

Thiol-addition reactions and their applications in thiol recognition

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Because of the biological importance of thiols, the development of probes for thiols has been an active research area in recent years. In this review, we summarize the results of recent exciting reports regarding thiol-addition reactions and their applications in thiol recognition. The examples reported can be classified into four reaction types including 1,1, 1,2, 1,3, 1,4 addition reactions, according to their addition mechanisms, based on different Michael acceptors. In all cases, the reactions are coupled to color and/or emission changes, although some examples dealing with electrochemical recognition have also been included. The use of thiol-addition reactions is a very simple and straightforward procedure for the preparation of thiol-sensing probes.

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Introduction

Thiol-containing small molecules such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) play important roles in

living organisms, and are involved in a number of biological processes. It has also been suggested that small biothiols are useful biomarkers, and it has been reported that abnormal levels of these species are closely related to certain diseases.^{1–7} For example, Cys deficiency is involved in many syndromes such as slow growth in children, hair depigmentation, edema, lethargy, liver damage, loss of muscle and fat, skin lesions, and weakness.⁸ At elevated levels in plasma, Hcy is a risk factor for Alzheimer's disease,⁹ folate and cobalamin (vitamin B12) deficiencies,^{10,11} and cardiovascular diseases.¹² Plasma total Hcy concentrations have also been linked to birth defects¹³ and cognitive impairment in the elderly.¹⁴ GSH, which is the most abundant intracellular non-protein thiol,¹⁵ serves many cellular functions, including maintenance of intracellular redox

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activities, xenobiotic metabolism, intracellular signal transduction, and gene regulation.^{16,17} More specifically, GSH can keep the Cys thiol group in proteins in the reduced state, and also protect cells from oxidative stress by trapping free radicals, which damage DNA and RNA.¹⁸ Thiophenols, in spite of their broad synthetic use, are a class of highly toxic pollutants and, generally, thiophenols are more toxic than aliphatic alcohols. Symptoms of exposure include a burning sensation, coughing, wheezing, laryngitis, shortness of breath, and headache.¹⁹

In view of the biological, clinical, and environmental importance of thiols, it is not surprising that there has been increasing interest in the design of analytical methodologies for their detection for a number of applications. Many instrumental methods for the detection of thiol levels have been reported, *e.g.*, HPLC,^{20–23} capillary electrophoresis,^{24,25} MS,^{26,27} and electrochemical methods.^{28,29} However, these methods generally have some limitations, *e.g.*, high equipment costs, complexity, sample processing, and run times, which make them impractical

for some applications such as high-throughput routine clinical or research purposes.

As an alternative to such instrumental methods, the development of optical probes for thiols has been explored and has become an active research area in recent years. In this context, fluorescence and/or colorimetric chemosensors for thiols based on unique reaction mechanisms between probes and thiols have been reported. These include Michael addition reactions,^{30,31} cyclization with aldehydes,^{32–34} cleavage of sulfonamide and sulfonate esters,^{35–37} cleavage of selenium–nitrogen bonds,^{38–40} cleavage of disulfide bonds,^{41–43} oxidation–reduction processes in metal complexes,^{44–46} displacement protocols,⁴⁷ and use of nanoparticles.^{48–53} Yoon's group has recently published a review focusing on fluorescence or colorimetric sensors for thiols based on the unique reactions between sensors and thiols.⁵⁴

Among all these tested procedures, this review will focus on the use of addition reactions for thiol recognition and sensing. Although Michael-addition-type probes have been very actively



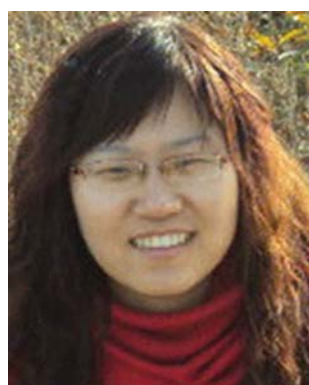
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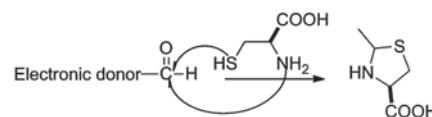
developed in recent years, it was in 1981 that Sippel reported the use of *N*-[4-(7-diethylamino-4-methylcoumarin-3-yl)phenyl]-maleimide as one of the first examples of a thiol probe; the probe was based on thiol-addition to a maleimide moiety. Since then, a number of researchers have used Michael addition reactions in thiol detection. Michael-addition-type probes have been actively developed in recent years,^{55–59} and a number of excellent Michael acceptors such as maleimides, squaraines, 7-oxanorbornadiene, quinones, chromenes, propiolates, acrylic acid, α,β -unsaturated aldehydes, ketones, diesters, and malonitrile have been exploited for the design of chromo- and fluoro- probes for thiol sensing.

All these probes can be classified into four types of addition reactions, according to their mechanisms.

1,1-Addition reactions

It is known that certain thiol-containing amino acids undergo 1,1-addition reactions with carbonyls to form new ring structures. Based on this concept, it is possible to design probes containing aldehydes coupled with suitable fluorophores or dyes, with which 1,1-addition reactions with thiols can take place. As a general example, Scheme 1 shows the reaction of Cys with an aldehyde and the formation, in this case, of a thiazolidine ring. Using this general approach, aldehyde derivatives based on phenanthrene, imidazole, dendritic chromophores, naphthalimide, fluorescein, rhodamine, coumarin, and metal complexes have been developed as thiol probes.

The phenanthrene-based probe **1** was used by Tanaka *et al.* as a 1,1-addition chemosensing ensemble for thiol recognition.⁶⁰ The authors tested probe **1** with a series of amino acids (Cys, methionine, serine, lysine, proline, and histidine) and GSH in acetonitrile:water mixtures. The reaction with Cys resulted in an increase in fluorescence ($\lambda_{\text{ex}} = 250$ nm, $\lambda_{\text{em}} = 380$ nm), whereas the presence of other amino acids and glucose did not change the emission profile of the probe. The reaction with GSH resulted in only a small increase in fluorescence. When the fluorescence intensity was measured after 30 min, only the reaction with Cys provided values meaningfully higher than that of the blank (without an amino acid). These results indicate that this fluorescence assay selectively



Scheme 1 Mechanism of 1,1 addition of thiols.

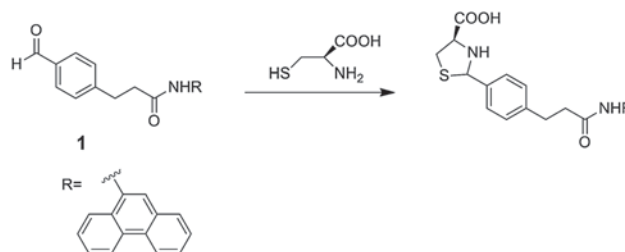


Fig. 1 Reaction of the aldehyde **1** and Cys.

detects Cys, and is also able to discriminate this amino acid from other simple thiols. The selectivity observed was the result of a thiol-addition reaction and formation of the corresponding thiazolidine ring in the presence of Cys (Fig. 1). The probe was also used in bioimaging applications.

Lin *et al.* designed 4-(1*H*-phenanthro[9,10-*d*]imidazol-2-yl)benzaldehyde (**2**)⁶¹ as a new ratiometric fluorescence probe for Cys and Hcy. Probe **2** consists of a phenanthroimidazole moiety and an aldehyde group. The electron-rich phenanthroimidazole moiety was selected to act as both a fluorescent dye and as an electron donor in the intramolecular charge-transfer (ICT) process. The optical response of probe **2** to various species was investigated in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.4/DMF (v/v, 1:3) at room temperature. Probe **2** was treated with 100 equiv. of a series of amino acids, glucose, reduced GSH, or 2-mercaptoethanol (ME). The authors found that the reactions of Cys and Hcy with the probe resulted in a decrease in the absorption band at 376 nm and formation of a new hypsochromic absorption band with a maximum at 330 nm. In contrast, no visible changes in the UV-vis profile of **2** were observed on addition of the other compounds tested, indicating that the aldehyde group specifically reacted with the β - and γ -aminoalkylthiol moieties of Cys and Hcy, respectively (see Fig. 2). In good agreement with the changes in the absorption studies, Cys/Hcy elicited selective large blue-shifts (125 nm) in the emission spectra, and color changes from green to blue (λ_{ex} 325 nm).

Two azo dyes (**3** and **4**) containing an aldehyde group were reported by Huang and Li *et al.*⁶² to act as colorimetric probes for Cys and Hcy. The addition of increasing concentrations of Cys or Hcy to DMF solutions of **3** resulted in a blue-shift of the absorption band from 465 to 442 nm. The addition of Cys to DMF:water solutions of **4** (9:1 v/v) at pH 7 (HEPES buffer) induced a decrease in the absorption intensity at 515 nm, with the concomitant appearance of a blue-shifted peak at 475 nm, corresponding to a color change from pink to yellow. The optical properties of **4** in DMF:water (9:1 v/v) on addition of other amino acids (Phe, Thr, Arg, His, Asn, Leu, Ala, Pro, Val,



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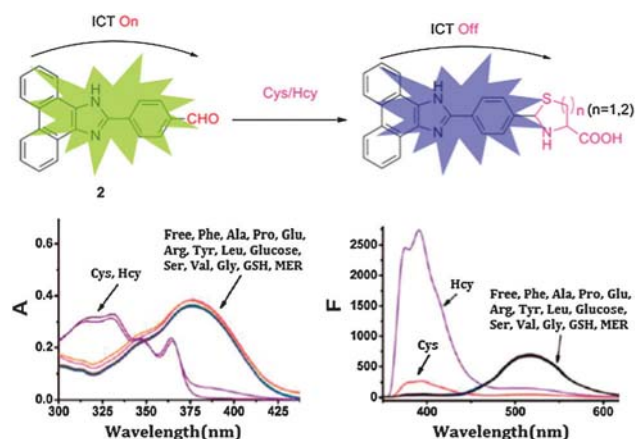


Fig. 2 Above: reaction of **2** with Cys and Hcy. Bottom: absorption (left) and emission (right) spectra of probe **2** with different species. Reprinted with permission from *Org. Lett.*, 2008, **10**, 5577–5580. Copyright 2008, American Chemical Society.

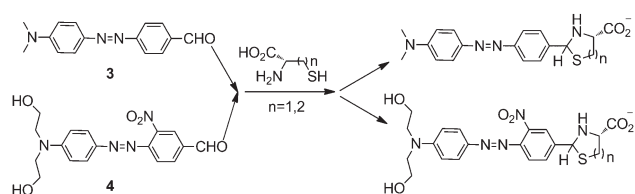


Fig. 3 Reaction of probes **3** and **4** with Cys and Hcy.

Gly, Lys, Gln, Met, Ser, Ile, Tyr, Trp, Glu, and Asp) were also investigated, but no obvious spectral changes similar to those found in the hypsochromic shift of **4** with Cys and Hcy were observed, indicating selective formation of thiazolidine **4a** and thiazinane **4b** derivatives (Fig. 3).

Huang *et al.* reported the design of the two-photon-active fluorophore **5**, using an imidazole core as a selective probe for Cys and Hcy in DMF solutions (Fig. 4).⁶³ Fluorophore **5** showed intense single-photon-absorption bands with a maximum absorption wavelength at 410 nm. Moreover, **5** in DMF showed intense single-photon-excited fluorescence (SPEF) emission at 535 nm. Upon addition of Cys/Hcy, the emission band of **5** at 535 nm gradually blue-shifted to 498 nm, corresponding to a fluorescence color change from green to cyan, and the quantum yield of **5** in the presence of Cys decreased from 0.66 to 0.31. The two-photon-excited fluorescence (TPEF) emission spectrum

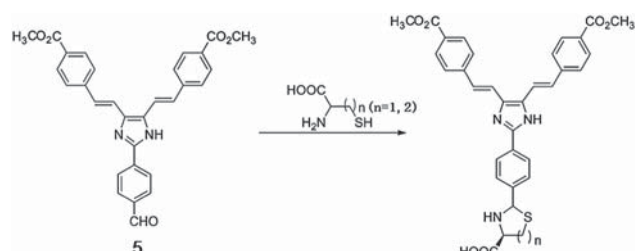


Fig. 4 Reaction of **5** and Cys and Hcy to afford the corresponding thiazolidine or thiazinane derivatives.

of **5** in the absence and presence of 40-fold Cys under excitation by a femtosecond laser at 787 nm showed that the intensity of the TPEF of **5** was more than 10 times stronger than that of a mixture of **5** and Cys (**5-Cys**), with a concomitant blue-shift of about 30 nm. The TPA cross-section maximum σ value of **5-Cys** was 246 GM at 787 nm, which was less than that of **5**, *i.e.*, 1556 GM. The TPEF spectrum of **5-Cys** was almost the same as the SPEF of **5-Cys**, which suggested the interesting possibility that **5** could act as an efficient TPEF probe for Cys/Hcy. Fluorescence spectra of **5** in DMF were also investigated on addition of other amino acids (Ala, Arg, Asn, Gln, Gly, His, Ile, Leu, Lys, Met, Pro, Ser, Thr, Tyr and Val). No obvious fluorescence changes were observed on addition of other amino acids, compared with the hypsochromic shift of **5** with Cys and Hcy, indicating that the reaction of the CHO group with Cys and Hcy was the key to their selective recognition. Furthermore, no response of **5** was observed on addition of reduced GSH. These results suggested that **5** displayed high selectivity in sensing Cys and Hcy. The transformations of **5** to the corresponding thiazolidine or thiazinane derivatives were confirmed by ¹H NMR spectroscopy.

The dendritic chromophore **6**, with ICT and a strong two-photon absorption, was designed as a turn-on fluorescent probe for Cys and Hcy by Loh *et al.* (Fig. 5).⁶⁴ The probe displayed an absorption at 410 nm in DMSO. Upon addition of Cys, the absorption at 410 nm slightly decreased and was blue-shifted (27 nm). The color of the solution changed from yellow to nearly colorless. An emission band at 625 nm ($\Phi = 1.6\%$) was observed for probe **6** in DMSO. The emission of the **6-Cys** mixture gradually increased in intensity and was blue-shifted (165 nm). After about 1 h, the **6-Cys** mixture exhibited sky-blue emission under illumination with UV light. Control experiments in the presence of other common amino acids revealed that **6** exhibited good selectivities for Cys and Hcy.

The fluorescence probe *N*-butyl-4,5-(*p*-aldehyde)phenyl-1,8-naphthalimide **7**, was designed and used for the detection of Cys (Fig. 6).⁶⁵ The addition of Cys to ethanol:water (60:40 v/v, HEPES buffer, pH 7.2) solutions of **7** resulted in a shift of the absorption band from 375 nm to 370 nm. Also, the emission intensity was enhanced and red-shifted from 455 to 480 nm, resulting in a color change from blue to cyan. The fluorescence quantum yield of **7** increased from 0.25 to 0.40. These changes were attributed to the reaction of the aldehyde groups in **7** with Cys to form very stable thiazolidine derivatives. The emission

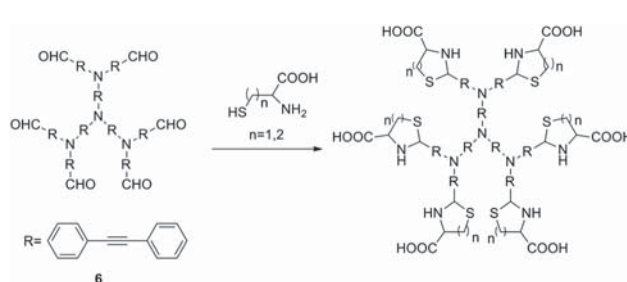


Fig. 5 Reaction between **6** and Cys.

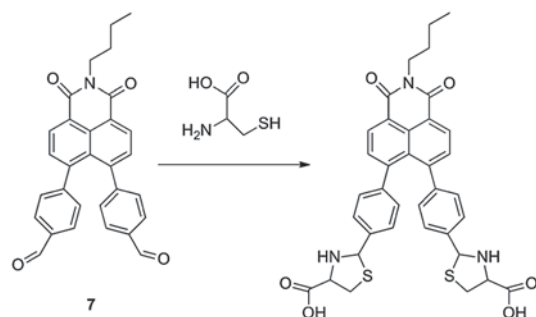


Fig. 6 Reaction of 7 with Cys.

spectrum of 7 was also studied in the presence of other amino acids, but only slight fluorescence quenching was observed.

The naphthalimide-based glyoxal hydrazone **8** was used for fluorescence turn-on detection of Cys and Hcy.⁶⁶ Probe **8** showed a very weak fluorescence at 524 nm in DMSO (λ_{ex} 439 nm, Φ_{F} = 0.012). However, on addition of Cys, the fluorescence intensity of the probe increased significantly, giving a strong green emission. The change in the emission intensity nearly became constant when the amount of Cys added reached 400 equiv. (Φ_{F} = 0.625 for **8**-Cys), and an approximately 52-fold fluorescence enhancement was observed. A similar behavior was found for Hcy. Probe **8** in DMSO was also treated with 100 equiv. of various amino acids, namely 3-mercaptopropionic acid (MPA), ethyl 2-mercaptoacetate, and GSH, but no emission changes were observed. The sensing mechanism involves cyclization of the aldehyde with Cys or Hcy, leading to formation of the corresponding thiazolidine or thiazinane **8**-Cys/**8**-Hcy derivatives. The creation of these rings favors the formation of intramolecular hydrogen-bonds between the thiazolidine/thiazinane NHs and the imine N, which prevents the C=N bond isomerization that caused the low emission of the probe (Fig. 7). The authors also used the probe for biological imaging of Cys or Hcy inside living cells.

The fluorescein derivative **9** was reported by Strongin *et al.* as a suitable probe for thiols.⁶⁷ Solutions of **9** in water (pH 9.5) are bright yellow as the result of absorbance at 480 nm. Upon addition of Cys or Hcy, a color change from bright yellow to brownish-orange was observed as a result of a red-shift of the absorption band from 480 to 500 nm. Addition of Cys or Hcy to solutions of **9** resulted in fluorescence quenching of the emission band at 510 nm (λ_{ex} = 460 nm). Other common thiols (methionine, mercaptoethanol, GSH), other amino acids (Gln, Ser, Gly, Glu), and amines (glucosamine hydrochloride) induced negligible changes, confirming the selectivity of **9** for Cys and Hcy. As in the above cases, the signaling mechanism involved the reaction of the aldehyde group in **9**, and formation of the corresponding thiazolidine or thiazinane derivatives (Fig. 8 top). Probe **9** was also used to determine aminothiols concentrations in plasma samples (Fig. 8 bottom).

Subsequently, Strongin's group developed another fluorescein derivative **10**, which was also used for signaling thiols (Fig. 9). The monoaldehyde **10** exhibited similar fluorescence quenching to those found for **9** in the presence of Cys and Hcy.

