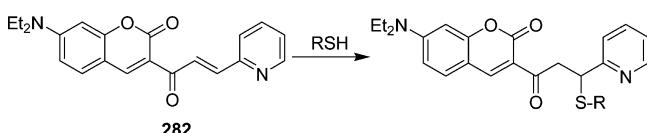


Figure 69. Reaction of **282** with a thiol.

and its reaction with a thiol shows a greatly enhanced fluorescence due to the elimination of the ICT process.⁶⁷⁶ Moreover, the fluorescence response is selective and sensitive for thiols with a detection limit of 9.25×10^{-7} M in an aqueous buffer of pH 7.4. These characteristics enable **282** to be used in potential biosystems.

A nonfluorescent coumarin-malononitrile derivative **283** was also proposed as a thiol probe, whose fluorescence can be selectively turned on through Michael addition of a thiol (Figure 70).⁶⁸⁴ The probe exhibits a highly selective fluorescence response toward biothiols (Cys, Hcy, GSH) with micromolar sensitivity both in vitro and in vivo, and has been applied for detection of intracellular thiols.

The selective cyclization reactions of aldehydes with Cys or Hcy were exploited to develop chromogenic and fluorogenic probes for Cys and Hcy.^{687–693} Interestingly, the probes with

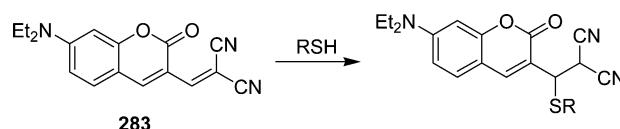


Figure 70. Reaction of **283** with a thiol.

aldehyde groups can form a five- or six-membered ring with Cys or Hcy, instead of the other biothiols like GSH. For example, probe **284**, which contains an aldehyde group, only reacts with Cys and Hcy (Figure 71),⁶⁹⁴ resulting in a marked

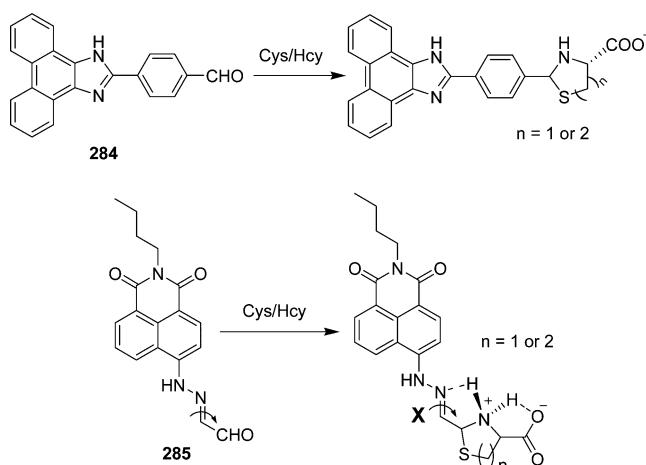


Figure 71. Reactions of **284** and **285** with Cys/Hcy.

decrease of emission intensity at 519 nm and concurrently a dramatic enhancement of emission intensity at 394 nm. This allows **284** to be employed for the quantitative detection of Cys or Hcy in a ratiometric mode. A naphthalimide-based probe **285** was reported for the detection of Cys/Hcy.⁶⁹⁵ The cyclization of the aldehyde group of **285** with Cys/Hcy (Figure 71) causes a large fluorescence enhancement and an obvious color change from black to green, which is attributed to the inhibition of the C=N isomerization-induced quenching process via the formation of an intramolecular hydrogen bond. The probe was applied to the imaging of Cys or Hcy inside living cells.

Compound **286** shows a selective ratiometric fluorescence response to Cys rather than Hcy and GSH by forming a 1:2 adduct with Cys (Figure 72),⁶⁹⁶ which arises from a much faster reaction rate of **286** for Cys than those for Hcy and GSH. The emission ratio (I_{487}/I_{557}) is directly proportional to the amount of Cys (2–900 μ M) with a detection limit of 7.5×10^{-7} M in pH 7.4 phosphate buffer containing 10% (v/v) CH₃CN. The probe is applicable for ratiometric imaging of the change of Cys levels in living cells.

Compound **287** was reported as a thiol probe in which malononitrile serves as a thiol-reactive moiety.⁶⁹⁷ The probe displays a selective colorimetric and ratiometric fluorescence response toward thiols, and its sensing mechanism, that is, multiaddition of a thiol molecule (e.g., Cys) to the C=C double bond and the –CN group (Figure 73), has been confirmed by mass spectral analysis. The detection limit of the probe for Cys is 6.02×10^{-7} M.

In a recent study, nitroolefin-functionalized BODIPY (**288**) was reported, which can rapidly react with Cys through an unexpected addition to the BODIPY moiety (Figure 74).⁶⁹⁸ **288** is weakly fluorescent; however, its reaction with Cys

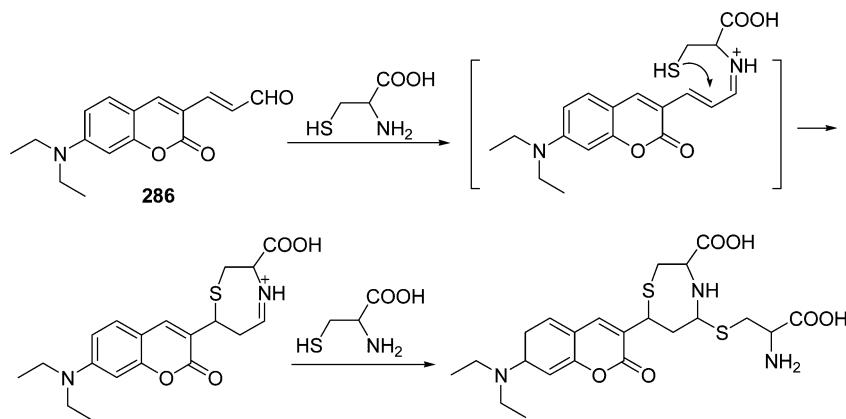


Figure 72. Reaction 286 with Cys.

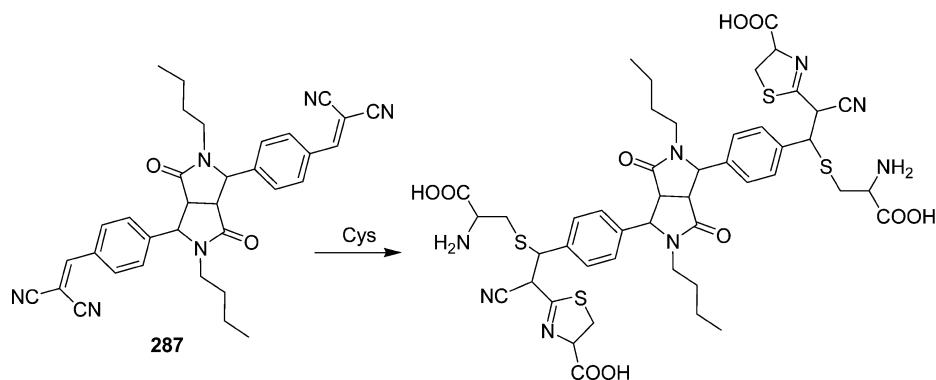


Figure 73. Reaction of 287 with Cys.

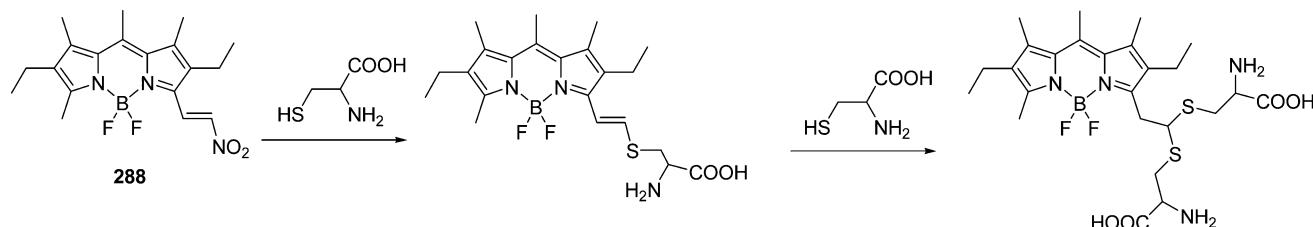


Figure 74. Reaction of 288 with Cys.

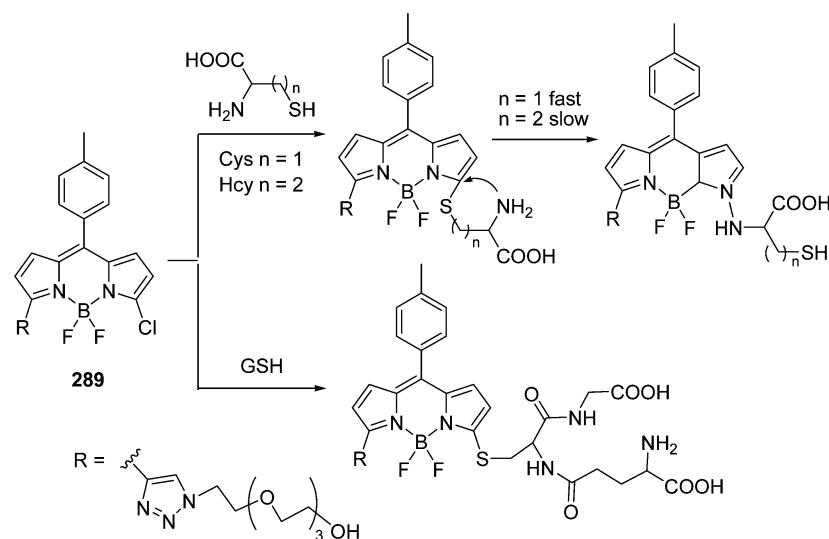


Figure 75. Reaction mechanisms of 289 with Cys/Hcy and GSH.

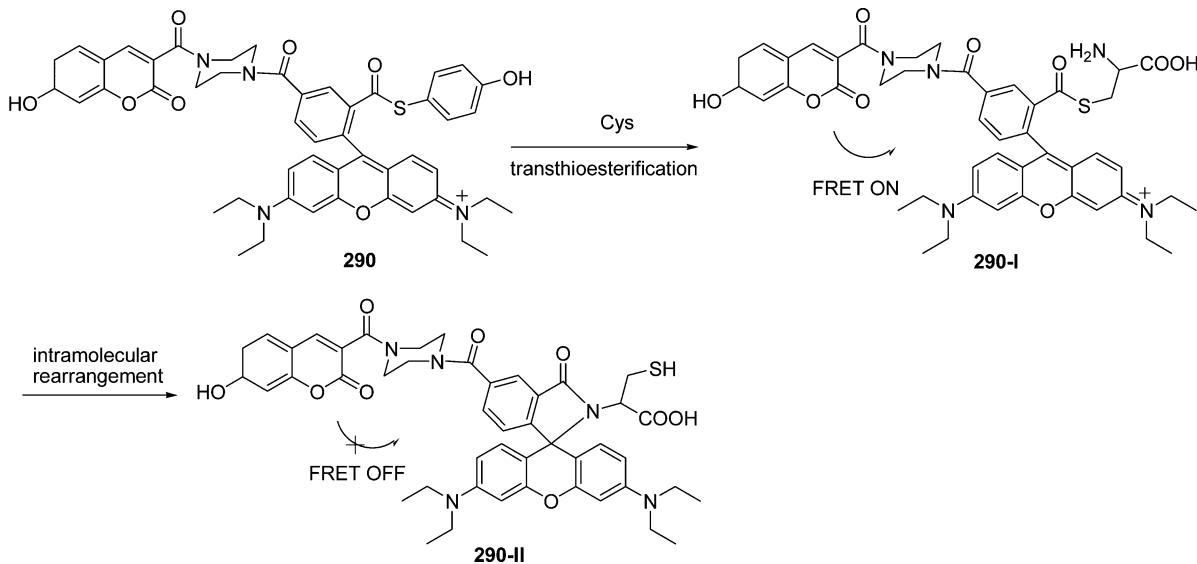


Figure 76. Reaction mechanism of **290** with Cys.