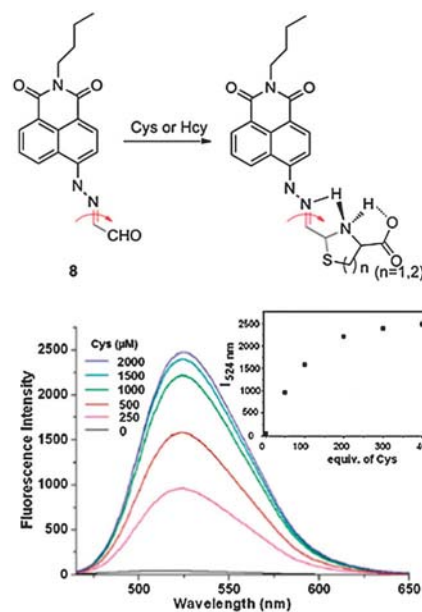


Fig. 7 Reaction of **8** with Cys and Hcy (above) and fluorescence spectral changes of **8** upon addition of Cys in DMSO (bottom). Reprinted with permission from *Org. Lett.*, 2012, **14**, 520–523. Copyright 2012, American Chemical Society.

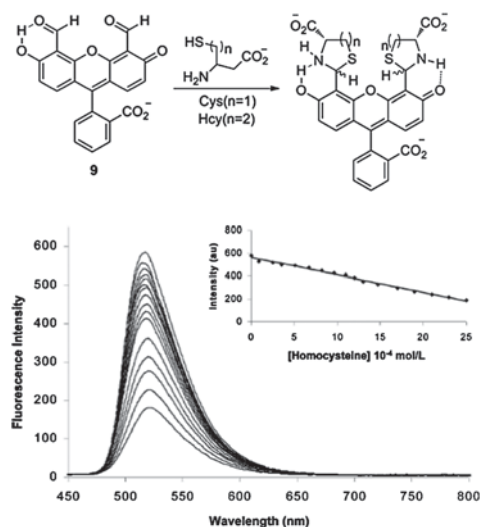


Fig. 8 Reaction of **9** with Cys, Hcy and fluorescence emission spectra of **9** in the presence of L-homocysteine in deproteinized human plasma excited at 460 nm (pH 9.5). Reprinted with permission from *J. Am. Chem. Soc.*, 2004, **126**, 438–439. Copyright 2004, American Chemical Society.

Strongin *et al.* also used the commercially available aldehyde **11** for the detection of Cys, based on formation of the corresponding five-membered rings.⁶⁸ Aldehyde **11** has a maximum absorbance at 400 nm. When Cys was added (carbonate buffer, pH 9.5), the absorbance at 400 nm decreased, and a color change from yellow to colorless was observed. Interestingly, Hcy did not cause any color change in **11** under similar conditions. The above results were explained bearing in mind that Cys may form generally more favored 5- (or possibly 7-) membered ring heterocycles, as compared to Hcy (6- or possibly

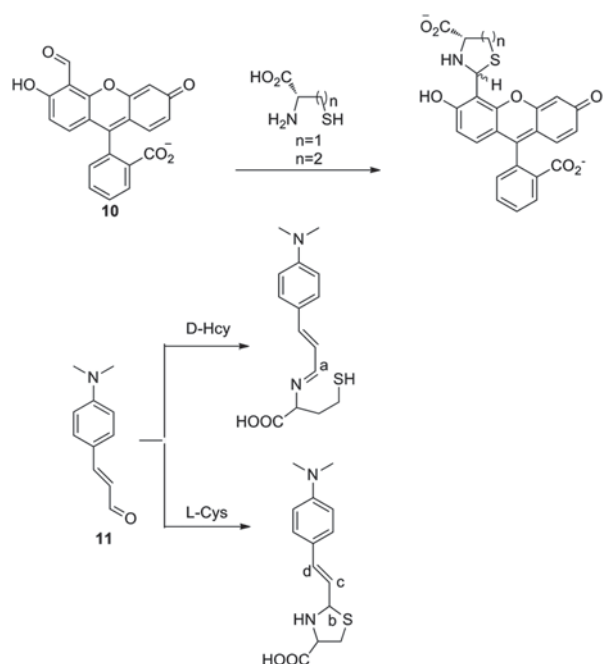


Fig. 9 Reaction of **10** with Cys, Hcy and **11** with Cys.

8-membered ring formation, respectively), upon reaction with α,β -unsaturated aldehydes. The authors studied the complete conversion of Cys (0.46 M) and **11** (3.4×10^{-2} M) in D_2O (containing 30% NaOD) to **11**-Cys by 1H NMR after 1 h of reaction. This transformation is confirmed *via* the disappearance of the aldehyde signal at 9.5 ppm and the subsequent appearance of the thiazolidine resonances (proton *b*) centered at δ 6.7 ppm. Olefin proton resonance *c* can be clearly observed appearing 0.4 ppm upfield relative to the corresponding resonance in **11**. Under similar conditions, Hcy reacts with **11** to form imine **11**-Hcy in trace amounts as the only observed product by 1H NMR. The NMR resonance for the Schiff base proton (carbon *a*) is centered at δ = 8.01 ppm, in agreement with analogous imine-forming controls using **11** and other substrates.

Strongin *et al.* also obtained the fluorescein derivative **12** (Fig. 10).⁶⁹ This probe showed a maximum absorption at 501 nm and displayed no emission in water (phosphate buffer, pH 7.4). Emission studies of **12** in the presence of various thiols (λ_{em} = 524 nm) demonstrated that compound **12** displayed

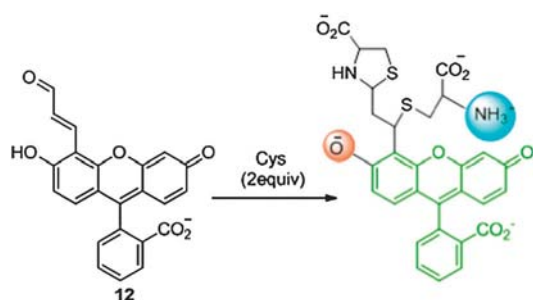


Fig. 10 The reaction of **12** with Cys.

selectivity towards Cys and Hcy. Compound **12** also showed a preference for Cys over Hcy because the Cys reaction product was formed at a faster rate (as much as 2.9-fold earlier in the course of the reaction). A greater than two-fold-higher selectivity towards Cys was observed for concentrations as low as 100 μM , and the limit of detection for Cys was found to be about three times less than that for Hcy (39 μM versus 114 μM after reaction for 20 min). The reaction of **12** with an excess of Cys involves both conjugate addition and thiazolidine formation. In the case of other thiols such as mercaptoethanol and mercaptopropionate, the emission enhancement was lower. The observed trend in emission enhancement of **12** after complete reaction with an excess of the corresponding thiol was Cys (40.98-fold) > Hcy (28.73-fold) > mercaptoethanol (6.04-fold) > mercaptopropionate (1.66-fold). In all cases the thiol attacked the β carbon of aldehyde **12**, thereby restoring xanthene emission. Once formed, the covalent complexes can undergo further interaction with the chromophore thereby affording increased selectivity by affecting the ionization state of the phenolic hydroxyl. Energy-minimized simulations (Sybyl 8.0, Tripos Inc.) indicated that, depending on the length of the alkyl chain, electrostatic interactions between the NH_3^+ group with both the phenolate and the carboxylate moieties of **12** occur (Fig. 10 bottom).

The rhodamine-based derivative **13** is colorless and weakly fluorescent (λ_{em} = 552 nm, λ_{ex} = 500 nm). However, on addition of Cys, the fluorescence emission was enhanced. For instance, when 200 μM Cys was added to **13**, an approximately 20-fold enhancement in fluorescence was observed at 37 $^\circ C$ in ethanol: water (3 : 7 v/v, 0.1 M phosphate-buffered saline (PBS), pH = 7.0) solutions. The fluorescence emission of **13** was linearly proportional to the amount of Cys added, and a limit of detection of 7.35×10^{-8} M was determined. The authors also found that the fluorescence of **13** was pH independent at pH > 5, demonstrating that it is possible to use the probe over a wide physiological pH range. Upon addition of other amino acids, including the structurally similar Hcy and GSH, to solutions of **13**, no obvious fluorescence changes were observed. Moreover, the competition experiments showed that adding diverse bioanalytes does not influence the fluorescence enhancement of Cys except for Hcy which induces a small decrease of the fluorescence intensity. The results clearly demonstrate that **13** was an excellent Cys-specific fluorescence chemodosimeter. The sensing mechanism is as follows. Upon addition of Cys to the colorless solution of **13**, thiazolidine **13'** (an unstable intermediate) was formed, followed by a ring-opening reaction that promoted hydrolysis of **13''** to **13'''**, which induced a strong fluorescence enhancement accompanied by a color change from colorless to pink (Fig. 11). The formation of **13'''** was confirmed by TOF-MS and 1H NMR spectroscopy. In the case of Hcy, the reaction with **13** produced **13'''**, which was colorless and non-fluorescent. For GSH, no reaction occurred.⁷⁰

Several examples of metal complexes containing aldehydes as reactive sites have been reported. In particular, the platinum(II) complex $Pt(phen)C\equiv CC_6H_4CHO$ (**14**) was found to be a highly selective phosphorescence chemodosimeter for Cys and Hcy (Fig. 12).⁷¹ Addition of Hcy to a solution of **14** in

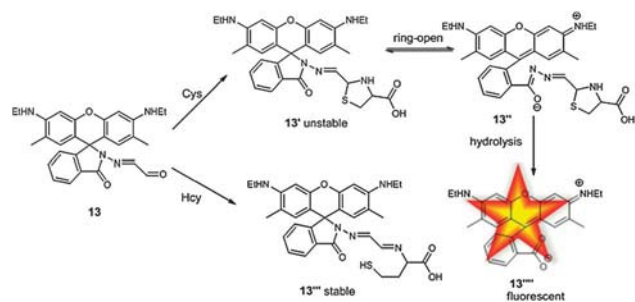


Fig. 11 Proposed mechanism for the discrimination of Cys from Hcy using **13**.

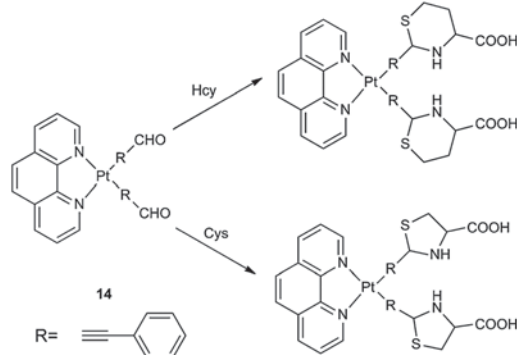


Fig. 12 Reaction of **14** with Hcy and Cys.

acetonitrile : water (4 : 1 v/v, pH 7.2) resulted in clear changes in the absorption spectrum. The high-energy band at 330 nm, assigned to the $n \rightarrow \pi^*$ transition, as a result of the presence of the aldehyde group, decreased gradually on addition of Hcy. At the same time, there was an increase in the band centered at 270 nm corresponding to $\pi \rightarrow \pi^*$ intraligand transitions. Similar phenomena were observed for the addition of Cys. Upon addition of Hcy, the emission band was red-shifted from 510 nm to 555 nm, corresponding to a luminescence color change from green to orange. The reactions of **14** with Cys and Hcy were confirmed by ^1H NMR spectroscopy and density functional theory calculations. To validate the selectivity of **14** in practice, in addition to Cys and Hcy, various other natural amino acids, namely His, Leu, Asp, Arg, Tyr, Thr, Pro, Ile, Try, Met, Val, Ala, Phe, Glu, Ser, Hyd, Lys, and Gly, were added to acetonitrile:water solutions of **14**; however, no obvious changes were observed in the emission spectra of **14**. No effect was observed in the presence of GSH. In addition, the changes in the emission spectra of **14** induced by Hcy were not influenced by the simultaneous addition of 100 equiv. of other amino acids or GSH. A similar selective behavior was observed for **14** in the presence of Cys.

The iridium(III) complex $\text{Ir}(\text{pba})_2(\text{acac})$ [$\text{Hpba} = 4$ -(2-pyridyl)-benzaldehyde; $\text{acac} = \text{acetylacetonate}$] (**15**) was used as an Hcy-selective probe (Fig. 13).⁷² The absorption spectrum of **15** showed some intense high-energy absorption bands in the UV region and a weak absorption at 510 nm. Complex **15** also showed a weak emission band at 615 nm, which resulted in a deep red photoluminescence color. The luminescence

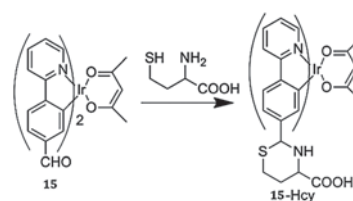


Fig. 13 Reaction of **15** with Hcy.

quantum yield of **15** in air-equilibrated solutions was *ca.* 0.003. The addition of increasing amounts of Hcy to a solution of DMSO–HEPES (pH 7.2, 9 : 1 v/v) containing **15** led to obvious changes in its absorption spectrum. In particular, the absorption band at 510 nm decreased gradually on addition of Hcy, resulting in a color change from orange to yellow. Moreover, in the luminescence spectra of **15**, the addition of Hcy induced the growth of a new emission band at 525 nm, which resulted in a change in the emission color from deep red to green that could be observed by the naked eye. Upon addition of Cys, the absorption band at 510 nm decreased. However, when an excess of Cys was added to a solution of **15**, a weak enhancement (two-fold) of the luminescence intensity at 525 nm was observed, although the intensity of the emission band at 615 nm decreased four-fold. This indicates that **15** is a UV-vis probe for Hcy and Cys, but it is a phosphorescence probe for Hcy. Upon addition of amino acids such as His, Leu, Asn, Arg, Tyr, Thr, Pro, Ile, Trp, Met, Val, Ala, Phe, Gln, Glu, Ser, Hyp, Lys, and Gly, no obvious changes were observed, either in the absorption or in the emission spectra. Also, no obvious enhancement of the phosphorescence emission of **15** was observed on addition of the thiol-related peptide GSH. These results are related to the selective reactions of the aldehyde moiety with the β - and γ -aminoalkylthiol groups in Cys and Hcy. To further understand the effect of the aldehyde (CHO) group on the photophysical properties, calculations based on DFT for **15** and **15**-Hcy were performed. Both complexes were confirmed to be genuine minima on the PES. Orbital analysis revealed that, for the two complexes, no obvious change was observed for the highest occupied molecular orbital (HOMO) distributions, and the HOMO primarily resided on the iridium center and phenyl part of the cyclometalated ligands, similar to most of the iridium(III) complexes. However, the lowest unoccupied molecular orbital (LUMO) distributions of **15** and **15**-Hcy are strikingly different. For **15**, the LUMO distribution is evenly localized on the two pba ligands. In contrast, the LUMO distribution of **15**-Hcy is partially located on the 2-phenylpyridine moiety of one ligand. As a result, the LUMO energy level of **15**-Hcy is higher. The HOMO–LUMO energy gaps were calculated as 3.21 and 3.46 eV for **15** and **15**-Hcy, respectively, which is in agreement with the remarkable blue-shift in the absorption and luminescence spectra of **15**-Hcy compared with those of **15**.

When Cys or Hcy was added to solutions of **16** in HEPES,⁷³ the absorbances at 320 and 428 nm decreased gradually. The influence of Cys and Hcy on the emission spectra of **16** was also investigated. The aldehyde group in complex **16** was a strong

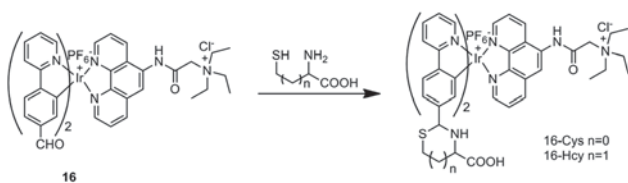


Fig. 14 Reaction of **16** with Cys and Hcy.

electron-withdrawing group that effectively quenched the luminescence of the complex (Fig. 14). Complex **16** therefore exhibited weak emissions in HEPES. The phosphorescence emission intensity of **16** at 565 nm was significantly enhanced by addition of Cys and Hcy. No detectable change in the phosphorescence emission of complex **16** was observed on addition of other amino acids such as Ala, Arg, Asp, Gln, Glu, Gly, GSH, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. As in the examples above, the changes observed are the result of the reaction of the aldehyde in complex **16** with Cys or Hcy to form the corresponding thiazolidine/thiazinane derivatives. Compound **16** has been used to monitor changes in Cys or Hcy concentrations in living cells *via* monitoring red luminescence after incubating with 20 mM solution of complex **16** at 37 °C for 30 min.

The nanoprobe **17**, based on the use of mesoporous silica nanoparticles as carriers and an iridium(III) complex as a signaling unit, was developed for the detection of thiol-containing derivatives (Fig. 15 top).⁷⁴ Solid **17** showed a broad and structureless photoluminescence emission band at 545 nm as a result of the presence of the iridium(III) complex. Upon addition of Hcy or Cys to solutions of **17** in water (PBS, pH 7.4) an intense emission band appeared at 531 nm (emission color change from orange to bright green). Control experiments were carried out with other amino acids such as His, Thr, Leu, Asp, Arg, Tyr, Pro, Iso, Try, Met, Val, Lys, Ala, Gln, Gly, Glu, Ser, L-OH-Pro, and Phe. These studies revealed that **17** exhibited good selectivities for Hcy and Cys. The recognition mechanism involves the reaction of the aldehyde group in the Ir(III) complex with the β - and γ -aminoalkylthiol groups of Cys and Hcy to form the corresponding thiazolidine/thiazinane derivatives. To further understand the response mechanism, the effect of the aldehyde group on the photophysical properties was studied using time-dependent density functional theory calculations. The probe was also used in the human nasopharyngeal epidermal carcinoma cell line (KB cell) bioimaging experiments (Fig. 15 bottom).

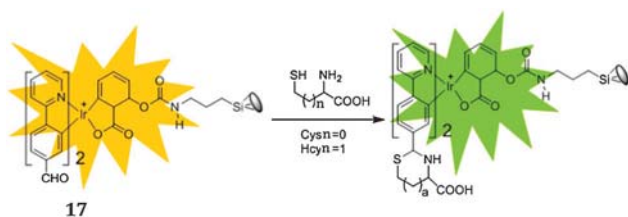


Fig. 15 Reaction of **17** with Cys/Hcy.

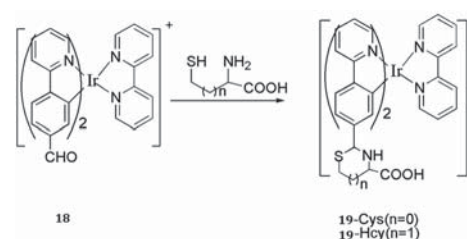


Fig. 16 Reaction of **18** with Cys and Hcy.

The complex $[\text{Ir}(\text{pba})_2(\text{bpy})][\text{PF}_6]$, [pba = 4-(2-pyridyl)benzaldehyde, bpy = bipyridine] (**18**), containing aldehyde groups, was designed as a novel Hcy/Cys-selective probe for ratiometric phosphorescence imaging in living KB cells.⁷⁵ On addition of increasing amounts of Hcy or Cys to a solution of **18** in DMSO:water (9:1 v/v, HEPES, pH 7.2), the absorbances at 435 and 301 nm decreased gradually, whereas the absorbance at 390 nm increased. Also, on addition of Hcy or Cys, the emission band progressively shifted to 586 nm (a red-shift of *ca.* 40 nm), with a decrease in intensity, resulting in an emission color change from yellow to red. Amino acids such as His, Leu, Asn, Arg, Tyr, Thr, pro, Ile, Trp, Met, Val, Ala, Phe, Gln, Glu, Ser, Lys, and Gly, and the small peptide GSH, did not induce detectable spectral changes. As in the above cases, the sensing mechanism was attributed to formation of the corresponding thiazinane or thiazolidine groups by reaction with Hcy or Cys, as shown in Fig. 16.

In another study, the Ru(II) complex $[\text{Ru}(\text{CHO-bpy})_3](\text{PF}_6)_2$ (CHO-bpy: 4-methyl-2,2'-bipyridyl-4'-carboxaldehyde) (**19**) was designed and used for the selective and sensitive detections of Cys and Hcy.⁷⁶ Compound **19** exhibited two strong absorption bands at 300 and 485 nm. In the presence of Cys or Hcy (DMSO:water, HEPES buffer), the two absorption bands of the complex were shifted to 290 and 465 nm, respectively, accompanied by a color change from orange to yellow. The emission spectrum of **19** also displayed a large blue-shift from 720 to 635 nm, accompanied by a significant increase in the luminescence intensity. Other amino acids, proteins, DNA, ethanolamine, and cysteamine were unable to induce emission changes in **19**. The reaction of **19** with Cys or Hcy resulted in the formation of highly luminescent thiazolidine or thiazinane derivatives, as shown in Fig. 17.

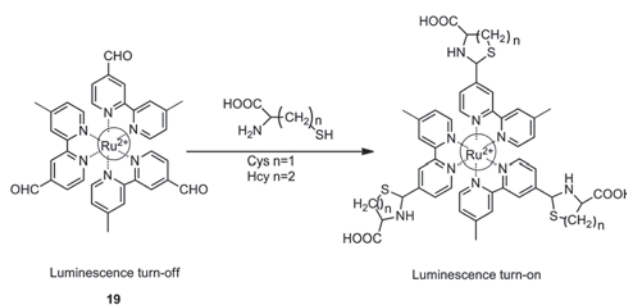


Fig. 17 Reaction of **19** with Cys and Hcy.

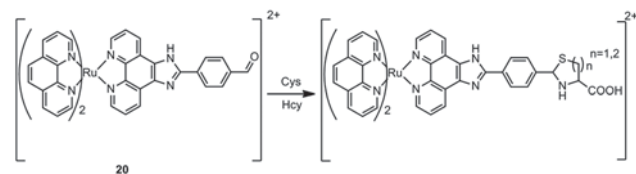


Fig. 18 Reaction of **20** with Cys and Hcy.

The ruthenium polypyridine complex $[\text{Ru}(\text{phen})_2(\text{IPBA})](\text{PF}_6)_2$ (**20**) (IPBA = 4-(1*H*-imidazo[4,5-*f*][1,9]phenanthroline-2-yl)benzaldehyde), was used as a probe for thiol detection (Fig. 18).⁷⁷ When increasing amounts of Cys or Hcy were added to DMF:water (10:1 v/v, HEPES) solutions of **20**, the bands in the 325–400 nm range decreased in intensity. In the presence of Cys or Hcy, enhancement of the emission was observed along with the appearance of a purple luminescence color. Other amino acids, as well as thiol biomacromolecules such as 2-thiobarbituric acid, thioglycol, and GSH, were also investigated; however, no optical changes in **20** were detected. The authors suggested that the poor reactivity found for GSH, which hardly reacted with **20**, may be the result of steric hindrance.

1,1-Addition reactions of thiols on several coumarin-based probes have also been reported. For instance, the coumarin-based probe **21** (7-dimethylamino-1,4-benzoxazin-2-one aldehyde) was designed as a fluorescence chemodosimeter for Cys and Hcy.⁷⁸ Free **21** showed an absorption band at 500 nm and was weakly fluorescent in the 3–8 physiological pH range. Upon addition of Cys, the fluorescence increased rapidly in acetonitrile:water (3:7 v/v, HEPES, pH 7.4). For instance, when 50 equiv. of Cys were added to **21**, an approximately five-fold emission enhancement at 560 nm was observed. At the same time, the absorption band of **21** at 500 nm gradually decreased and a new absorption at 430 nm appeared, resulting in a color change from orange to yellow. The emission intensity of **21** at 560 nm showed a good linear relationship within the concentration range usually found for Cys under physiological conditions (the total concentration of Cys in healthy plasma is in the range 240–360 μM). Using probe **21**, a detection limit for Cys of 6.8×10^{-7} M was determined. The fluorescence responses of **21** to various amino acids such as Gly, Ala, Val, Leu, Ile, Pro, Ser, Thr, GSH, Glu, Arg, Lys, and thiol biomacromolecules were studied. However, none of the tested species, except Hcy and Cys, induced any fluorescence emission enhancement. The recognition mechanism is based on the cyclization reactions of Cys and Hcy with the aldehyde group in **21**, as shown in Fig. 19.

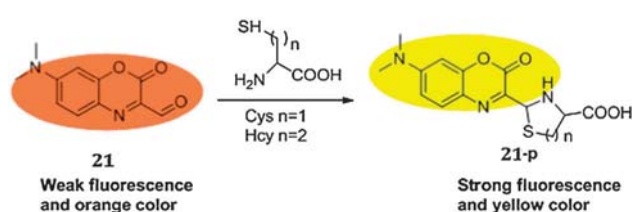


Fig. 19 Reaction of aldehyde **21** with Cys or Hcy.

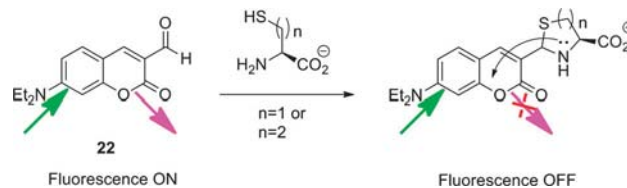


Fig. 20 Proposed fluorescence sensing mechanism of **22** upon reaction with Cys or Hcy.

Another coumarin-based fluorescence sensor was reported by Kim *et al.* for the selective detection of Cys and Hcy. This “push–pull” type of coumarin-based probe⁷⁹ showed a strong fluorescence emission at 488 nm. When Hcy or Cys was added to a solution of **22** in ethanol, the emission intensity clearly decreased. Some changes were also observed in the presence of GSH. Typically, the ratios F_0/F on addition of 500 equiv. of these analytes were 54, 43, and 1.5 for Hcy, Cys, and GSH, respectively. Studies were also carried out in the presence of Gly, Ala, Val, Leu, Ile, Pro, Ser, Thr, DTT, GSH, Glu, Arg, and Lys, but no changes were found. The observed behavior was the result of the reactions of Cys and Hcy with **22**, which induced formation of the corresponding thiazinane/thiazolidine rings (Fig. 20). The authors attributed the decrease in the emission intensity to the presence of effective photoinduced electron transfer (PET) from the neighboring nitrogen atom of the thiazinane/thiazolidine ring in the final products, which did not occur in **22**.

Kim and Hong *et al.* reported the use of the *o*-hydroxy-aldehyde-functionalized coumarin **23** as a selective turn-on probe for Hcy and Cys (see Fig. 21).⁸⁰ The probe itself was not fluorescent in aqueous HEPES solutions (pH 7.4). However, on addition of Hcy, the emission intensity at 450 nm increased more than 100-fold. An emission color change of the solution from colorless to blue was also observed. A similar fluorescence increase was found for Cys, with a 49-fold enhancement at 450 nm. However, the authors found that other amino acids such as *N*-Boc-Cys, Met, Ser, Thr, Lys, Trp, Asp, Pro, Glu, Leu, Ile, Gly, Ala, and GSH did not cause any significant changes in the emission of **23**. The authors proposed that two factors affected the fluorescence enhancement upon addition of Hcy or Cys to probe **23**. First, a hydrogen bond between the phenol proton and thiazinane nitrogen atom of **23**-Hcy is assumed to inhibit the possible PET quenching observed in the previous thiazinane coumarin complex. Computational structure of **23**-Hcy explicitly shows the plausible hydrogen bonding between the phenol proton and nitrogen atom. Secondly, the

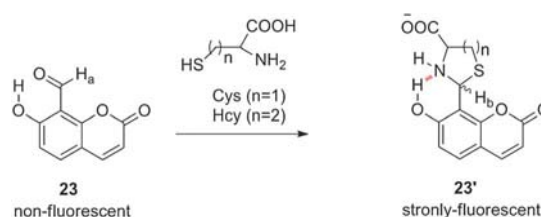


Fig. 21 Probe **23** and its reaction with Hcy or Cys.

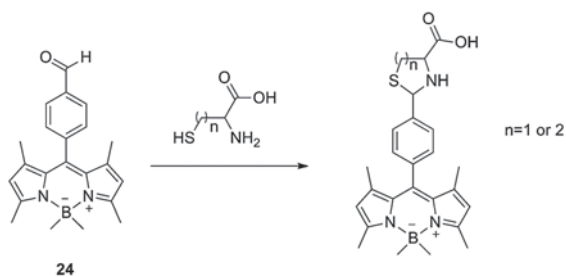


Fig. 22 Reaction of **24** with Hcy and Cys.

fluorescence quenching due to a possible charge transfer from the coumarin HOMO to the aldehyde carbonyl LUMO in **23** will be depressed in the **23**-Hcy complex, where the sp^2 hybrid carbonyl is transformed to an sp^3 hybrid carbon owing to the thiazinane ring formation. Therefore, the excited electrons in **23**-Hcy will decay by emitting a strong fluorescence. The authors also demonstrated the use of probe **23** to determine submillimolar concentrations of Hcy in blood samples.

The fluorescence response of compound **24** to various amino acids and reduced GSH was investigated in water:acetonitrile (2:3 v/v, HEPES, pH 7.4).⁸¹ The reaction of Hcy or Cys with the probe resulted in significant fluorescence enhancement at 513 nm. In contrast, other amino acids, including Ala, Thr, Arg, Gly and GSH, induced no discernible changes in the emission profile of **24**. As above, the response observed was caused by formation of the corresponding thiazinane or thiazolidine rings upon reaction of Hcy or Cys with **24** (Fig. 22).

Two new two-photon excitation fluorescence (TPEF) probes (**25** and **26**) for the selective sensing of Cys were developed by Yang *et al.*⁸² The addition of Cys to **25** in acetonitrile:water solutions, buffered with tris(hydroxymethyl)aminomethane (Tris), resulted in a color change from colorless to bright yellow as a result of the appearance of a new band at 525 nm. Interestingly, the addition of other bioanalytes (Ile, L-Ala, Leu, Lys, Met, Nac, Phe, Pro, Ser, Thr, Trp, Tyr, Val, His, Gly, Glu, Gln, DTT, β -Ala, Asp, Arg, GSH, and Hcy) did not induce color changes in **25**. In acetonitrile:water (buffered with Tris) solutions, the addition of Cys to **26** induced a color change from colorless to pale yellow as a result of the shift of the original absorbance of **26** at 350 nm to 420 nm. In this medium, the addition of *N*-acetyl-L-cysteine (NAC), GSH, and His also induced some absorbance changes. However, the addition of other bioanalytes (Ile, L-Ala, Leu, Lys, Met, NAC, Phe, Pro, Ser, Thr, Trp, Tyr, Val, His, Gly, Glu, Gln, DTT, β -Ala, Asp, Arg, and Hcy) caused no absorbance changes. Compound **25** was weakly fluorescent, whereas the SPEF of **25** + Cys was notably enhanced, with a red-shift of 84 nm (from 493 to 577 nm), accompanied by a fluorescence color change from dark to deep red. Upon addition of *N*-acetylcysteine (NAC), the SPEF intensity of **25** also increased slightly, although the intensity of **25** + Cys was 6.3 times stronger than that of **25** + NAC. Hcy did not alter the emission of **25**. The SPEF intensity of **26** + Cys was 2.5 times larger than that of **26** + NAC. The TPEF intensities of **25** + Cys at 630 nm were 11, 67, and 60 times stronger than those of

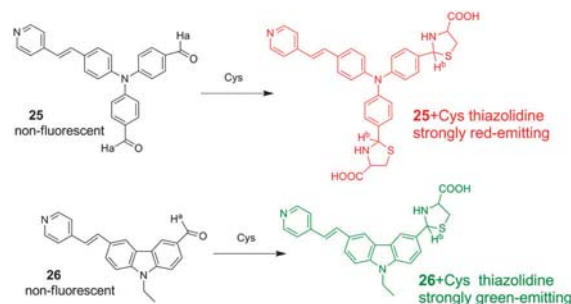


Fig. 23 Reaction of **25** and **26** with Cys.

25 + NAC, **25** + Hcy, and **25**, respectively. Under similar conditions, the corresponding enhancements of **26** + Cys at 551 nm were 2.64-fold, 77-fold, and 59-fold, respectively. The σ value of **25** + Cys was 1700 GM at 920 nm and that of **26** + Cys was 90 GM at 810 nm. The above results can be explained by taking into account the reaction of Cys with **25** and **26**, which results in the formation of thiazolidine rings and the transformation of an A- π -A electronic structure to an A- π -D motif (Fig. 23).

Compound **27** proved to be a suitable probe for Cys in DMSO:water (buffered with HEPES) mixtures.⁸³ The probe consisted of two aldehyde groups as reactive sites and a dimethyl tetraphenyl silole core as the signaling moiety (Fig. 24). Compound **27** has moderate solubility in DMSO:water. When Cys was added to the solution, it readily reacted with **27**, leading to a thiazolidine derivative, which has lower solubility (formation of aggregates). In particular, upon addition of Cys, the emission intensity at 479 nm increased slowly, and a new band at 424 nm appeared. The intensity of the new band grew quickly with increasing amounts of Cys (aggregation-induced emission effect). Also, the reaction mixture turned from transparent to turbid. To examine the specificity of **27** for Cys, the responses of the probe to other amino acids (Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Tyr, Val, Pro, Hcy, Pgl, and GSH), glucose, and protected Cys (Cya and Cyt) were investigated. Among all the tested analytes, Cys gave the highest fluorescence response. Moreover, emission enhancements of

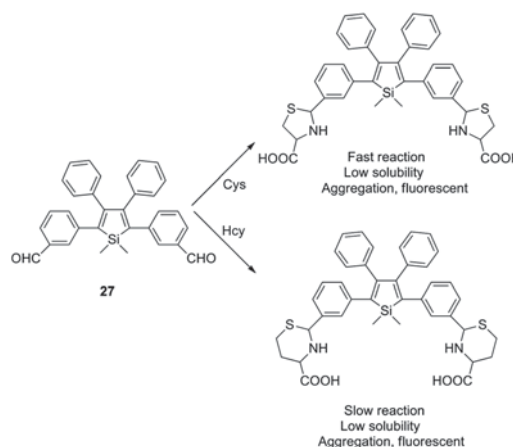


Fig. 24 Reaction of probe **27** with Cys or Hcy.

ca. four-, 1.7-, and one-fold were recorded for Cyt, Hcy, and Lys, respectively. The authors also observed that Hcy displayed a similar blue-shifted emission peak as that found for Cys. It was easy to distinguish between Cys and Hcy by checking the emission intensities and the rate of formation of a white precipitate (turbid solutions), which was quick for Cys but slow for Hcy.

Two compounds (**28** and **29**), functionalized with two aldehyde groups, were developed as probes for Cys and Hcy. Compound **28** in DMSO:water (HEPES, pH 7.4) displayed a low-intensity emission band at 501 nm. When Cys was added to a solution of **28**, the transparent reaction solution turned turbid in a few minutes, and the process could be conveniently observed by the naked eye. The weakly emissive probe solution gradually became intensely fluorescent (5.7-fold enhancement), and the emission color changed from green to blue (*i.e.*, the emission at 501 nm increased gradually, and a new band emerged at 425 nm). Different behavior was observed in the presence of Hcy. In this case, only small changes were observed after several hours. After 3 d, a white precipitate appeared and the suspension showed a strong blue fluorescence. The responses of probe **28** to other amino acids and glucose were investigated. Among all the tested analytes, Cys exhibited the highest fluorescence response: in 1 h, a four-fold emission enhancement was observed for Cys, whereas a 0.27-fold enhancement was recorded for Hcy, and less than 0.2-fold enhancements for the rest of the analytes. These results indicate that although the emission enhancement was not very high, the probe had a high specificity for Cys. Probe **29** in DMSO:HEPES (pH 7.4) displayed a low-intensity emission band at 501 nm. In the presence of Cys, the reaction solution changed from transparent to turbid, the emission became more intense (15.7-fold enhancement), and an emission color evolution from green to purple/blue was observed (*i.e.*, the emission at 501 nm increased gradually, and a new band at 421 nm emerged). In the presence of Hcy, probe **29** showed no response for hours; however, after 3 d, a white solid precipitated and the emission increased slightly. Compared with **28**, the dialdehyde-substituted silole derivative **29** amplified the differences between Cys and Hcy by shortening the response time for Cys and prolonging the time during which Hcy induced some changes. The reaction mechanisms of **28** and **29** with Cys and Hcy involve formation of the corresponding thiozolidine and thiazinane derivatives (Fig. 25). The strong hydrogen-bonding interactions between the carboxylic and thiazolidine/thiazinane groups in the final products probably cause aggregate formation. The emission enhancement was ascribed to the resulting aggregates (aggregation-induced emission). The large time differences between Cys and Hcy for fluorescence turn-on imply that the reaction of Hcy with the probes is quite slow compared with that of Cys; the reason for the kinetic difference was associated with the different cyclization rates.⁸⁴

Probes **30** and **31**, bearing pendant aldehyde functional groups, were used to sense Cys and Hcy.⁸⁵ Probe **30** showed an absorption band at 397 nm and ICT emission at 528 nm ($\lambda_{\text{ex}} = 397 \text{ nm}$, $\Phi = 0.0066$). Upon addition of increasing amounts of Cys or Hcy to a solution of **30** in DMSO:water (3:2 v/v, HEPES, pH 7.4),

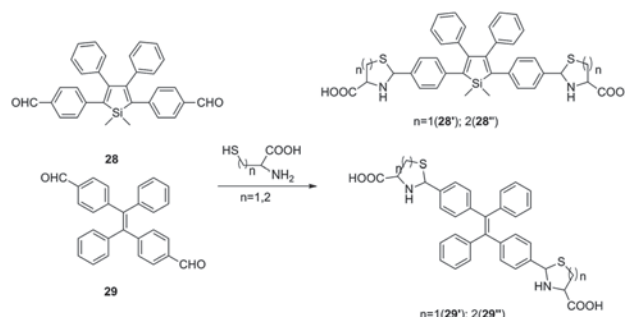


Fig. 25 Reaction of probe **28** and **29** with Cys or Hcy.

the absorption band decreased gradually, with a concomitant increase in the intensity of a new band at 464 or 462 nm, for Cys and Hcy, respectively. These changes were associated with a visually detectable change in color from yellow to orange. An appreciable red-shift in the emission band maximum from 528 to 598 nm ($\lambda_{\text{ext}} = 397 \text{ nm}$) was also observed, and was associated with a visually detectable change in the solution fluorescence from yellow to red. In DMSO:water (8:2 v/v, HEPES, pH 7.4), probe **31** showed an absorption band at 346 nm and a strong ICT emission at 506 nm ($\lambda_{\text{ex}} = 346 \text{ nm}$, $\Phi = 0.45$). The addition of Cys or Hcy to **31** resulted in the development of a new blue-shifted ($\Delta\lambda \approx 70 \text{ nm}$) band maximum at 276 nm, and the original spectral band of **31**, with a maximum at 346 nm, disappeared. It should be noted that for **31**, the spectral changes were restricted to the UV region, and therefore no associated color change was detected visually. The addition of increasing amounts of Cys or Hcy to solutions of **31** resulted in a new blue-shifted emission band at 438 nm ($\lambda_{\text{ext}} = 346 \text{ nm}$), with a concomitant decrease in the emission intensity at 506 nm. Neither **30** nor **31** showed any significant changes in their absorption or emission spectral patterns on addition of excess Gly, Leu, Asp, Pro, Arg, Lys, Val, GSH, Ala, Thr, His, Trp, Tyr, Ser, Met, cystine, calf-thymus DNA, $\text{NH}_2\text{CH}_2\text{CH}_2\text{OH}$, or ME. However, some changes were found in the presence of $\text{NH}_2\text{CH}_2\text{CH}_2\text{SH}$. The above spectral changes are based on reactions between aldehyde groups with the β - γ -aminothiols in Cys/Hcy to yield the corresponding thiazolidine/thiazinane derivatives (Fig. 26). The two probes were used for the detection of Cys/Hcy in complex biological fluids and in the *in vivo* imaging of cellular thiols.