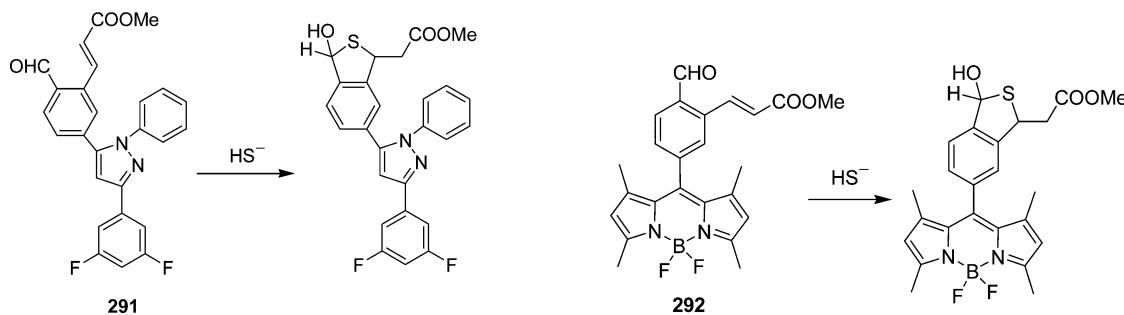


Figure 77. Reactions of **291** and **292** with sulphide.

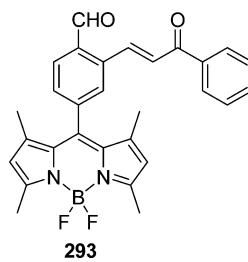
generates a highly fluorescent product in aqueous buffer of pH 7.4 with a detection limit of 8.77×10^{-7} M. The reaction is highly selective for Cys, and has been applied for detecting Cys in living cells.

Monochlorinated BODIPY (**289**) has recently been proposed as a ratiometric fluorescent probe for highly selective detection of GSH over Cys/Hcy.⁶⁹⁹ The chlorine of **289** can be rapidly replaced by biothiols through thiol–halogen nucleophilic substitution. The amino group of Cys/Hcy further replaces the thiolate to form amino-substituted BODIPYs, which exhibit dramatically different spectroscopic properties as compared to the sulfur-substituted BODIPY from GSH (Figure 75), thereby permitting the discrimination of GSH from both Cys and Hcy. The probe has a detection limit of 8.6×10^{-8} M for GSH, and has been applied for detection of GSH in live cells.

Compound **290** is a selective FRET-type probe for aminothiols over other thiols.⁷⁰⁰ Treatment of **290** with aminothiols (e.g., Cys) affords the intermediate thioester (**290-I**) by the reversible transthioesterification reaction (Figure 76), which might further undergo irreversible intramolecular nucleophilic attack by the nearby amino group to form the spirolactam (**290-II**). Because the FRET is on and off in **290** and **290-II**, respectively, a very large variation (up to 160-fold) in the fluorescence ratio (I_{458}/I_{603}) is produced. Importantly, probe **290** can be used to ratiometrically detect aminothiols in real biological samples such as newborn calf and human serum,

and is also suitable for ratiometric imaging of aminothiols in living cells.

4.2.2. Probes for Anions. **4.2.2.1. Probes for Sulphide.** He et al. developed a selective sulphide-trapping strategy involving sulphide addition to an aldehyde, and the resulting hemithioacetal underwent a Michael addition reaction with an adjacent unsaturated acrylate ester to further form a thioacetal at neutral pH in aqueous solution.⁷⁰¹ Employing this new strategy, two sulphide-selective fluorescent probes, **291** and **292**, were synthesized on the basis of two different fluorochromes (Figure 77).⁷⁰¹ Both probes exhibit fluorescence off-on response to HS⁻ (**291** also shows emission maximum shift) under physiological conditions. The utility of the probes for the selective detection of sulphide and their capacity to image free sulphide in living cells have been demonstrated. Compound **293** shows faster reaction with sulphide,⁷⁰² and has been successfully applied for the measurement of sulphide concentration in mice blood plasma and brain tissues.



4.2.2.2. Probes for Hydrogensulfite and Sulfite. Compound **294** was reported as a turn-on fluorescent probe for the detection of hydrogensulfite.⁷⁰³ The probe shows a very weak fluorescence at 535 nm, but the fluorescence can be greatly enhanced upon reaction with HSO_3^- , which is ascribed to the inhibition of the $\text{C}=\text{N}$ isomerization in the product (Figure 78). With the usage of 2 μM probe, a detection limit of 0.1 μM was obtained for HSO_3^- . Probe **294** was applied to detection of hydrogensulfite in granulated sugar.

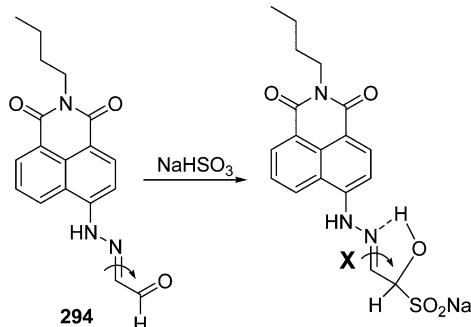


Figure 78. Reaction of **294** with bisulfite.

Zeng et al. constructed **295** as a fluorescent off-on probe for the detection of sulfite anions.⁷⁰⁴ The probe itself shows very weak fluorescence due to the PET between anthracene and aldehyde. However, sulfite can react selectively and quickly with the aldehyde moiety to inhibit the PET process, and the strong fluorescence of the anthracene moiety could be recovered (Figure 79). With the usage of 10 μM of **295**, SO_3^{2-} down to

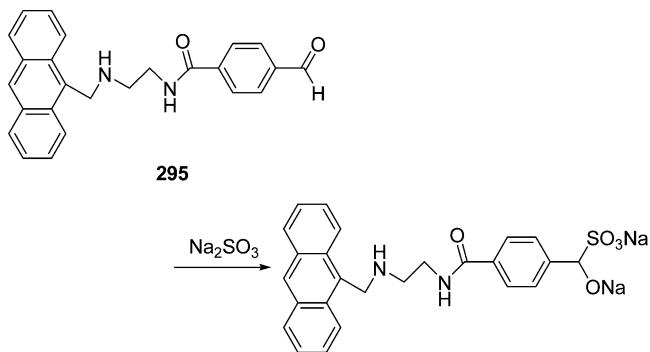


Figure 79. Reaction of **295** with sulfite.

10 nM ($S/N = 3$) can be detected. The probe has been used for detecting sulfite in beer and red wine. Interestingly, the probe can also serve as an indirect indicator for detecting sulfur dioxide content in air.

4.2.2.3. Probes for Cyanide. Nucleophilic addition of cyanide (CN^-) is often used to design its fluorescent probes. Although this reaction has led to the development of many fluorescent probes for CN^- detection, only a few of them work well in aqueous solution.^{480,705–708} Compound **296** was proposed as a selective fluorescent off-on probe for CN^- .⁷⁰⁹ In this probe, the aldehyde group is activated by the adjacent phenolic hydrogen through the formation of an intramolecular hydrogen bond, which facilitates the nucleophilic addition of CN^- (Figure 80). The addition of CN^- could occur in aqueous media of pH 7, resulting in a great increase in fluorescence, whereas other anions did not show this behavior.

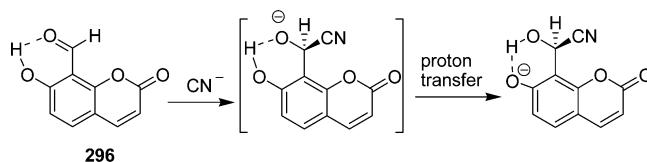


Figure 80. Reaction of **296** with CN^- .

Compound **297** is a spirocyclic derivative, which can serve as a selective and sensitive chromogenic probe for CN^- in aqueous media of pH 9.3 in the presence of 50% (v/v) CH_3CN under UV-irradiation.⁷¹⁰ The colorless spirocyclic form of **297** can be isomerized to the colored merocyanine when irradiated with UV light. The nucleophilic addition of CN^- to the positively charged spirocarbon of merocyanine then produces the **297-CN**⁻ species with a new absorption at 421 nm, which enables CN^- to be determined by spectrophotometry (Figure 81). By incorporating coumarin into spirocyclic, a fluorescent off-on probe for CN^- was developed, which permits the determination of CN^- at much lower levels.⁷¹¹

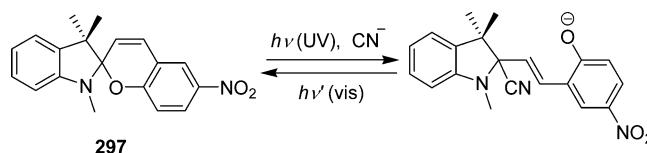


Figure 81. Reaction of **297** with CN^- .

On the basis of the addition of CN^- to trifluoromethyl-activated carbonyl, Li et al. reported **298** as a chromogenic and fluorogenic probe for CN^- (Figure 82).⁷¹² Probe **298** reacts

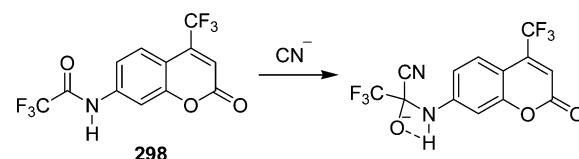


Figure 82. Reaction of **298** with CN^- .

rapidly (<30 s) with CN^- in $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ (4:1, v/v) media, producing a large fluorescence enhancement due to the improvement of ICT process. However, the detection limit of the fluorescence assay for CN^- is only 0.3 mM. Moreover, the reactions of CN^- with trifluoromethyl-activated carbonyl groups usually proceed in the media with high percentage of organic solvents,⁷¹³ and some even require pure organic solvents, which may limit their practical use.