1,2-Addition reactions

When an alkene or alkyne group is connected to an electron-withdrawing group, a Michael addition can take place in the presence of nucleophilic thiols. This basic reaction has been coupled with chromo- or fluorogenic groups to design probes for thiols. Using this general approach, several alkene or alkyne scaffolds connected to electron-withdrawing groups such as cyano, nitro, and carbonyl groups, and to signaling units such as coumarin, maleimide, fluorescein, and dansyl, have been reported as thiol probes (Scheme 2).

Most of the examples using this approach are based on the use of coumarins, usually leading to strong fluorescence

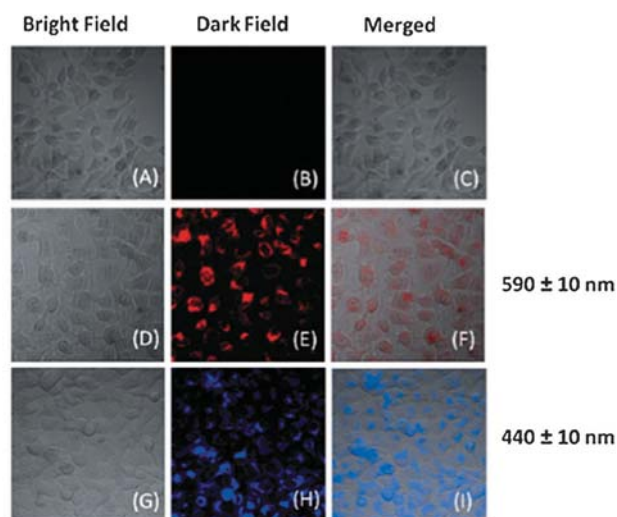
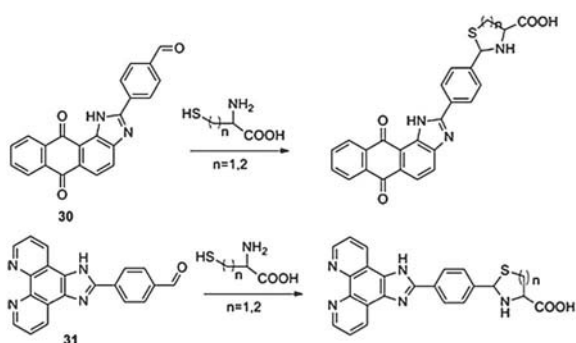
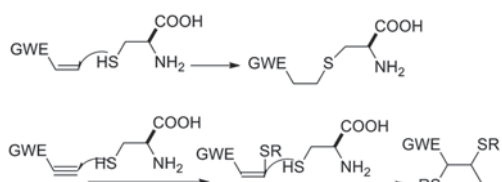


Fig. 26 Reaction of probes **30** and **31** with Cys or Hcy (above) and the use of the probe in human cervical cancer cells (HeLa cells): bright-field transmission image of HeLa cells in the absence of **30** and **31** (A); fluorescence image of HeLa cells in the absence of **30** and **31** (B), bright-field transmission images of HeLa cells incubated with **30** (D) and **30** (G); fluorescence images of HeLa cells incubated with **30** (from the red channel; E) and **31** (from the blue channel; H); incubation was performed at 37 °C for 50 min and [**30**] or [**31**] = 20 μM. Reprinted with permission from *Chem.–Eur. J.*, 2012, **18**, 15382–15393. Copyright 2012, John Wiley and Sons.



Scheme 2 Mechanism of 1,2 addition.

enhancements. For instance, Pavez *et al.* reported compounds **32** and **33** as fluorescence probes for the detection of Cys, Hcy, GSH, and Cys-Gly.⁸⁶ When **32** in HEPES buffer was excited at 340 nm, an emission was observed at 430 nm ($\Phi = 0.00083$). Upon addition of Cys, Hcy, GSH, and Cys-Gly, emission increments were observed in the order Cys > GSH > Hcy ≥ Cys-Gly. For instance, on addition of 10 equiv. of Cys or GSH, the emission was enhanced, with a change in the quantum yield to 0.0013 or 0.0010, respectively, with no band shift. A similar experiment with **33** ($\lambda_{\text{max}} = 435$ nm, $\Phi = 0.0014$) led to increases

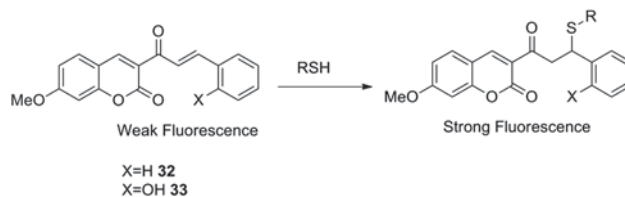


Fig. 27 Reaction of **32** and **33** with thiols.

in the quantum yields to 0.0022 and 0.0016, respectively. The selectivities of **32** and **33** towards these biothiols were investigated by incubating these probes with different species, including some other amino acids (Asp, Lys, Ser, His, Pro, Phe, and Val). None of these compounds interfered to any obvious extent in the detection of the above-mentioned biothiols, even at a 100 : 1 molar ratio with respect to the probe. Moreover, **33** was found to be more active than **32**, probably as a consequence of the proton-donating ability of the hydroxy group in **33** to the vinyl carbon neighboring the carbonyl group, making the reaction site more prone to attack by thiols (Fig. 27). In addition, **32** and **33** were used to detect biothiols in SH-SY5Y cells.

Michael addition of a thiol to a nitroolefin was used as a sensing mechanism in **34** for the detection of thiols (Fig. 28). Upon addition of Cys to **34** in acetonitrile:water (1 : 1 v/v, HEPES, pH = 7.4) the absorption of **34** at 483 nm gradually decreased and a new band appeared at 400 nm (color change from orange to yellow).⁸⁷ Kinetic studies showed that the reaction was complete within 30 s. Probe **34** was weakly fluorescent, but on addition of Cys, the emission intensity increased significantly and a fluorescence enhancement factor of approximately six-fold at 480 nm ($\lambda_{\text{ex}} = 410$ nm) was found. This emission enhancement resulted in an emission color change from dark to blue-green. The distinct fluorescence enhancement of probe **34** in the presence of Cys was explained by the formation of a **34**-Cys adduct, which blocked the PET quenching of the nitroolefin observed in **34**. To investigate the selectivity shown by the probe, **34** was treated with other natural amino acids and similar responses to that for Cys were observed for Hcy and GSH. However, the reactions of **34** with Hcy and GSH took *ca.* 2 min and 6 min, respectively. The observed order reactivity, *i.e.*, Cys > Hcy > GSH, can be rationalized on the basis of steric-hindrance effects. The probe was used in thiol detection in human blood samples and in living cells.

Probe **35** exhibited an absorption band at 466 nm and was poorly fluorescent ($\Phi = 0.0002$) in phosphate buffer solutions (pH 7.4) (Fig. 29).⁸⁸ The addition of increasing amounts of Cys elicited a gradual decrease in the absorption at 466 nm and a

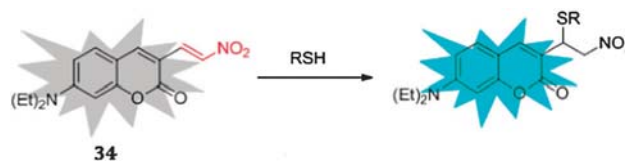


Fig. 28 Reaction of probe **34** with thiols.

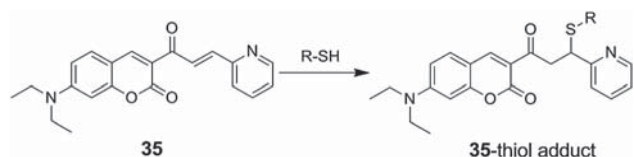


Fig. 29 Reaction of probe 35 with thiols.

progressive increase in a new absorption band centered at 444 nm. Cys also induced the appearance of a new strong emission peak at 496 nm, with a fluorescence enhancement of up to 211-fold. This resulted in an emission color change from dark to green. The detection limit for Cys was determined to be 9.25×10^{-7} M under these experimental conditions. To investigate the selectivity, probe 35 was treated with various biologically relevant analytes such as representative amino acids, glucose, metal ions, reactive oxygen species, reducing agents, nucleosides, and small-molecule thiols. The authors found that the presence of Cys, Hcy, and GSH induced optical changes, whereas no changes were found in the presence of the other species studied. The spectral changes were attributed to the reaction of the thiol with the β -unsaturated ketone moiety, which inhibited conjugation between the pyridine moiety and the coumarin dye, resulting in a significant emission enhancement. The probe was used for fluorescence sensing of thiols in a newborn-calf serum sample.

Probe 36 was poorly fluorescent ($\Phi_0 = 0.001$); however, on addition of GSH, a 470-fold turn-on fluorescence response was observed ($\Phi = 0.47$). The authors found that this change in emission increased linearly with the concentration of GSH up to a ratio of 1 : 1. A similar response was also found for Cys and β -mercaptoethanol. No obvious changes in 36 were observed on addition of other natural amino acids. Glutathione disulfide (GSSG) and cystine did not react with 36 under similar experimental conditions. Furthermore, GSH could be detected at least down to 0.5 nM (0.1 pmol) when 36 was used at a concentration of 1.0×10^{-7} M in aqueous buffered solutions. The turn-on of the emission was explained by assuming that the reaction of the thiol with the double bond inhibited the PET quenching mechanism that was active in 36 (Fig. 30). The probe was also used in fast detection/labeling of thiol-containing proteins.⁸⁹

Derivative 37 (ref. 90) contains a coumarin group as a signaling unit, a conjugated enone as a reactive site, and an *o*-hydroxyl group as an activating unit *via* hydrogen-bonding interactions (Fig. 31). Probe 37 showed an absorbance at 434 nm, in DMF:water (3 : 1 v/v, HEPES, pH 7.4). On addition of GSH, the absorbance at 434 nm decreased and a new band at 404 nm evolved. A color change from yellow to colorless was observed. The fluorescence of 37 underwent a 22-fold increase in intensity at 480 nm ($\lambda_{\text{ex}} = 417$ nm), resulting in a strong blue fluorescence. Similarly, Cys induced a significant fluorescence turn-on in 37 (16-fold). Other natural amino acids tested did not cause any significant fluorescence changes.

The addition of Cys to 38 in aqueous solution (PBS buffer, pH 7.4, 10% DMSO) induced a 62 nm hypsochromic shift of the

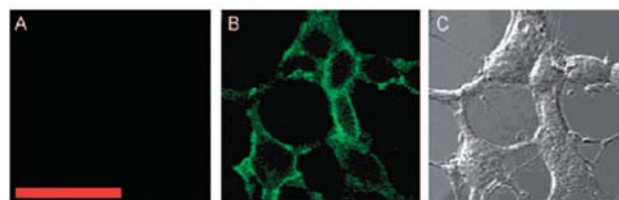
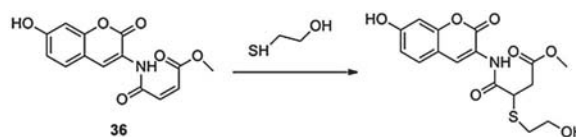


Fig. 30 Reaction of probe 36 with β -mercaptoethanol and its human embryo kidney cells (HEK-293): fluorescence image before (A) and after (B) treating the cells with 36 (1.0 μM) for 5 min at room temperature. (C) Brightfield image of the cells. Scale bar = 50 μm . Reprinted with permission from *Angew. Chem., Int. Ed.*, 2009, 48, 4034–4037. Copyright 2009, John Wiley and Sons.



Fig. 31 Reaction of probe 37 with thiols.



Fig. 32 Reaction of probe 38 with thiols.

absorption maximum at 426 nm, resulting in a perceived color change from dark orange to green (Fig. 32). Besides, the fluorescence maximum at 502 was enhanced *ca.* 107-fold ($\Phi_{\text{f}} = 0.3268$). A kinetic analysis, based on a single exponential decay model, showed that 38 reacts with Cys 13- and 21-fold faster than with Hcy and GSH, respectively. The optical changes were attributed to the addition of thiols to 38 and rupture of the conjugation of the coumarin ring system to the α,β -unsaturated carbonyl framework. Potential fluorescence changes in 38 were also examined using various biologically relevant analytes, but the authors found that no reaction occurred in the presence of other amino acids (Ala, Arg, Asn, Asp, Gln, Gluc, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Tau, Thr, Trp, Tyr, and Val), biologically common metal ions (K^+ , Ca^{2+} , Mg^{2+} , Na^+ , Zn^{2+} , Fe^{2+} , and Fe^{3+}), redox agents (H_2O_2 and NADH), and glucose.⁹¹ The probe was also used in bioimaging.

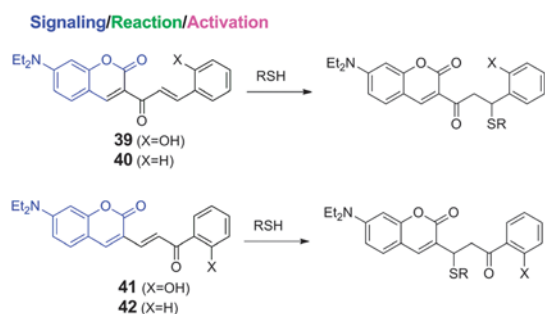


Fig. 33 Reaction of probes 39–42 with thiols.

The coumarinyl-enone-based derivatives 39–42 were designed as probes for thiols in DMSO:water (buffered with HEPES) solution (Fig. 33).⁹² The authors performed a kinetic study of the reactions of 39–42 with GSH. The study showed that the reaction rate of 39 was enhanced 2.8-fold compared with that of 40, and the rate of 41 was enhanced 26-fold compared with that of 42. This study showed the role played by the hydroxyl groups in the structure, and the effect of hydrogen-bond formation on the reaction rate. This is especially evident for 41, in which the oxyanion of 41 can clearly be stabilized by hydrogen-bonding interactions, resulting in a significant rate acceleration compared with that of 42. This effect was less evident for 39, for which it is structurally difficult to adopt a suitable conformation that favors hydrogen-bonding interactions, so the hydroxyl group had little effect on the reaction rate. In addition, 39 was fairly soluble in HEPES buffer (pH 7.4) and could be used for GSH detection in water. On addition of GSH, the fluorescence intensity of 39 at 500 nm ($\lambda_{\text{ex}} = 470$ nm) was significantly enhanced, and almost saturated at around 500 equiv. of GSH. The limit of detection for GSH was determined to be 0.14 mM. According to the Job's plot, the binding stoichiometry between 39 and GSH was 1:1. Other natural amino acids (Ala, Phe, Ser, Lys, and Asp) did not elicit photophysical changes in 39, although Cys did.

The coumarin aldehyde derivative 43 displayed an emission band at 557 nm with a fluorescence quantum yield of 0.12. In the presence of Cys, a marked decrease in the emission intensity at 557 nm and a concurrent enhancement of the emission intensity at 487 nm were observed (quantum yield 0.25). The emission ratio (I_{487}/I_{557}) changed from 0.054 in the absence of Cys to 6.24 in the presence of Cys. These changes occurred in parallel with a clear emission color change from green to blue. To investigate its selectivity, 43 was subjected to various potential analytes, namely amino acids (Arg, Cys, GSH, Hcy, Phe, Pro, Tyr, Val, Ala, Gly, Lys, Leu, Glu, and Ser), glucose, metal ions (Na^+ , K^+ , Ca^{2+} , and Mg^{2+}), a reactive oxygen species (H_2O_2), reducing agents (NADH, vitamin C), and proteins [bovine serum albumin (BSA), Hela cell total protein, and HepG2 cell total protein]. Significantly, the probe was highly specific for Cys over other biospecies, including Hcy and GSH. The sensing mechanism involves the reaction of Cys with the aldehyde group to afford the imine intermediate M1, which undergoes ring closure to form the intermediate M2.

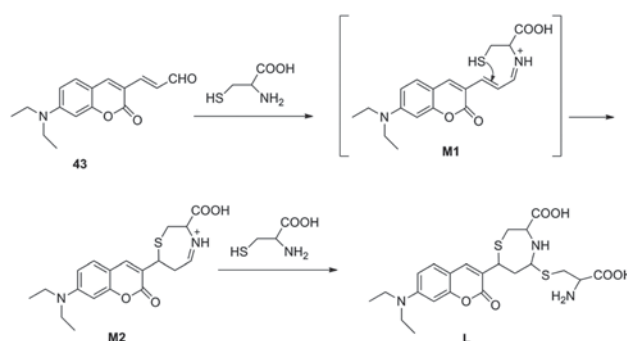


Fig. 34 Proposed sensing mechanism for Cys when using probe 43.

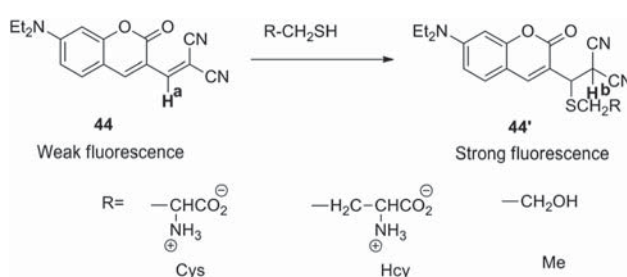


Fig. 35 Reaction of probe 44 with thiols to give 44'.

Finally, addition of another Cys molecule to the intermediate M2 provides the final 1:2 adduct (Fig. 34). Probe 43 was used for ratiometric imaging applications in MCF-7 cells.⁹³

Probe 44 has a coumarin group as a fluorophore and an α,β -unsaturated malonitrile moiety as the Michael acceptor, which is susceptible to attack by nucleophilic thiols (Fig. 35).⁹⁴ The malonitrile moiety serves not only as a tunable electrophile but also as a quencher of the coumarin fluorophore. On addition of a thiol to the double bond situated between the fluorophore and the quencher, the malonitrile group is no longer effective as a fluorescence quencher, resulting in emission enhancement of the system. In fact, both the UV-vis and fluorescence spectra changed significantly when probe 44 was treated with ME in DMSO:water (1:2 v/v, HEPES, pH 7.4), which was used as a model biorthiol derivative. In particular, addition of ME to 44 resulted in the disappearance of the absorbance at 524 nm, and growth of a new band at 406 nm. The noticeable hypsochromic shift indicated that the conjugation between the coumarin and the malonitrile was broken as a result of Michael addition of ME to 44. The rate constant under pseudo-first-order reaction conditions was calculated to be $k_{\text{obs}} = 4.03 \times 10^{-4} \text{ s}^{-1}$ ($\tau = 28.6$ min). Motivated by the model reaction, the authors screened the selectivity of 44 for amino acids. The authors found that Cys showed a large increase (19-fold) in the fluorescence intensity at 475 nm ($\lambda_{\text{ex}} = 394$ nm). Hcy and GSH behaved in a similar way and enhanced the fluorescence intensity of 44 12- and 5.6-fold, respectively. The prominent fluorescence changes in 44 were observable by the naked eye. Other natural amino acids did not induce any fluorescence changes in 44. Probe 44 was also used for cellular studies. The Michael reaction was also observable by ^1H NMR

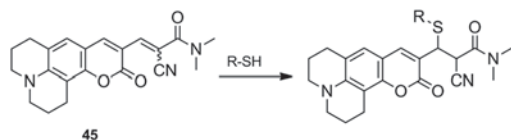


Fig. 36 Reactions of the cyanoacrylic electrophilic group in **45** with thiols.

spectroscopy. Upon addition of β -mercaptoethanol (ME), a vinyl proton (H^a) of **44** around δ 8.6 ppm dramatically disappears with the concomitant appearance of a new peak (H^b) at 5.45 ppm. In contrast, the slight chemical shifts of aromatic protons indicate that the reaction occurs at the peripheral rather than at the aromatic regions of **44**.

The coumarin derivative **45**, with a conjugated cyanoacrylamide group, was used to detect thiols (Fig. 36).⁹⁵ Probe **45** showed an absorption at 500 nm, and it was poorly fluorescent, displaying an emission band at 585 nm with a quantum yield of 0.0095 ($\lambda_{\text{ex}} = 520$ nm). Treating compound **45** with increasing concentrations of 2-sulfanylethanol (β -SE) caused a gradual reduction in the absorption band at 500 nm and the appearance of a new band at 425 nm. The addition of β -SE to compound **45** also induced significant fluorescence changes, *i.e.*, the band at 585 nm decreased in intensity and a new band at 510 nm grew, giving a final quantum yield of 0.28. Similar spectral changes were observed in the presence of Cys and GSH. When **45** was treated with ethanolamine, cystamine, or GSSG (up to 100 mM) at neutral pH, no optical changes were observed. The color and emission changes originated from addition of the thiol derivatives to the double bond in the conjugated cyanoacrylamide group.

A benzoxazine-hemicyanine based probe for the colorimetric and ratiometric detection of biothiols was developed by Sun's group.⁹⁶ Fig. 37 shows the changes in the absorption spectra when Cys was added to a CH_3CN -Tris-HCl buffered solution (50 mM, pH 7.4, 1:4 v/v) of probe **46** (10 μM). The absorption peak at 600 nm gradually decreased and a new peak appeared at 457 nm when the concentration of Cys was increased (0–1120 equiv.). A blue-shift (143 nm) was observed and the color instantly switched from blue to yellow (Fig. 37, inset) with a well-defined isosbestic point at 496 nm. The absorbance ratios at 457 nm and 600 nm were linearly related to the Cys concentration between 0–4.8 mM (0–480 equiv.). The changes in fluorescence emission spectra of probe **46** in the absence and presence of Cys are displayed in Fig. 37. Probe **46** (10 μM) exhibits a red emission peak at 679 nm in CH_3CN -Tris-HCl buffer with a fluorescence quantum yield of 0.031. After the treatment of 1120 equiv. of Cys, the solution exhibited a significant hypochromatic shift (109 nm) and a fluorescence

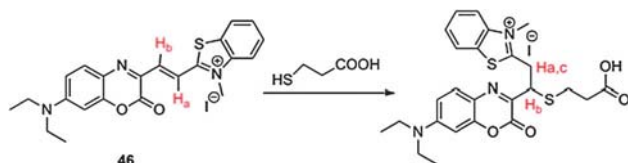


Fig. 37 Reactions of **46** with Cys.

enhancement factor of approximately 7-fold at 570 nm, and the fluorescence quantum yield increased to 0.435. The selectivity of probe **46** toward biothiol over other competitive species was also tested. The presence of Gly, Pro, Val, Ala, Arg, His, Ser, Leu, Thr, Lys, Glu, Phe in CH_3CN -Tris-HCl buffered solutions of **46** (50 mM, pH = 7.4, 1:4, v/v) did not indicate any obvious absorption and fluorescence changes. Under the identical test conditions, a similar response to that found for Cys was observed for other thiol-containing compounds (Hcy and GSH). The probe exhibited much lower reactivity toward GSH. The relative reactivity of the probe can be rationalized on the basis of the steric-hindrance effects on the thiol addition reaction. To explore the detection mechanism, 3-mercaptopropionic acid (MPA) was selected as thiol species for the mechanism study. The reaction between **46** and MPA was monitored by $^1\text{H NMR}$. Upon addition of 8.0 equiv. of MPA to $\text{DMSO}-d_6$ solutions of **46**, the vinylic protons in the $^1\text{H NMR}$ (H_a and H_b at δ 7.98, 8.11 ppm) of **46** disappeared and new peaks around 4.23 and 4.82 ppm in the $^1\text{H NMR}$ spectrum appeared, which indicates the reaction of MPA with the double bond. Mass spectral analysis of the resulting mixture showed corroborative evidence for the presence of **46**-MPA *via* the presence of a peak at 498.1500 m/z (calcd 498.1521 for $[\text{46}^+-\text{MPA}]^+$). The nucleophilic attack of thiol on the double bond between the two main molecular moieties would interrupt the π,π -conjugation, which would contribute to the visual color change of the solution from blue to yellow and the blue shift in the emission spectra, respectively. KB cell experiments suggested that probe **46** has a ratiometric response to biothiols in living cells.

A ferrocenyl enone (**47**) was used as a Michael acceptor for the development of a probe for Cys (Fig. 38).⁹⁷ Upon addition of Cys to **47** in DMSO :water (9:1 v/v, HEPES, pH 7.4), the absorption maxima at 375 nm and 490 nm decreased with an apparent rate constant of $k = 4.7 \times 10^{-4} \text{ s}^{-1}$, and a new band appeared at 472 nm. The carbonyl group in **47** was activated by the intramolecular hydrogen-bond of an *o*-hydroxyl group, which induced fast Michael addition of thiol groups. The selectivity of **47** for natural amino acids was screened; the relative absorbance ratio (A_{472}/A_{490}) of **47** was significantly changed only by Cys, and other amino acids possessing structurally similar, neutral, acidic or basic side chains did not cause any significant optical changes in **47**.

Some examples involved the use of maleimide groups as reactive sites. Maleimides are known to react fairly selectively with thiols *via* addition reactions involving their carbon-carbon double bond.

A new HPLC method for determining biological thiols has been developed using derivatization by ThioGlo™ maleimide

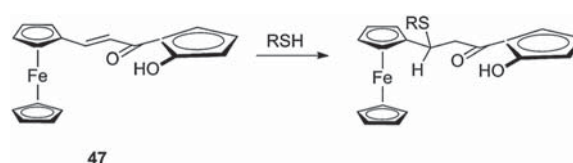


Fig. 38 Reaction of probe **47** with thiols.

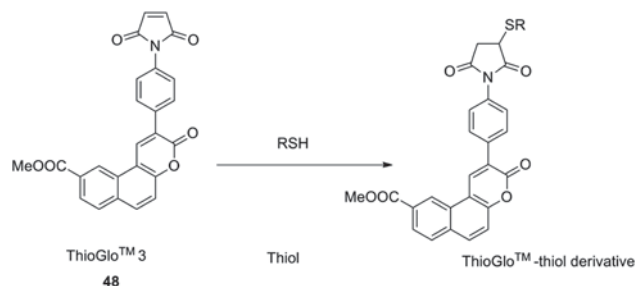


Fig. 39 Reaction of probe 48 with thiols.

(9-acetoxy-2-[4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)phenyl]-3-oxo-3H-naphtho[2,1-b]pyran) (**48**), using a mobile phase of 30% water and 70% acetonitrile, with 1 mL L⁻¹ acetic acid and 1 mL L⁻¹ phosphoric acid (Fig. 39).⁹⁸ Compound **48** reacted selectively and rapidly with thiols to yield fluorescent adducts that can be detected fluorometrically ($\lambda_{\text{ex}} = 365$ nm, $\lambda_{\text{em}} = 445$ nm). The within-run coefficient of variation for GSH ranged from 1.08 to 2.94%. GSH showed a linear range in the 2.5–1250 nM range, with a detection limit of 50 fmol. GSSG and Cys showed similar responses. The within-run precisions for GSSG and Cys were 0.85–7.29% and 0.58–4.13%, respectively. The between-run precisions were 3.02–7.64% for GSSG and 2.07–7.55% for Cys. GSSG and Cys had linear ranges of 2.5–1250 nM and 5.0–1250 nM, respectively. Probe **48** was also used to detect 2-mercaptoethanesulfonate fluorometrically, at an excitation wavelength of 365 nm and an emission wavelength of 445 nm, by reverse-phase HPLC using acetonitrile:water (75:25 v/v) with acetic acid and phosphoric acid (1 mL L⁻¹ each) as the mobile phase.⁹⁹

The chromenoquinoline-based receptor **49** was used as a fluorescence off-on thiol probe.¹⁰⁰ Probe **49** displayed an absorption band centered at 384 nm in HEPES buffer (pH = 7.4, 1.0% DMSO). Upon excitation at 384 nm, the probe displayed a poor emission band at 457 nm ($\Phi_{\text{F}} = 0.0035$). The fluorescence intensity enhancement on addition of Cys to probe **49** was approximately 223-fold. Similarly, 156- and 185-fold enhancements in the emission intensities were observed on addition of GSH and Hcy, respectively. A clear strong blue fluorescence color was observed. To evaluate the selective response of probe **49**, different amino acids were tested (Ala, Phe, Lys, Arg, His, Met, Tyr, Ser) but **49** displayed only small fluorescence enhancements. The sensing mechanism is related to Michael addition of the thiol derivatives to the maleimide group in **49** (Fig. 40). Cell permeability and live-cell imaging of thiols were also demonstrated by the authors.

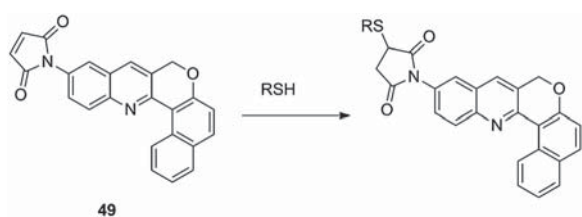


Fig. 40 Reaction of probe 49 with thiols.

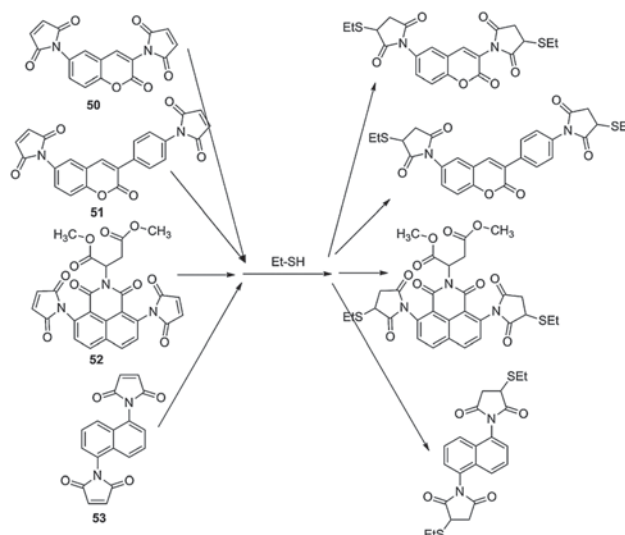


Fig. 41 Reaction of probes 50–53 with thiols.

Fluorophores **50**, **52**, **52**, and **53**¹⁰¹ contain two maleimide groups attached directly to a fluorophore core. These probes were characterized by their ability to undergo addition reactions with thiols. The addition of 3 mM ethanethiol to 1 mM solutions of **50**, **51**, or **52** in DMSO or 9:1 water–DMSO led to a marked increase in fluorescence. The increases in fluorescence intensity were studied for compounds **50** and **51**, and found to be directly proportional to the number of equivalents of thiol added, until a plateau was reached at *ca.* 2.2 equiv. of thiol. Similarly, fluorophore **53** reacted with ethanethiol in DMSO to form a dithiolated adduct that was more fluorescent than **53** by a factor of 20. However, **53** proved to be insoluble in water, even on addition of up to 10% DMSO, making it unsuitable for practical applications. The fluorescence increase mechanism is based on thiol–maleimide addition (Fig. 41). The authors used probes **50** and **51** for the fluorescence labeling of specific proteins.

Dimaleimide probes **54** and **55** were used as probes for thiols (Fig. 42).¹⁰² Probe **54** showed a weak fluorescence at 511 nm ($\lambda_{\text{ex}} = 365$ nm, $\Phi_{\text{F}} = 0.04$). Upon addition of MPA, the fluorescence increased to give a final quantum yield of 0.22. Similarly, on excitation at 365 nm, **55** displayed a poor emission band at 525 nm ($\Phi_{\text{F}} = 0.03$). Again, the presence of MPA induced significant emission enhancement to give a final product with a

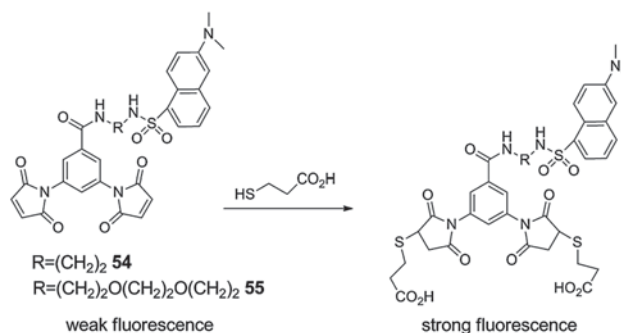


Fig. 42 Reaction of probes 54 and 55 with 3-mercaptopropanoic acid.

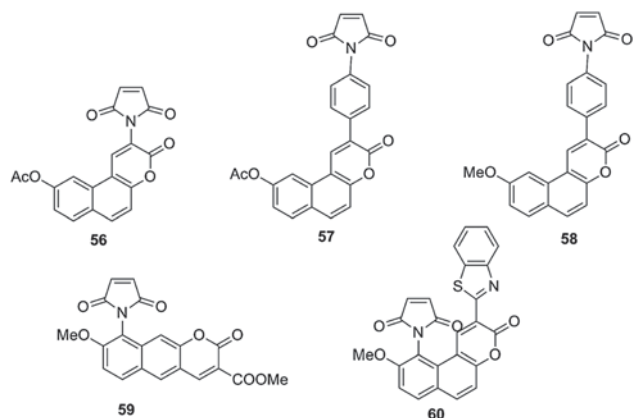


Fig. 43 The structures of thiol probes.

quantum yield of 0.11. These results are based on the reaction of MPA with the two maleimide groups in the probes. The probes were also tested in fluorescence protein labeling.

Probes 56–60 showed emission maxima at 430, 446, 501, 536, and 516 nm ($\lambda_{\text{ex}} = 365$ nm), respectively, and their corresponding fluorescence quantum yields were 0.023, 0.003, 0.061, 0.020, and 0.012, respectively. The authors found that these maleimide derivatives, with low quantum yields, became strongly fluorescent on reaction with thiols (Fig. 43). For instance, the reactions of 56–60 with GSH resulted in final products with quantum yields of 0.18, 0.48, 0.62, 0.12, and 0.66, respectively. These probes also reacted with simple thiols such as *N*-acetyl-L-cysteine and ME. The authors found that the reaction was instantaneous at physiological pH, without interference from aliphatic amines and non-thiol amino acids or proteins. Probes 57 and 58 were used to selectively detect GSH in viable Chinese hamster V79 cells.¹⁰³

N-[4-(7-diethylamino-4-methylcoumarin-3-yl)phenyl]maleimide (61) was also used to determine thiols selectively *via* addition to the maleimide group in water at pH 6.0 (Fig. 44). Compound 61 was used to stain protein-containing thiol groups.^{104,105}

Probes 62–64 containing the maleimide group were used as probes for thiol detection (Fig. 45).¹⁰⁶ In the absence of thiols, effective PET takes place in derivatives 62–64, and therefore they were poorly fluorescent. Michael addition of thiols to the electron-deficient alkene groups in 62–64 gave rise to emission enhancement ($\lambda_{\text{ex}} = 443$ nm, $\lambda_{\text{em}} = 525$ nm). The authors found that ME, *n*-butanethiol, and Cys induced large fluorescence enhancements in aqueous methanol solution (1 : 1 v/v, pH 7.2). No changes were observed in the absorption spectra of 62–64 on addition of thiols. The above examples are Michael addition reactions for the fluorescent probing of thiols.

A series of probes (65–68) bearing maleimide groups were synthesized by Corrie.¹⁰⁷ The probes reported in this work

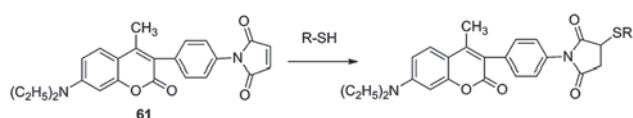


Fig. 44 Reaction of probe with tested biothiols (RSH).

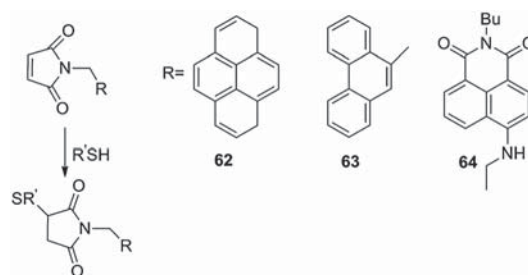


Fig. 45 Reaction of probes 62–64 with thiols.