Compound **299** is a coumarin–hemicyanine conjugate (Figure 83), which can serve as a ratiometric fluorescent probe for CN^- .⁷¹⁴ The nucleophilic attack of CN^- toward the indolium group of **299** interrupts the π -conjugation, thereby giving the fluorescence emission of the coumarin moiety. In 10 mM Tris-HCl buffer of pH 9.3 with 50% (v/v) MeOH , the

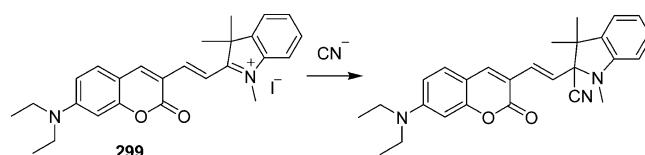


Figure 83. Reaction of **299** with CN^- .

probe displays a largely CN^- -induced increase in the fluorescence intensity ratio at 514/630 nm, which enables CN^- to be determined ratiometrically with a detection limit of 0.6 μM . Furthermore, the probe also shows high selectivity for CN^- over other anions including Cys.

Compound 300 is a BODIPY-based fluorescent CN^- probe (Figure 84).⁷¹⁵ Reaction of the probe with CN^- in aqueous

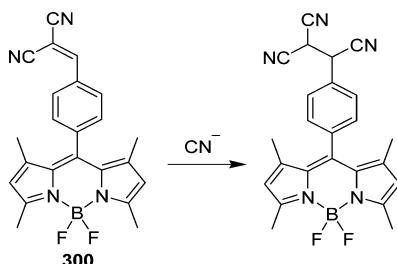


Figure 84. Reaction of 300 with CN^- .

media containing 1% (v/v) tetrahydrofuran leads to a 43-fold fluorescence increase. The probe is sensitive to micromolar levels of cyanide and has been applied to imaging CN^- in HeLa cells.

It should be noted that some CN^- probes may suffer from the interference of a thiol, because these two substances have similar nucleophilic reactivity. For instance, probes 283 and 300 with the same reacting site may be sensitive to both CN^- and a thiol via the Michael addition.

4.2.3. Probes for Metal Ions. Metal ion-catalyzed or promoted covalent bond formation reactions, such as Cu^{+} -catalyzed “click” reaction, and Hg^{2+} -promoted intramolecular cyclic guanylation of thiourea derivatives, provide routes to developing fluorescent probes for the corresponding metal ions.

4.2.3.1. Probes for Cu^+ and Cu^{2+} . The Cu^+ -catalyzed 1,3-dipolar cycloaddition of a terminal alkyne to an azide to form a triazole (“click” reaction)^{716–718} has led to the design of two fluorescent probes for Cu^+ (Figure 85). The first one (301), reported by Zhou and Fahrni, is a nonfluorescent alkynyl coumarin derivative, which however can be converted to a fluorescent triazole congener ($\lambda_{\text{ex/em}} = 328/415 \text{ nm}$) upon reaction with 4-azidomethylbenzoic acid in the presence of Cu^+ in aqueous buffer.⁷¹⁹ Later, a Eu^{3+} complex (302) has also been proposed for the luminescent detection of Cu^+ via click

chemistry.⁷²⁰ This detection system consists of a Eu^{3+} -propynylamide complex and a dansyl azide, which can be coupled to each other in the presence of the glutathione complex of Cu^+ in water. The resulting dansyl-triazole Eu^{3+} chelate shows a 10-fold increased luminescence ($\lambda_{\text{ex/em}} = 350/615 \text{ nm}$) with respect to its starting alkyne complex. However, neither of these probes has been utilized to detect Cu^+ in real samples. Moreover, the detection requires the interaction of three different species at appropriate concentrations, which might be a potential barrier for their use in complex biological environments.¹⁹²

An efficient chemical transformation of nonemissive azoanilines to highly fluorescent benzotriazoles has been exploited for the fluorescence off-on detection of Cu^{2+} in aqueous solution at room temperature.⁷²¹ As illustrated in Figure 86, compound 303 responds selectively to Cu^{2+} at ppm levels at pH 6–8 but remains inert in acidic (pH < 5.5) environment. 303 may be used in tracing Cu^{2+} in biological samples.

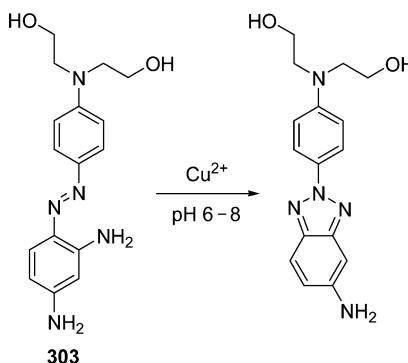


Figure 86. Reaction of 303 with Cu^{2+} .

4.2.3.2. Probes for Hg^{2+} . The intramolecular cyclic guanylation of thiourea derivatives has been utilized to develop fluorescent probes for Hg^{2+} ,^{722,723} as exemplified by probe 304.⁷²⁴ The probe can undergo a sulfur-to-nitrogen exchange reaction promoted by Hg^{2+} , forming a guanylated compound that emits fluorescence at a different wavelength (Figure 87). On the basis of this Hg^{2+} -promoted conversion, other fluorescent probes for Hg^{2+} have also been prepared, including 305,⁷²⁵ 306,⁷²⁶ and 307–309.⁷²⁷ However, the exchange

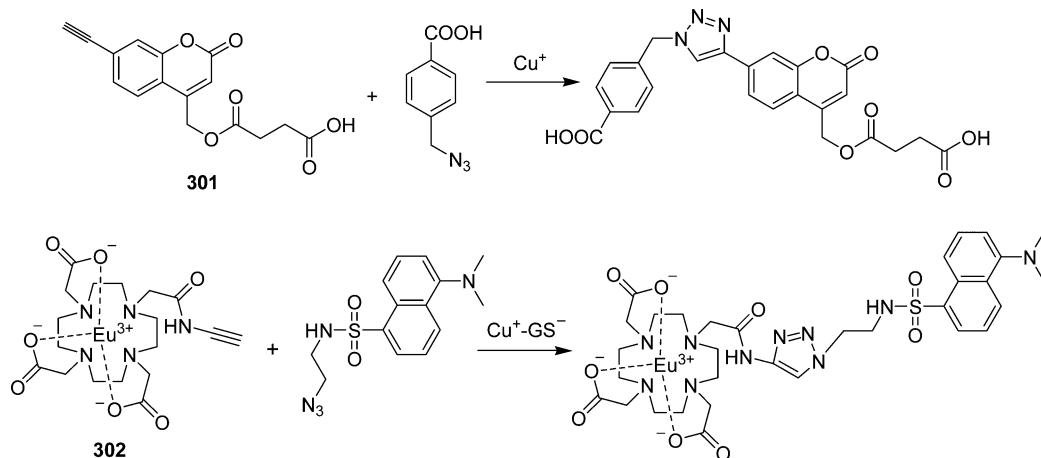


Figure 85. Cu^+ -catalyzed reactions of 301 and 302 with azides.

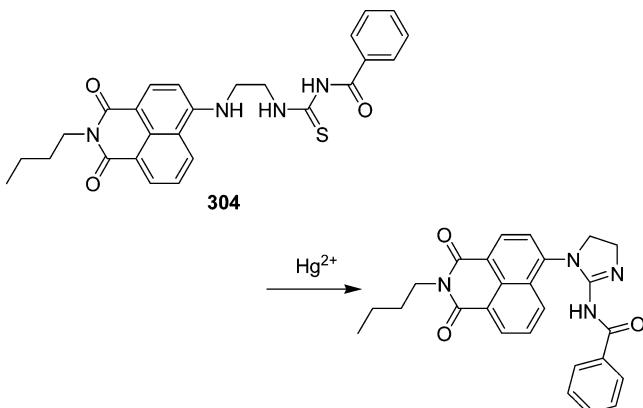
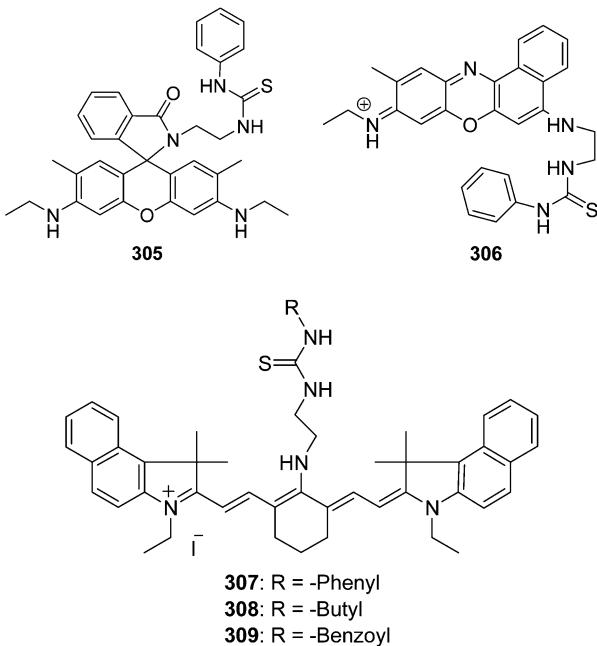


Figure 87. Reaction of 304 with Hg^{2+} .

reaction of these probes is slow, and organic solvents usually need to be used.



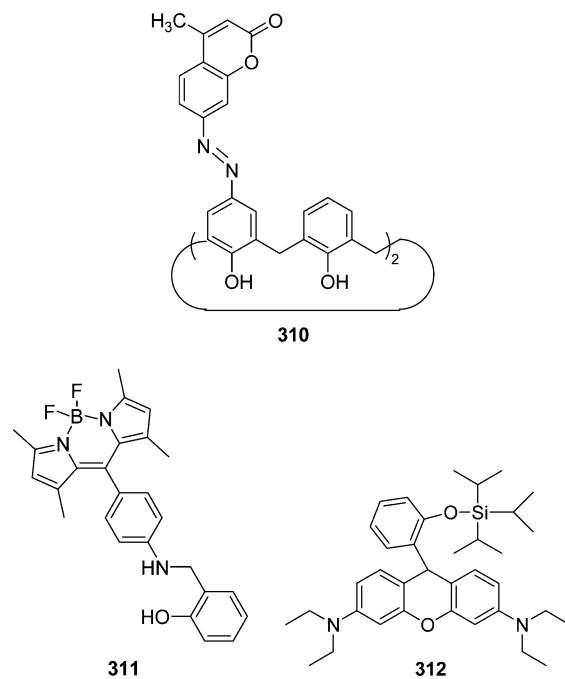
5. REDOX-BASED CHROMOGENIC AND FLUOROGENIC PROBES

In this section, we will review the probes that respond to analytes via redox reactions without the release of fluorochromes, which include the probes for metal ions, ROS, small molecules, and enzymes. These probes usually have the following response mechanism: redox reactions lead to the inhibition of a PET process or the conversion between a nonfluorescent molecule and a fluorescent one. Note that some redox reactions between probes and ROS or thiols can lead to the release of fluorochromes, and the probes featuring such reactions are included in sections 4.1.3 and 4.1.4.