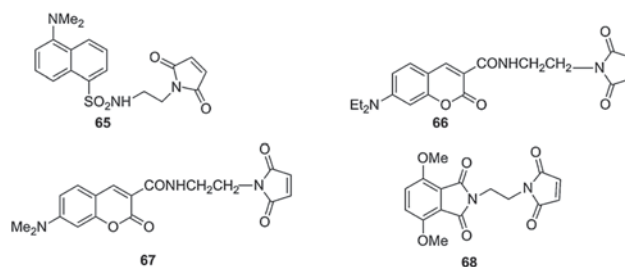


Fig. 46 Thiol probes 65–68 based on maleimide.

showed significant fluorescence enhancements (*e.g.*, 31-fold for 65, five-fold for 66 and 67, and seven-fold for 68) after addition of 2-sulfanylethanesulfonate to the maleimide double bond in ethanolic aqueous solution at pH 7.0 (phosphate buffer) (Fig. 46).

The fluorescence of probe 69 was strongly quenched by PET from the 4,4-difluoro-4-borata-3*a*-azonia-4*a*-aza-*s*-indacene (BODIPY) core to the maleimide moiety in a buffered aqueous solution (pH 7.4). However, in the presence of thiols, the fluorescence of BODIPY in 69 was restored, affording a 350-fold emission intensity increase (Fig. 47).¹⁰⁸ This probe

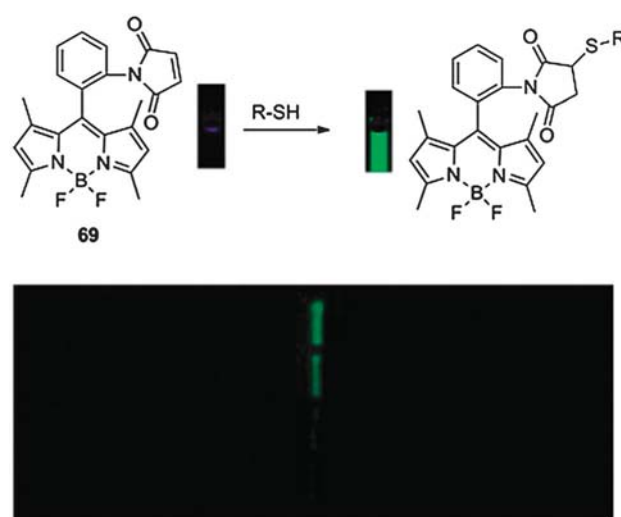


Fig. 47 Reaction of probe 69 with thiols (above) and fluorescence image of protein bands in SDS-PAGE gel, obtained with a FluorImager595 (Amersham), with excitation at 488 nm (bottom). Reprinted with permission from *Org. Lett.*, 2007, 9, 3375–3377. Copyright 2007, American Chemical Society.

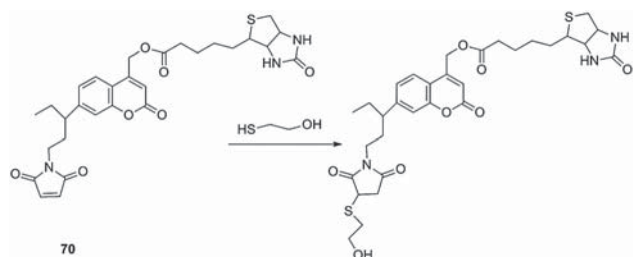


Fig. 48 Reaction of probe 70 with tested biothiols (RSH).

was also confirmed to be useful for detecting extremely low concentrations of proteins in gels after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The 480 nm ($\lambda_{\text{ex}} = 390$ nm) fluorescence intensity of probe 70 exhibited a 43-fold enhancement after 150 s by reaction with thiols in 6 : 4 methanol : water (Tris, pH 7.4). The fluorescence quantum yield recovered from $\Phi_f = 0.01$ for 70 to $\Phi_f = 0.43$ for the final product. The low fluorescence of the probe was a result of the maleimide ring effectively quenching the excited state of the coumarin dye by PET. As with other thiol-activable fluorescence probes, coumarin dyes are turned “off” by maleimides and “on” by thiols as a result of conversion of the maleimide to a succinimide *via* Michael addition (Fig. 48). To examine the selectivity offered by 70, the probe was treated with various biologically relevant compounds, namely thiol-containing amino acids (Cys, Hcy, and GSH), non-thiol-bearing amino acids (Glu, Phe, Ala, and Lys), glucose, reactive oxygen species (H_2O_2), a reducing agent (ascorbate), metal ions (Mg^{2+} , Fe^{3+} , Zn^{2+} , and Ca^{2+}), non-thiol-bearing proteins (transferrin, ovalbumin, lysozyme, and trypsin inhibitor), a thiol-bearing protein (BSA), a thiol-disabled protein (BSA + *N*-ethylmaleimide), and a protein containing reduced disulfide bonds [lysozyme + tris(2-carboxyethyl)phosphine hydrochloride]. The results revealed that both molecular and macromolecular thiols could enhance the emission of 70, whereas the other biologically relevant species could not.¹⁰⁹

A Tb(III)-cyclen maleimide was used to detect GSH (Fig. 49).¹¹⁰ The probe 71, containing a maleimide moiety, as a sulfhydryl acceptor, was poorly emitting in aqueous solution (pH 7) in the absence of a thiol. However, upon addition of thiols such as glutathione (GSH), large enhancements were observed at 545 nm, particularly within the physiological pH range. The reaction mixture was then analyzed using ESMS without carrying out any workup on the crude reaction mixture. The results confirmed that the probe was converted successfully under these experimental conditions to the desired probe-GSH

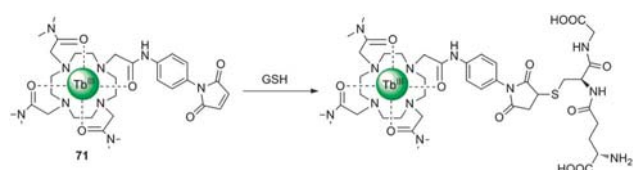


Fig. 49 Reaction of probe 71 with GSH.

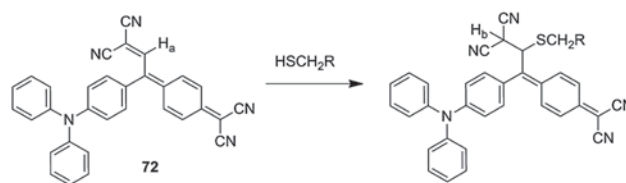


Fig. 50 Reaction of probe 72 with thiols.

product, where the ESMS (+mode) showed the presence of a peak at $m/z = 1119.38$, corresponding to a species with chemical formula [probe-GSH- $3\text{CF}_3\text{SO}_3\text{-}2\text{H}^+$]. The selectivity of probe 70 was studied. As expected, the Tb(III) emission of 70 (10 μM) was found to be modulated in the presence of the thiol based amino acids Cys and Hcy. However, no significant changes were observed in the Tb(III) emission using other naturally occurring amino acids (40 μM) including Ala, Asp, His, Arg, Phe, Ser, Val, Ile, GSSG, Pro, Thr, Tyr, Gly, Leu, Lys, Met, Glu, Sar and Asn. The probe can be used to monitor the enzymatic reduction of GSSG to GSH in real time.

The 7,7,8,8-tetracyanoquinodimethane-based triphenylamine derivative 72, which displays a strong ICT character, was designed as a colorimetric chemosensor for thiols (Fig. 50).¹¹¹ The recognition of Cys by 72 was investigated in acetonitrile : water solutions (1 : 1 v/v, HEPES, pH 7.4). The absorption spectrum of 72 showed an intense ICT band at 426 nm, with a second ICT absorption of lower energy appearing at 710 nm. Upon addition of Cys, the intensities of the two bands decreased gradually, and a band centered at 300 nm increased in intensity, with a slight red-shift. Meanwhile, a new absorption band at 610 nm emerged. A calibration graph, obtained by plotting the absorption intensities at 426 nm as a function of the Cys concentration, showed an excellent linear relationship. The thiol-containing derivatives Hcy and GSH gave rise to similar variations. However, the introduction of other natural amino acids such as Ala, Arg, Asp, Glu, Gly, Leu, iso-Leu, Lys, Met, Ser, Thr, Trp, Tyr, and Val did not cause any significant changes in the absorption spectra.

The iridium(III)-containing phosphorescent chemosensor $\text{Ir}(\text{ppy})_2(\text{L})(\text{PF}_6)$ (73; ppy = 2-phenylpyridine), containing a 2,2'-bipyridyl ligand functionalized with an α,β -unsaturated ketone (L), was used as a probe for the selective detection of thiols (Fig. 51).¹¹² The complex 73 in DMF : water (4 : 1 v/v, HEPES, pH 7.2) exhibited absorption bands at 280–320 nm and a broad band at 450 nm. Upon addition of Cys to solutions of 73, the

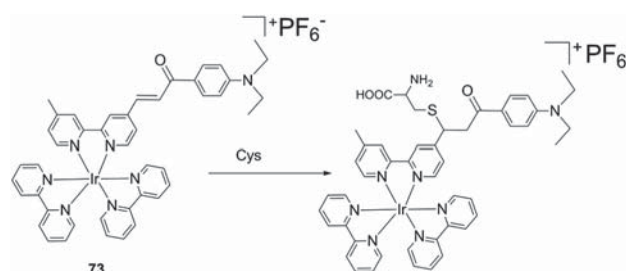


Fig. 51 Reaction of 73 with Cys.

absorption at 450 nm decreased gradually with concomitant growth of a band at 350 nm. A change in color from yellow to colorless was observed. A similar trend was also found on addition of Hcy to **73**, but in this case the band at 450 nm did not completely disappear, even when an excess of Hcy was added. The significant blue-shift of the lowest-energy absorption for **73** results from 1,2-addition of Cys or Hcy to the α,β -unsaturated ketone in **73**, leading to formation of a thioether. As stated above, a weak band still remained at 450 nm on titration of **73** with Hcy, probably as a result of the lower reactivity of Hcy, compared with that of Cys, with the α,β -unsaturated ketone. The response of complex **73** to thiols was also investigated using emission titrations. Probe **73** was weakly emissive at 587 nm. However, on addition of a thiol, the emission intensity gradually increased. For instance, the luminescence intensity of complex **73** showed a 20-fold enhancement when 80 equiv. of Cys were added. The luminescence intensity at 587 nm displayed a good linear relationship with the Cys concentration in the range $0\text{--}10^{-4}$ M. For Hcy, the maximum enhancement of the luminescence intensity was 14-fold, and the luminescence intensity at 587 nm also showed a good linear relationship with the Hcy concentration in the range 0 to 8×10^{-4} M. Probe **73** was also tested in the presence of Gly, Ala, Ser, Thr, Val, Leu, Ile, Met, Phe, Trp, Tyr, Asp, Asp, Glu, Gln, Lys, Arg, His, and Pro, but no optical changes were observed.

The fluorescein derivatives **74** and **75** were synthesized by Chen's group and used as thiol-selective probes (Fig. 52).¹¹³ After incubation with Cys for 10 min, probe **75** displayed significant fluorescence enhancement at 515 nm and the appearance of a new absorption band at 490 nm, in ethanol:water (2:8 v/v, phosphate buffer, pH 7.4), whereas amino acids without thiol groups (Phe, Ser, Glu, Arg, Ala, His, Lys, Gln, Gly, Tyr, and Met) induced negligible optical changes in **74** under similar conditions. Interestingly, the addition of Hcy or GSH did not show significant emission enhancement and colorimetric changes like those induced by Cys. In contrast, a solution containing **75**, bearing a single acrylate group, did not show any great selectivity for Cys, and in this case Hcy and GSH displayed similar changes in the emission

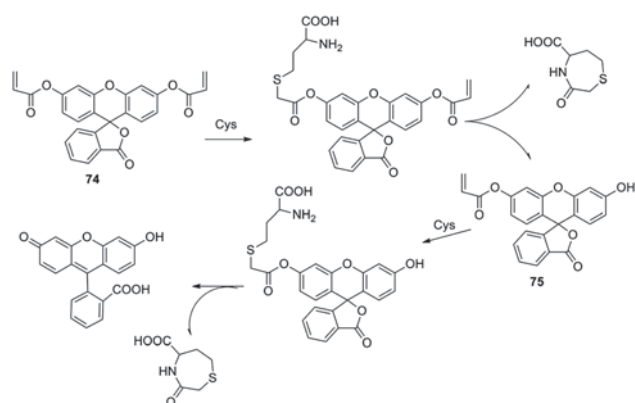


Fig. 52 Reaction of **74** and **75** with thiols.

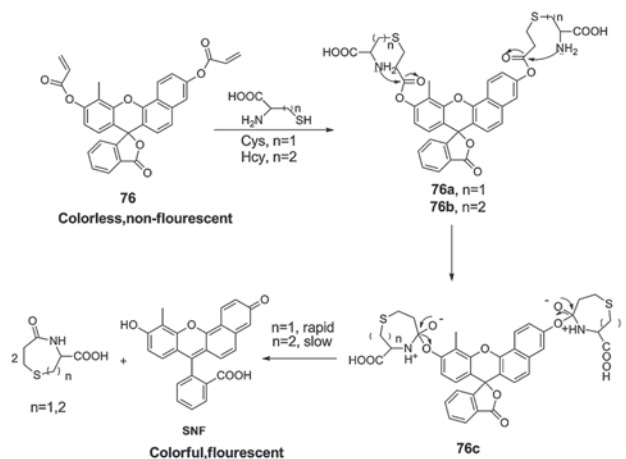


Fig. 53 Proposed mechanism of the discrimination between Cys and Hcy when using probe **76**.

and absorption spectra. The sensing mechanism is based on the addition of thiol to the acryloyl group, followed by cleavage of the ester bond to form fluorescein. Probe **74** was used in bioimaging studies. Compared to **75**, the higher selectivity of **74** to Cys over Hcy and GSH should be attributed to the dual addition-cleavage processes in the reaction between **74** and Cys.

The semi-naphthofluorescein bisacrylate **76** was synthesized for Cys recognition (Fig. 53).¹¹⁴ Probe **76** was non-fluorescent ($\lambda_{\text{ex}} = 550$ nm) in 1.0 mM cetyltrimethylammonium bromide buffered at pH 7.4, but its emission intensity at 621 nm increased with increasing Cys concentration. The observed emission was nearly proportional to the Cys concentration up to $10 \mu\text{M}$. Cys concentrations as low as $0.2 \mu\text{M}$ were readily detected. Some kinetic studies were also performed. The authors observed that the emission upon reaction with Cys increased with time and reached a plateau at 20 min. In contrast, no significant fluorescence growth was observed for Hcy and GSH in this time period. When the reaction time was prolonged, fluorescence increased for Hcy but not for GSH. To further evaluate the selectivity of **76** for Cys, changes in the fluorescence intensity of **76** caused by other analytes were also measured. It was observed that other amino acids and biothiols promote almost no emission changes under the same experimental conditions, demonstrating the high selectivity of **76** for Cys over other analytes. The mechanism was explained as follows. On mixing Cys with **76**, the conjugate addition product **76a** was formed, which underwent a rapid cyclization reaction to produce **76c**, with release of free SNF. In the case of GSH, 1,2-addition of thiols to the α,β -unsaturated carbonyl moieties of **76** can occur. However, an ensuing intramolecular cyclization similar to that with Cys cannot proceed because the free amine does not attack the ester moiety in the **76**-GSH adduct, apparently because of the entropic considerations involved in large-ring formation. Compound **76** was used for quantitative measurement of Cys in a human plasma sample.

A highly selective ratiometric near-IR (NIR) cyanine-based probe (**77**) for Cys over Hcy and GSH was developed (Fig. 54).¹¹⁵ On addition of Cys to **77**, the absorption at 775 nm decreased

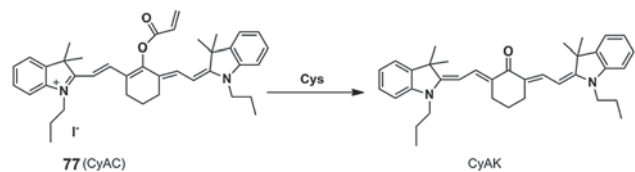


Fig. 54 Reaction of probe 77 with Cys.

sharply and a new band at 515 nm appeared (color change from light blue to red). The authors confirmed the transformation from 77 to CyAK on reaction with Cys. The sensing mechanism involves the addition of Cys to the acrylic unit to generate the corresponding thioether, and a further intramolecular cyclization to yield CyAK. The fluorescence response of 77 was also studied in the presence of other amino acids, but no responses were observed. Remarkably, the significant responses of 77 to Cys were not observed for Hcy and GSH. This difference can be attributed to very different kinetic rates of the intramolecular cyclization reactions. The intramolecular cyclization reaction to form the seven-membered ring with Cys should be kinetically favored compared with the eight-membered ring that must be formed for Hcy. In the case of GSH, only the conjugated thioether was generated, since the bulkiness of its tripeptide significantly hindered the intramolecular cyclization. The probe was used in bioimaging studies.

Oxanorbornadienedicarboxylates (ONDs) are electrophiles that have proved to be readily available compounds with excellent water stability, while retaining rapid and selective reactivity towards thiol groups in small molecules, peptides, and proteins. Based on this concept, probes 78–81 were designed (Fig. 55). The probes also contain a dansyl fluorescence moiety as a signaling subunit. These derivatives are poorly fluorescent, but the dansyl emission was restored on thiol-addition to the OND fragment. For instance, 78 showed an emission band at 550 nm ($\lambda_{\text{ex}} = 332$ nm) in phosphate-buffered aqueous solutions (pH = 7, 1% DMSO). Incubation with Cys and GSH gave rise to an immediate and strong increase in the emission, whereas Lys and His induced a very small (<2%) increase in the fluorescence, and other amino acids, as well as the disulfide cystine, induced negligible changes. GSH and Cys were also found to react with 78–81 at high rates (second-order rate constants in the range 40–200 $\text{M}^{-1} \text{s}^{-1}$) to give adducts that exhibit enhancements of fluorescence intensity up to 180-fold.¹¹⁶

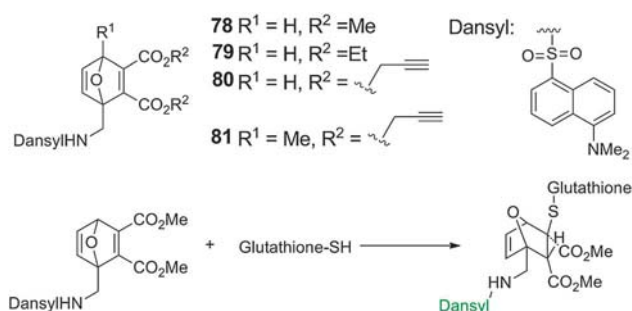


Fig. 55 Reaction of probes 78–81 with GSH.

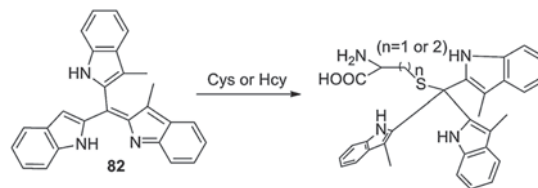


Fig. 56 Reaction of probe 82 with thiols.

The tris(indolyl)methene receptor 82 showed a strong and broad absorption band in the 400–700 nm range in acetonitrile:water (1:1 v/v) solutions at neutral pH (Fig. 56). In the presence of 200 equiv. of Cys or Hcy, the absorption band almost disappeared and a new absorption centered at 286 nm appeared (color change from violet to colorless). In contrast, no obvious color changes occurred in the presence of Gly, Ala, Val, Leu, Ile, Pro, Phe, Tyr, Trp, Ser, Thr, Met, Asp, Glu, Lys, Arg, Cys, Hcy, His, and GSH. Based on this behavior, the authors carried out quantitative determinations of Cys and Hcy, using flow injection analysis. An acetonitrile:water (1:1 v/v) solution of receptor 82 at neutral pH, buffered with HEPES–NaOH was used as the reagent stream. The results obtained for Cys and Hcy indicated that the method was simple, rapid, and highly sensitive. The procedure also showed a wide linear range (2.0×10^{-5} to 2.0×10^{-3} M for Cys and 1.4×10^{-5} to 3.0×10^{-3} M for Hcy) and good precision (relative standard deviation = 1.4% for Cys and 0.9% for Hcy). The limits of detection were 2.0×10^{-5} M and 1.4×10^{-5} M for Cys and Hcy, respectively.¹¹⁷

The α,β -unsaturated acyl sulfonamide 83 was used as a probe to detect thiols (Fig. 57).¹¹⁸ The probe displayed almost no fluorescence in PBS buffer (pH = 7.4); however, on addition of Cys, a gradual increase in the emission at 520 nm ($\lambda_{\text{ex}} = 350$ nm) was observed. An approximately 30-fold fluorescence enhancement was detected when the reaction was complete. Subsequent data analysis revealed an excellent linear relationship between the normalized fluorescence signal at 520 nm and the Cys concentration. The detection limit for Cys was determined to be 1.67×10^{-6} M. The probe was also incubated with 20 natural amino acids. Significant increases in fluorescence intensity were only observed with Cys, GSH, and Hcy, in the order Cys > GSH > Hcy. The fluorescence increases were attributed to formation of the corresponding thiol adducts *via* Michael addition to the carbon–carbon double bond in 83. The probe was used to detect thiols in living human lung adenocarcinoma (A549) cells.

The simple derivative ethyl propiolate (84) was proposed as a suitable probe for detecting thiols (Fig. 58).¹¹⁹ The probe has no

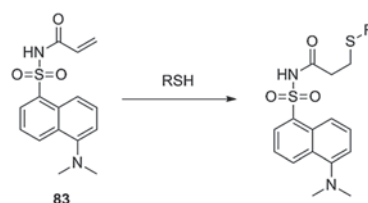
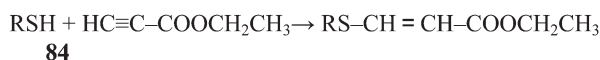


Fig. 57 Reaction of probe 83 with thiols.

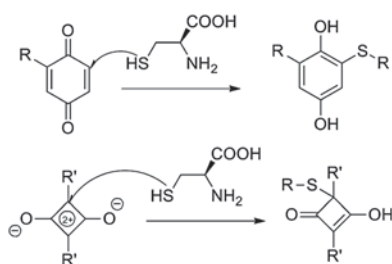
Fig. 58 Reaction of **84** with thiols.

absorption in the UV region; however, when **83** was cultivated with thiol-containing compounds, namely captopril (Cap) or Cys, for 5 min, prominent UV absorbance in the range 270–310 nm was observed. The reaction mechanism is based on nucleophilic attack of the thiol on the carbon atom of the triple bond, resulting in a stable alkylthioacrylate derivative that absorbs in the UV region. The authors additionally proved the selectivity of **84** towards thiols *via* a study of the interaction of **84** with other amino acids and disulfides (Gly and cystine were selected as model compounds), but no reactions were observed. The probe was used to detect the Cap contents of tablets.

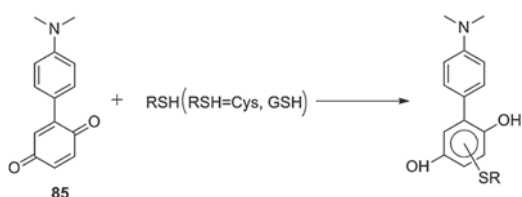
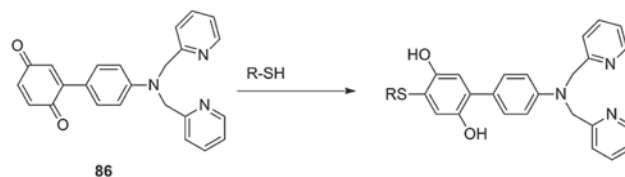
1,3-Addition reactions

1,3-Addition reactions have also been used for signaling the presence of thiols. In this section, the addition of thiols to quinone- and squaraine-like derivatives is reported (see Scheme 3).

Compound **85**, containing *N,N*-dimethylaniline and quinone units, was used as a colorimetric probe for certain thiols by Zhang *et al.* (Fig. 59).¹²⁰ Probe **85** shows a strong ICT band centered at 582 nm. After addition of Cys, the intensity of the ICT band started to decrease as a result of reaction of the thiol group with the quinone unit of **85**, resulting in transformation of the quinone into the corresponding hydroquinone. Similarly, the intensity of the ICT band of **85** decreased after the addition of GSH. The authors also found that compared with that with Cys, the intensity of the ICT band of **85** was reduced more slowly by reaction with GSH; this may be the result of steric hindrance associated with the latter.



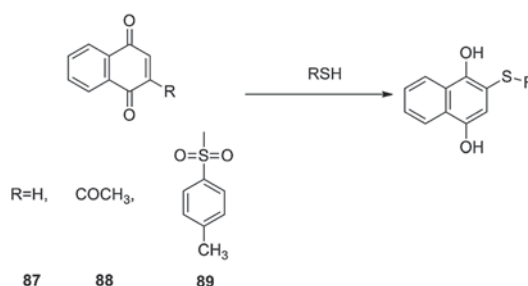
Scheme 3 1,3-Addition reaction of Cys to quinone- and squaraine-like derivatives.

Fig. 59 Reaction of **85** with thiols.Fig. 60 Reaction of **86** with thiols.

Compound **86**, containing *N,N*-bis(pyridin-2-ylmethyl)aniline and quinone units, shows a strong ICT absorption band at 532 nm in acetonitrile:water (1:1 v/v). Upon addition of increasing amounts of Cys, the ICT band started to decrease gradually. Compound **86** also displayed a similar response in the presence of GSH. To examine the selectivity of compound **86**, competitive experiments were also carried out, but the authors did not observe any optical changes in the presence of the other tested amino acids (Ala, Arg, Asp, His, Lys, Ser, and Tyr). The sensing mechanism involves the reaction of the thiol with the quinone unit of **86**, resulting in the formation of the corresponding hydroquinone (Fig. 60).¹²¹

The naphthoquinone derivatives **87–89** (Fig. 61) were used to detect thiols by cyclic voltammetry using screen-printed carbon electrodes. In these studies, the naphthoquinone group was subjected to a 1,3-Michael addition by the nucleophilic thiol, resulting in generation of the corresponding reduced conjugate.¹²² In particular, on addition of GSH, the electrode potentials of these derivatives change as a consequence of the nucleophilic addition of the thiol to the quinone indicator. The magnitude of the reduction peak decreased within increasing thiol concentration. The key analytical parameters underpinning the selective and sensitive determination of thiols were assessed by the authors, and the clinical efficacy of the approach was demonstrated through its application to the analysis of human plasma. The results were compared, and the ways in which the system can be adopted within mainstream biomedical environments were highlighted.¹²³

The quinone derivatives **90** and **91**, were also used for the electrochemical detection of certain thiols (Fig. 62).¹²⁴ The results additionally showed that the two compounds promoted a highly selective and sensitive response towards Cys. In the absence of Cys, a single reduction peak was observed for **90** at +0.02 V. On addition of Cys, a second reduction process was observed at +0.24 V, as an ill-defined shoulder on the main **90**

Fig. 61 Reaction of probes **87–89** with thiols.

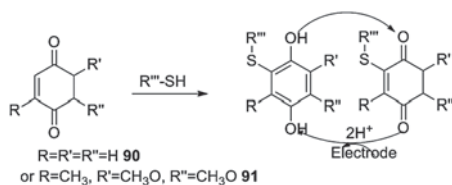


Fig. 62 Reaction of probes **90** and **91** with thiols.

reduction peak; this corresponds to the reduction of the **90**-Cys adduct. Further addition of Cys resulted in an increase in the magnitude of the adduct peak and the emergence of a third reduction process at -0.22 V. The latter is ascribed to the reduction of species that have undergone multiple additions. This has the effect of splitting the Cys signal among those from single- and multi-substituted species, and limits both the linearity and sensitivity of the protocol. Probe **91** possesses a single reaction site at the 6-position on the ring, and it was thought that by reducing the number of sites at which Cys could react, a clear, unambiguous signal could be obtained. An analogous set of experiments was conducted. In the absence of Cys, probe **91** showed a single reduction peak at -0.11 V. The introduction of Cys led to the emergence of a single, well-resolved peak at $+0.12$ V. This was found to increase linearly with Cys concentration in the range 5 – 47 μM , providing a limit of detection of 4 μM . The effects of other thiols (Hcy and GSH) were also studied, but no appreciable changes in the voltammetric profiles were found. The practical use of the approach was investigated by examining the response to Cys in urine.

Squaraines typically contain an electron-deficient central four-membered ring and two electron-donating groups arranged in a donor-acceptor-donor (D-A-D) pattern. The electron-deficient central cyclobutene ring of squaraine dyes is susceptible to nucleophilic attack; this breaks the conjugation of the dye, resulting in bleaching. Another interesting aspect in relation to squaraines is that they show a very intense band at long wavelengths, and are usually highly fluorescent even in aqueous environments.

The di-*N*-methylhydroxyethylaniline squaraine **92** was reported by Guang *et al.* as a sensitive colorimetric probe for Cys (Fig. 63). Probe **92** showed two absorption bands, at 289 and 646 nm, in aqueous PB (pH 7.5). The first weak absorption (289 nm) is attributed to the aniline moiety, and the second strong and sharp absorption (646 nm) arises from the presence of the π -conjugated system. Upon addition of Cys, the peak

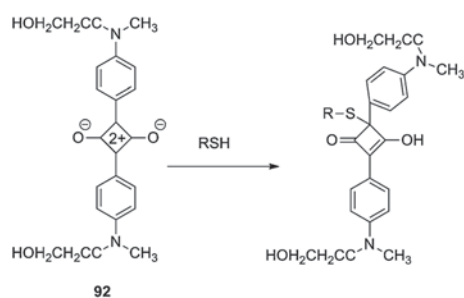


Fig. 63 Reaction of **92** with thiols.

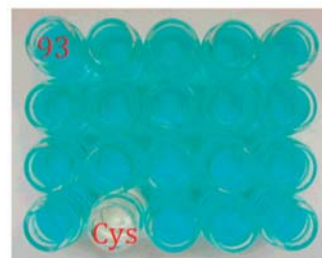
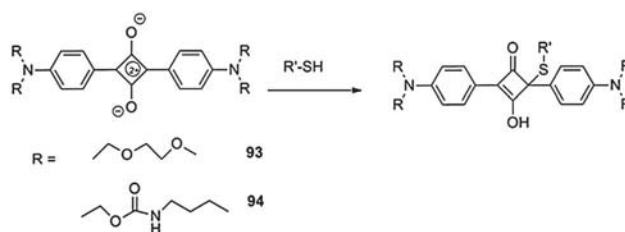


Fig. 64 Reaction of **93** and **94** with thiols and photograph showing the color change in acetonitrile-water, 20 : 80 v/v, at pH 6 (MES 0.01 mol dm^{-3}) of **93** (1.21×10^{-5} mol dm^{-3}) in the presence of 10 equiv. of certain amino acids. Reprinted with permission from *J. Am. Chem. Soc.*, 2004, **126**, 4064–4065. Copyright 2004, American Chemical Society.

intensity at 289 nm increased and that at 646 nm decreased. The authors tested the response of **92** in the presence of common metal ions and biological macromolecules without sulfhydryl groups, but found no effect on the optical characteristics of the probe. Moreover, the colorimetric responses of **92** to the 19 natural bio- α -amino acids were studied but no optical modulations were found except for Cys (see above). The sensing mechanism is related to the addition reaction of the nucleophilic thiol to the electron-deficient cyclobutene ring in **92**. To further evaluate the feasibility of using the probe, the authors used it to determine Cys in human serum.¹²⁵

The two water-soluble squaraine derivatives **93** and **94** were used to recognize Cys (Fig. 64).¹²⁶ Both **93** and **94** showed an absorption band at *ca.* 640 nm and had a quantum yield in acetonitrile : water [20 : 80 v/v, 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6] of *ca.* 0.1. The reactivities of the probes were studied with different natural α -amino acids (Phen, The, Arg, His, Asn, Leu, Ala, Pro, Val, Gly, Lys, Gln, Met, Ile, Ser, Cys, Try, Glu, and Asp). Both **93** and **94** gave selective bleaching in the presence of Cys. As in the above case, the sensing mechanism is based on addition of the nucleophilic thiol to the four-membered rings of squaraines **93** and **94**. The total Cys and Cys derivatives in human plasma were determined using these two probes by a simple colorimetric addition standard method, with good results.

A NIR colorimetric chemodosimeter based on a croconium dye (**95**) was developed by Cheng and co-workers for the selective and sensitive detection of certain thiols in ethanol : water (7 : 3 v/v, MES, pH 5.7).¹²⁷ Probe **95** displayed a sharp and intense absorption band in the NIR region, at 823 nm, which disappeared in the presence of Cys. A color change from light yellow to colorless was observed. Similar responses were found for Hcy and for Cys-Gly. In contrast, no discernible color change was found on addition of other amino acids. Additionally, ethanethiol, GSH, ME, and mercaptoacetic acid (10 equiv.)

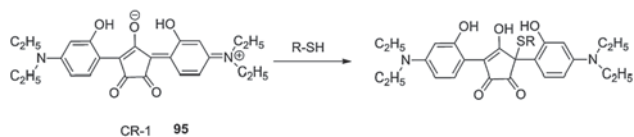


Fig. 65 Reaction of probe **95** with thiols.

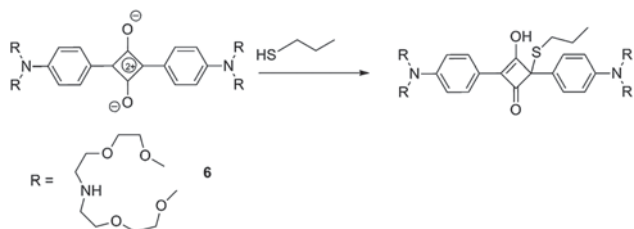


Fig. 66 Reaction of **96** with mercaptopropionic acid.

were unable to completely bleach solutions of **95**. The sensing mechanism involves nucleophilic attack of the thiols on the central croconic ring (Fig. 65).

The blue **96** has been reported to react with mercaptopropionic acid to give a colorless solution as a result of the addition of the thiol group to the electron-deficient central cyclobutene ring of the squaraine (Fig. 66). The product reacts with Hg^{2+} to give the corresponding squaraine derivative **96**, resulting in a highly selective chromo- and fluorogenic switching-on process indicative of the presence of Hg^{2+} ions.¹²⁸ Using a similar approach, a similar squaraine derivative was used to differentiate a series of metal ions and a series of thiols.¹²⁹ Probe **96** coupled with the use of mesoporous materials was also used to design solid supports for the removal and detection of Hg^{2+} (ref. 130) and for the detection of methylmercury.¹³¹

The new fluorimetric probe **97** was used to detect physiologically significant thiols.¹³² The addition of thiols to **97** in DMSO:water (3:7 v/v, Tris, pH 7.0) resulted in large increases in the emission at 595 nm ($\lambda_{\text{ex}} = 500$ nm). The authors observed this behavior for GSH, Hcy, and Cys. In contrast, the fluorescence intensity of the coumarin was not influenced by the presence of other thiols, even after incubation for 1200 s. The fluorescence signal was the result of a tandem reaction: thiol-addition to the quinone moiety in **97** and a quinone-methide-type rearrangement to release the fluorogenic coumarin (Fig. 67). The authors also demonstrated the use of **97** in the determination of thiol concentrations in human plasma.

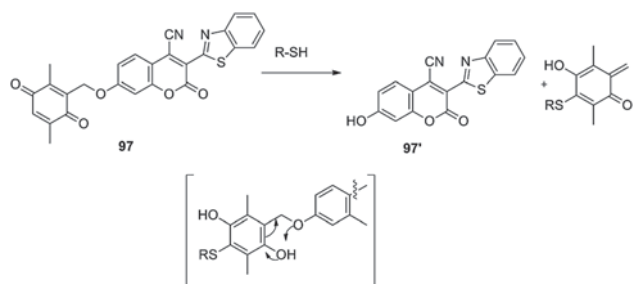
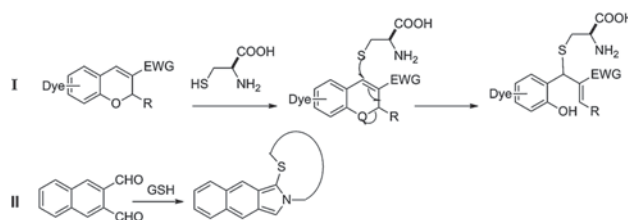


Fig. 67 Reaction of probe **97** with thiols.

1,4-Addition reactions

Reported examples of 1,4-addition reactions for the detection of thiols are based on the use of chromene cores containing electron-withdrawing groups (EWG) or 2,3-naphthalenedicarboxaldehyde. In the first case a ring opening occurs whereas in the second the thiol addition induced the formation of a pyrrole ring (see Scheme 4).

The chromene derivative **98** (see Fig. 68) proved to be a suitable probe for the detection of Cys, Hcy, and GSH in aqueous solutions buffered with HEPES (pH 7.0, 0.1% ethanol). For instance, on addition of Cys to **98**, the original absorption band at 292 nm gradually decreased, and a new band appeared at 405 nm (red-shifted by 113 nm and a color change from colorless to yellow).¹³³ The system exhibited higher selectivity for Cys than for other amino acids; Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val induced no color changes in **98**. The above facts speculated that this may be a result of the release of the 4-nitrophenolate group



Scheme 4 Mechanism of 1,4 addition.