5.1. Probes for Metal Ions

Most of the redox-based probes for metal ions are water-insoluble, but a few for Cu^{2+} can function in aqueous media.^{728–732} For instance, Ma's group developed a calix[4]-arene-based fluorescent Cu^{2+} probe (**310**) by combining multiple selective factors (the shielding action of calixarene

skeleton, high affinity of Cu^{2+} for nitrogen atoms, and redox reactivity of Cu^{2+}).⁷²⁹ In basic aqueous media, the probe may be oxidized to an azoxy or arylnitramine compound by Cu^{2+} , accompanied by its fluorescence quenching and solution fading. Importantly, the spectroscopic response of **310** to Cu^{2+} is highly sensitive (detection limit, 6.0 nM), and is not influenced by the presence of 20–10 000-fold excesses of alkali, alkaline earth, and other transition metal ions. As a result, trace Cu^{2+} in real biological fluids, such as cerebrospinal fluid and microdialysates, can be sensitively and selectively determined with the probe. Similarly, based on the Cu^{2+} -induced oxidation, compounds **311**⁷³⁰ and **312**⁷³¹ have been proposed as Cu^{2+} -selective fluorescent probes. For both of the probes, Cu^{2+} -induced oxidation results in a great increase in fluorescence intensity, and **312** shows a higher selectivity and sensitivity toward Cu^{2+} with a detection limit of 2.61×10^{-7} M, which is lower than the typical concentration of blood copper in normal individuals. Moreover, probe **312** is cell-membrane permeable and can be used for the fluorescence imaging of Cu^{2+} in living cells.



5.2. Probes for Reactive Oxygen Species

5.2.1. Probes for Hydrogen Peroxide. Triarylphosphines can react with H_2O_2 to give the corresponding phosphine oxides under mild conditions. On the basis of this reaction, several H_2O_2 probes have been designed by linking arylphosphine to a fluorochrome.^{733–735} Compounds **313**⁷³⁴ and **314**⁷³⁵ are such examples, in which 2,1,3-benzoxadiazole and 7-hydroxycoumarin skeletons serve as fluorochromes, respectively (Figure 88). Reaction of both probes with H_2O_2 produces a phosphine oxide, concomitant with fluorescent off-on response. However, these probes suffer from the interference from other hydroperoxides.

By using naphthalimide as a fluorochrome and *p*-anisidine as both a redox-active moiety for H_2O_2 and a PET quencher, compound **315** was proposed as a H_2O_2 probe.⁷³⁶ The probe has very weak fluorescence and a large Stokes' shift ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 455/536$ nm). Upon oxidation by H_2O_2 , the PET effect in **315** is suppressed, causing a greatly enhanced fluorescence. In

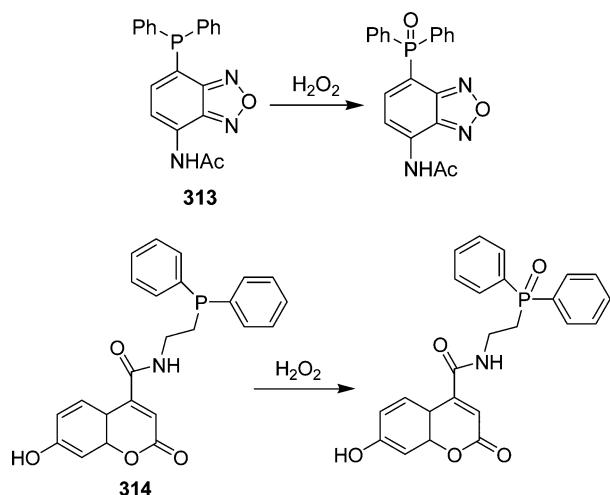
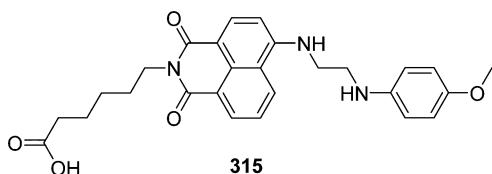


Figure 88. Reactions of 313 and 314 with H₂O₂.

combination with horseradish peroxidase, 315 displays quick response to H₂O₂ with a detection limit of 280 nM, which enables sensitive enzymatic assays of oxidase substrates in a kinetic format. The oxidation mechanism of 315 by H₂O₂ is unclear, but the electrochemical oxidation of *p*-anisidine is known to include the generation of an unstable radical cation,⁷³⁷ which may be a possible mechanism for the fluorescence response of 315.



Chang et al. designed a reversible fluorescent probe 316 for repetitive detection of H₂O₂ (Figure 89).⁷³⁸ Oxidation of 316

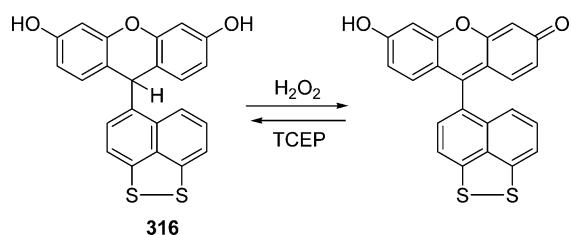


Figure 89. Reversible response of 316 with H₂O₂.

by H₂O₂ causes a large increase in fluorescence intensity, but the resulting oxidized product can be reversibly reduced back to 316 in the presence of tris(2-carboxyethyl)phosphine (TCEP). The biological applicability of the probe has been demonstrated by imaging live HEK 293 cells. As expected, treatment of the 316-loaded HEK 293 cells with H₂O₂ shows a marked increase in fluorescence, but the observed intracellular fluorescence decreases slowly due to the native reducing environment of the cells. This indicates that 316 is suited for imaging reversible redox cycles in living cells.

5.2.2. Probes for Singlet Oxygen. For ¹O₂, most probes were designed by taking advantage of its reactivity with an anthracene moiety. The commonly used chromogenic probe for ¹O₂ is 9,10-diphenylanthracene, which reacts with ¹O₂ to form an endoperoxide product.⁷³⁹ However, 9,10-diphenylan-

thracene is not very sensitive because the detection is based on the measurement of absorbance. To improve sensitivity, some fluorescent ¹O₂ probes have been developed on the basis of 9,10-diphenylanthracene-conjugated xanthene derivatives. Compound 317 is such a probe, which shows low background fluorescence.⁷⁴⁰ However, its reaction with ¹O₂ under neutral conditions (pH 7.4) results in a large increase in fluorescence. This response is caused by the endoperoxide formation between ¹O₂ and the anthracene moiety, which inhibits the PET process (Figure 90). Probe 317 has a great potential for ¹O₂ detection in biosystems.

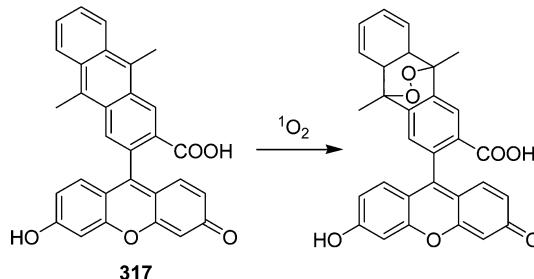


Figure 90. Reaction of 317 with singlet oxygen.

Compound 318 has been proposed as a highly selective and sensitive CL probe for singlet oxygen.¹⁰ The design strategy for the probe was directed by the idea of PET process and carried out through the incorporation of electron-rich tetrathiafulvalene unit into the reactive fluorochrome of anthracene specific for ¹O₂. The probe exhibits a highly selective response to ¹O₂, with respect to other ROS. This remarkable CL property permits ¹O₂ to be distinguished easily from the other ROS and makes the probe possible to be used widely for ¹O₂ detection in many chemical and biological systems.⁷⁴¹ The CL reaction mechanism of 318 is shown in Figure 91: the anthracene moiety activated by tetrathiafulvalene first traps ¹O₂ to yield an unstable endoperoxide, whose decomposition causes not only the excitation of the anthracene core, which in turn emits light through radiative deactivation, but also the oxidation of the electron-rich tetrathiafulvalene moiety to turn into a cation species. In such a tandem reaction, the formation of the final cation species promotes the proceeding of CL reaction forward and meanwhile cleaves the PET process between tetrathiafulvalene and the excited anthracene unit, thus enhancing CL. The detection limit of the probe for ¹O₂ is 76 nM. Compound 319, bearing two anthracenes and a tetrathiafulvalene group,⁷⁴² also exhibits high sensitivity and selectivity for ¹O₂ (detection limit of 81 nM), and has been successfully used to characterize H₂O₂-dependent generation of ¹O₂ from saliva in real time.⁷⁴³ To improve water solubility, 320 containing hydrophilic chains was further designed and synthesized, which is capable of detecting ¹O₂ in aqueous buffer (pH 7.0) containing 20% (v/v) methanol.⁷⁴⁴

Yuan et al. prepared a series of lanthanide complex-based probes (321–323) for time-resolved luminescence detection of ¹O₂.^{745–747} The almost nonluminescent probes can react specifically with ¹O₂ to yield the corresponding endoperoxides with a great increase of the luminescence intensity. These probes are also highly sensitive (with detection limits ranging from 2.8 to 10.8 nM), and are expected to be useful for the monitoring of ¹O₂ in some biosystems.

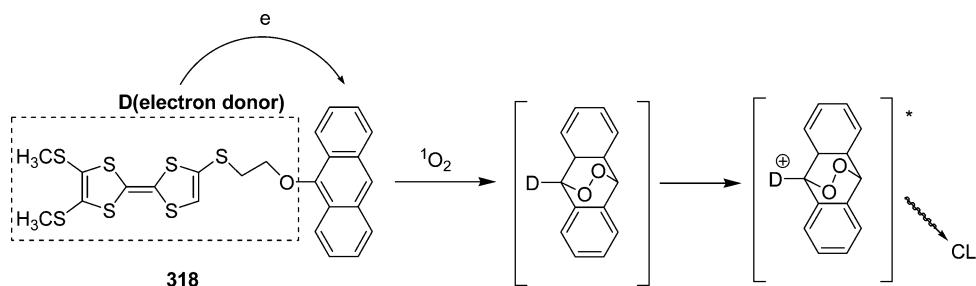
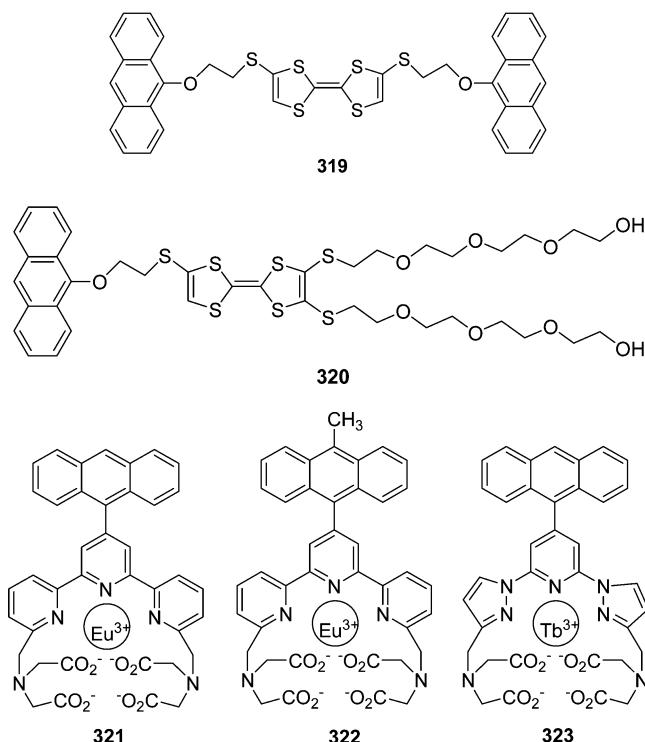


Figure 91. Reaction mechanism of 318 with singlet oxygen.



Recently, a mitochondrial-targeted NIR fluorescent $^1\text{O}_2$ probe (324) has been reported, which is based on the reaction between histidine and $^1\text{O}_2$ (Figure 92).⁷⁴⁸ The response of 324 to $^1\text{O}_2$ is rapid, sensitive, and selective. Thus, the probe could be applied to detecting and imaging $^1\text{O}_2$ in living cells and tissues.

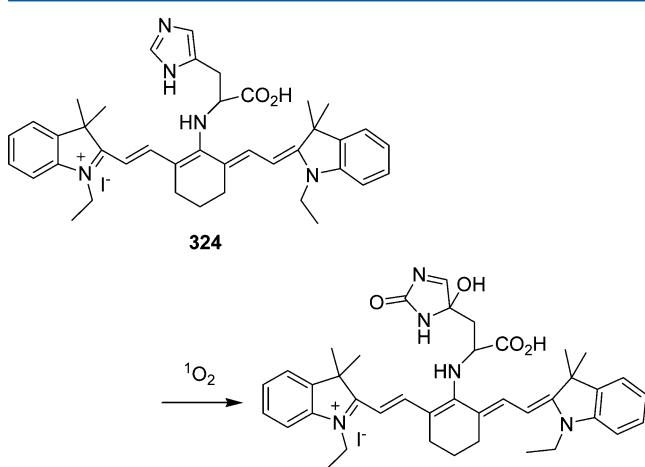


Figure 92. Reaction of 324 with singlet oxygen.

5.2.3. Probes for Superoxide Radical. The reaction between $\text{O}_2^\bullet-$ and hydroethidine has been used for developing $\text{O}_2^\bullet-$ probes.^{749,750} Compound 325 is such a probe, whose oxidation by $\text{O}_2^\bullet-$ forms a fluorescent product 2-hydroxyethidium (Figure 93).⁷⁴⁹ Importantly, other ROS cannot oxidize

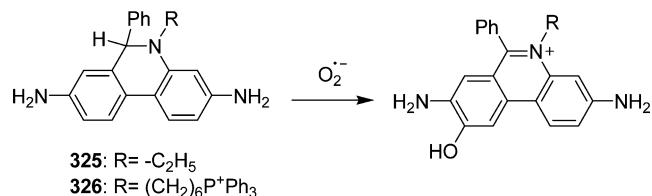


Figure 93. Reactions of 325 and 326 with superoxide radical.

325 to the same product. When bovine aortic endothelial cells were pretreated with different concentrations of menadione as an intracellular $\text{O}_2^\bullet-$ generator and were further treated with 325, a dose-dependent increase in fluorescence was observed. However, probe 325 is vulnerable to autoxidation. Another fluorescent $\text{O}_2^\bullet-$ probe (326) containing the triphenylphosphonium cation as the target moiety for mitochondria has been developed (Figure 93),⁷⁵⁰ which can be used for the detection of mitochondrial $\text{O}_2^\bullet-$.

2-(2-Pyridyl)benzothiazoline (327) was proposed as a $\text{O}_2^\bullet-$ probe,⁷⁵¹ whose oxidation by $\text{O}_2^\bullet-$ gives a strongly fluorescent product (Figure 94). In alkaline $\text{Na}_2\text{S}_2\text{O}_4$ (a superoxide radical

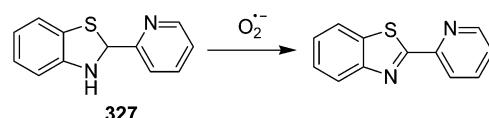
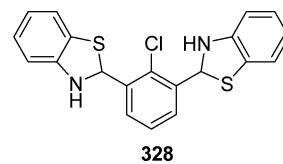


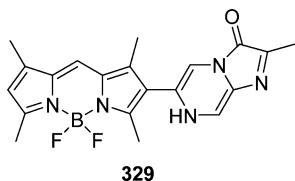
Figure 94. Reaction of 327 with superoxide radical.

source) solution, 327 displays a large increase in fluorescence intensity at 528 nm. The probe also exhibits good selectivity for $\text{O}_2^\bullet-$ over H_2O_2 and $\cdot\text{OH}$, and has been employed to determine superoxide dismutase activity in onion. Compound 328 reacts with $\text{O}_2^\bullet-$ following the same mechanism.⁷⁵²



Imidazopyrazine is known as a CL emitter upon oxidation under neutral pH conditions, and this moiety has been introduced to BODIPY, yielding 329,⁷⁵³ which shows the strongest CL response toward $\text{O}_2^\bullet-$ among the various ROS

tested. 329 has been used for the detection of $O_2^{\bullet-}$ generated from stimulated cells.



Murthy's group has found that hydrocyanines, generated from the reduction of the cyanine dyes with $NaBH_4$, could be used to detect $O_2^{\bullet-}$ and $\cdot OH$.⁷⁵⁴ These compounds are weakly fluorescent because of their disrupted π conjugation; however, oxidation of these compounds such as 330 by either $O_2^{\bullet-}$ or $\cdot OH$ increases their fluorescence dramatically through the regeneration of an extended π conjugated product (Figure 95). The hydrocyanines have excellent stability to autoxidation and nanomolar sensitivity to ROS, and can be used to image $O_2^{\bullet-}$ and $\cdot OH$ in cell culture and tissue explants *in vivo*.

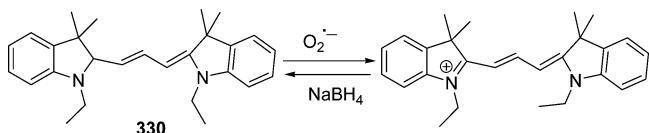


Figure 95. The redox reaction of 330.

5.2.4. Probes for Hypochlorite. Yang and co-workers developed 331 as a HOCl-selective fluorescent probe,⁷⁵⁵ whose design strategy is based on the oxidation of *p*-methoxyphenol to benzoquinone by hypochlorous acid (Figure 96). Treatment of

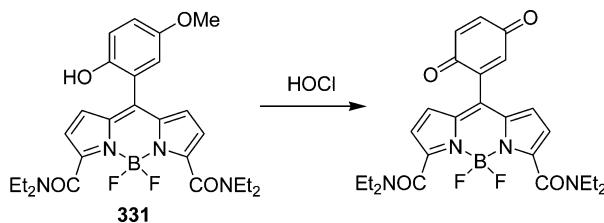


Figure 96. Reaction of 331 with HOCl.

331 in aqueous buffer with HOCl results in an increase in fluorescence intensity due to the inhibition of PET. By using 10 μM of the probe, a linear relationship was found between the emission intensity and the concentration of HOCl in the range of 3–8 μM . Moreover, the probe is highly selective for HOCl, and has been used to monitor HOCl generation in both an

abiotic system and an enzymatic system (myeloperoxidase/ H_2O_2/Cl^-) as well as in living macrophage cells upon stimulation.

5.2.5. Probes for Hydroxyl Radical. By employing $\cdot OH$ -induced aromatic hydroxylation, two coumarin derivatives^{756,757} and a Tb^{3+} complex⁷⁵⁸ have been proposed as luminescent probes for $\cdot OH$ detection. These probes all exhibit turn-on response to $\cdot OH$, and one of them (332) relies on the hydroxylation of trimesate by $\cdot OH$ and subsequent chelation by Tb-DO3A (Figure 97).⁷⁵⁸ Moreover, probe 332 exhibits high sensitivity (detection limit at fM level), high selectivity over competing ROS, and long luminescence lifetime, which makes it useful for the study of $\cdot OH$ in biological processes.

Compound 333 is a $NaBH_4$ -reduced coumarin-cyanine derivative (Figure 98),⁷⁵⁹ which can be oxidized by hydroxyl

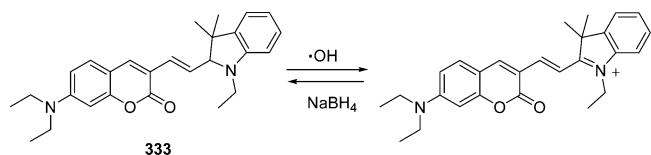


Figure 98. Reaction of 333 with $\cdot OH$.

radical, regenerating the extended π -conjugation of coumarin-cyanine. The reaction causes a large emission shift from 495 to 651 nm and a great ratiometric fluorescence enhancement (210-fold) of $I_{651}/I_{495\text{ nm}}$, which enables it to be used for ratiometric fluorescence detection of hydroxyl radicals in living cells.

5.2.6. Probe for Ozone. Compound 334 was reported as an NIR fluorescent probe for ozone (Figure 99).⁷⁶⁰ Reaction of 334 with O_3 produces an increase in fluorescence, and this fluorescence response is rapid, highly selective, and sensitive to O_3 with a detection limit of 17 nM. The probe has been successfully applied to detecting and imaging O_3 derived from living cells.

5.2.7. Probes for Nitric Oxide. Numerous fluorescent probes for NO assay have been reported, which mainly fall into the following two types: *ortho* aromatic diamines and transition-metal complexes.^{415,592,761–763}

In *ortho* aromatic diamines, the electron-rich amino groups effectively quench the fluorescence of the fluorochromes by a PET process. Upon reaction with NO under aerobic conditions, the vicinal aromatic diamines are transformed into an electron-deficient triazole, which terminates the PET process and restores the fluorescence (Figure 100).^{588,592} By using fluorescein,^{764–766} rhodamine,⁷⁶⁷ BODIPY,^{768,769} and cyanine⁷⁷⁰ as fluorochromes, a series of *ortho* aromatic diamine-

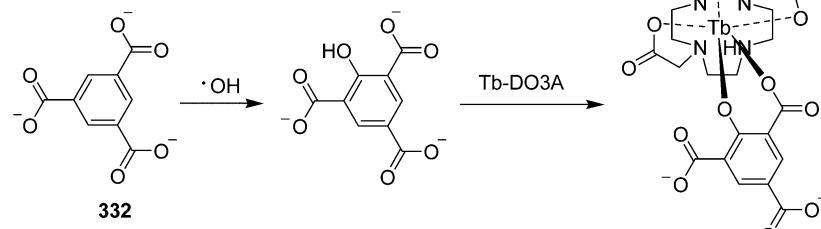


Figure 97. Reaction of 332 with $\cdot OH$.

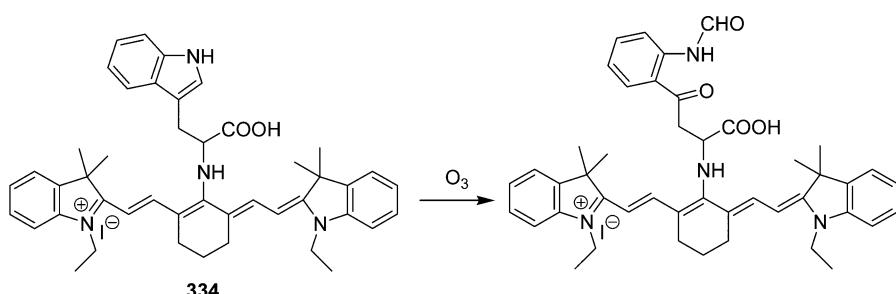


Figure 99. Reaction of 334 with O₃.

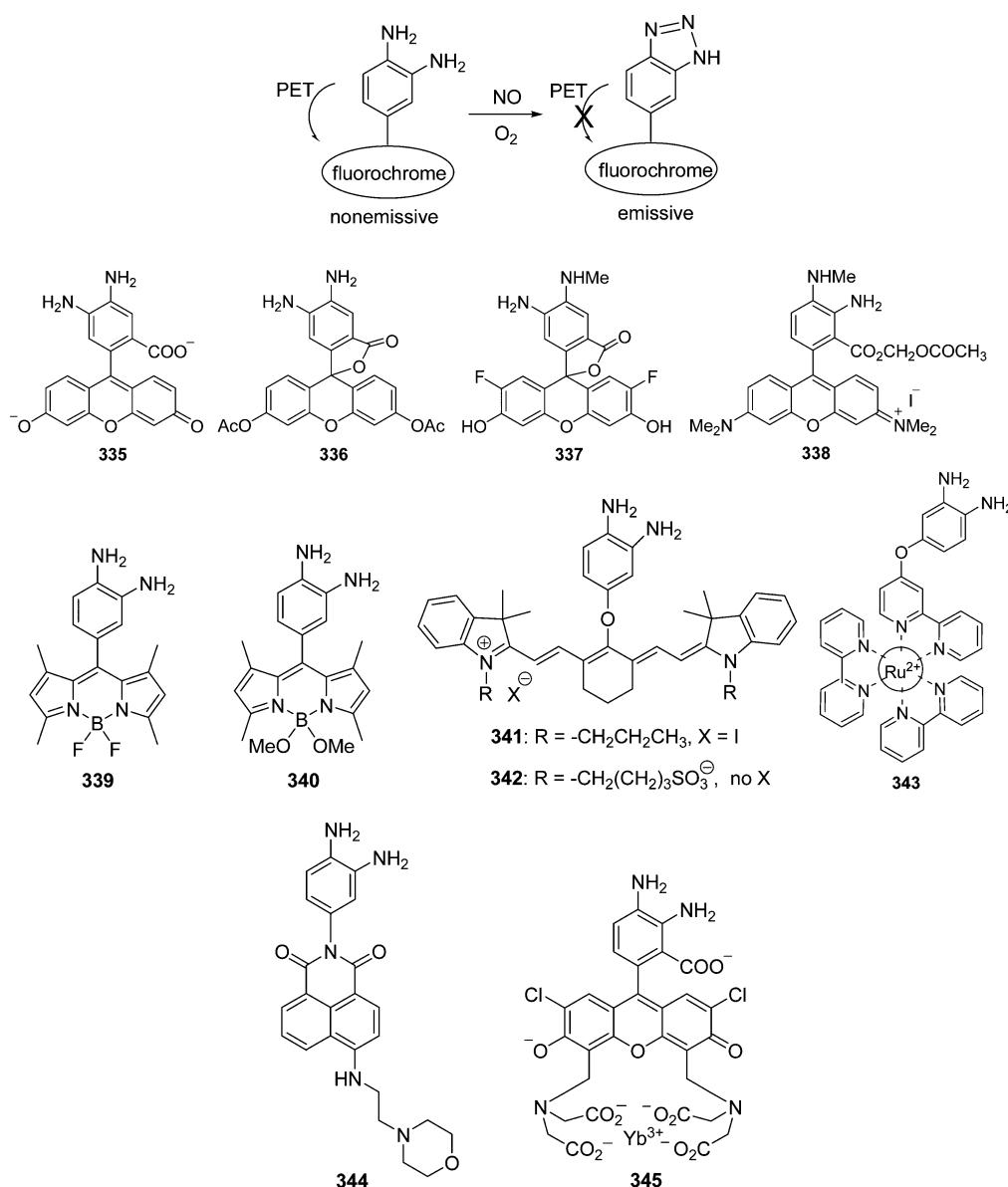


Figure 100. Reaction mechanism of *ortho* aromatic diamines with NO and structures of 335–345.

based probes have been synthesized. Diaminofluorescein derivatives 335–337 were prepared as the first-generation fluorescent NO probes (Figure 100).^{764–766} However, fluorescence intensities of most of the triazole products decrease dramatically in acidic solutions, except the reaction product of 337, whose fluorescence is stable and intense over a wide pH range. Probe 337 was attempted to detect intracellular

NO release in biological specimens.⁷⁶⁶ To obtain higher photostability and longer excitation wavelengths, a diamino-rhodamine derivative 338 was synthesized as a fluorescent NO probe (Figure 100).⁷⁶⁷ Reaction of 338 with NO induces 840-fold fluorescence enhancement, and the fluorescence shows no pH dependency above pH 4. Probe 338 has a detection limit of 7 nM for NO, and has been applied to imaging of NO

production in bovine aortic endothelial cells. Similarly, reaction of BODIPY-based fluorescent probes (**339** and **340**) with NO converts the diamino form to the triazole structure, resulting in a large fluorescence increase due to the inhibition of PET.⁷⁶⁸ As compared to **339**, probe **340** exhibits higher water solubility, and its fluorescence intensity shows greater pH-independency.⁷⁶⁹ Compounds **341** and **342** are NIR probes, which are expected to be applicable to not only cellular but also *in vivo* NO imaging.⁷⁷⁰ Ruthenium(II) complex **343** was also reported as a probe for NO with a detection limit of 2.7×10^{-7} M, which has been applied in luminescence imaging of exogenous NO in mouse macrophage cells and endogenous NO in gardenia cells.⁷⁷¹ A lysosome-specific TP fluorescent probe, **344**, was developed,⁷⁷² which shows high selectivity and sensitivity toward NO. With the usage of 11 μ M **344**, a linear correlation was found between fluorescence intensity and NO in the concentration range of 1–1.3 μ M with a detection limit of 5 nM. Employing this probe, the capture of NO within lysosomes of macrophage cells has been achieved by both TP fluorescence microscopy and flow cytometry. Nagano et al. recently developed **345** as an NIR luminescent probe for NO⁷⁷³ by conjugating a Yb³⁺ chelate to an established fluorescein-based NO probe.⁷⁶⁵ Upon reaction with NO in aqueous buffer, the diamine complex **345** shows an approximately 50-fold increase in luminescence intensity at 980 nm with a detection limit of 90 nM, which is ascribed to the formation of the corresponding triazole product.

Although the past several years have witnessed great progress in *ortho* diamine fluorescent probes, they still have some shortcomings. First, vicinal aromatic diamine probes are susceptible to the interference by both oxidants and antioxidants;⁷⁷⁴ for example, dehydroascorbic acid and ascorbic acid can react with this kind of probes and turn on the fluorescence.^{775,776} Second, some common influencing factors such as light,⁷⁷⁴ Mg(II),⁷⁷⁷ and Ca(II)⁷⁷⁸ also interfere with the detection of NO.

In transition-metal complex probes, either the paramagnetism of the metal or the heavy-atom effect can quench the fluorescence of an appended fluorochrome. Upon reaction with NO, however, the quenching effect could be eliminated by the metal reduction, resulting in the recovery of fluorescence. A number of such probes have been reported for NO detection.^{415,763,779–786} Compound **346** is a fluorescein derivative with a pendant aminoquinoline moiety for binding Cu(II).^{781,782} Upon treatment with NO, Cu(II) is reduced to Cu(I) with concomitant nitrosation of the secondary amine (Figure 101), leading to a rapid fluorescence enhancement (up to 16-fold, dependent on NO concentration). Moreover, **346** shows high selectivity for NO over other ROS, and has been

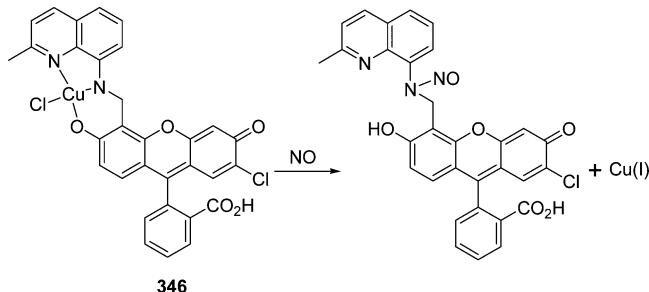
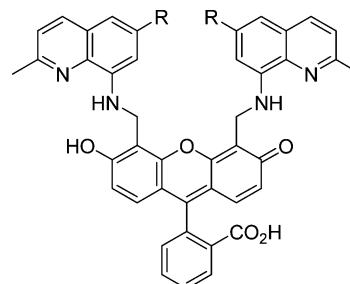


Figure 101. Reaction of the Cu(II)-mediated NO probe **346**.

successfully used to detect NO produced by both constitutive and inducible NO synthases in live neurons and macrophages.⁷⁸² Modification of **346** produces symmetric fluorescein derivatives (**347**–**349**) with improved dynamic range, and the copper complex of **348** can be hydrolyzed intracellularly to form the copper complex of **349**.^{783,784} In addition, **350** is developed as a fluorescent NO probe based on the Cu(II)-mediated N-nitrosation,⁷⁸⁶ whereas **351** is a chromogenic and fluorogenic NO probe with Cu²⁺ as a promoter.⁷⁸⁷ The Cu²⁺–**351** complex exhibits a significant fluorescence increase and a large blue shift of 200 nm upon addition of NO in neutral buffer solution, and this response is highly sensitive and selective for NO over other biological ROS with a detection limit of 30 nM.

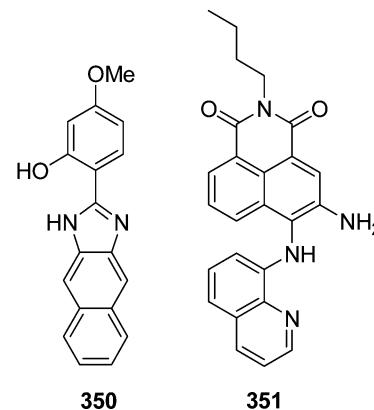
Despite the progress in the metal-based complex probes, there still exist several disadvantages such as biological incompatibility,⁷⁸⁰ easy leakage from cells,⁷⁶³ or side effect of the metal ion.⁷⁷⁴



347: R = H

348: R = OCH₂CO₂Et

349: R = OCH₂CO₂H



Recently, Anslyn and co-workers have developed **352** as a highly selective fluorescent probe for NO over various substances including other ROS.⁷⁸⁸ The probe employs a single reactive amino group rather than highly electron-rich *ortho* diamines to react with NO, and this reaction results in the formation of a six-membered diazo ring structure (Figure 102). Moreover, the fluorescence of the reaction product is not affected by pH.

5.2.8. Probes for Nitroxyl. Lippard et al. reported a BODIPY derivative (**353**), which bears a DPA chelator and a triazole bridge.⁷⁸⁹ The fluorescence of **353** is strongly quenched upon formation of the 1:1 Cu(II)–**353** complex, supposedly by PET from excited BODIPY to the bound Cu(II) ion. In the presence of HNO, however, reduction of the Cu(II)–**353** complex generates NO and Cu(I)–**353** with concomitant

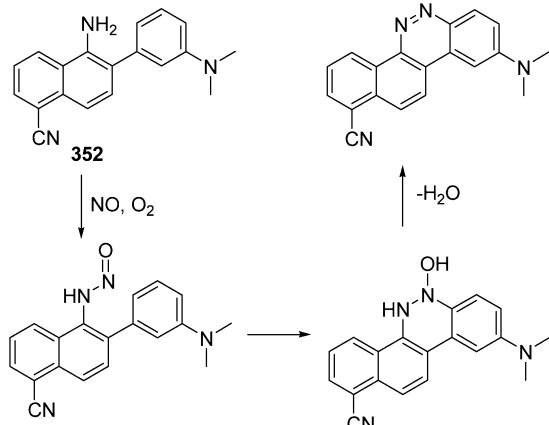
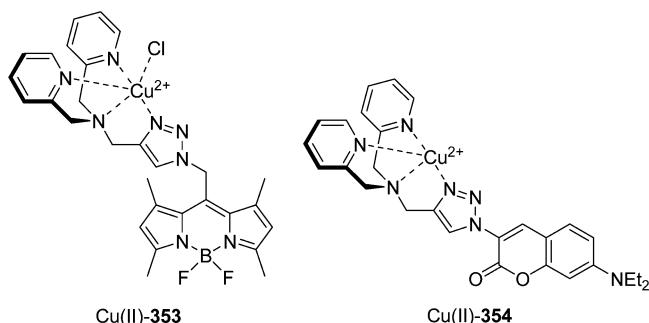


Figure 102. Reaction mechanism of 352 with NO.

restoration of BODIPY fluorescence. This Cu(II) complex can be used for the direct detection of HNO in aqueous solution, and is potentially valuable for studying the individual roles of NO and HNO in biology. Complex Cu(II)-354, with a similar structural feature to Cu(II)-353, could act as a dual-response probe to HNO for both fluorescence and electron paramagnetic resonance detection.⁷⁹⁰ Moreover, the probe shows high selectivity for HNO over other ROS, and has been used for monitoring the change of HNO levels in living cells.



5.2.9. Probes for Peroxynitrite. Taking advantage of the strong nitration of ONOO^- , compounds 355 and 356 were synthesized as probes for detection of ONOO^- (Figure 103).⁷⁹¹ Reaction of the probes with ONOO^- leads to a

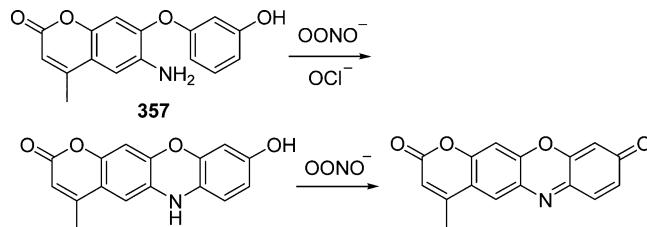


Figure 104. Reaction mechanism of 357 with peroxynitrite.

transformed into a red-emitting resorufin derivative via an orange-emitting intermediate. However, oxidation of 357 by OCl^- only produces the orange-emitting intermediate. This enables 357 to differentiate ONOO^- from OCl^- . The probe has been used to selectively detect peroxynitrite in cellular studies.

Some redox-reversible fluorescent probes have been developed for monitoring the redox cycle between oxidative stress of ONOO^- and thiol repair.^{793–796} For example, probe 358 (Figure 105)⁷⁹³ undergoes ONOO^- -promoted oxidation

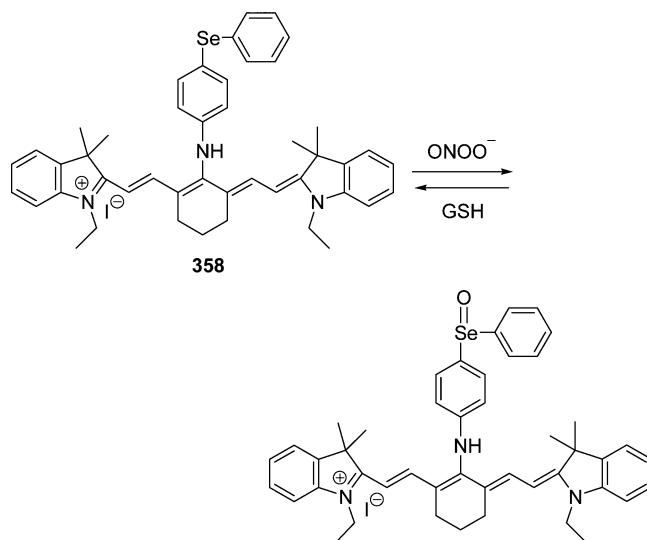


Figure 105. Reaction of 358 with peroxynitrite.

of diphenyl selenide to selenoxide resulting in a significant increase in fluorescence, and the oxidized probe can be reduced by GSH to its original form along with the decrease of the fluorescence to the starting level. The probe has been used to visualize the change of ONOO^- added in cells with negligible background fluorescence. Similarly, other selenide-containing fluorescence probes (359 and 360) were reported, which may also be used for monitoring the redox cycle between ROS species (e.g., OCl^- and ONOO^-) and a thiol.^{794,795}

The redox property of Te atom leads to the development of 361 as a reversible NIR fluorescent probe for ROS.⁷⁹⁷ 361 can react with several ROS, such as $\cdot\text{OH}$, ONOO^- , and HOCl . The redox cycle between 361 and its oxidized product can be run repeatedly by ROS and GSH (Figure 106), which was confirmed to work in living cells. The reversible redox character of 361 makes it useful for monitoring the dynamics of ROS production *in vivo* continuously.

Similar to the above probes for transition metal ions, most of the current ROS probes are unable to detect the basal levels of ROS in biosystems, and detection studies have to be conducted

Figure 103. Reaction of 355 or 356 with peroxynitrite.

large increase in fluorescence, due to the inhibition of PET. The probes are highly selective for peroxynitrite over other ROS, and can be applied for fluorescence imaging of ONOO^- in living cells.

Compound 357, a green-emitting coumarin derivative, was rationally designed and synthesized as a fluorescent probe for ONOO^- (Figure 104).⁷⁹² Upon oxidation by ONOO^- , 357 is

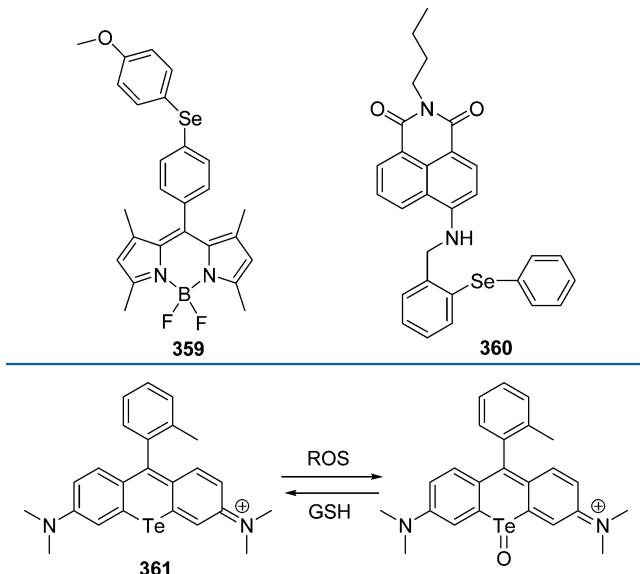


Figure 106. The redox reaction of 361.

by various stimuli or the addition of ROS into the related samples.

5.3. Probes for Small Molecules

5.3.1. Probe for Carbon Monoxide. Chang et al. recently reported a fluorescent off-on probe 362 for selective detection of CO.⁷⁹⁸ The cyclopalladated probe reacts with CO to trigger a fluorogenic carbonylation reaction, resulting in a dose-dependent response down to 28 nM of CO. The probe is capable of detecting CO both in aqueous buffer and in live cells with high selectivity over a wide range of biologically relevant reactive species, providing a potential for studying CO functions in biosystems (Figure 107).

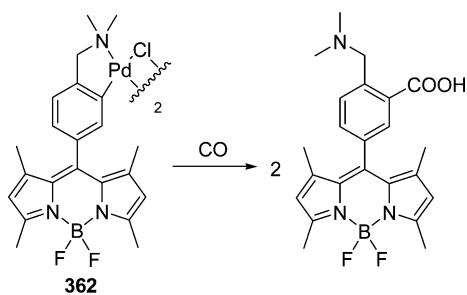
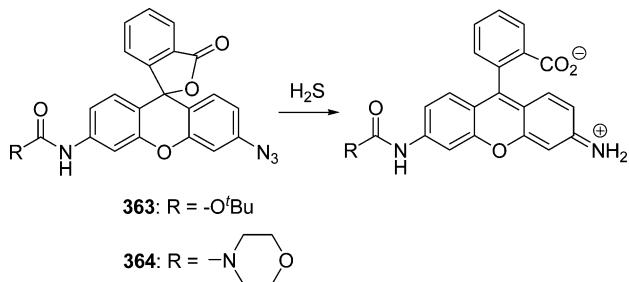


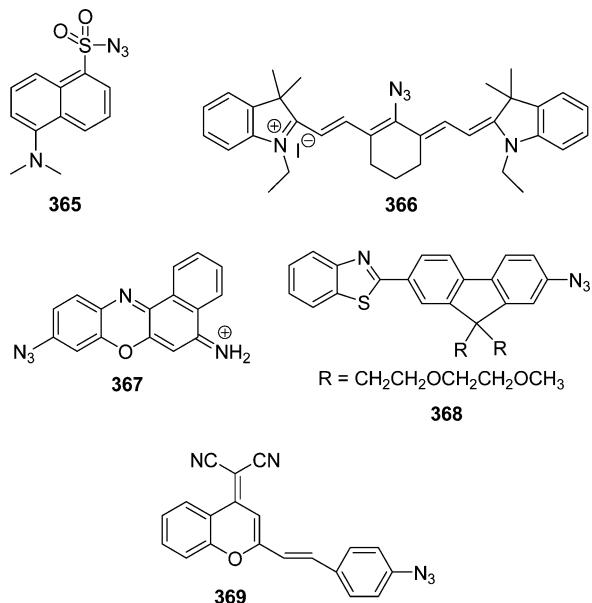
Figure 107. Reaction of 362 with CO.

5.3.2. Probes for Hydrogen Sulfide. H₂S-mediated reduction of azides to amines has been recently adopted for the design of selective fluorogenic H₂S probes.^{799–805} Chang's group developed azide-containing compounds 363 and 364 as a pair of H₂S-sensitive fluorescent probes (Figure 108).⁷⁹⁹ These probes, showing a fluorescence off-on response to H₂S with a detection limit of 5–10 μM, are highly selective for H₂S in aqueous media and can be used to detect H₂S in both water and live cells.

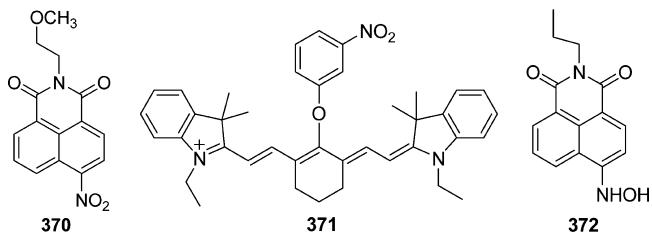
Additionally, other azide-containing compounds were also proposed as fluorescent H₂S probes.^{800–806} Among these probes, 365 has been used in the detection of H₂S in mouse blood,⁸⁰² whereas 366 and 367 have been applied for ratiometric imaging of H₂S in live cells,^{803,804} revealing that the concentration of endogenous H₂S in HeLa cells is lower

Figure 108. Reaction of 363 or 364 with H₂S.

than 0.1 μM (the detection limit of 367). In particular, probe 367 exhibits good stability, and its ratiometric response to H₂S functions well under physiological conditions. 367 has been applied to imaging H₂S in zebrafish.⁸⁰⁴ 368 is a TP fluorescent probe, which shows a 21-fold fluorescence enhancement when reacting with H₂S with a detection limit of 5–10 μM and can be used to selectively detect H₂S in a rat hippocampal slice at a depth of 90–190 μm by using TP microscopy.⁸⁰⁵ Recently, another TP probe (369) with NIR emission was reported for H₂S.⁸⁰⁶ The probe has a detection limit of 3.05 μM, which makes it suitable for H₂S detection in bovine serum. Moreover, 369 has been utilized to image H₂S in living mice.



The reduction of nitro group or hydroxylamine to amine has been employed to develop fluorescent H₂S probes, such as probes 370–372,^{800,807,808} which all show fluorescence enhancement upon reaction with H₂S. However, the reduction reactions proceed very slowly at room temperature, which may limit the application of the probes for real-time monitoring of H₂S.



5.3.3. Probe for Benzenethiols. Compound 373 is a thiol-sulfoxide-based ratiometric probe for benzenethiols.⁸⁰⁹ Upon reduction by benzenethiols (Figure 109), the ratio of emission

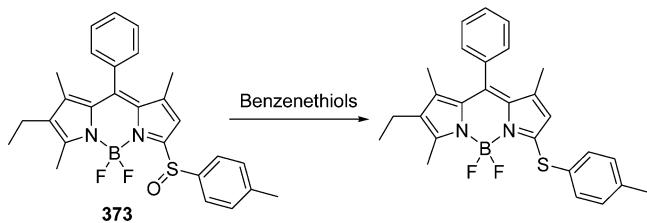


Figure 109. Reaction of 373 with benzenethiols.

intensities (I_{568}/I_{536}) of 373 increased largely (detection limit of $0.74 \mu\text{M}$), and this response to benzenethiol was not affected by pH over a wide range of 1–10. The probe is suitable for quantitative determination of benzenethiol in water samples.

5.4. Probes for Enzymes

5.4.1. Probes for Tyrosinase. We have developed an NIR cyanine probe (374) for monitoring tyrosinase activity.⁸¹⁰ The probe was designed on the basis of the fact that tyrosinase catalyzes the hydroxylation of phenol to catechol, followed by further oxidation to the corresponding *o*-quinone product (Figure 110). Quinone is usually a fluorescence quencher.

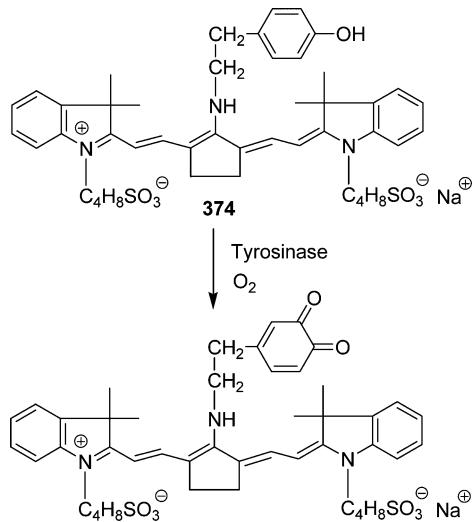


Figure 110. Tyrosinase-catalyzed oxidation of 374.

Therefore, the enzyme-catalyzed oxidation led to both the fluorescence quenching and the color change of 374 from blue to purple. The probe can be used for monitoring tyrosinase activity and for the screening of potential inhibitors by fluorescence or the naked-eye. The above compound 136, a colorimetric Pb^{2+} probe,³³⁰ has also been proposed as a fluorescent probe for tyrosinase assay.⁸¹¹ The fluorescence of the probe was quenched due to PET from the catechol moiety to the excited BODIPY. However, the tyrosinase-catalyzed oxidation can suppress the PET process and result in enhanced fluorescence. Probe 136 has been used for imaging tyrosinase activity in living cells.

5.4.2. Probes for Other Redox Enzymes. Compound 375 was developed as a fluorescent probe for 3α -hydroxysteroid dehydrogenases (3α -HSD).⁸¹² In this probe, the ketone carbonyl is designed as a part of the push–pull system.

Reduction of the carbonyl group to an alcohol converts an electron-withdrawing group (and often a quenching group) to an electron-donating group, resulting in a profound electronic change of the system, which in turn may lead to a change in the emission profile (Figure 111). Specifically, a large fluorescence

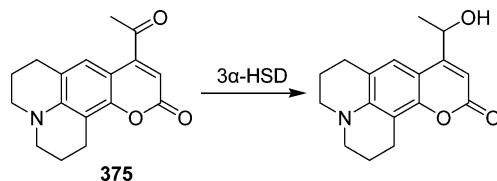
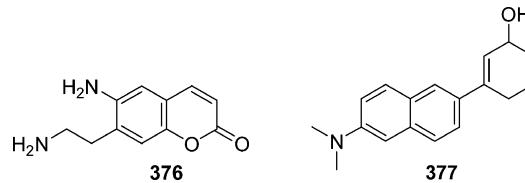


Figure 111. 3α -HSD-catalyzed reduction of 375.

enhancement was observed when the carbonyl group of 375 was reduced to hydroxyl group by the catalysis of 3α -HSD. 375 might be used as a redox imaging probe for *in vitro* and *in vivo* studies. Following the same reduction mechanism, several fluorescent probes containing carbonyls have been reported for other reductases.^{813–815} In addition, on the basis of the oxidation of amino or hydroxyl group to aldehyde, compounds 376 and 377 have been proposed as fluorescent probes for assaying the activity of monoamine oxidases⁸¹⁶ and 17β -hydroxysteroid dehydrogenase,^{817,818} respectively.



6. CONCLUSIONS AND FUTURE PERSPECTIVES

In this Review, we have summarized the most often used strategies in designing small molecular chromogenic and fluorogenic probes for various analytes, which include protonation–deprotonation, complexation, cleavage/formation of covalent bonds, and redox reactions. From the aforementioned cases, it can be seen that in the past 10 years most of the chromogenic and fluorogenic probes were constructed by using the fluorochromes such as anthracene, benzofurazan, BODIPY, coumarin, cyanine, naphthalene, quinoline, squaraine, and xanthene, and a lot of them exhibit fluorescence off–on or ratiometric (wavelength shifts) responses to an analyte via the photophysical processes like PET, ICT, or FRET. Fluorescence off–on probes, as compared to on–off or ratiometric probes, have the advantage of the ease detection of low-concentration contrast relative to a “dark” background, which is rather suited for qualitative analysis due to the high sensitivity; whereas ratiometric probes, despite the disadvantage of time-consuming ratio calculation/measurement, can eliminate the influence of several variants (e.g., probe concentration, instrumental efficiency, and environmental conditions) by built-in correction of two excitation or emission bands, which is rather suited for accurate quantitative analysis. There is no doubt that the combination of the above reactions with a rationally functionalized fluorochrome may still serve as effective strategies to design new chromogenic and fluorogenic probes. Moreover, the new probes with the following performances are desired.^{1–4,819–821} First, for practical applications of the probes in biochemical studies (especially for cell imaging studies),

good water solubility is essential, because the usage of organic solvents usually destroys the normal function of biomolecules. Second, more probes with NIR features should be exploited. Light in the NIR region (650–900 nm) possesses the advantages such as good tissue penetration, low autofluorescence, and biological damage, and is thus favorable for *in vivo* imaging. Unfortunately, most of the existing NIR fluorescent probes are derived from cyanine dyes, which suffer from poor stability. Therefore, NIR chromogenic and fluorogenic probes with high stability and quantum yield as well as good water solubility are still expected. We believe that, with the development of new design strategies, more chromogenic and fluorogenic probes with excellent properties will appear, and they will find a wider and deeper analytical use in various biosystems.

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Notes

The authors declare no competing financial interest.

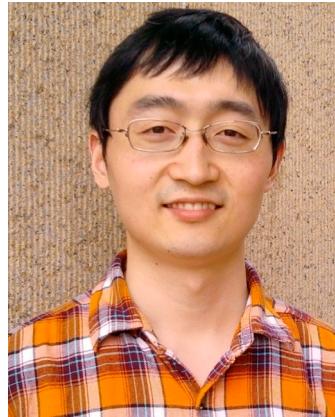
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ABBREVIATIONS

AM	acetoxyethyl
ATP	adenosine triphosphate

BAPTA	1,2-bis(<i>o</i> -aminophenoxy)ethane- <i>N,N,N',N'</i> -tetraacetic acid
BETA	bis{2-[2-(2-ethylthio)ethylthio]ethyl}amine
BODIPY	boron dipyrromethene difluoride
BPO	benzoyl peroxide
CL	chemiluminescence
CTEA	<i>N,N</i> -bis[2-(carboxymethyl)thioethyl]amine
Cys	cysteine
DNBS	2,4-dinitrobenzenesulfonyl group
DPA	di-2-picolylamine
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ESI-MS	electrospray-ionization mass spectrometry
FRET	fluorescence resonance energy transfer
GSH	reduced glutathione
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hcy	homocysteine
HOMO	highest occupied molecular orbital
8-HQ	8-hydroxyquinoline
3 α -HSD	3 α -hydroxysteroid dehydrogenases
ICT	internal charge transfer
K_a	association constant
K_d	dissociation constant
LUMO	lowest unoccupied molecular orbital
MMP	matrix metalloproteinase
NBD	4-nitrobenzo[1,2,5]oxadiazole derivative
NIR	near-infrared
NOC13	1-hydroxy-2-oxo-3-(3-aminopropyl)-3-methyl-1-triazenes
PCR	polymerase chain reaction
PET	photoinduced electron transfer
PPi	pyrophosphate
ROS	reactive oxygen species
SNARF	seminalphthorhodafluor
TBET	through-bond energy transfer
TCEP	tris(2-carboxyethyl)phosphine
TP	two-photon
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride

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