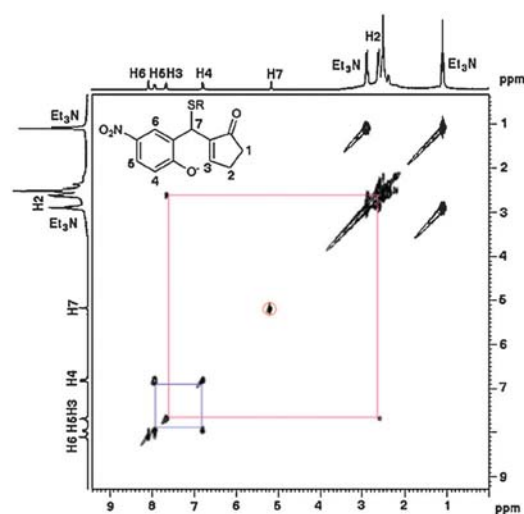
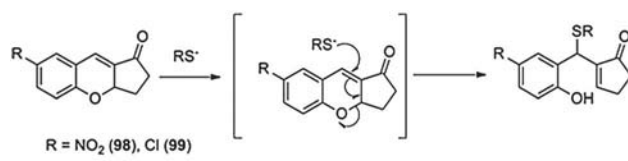


Fig. 68 Reaction of probes **98**, **99** with thiols and 2D ^1H - ^1H COSY (300 MHz) correlation experiment of the proposed **98**-SR in DMSO- d_6 . Reprinted with permission from *Org. Lett.*, 2010, **12**, 4756–4759. Copyright 2010, American Chemical Society.

(Fig. 68 top) through chromene ring-opening, based on a nucleophilic attack on the sulfhydryl group by probe **98** (Michael addition). The 2D COSY of **98**-SR correlation experiments provided direct evidence for the above statement and proposed mechanism. As shown in Fig. 68 bottom, the COSY spectrum suggested that this may be a result of the release of the 4-nitrophenolate group through chromene ring-opening, based on a nucleophilic attack on the sulfhydryl group by probe **98** (Michael addition). The COSY spectrum exhibits clear cross-peaks between H3 (=CH_{cyclopent-2-enone}) and H2 (the alkyl protons) indicated by a red square, with the blue square showing the interrelation of H4 and H5. The proton H7 (red circle) was isolated and did not relate to any other protons.

The derivative **99** was also used as a probe for thiols. In particular, addition of Cys to **99** in an aqueous solution buffered with Tris (pH 8.0) resulted in decreases in the absorptions at 250, 302, and 377 nm, and growth of a new band at 227 nm. Also, the emission band of **99** at 550 nm ($\lambda_{\text{ex}} = 380$ nm) decreased rapidly on addition of Cys. The authors found that GSH and Hcy caused similar responses to that of Cys, whereas other amino acids did not affect the optical properties of **99**. The observed behavior in the presence of thiols of **98** and **99** was related to thiol-addition to the double bond in the chromene unit and ring opening.¹³⁴

Another coumarin–chromene was employed to detect thiol by Yin *et al.*¹³⁵ Selectivity studies of the probe for thiol were carried out by absorbance and monitoring fluorescence changes that **100** undergoes upon the addition of various amino acids, including Cys, Hcy, GSH, Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val and cystine in HEPES (10 mM, pH 7.4). Probe **100** displays UV-Vis spectral changes and a fluorescence enhancement ($\lambda_{\text{ex}} = 365$ nm, $\lambda_{\text{em}} = 455$ nm) in the presence of the thiol-containing compounds Cys, Hcy and GSH. However, analytes without thiols induced no changes in UV-Vis spectra or in the fluorescence emission properties under the same conditions. It was also confirmed that for other thiols, such as mercaptopropionic acid (MPA) and mercaptoethanol (ME), probe **100** showed UV-Vis and fluorescence responses similar to Cys, Hcy and GSH. As a typical biological thiol, Cys was used to further examine the UV-Vis response of probe. When Cys was added to HEPES (10 mM, pH 7.4) solution containing **100** (35 μM), with increasing Cys concentration (0–1 equiv.), the absorption peak at 320 nm gradually decreased and a new peak appeared at 372 nm (red shifted 52 nm). A well-defined isosbestic point was noted at 350 nm, which may indicate the formation of single new species. When changes at 320 nm and 372 nm were monitored, the relative absorption values were linearly related to the Cys concentration between 0 and 35 μM , respectively. The changes in the fluorescence spectra of probe **100** ($\Phi_{\text{f}} = 0.1386$) (10 μM) in the absence or presence of Cys (0–10 μM) in HEPES buffer were also studied. It was observed that the addition of Cys caused changes in the emission spectrum; *i.e.* an enhancement of the emission intensity at 455 nm was observed ($\Phi_{\text{f}} = 0.2297$). It is interesting to note that

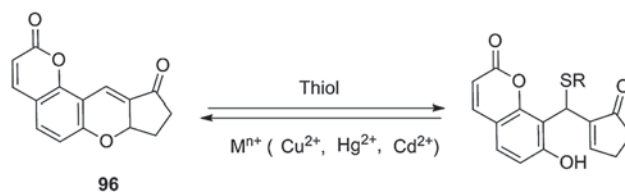


Fig. 69 Reaction of probes **100** with thiols and cell imaging.

probe **100** was applied in cellular imaging of thiols, and that the **100**-thiol system could also be applied in the design of heavy metal ions regenerative chemodosimeters. The mechanism of thiol detection is based on a thiol–chromene “click” ring-opening reaction. Moreover it was found that some metal ions promoted desulfurization. The Michael addition was proved using ¹HNMR and time-dependent density functional theory (TD-DFT) calculations (Fig. 69).

The optical properties of the fluorescein chromene derivative **101** on addition of various analytes, including Cys, Hcy, GSH, Gly, Phe, Ser, Glu, Lys, Arg, His, Ala, Gln, Met, Tyr, and Cys, in aqueous HEPES-buffered solutions (pH 7.4, 1% acetonitrile) were studied by Yoon's group.¹³⁶ In the presence of thiol-containing analytes, *i.e.*, Cys, Hcy, and GSH, probe **101** displayed a decrease in the absorption peak at 454 nm and a progressive increase in a new absorption at 500 nm. Probe **101** also showed emission enhancement at 520 nm ($\lambda_{\text{ex}} = 485$ nm). The initial quantum yield of the probe was 0.04, and the addition of 10 equiv. of GSH, Cys, and Hcy resulted in quantum yields of 0.65, 0.91, and 0.47, respectively. The other amino acids studied induced minor color or emission changes. Other thiols such as ME and DTT cause optical changes in **101** similar to those observed for biothiols. The fluorescence enhancement and UV-vis changes are attributed to 1,4-addition of thiols to the α,β -unsaturated ketone in **101** to form the corresponding thioether (Fig. 70). The value of this system was demonstrated by using it in the detection of thiol species in cells and zebrafish.

Leroy *et al.* developed a fluorescence assay using a universal 96-well microplate format and probe **102** for the determination of GSH in yeast cells (Fig. 71).¹³⁷ The microplate reader was equipped with filters selecting excitation and emission wavelengths at 485 nm and 530 nm, respectively. A borate buffer of pH value close to 9 was used as the reaction medium. Upon addition of GSH to **102**, the emission at 528 nm increased. Full selectivity was observed *versus* other endogenous thiols (except for γ -glutamylcysteine), GSSG, and enzymatic reducing reagents

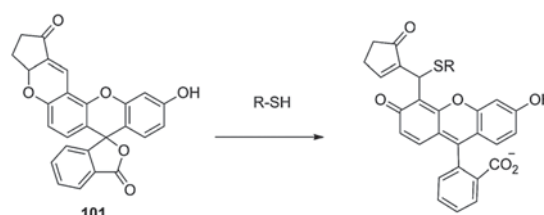


Fig. 70 Reaction of probe **101** with thiols.

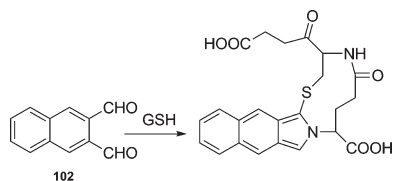


Fig. 71 Reaction of probe **102** with GSH.

of GSSG. A linear response in the 0.3–6.5 μM range was verified and the limits of quantification and detection were 0.3 and 0.05 μM , respectively. The method relies on the reaction between GSH and the highly selective fluorogenic probe **102**. The method was then used to monitor GSH in the yeast strain *Kluyveromyces lactis* during its normal growth period, and in the presence of an inhibitor of GSH biosynthesis.

Concluding remarks

In this review, we have summarized recent exciting reports regarding thiol-addition reactions and their applications in thiol recognition. The examples reported can be classified into four reaction types according to their mechanisms, based on different Michael acceptors. 1,1-Addition reactions of thiols occur with probes containing aldehydes, and this is a general approach that has been widely used for the detection of thiol-containing derivatives. 1,2-Addition reactions of thiols take place mainly with probes containing units such as coumarin and maleimide; 1,3- and 1,4-addition reactions generally occur with quinone, squaraine, and chromene derivatives. In most cases, the reactions are accompanied by color and/or emission changes. Several examples dealing with electrochemical recognition of thiols that follow a similar reaction path have also been included. Because of accompanying color changes, the process can be monitored by the naked eye, which will provide great convenience for practical application.^{61,121,126} Moreover probes with emission changes upon addition thiols will be used for cell imaging and protein labeling.^{73,74,85,89–100,108,118,136} In addition, some near-infrared (NIR) dyes as thiol probes have the unique advantage of tracing molecular activity *in vivo* because NIR photons can penetrate relatively deeply into tissues with low auto-fluorescence background additionally causing less damage to biological samples.¹¹⁵ Moreover many examples behave as ratiometric fluorescent probes.^{61,75,93,96,115} By this token, the use of thiol-addition reactions is a very simple and straightforward procedure for the preparation of probes for thiol recognition. Most of the reported examples are designed to detect biothiols such as Cys, Hcy, and GSH. However, the similarities among the structures of these analytes make it difficult to distinguish them from each other. In fact, the design of highly selective probes for Cys, Hcy, and GSH is still a challenge and very few examples have been reported so far.^{72,93,115} We hope that this review will help to inspire in the future the design of highly selective simple colorimetric or fluorimetric turn-on probes for target thiols.

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Notes and references

- D. M. Townsend, K. D. Tew and H. Tapiero, *Biomed. Pharmacother.*, 2003, **57**, 145–155.
- L. A. Herzenberg, S. C. De Rosa, J. G. Dubs, M. Roederer, M. T. Anderson, S. W. Ela, S. C. Deresinski and L. A. Herzenberg, *Proc. Natl. Acad. Sci. U. S. A.*, 1997, **94**, 1967–1972.
- C. Sofia, B. Chadefaux, M. Coude, O. Oaillard and E. Kamoun, *Clin. Chem.*, 1990, **36**, 2137–2138.
- F. J. T. Staal, S. W. Ela, M. Roederer, M. T. Anderson and L. A. Herzenberg, *Lancet*, 1992, **339**, 909–912.
- M. T. Heafield, S. Fearn, G. B. Steventon, R. H. Waring, A. C. Williams and S. G. Sturman, *Neurosci. Lett.*, 1990, **110**, 216–220.
- O. Nygard, J. E. Nordrehaug, H. Refsum, P. M. Ueland, M. Farstad and S. E. Vollset, *N. Engl. J. Med.*, 1997, **337**, 230–237.
- D. W. Jacobsen, *Clin. Chem.*, 1998, **44**, 1833–1843.
- S. Shahrokhian, *Anal. Chem.*, 2001, **73**, 5972–5978.
- S. Seshadri, A. Beiser, J. Selhub, P. F. Jacques, I. H. Rosenberg, R. B. D'Agostino, P. W. F. Wilson and P. A. Wolf, *N. Engl. J. Med.*, 2002, **346**, 476–483.
- D. G. Savage, J. Lindenbaum, S. P. Stabler and R. H. Allen, *Am. J. Med.*, 1994, **96**, 239–246.
- G. G. Klee, *Clin. Chem.*, 2000, **46**, 1277–1283.
- H. Refsum, P. M. Ueland, O. Nygard and S. E. Vollset, *Annu. Rev. Med.*, 1998, **49**, 31–62.
- J. G. Ray and C. A. Laskin, *Placenta*, 1999, **20**, 519–529.
- A. D. Smith, *Am. J. Clin. Nutr.*, 2002, **75**, 785–786.
- C. Hwang, A. J. Sinskey and H. F. Lodish, *Science*, 1992, **257**, 1496–1502.
- T. P. Dalton, H. G. Shertzer and A. Puga, *Annu. Rev. Pharmacol. Toxicol.*, 1999, **39**, 67–101.
- P. K. Pallela, T. Chiku, M. J. Carvan III and D. S. Sem, *Anal. Biochem.*, 2006, **352**, 265–273.
- C. K. Mathews, K. E. van Holde and K. G. Ahern, *Biochemistry*, Addison-Wesley Publishing Company, San Francisco, 2000.
- Anon, USA, Dangerous Properties of Industrial Materials Report, 1994, **14**, 92.
- G. L. Ellman, *Arch. Biochem. Biophys.*, 1959, **82**, 70–77.

- 21 C. Komuro, K. Ono, Y. Shibamoto, T. Nishidai, M. Takahashi and M. Abe, *J. Chromatogr.*, 1985, **338**, 209–212.
- 22 W. Chen, Y. Zhao, T. Seefeldt and X. Guan, *J. Pharm. Biomed. Anal.*, 2008, **48**, 1375–1380.
- 23 O. Nekrasova, N. S. Lawrence and R. G. Compton, *Talanta*, 2003, **60**, 1085–1095.
- 24 T. Inoue and J. R. Kirchhoff, *Anal. Chem.*, 2000, **72**, 5725–5730.
- 25 A. Zinellu, S. Sotgia, B. Scanu, M. F. Usai, A. G. Fois, V. Spada, A. Deledda, L. Deiana, P. Pirina and C. Carru, *Amino Acids*, 2009, **37**, 395–400.
- 26 N. Burford, M. D. Eelman, D. E. Mahony and M. Morash, *Chem. Commun.*, 2003, 146–147.
- 27 M. Fafii, R. Elango, G. Courtney-Martin, J. D. House, L. Fisher and P. B. Pencharz, *Anal. Biochem.*, 2007, **371**, 71–81.
- 28 K. Amarnath, V. Amarnath, K. Amarnath, H. L. Valentine and W. M. Valentine, *Talanta*, 2003, **60**, 1229–1238.
- 29 M. Wen, H. Liu, F. Zhang, Y. Zhu, D. Liu, Y. Tian and Q. Wu, *Chem. Commun.*, 2009, 4530–4532.
- 30 S. Sreejith, K. P. Divya and A. Ajayaghosh, *Angew. Chem., Int. Ed.*, 2008, **47**, 7883–7887.
- 31 M. E. Moragues, R. Martínez-Mañez and F. Sancenón, *Chem. Soc. Rev.*, 2011, **40**, 2593–2643.
- 32 Z. Yao, X. Feng, C. Li and G. Shi, *Chem. Commun.*, 2009, 5886–5888.
- 33 T. J. Tolbert and C. H. Wong, *Angew. Chem., Int. Ed.*, 2002, **41**, 2171–2174.
- 34 T. K. Kim, D. N. Lee and H. J. Kim, *Tetrahedron Lett.*, 2008, **49**, 4879–4881.
- 35 B. Tang, Y. Xing, P. Li, N. Zhang, F. Yu and G. Yang, *J. Am. Chem. Soc.*, 2007, **129**, 11666–11667.
- 36 H. Maeda, H. Matsuno, M. Ushida, K. Katayama, K. Saeki and N. Itoh, *Angew. Chem., Int. Ed.*, 2005, **44**, 2922–2955.
- 37 S. Ji, J. Yang, Q. Yang, S. Liu, M. Chen and J. Zhao, *J. Org. Chem.*, 2009, **74**, 4855–4865.
- 38 M. M. Pires and J. Chmielewski, *Org. Lett.*, 2008, **10**, 837–840.
- 39 J. Bouffard, Y. Kim, T. M. Swager, R. Weissleder and S. A. Hilderbrand, *Org. Lett.*, 2008, **10**, 37–40.
- 40 B. Tang, L. Yin, X. Wang, Z. Chen, L. Tong and K. Xu, *Chem. Commun.*, 2009, 5293–5295.
- 41 J. Zhu, I. Dhimitruka and D. Pei, *Org. Lett.*, 2004, **6**, 3809–3812.
- 42 P. K. Pallela, T. Chiku, M. J. Carvan III and D. S. Sem, *Anal. Biochem.*, 2006, **352**, 265–273.
- 43 A. M. Piggott and P. Karuso, *Anal. Chem.*, 2007, **79**, 8769–8773.
- 44 L. Nie, H. Ma, M. Sun, X. Li, M. Su and S. Liang, *Talanta*, 2003, **59**, 959–964.
- 45 S. Wang, H. Ma, J. Li, X. Chen, Z. Bao and S. Sun, *Talanta*, 2006, **70**, 518–521.
- 46 B. Rezaei and A. Mokhtari, *Spectrochim. Acta, Part A*, 2007, **66**, 359–363.
- 47 C. F. Chow, B. K. W. Chiu, M. H. W. Lam and W. Y. Wong, *J. Am. Chem. Soc.*, 2003, **125**, 7802–7803.
- 48 H. Wang, W.-S. Wang and H.-S. Zhang, *Talanta*, 2001, **53**, 1015–1019.
- 49 M. S. Han and D. H. Kim, *Tetrahedron*, 2004, **60**, 11251–11257.
- 50 N. Shao, J. Y. Jin, S. M. Cheung, R. H. Yang, W. H. Chan and T. Mo, *Angew. Chem., Int. Ed.*, 2006, **45**, 4944–4948.
- 51 Y. Fu, H. Li, W. Hu and D. Zhu, *Chem. Commun.*, 2005, 3189–3191.
- 52 X. F. Yang, P. Liu, L. Wang and M. Zhao, *J. Fluoresc.*, 2008, **18**, 453–459.
- 53 B. Han, J. Yuan and E. Wang, *Anal. Chem.*, 2009, **81**, 5569–5573.
- 54 X. Chen, Y. Zhou, X. Peng and J. Yoon, *Chem. Soc. Rev.*, 2010, **39**, 2120–2135.
- 55 S. Sreejith, K. P. Divya and A. Ajayaghosh, *Angew. Chem., Int. Ed.*, 2008, **47**, 7883–7887.
- 56 T. C. Owen, *Bioorg. Chem.*, 2008, **36**, 156–160.
- 57 J. Lee, S. Lee, D. Zhai, Y. Ahn, H. Y. Yeo, Y. L. Tan and Y. Chang, *Chem. Commun.*, 2011, **47**, 4508–4510.
- 58 G. Kim, K. Lee, H. Kwon and H. Kim, *Org. Lett.*, 2011, **13**, 2799–2801.
- 59 Q. Zuo, B. Li, Q. Pei, Z. Li and S. Liu, *J. Fluoresc.*, 2010, **20**, 1307–1321.
- 60 F. Tanaka, N. Mase and C. F. Barbas III, *Chem. Commun.*, 2004, 1762–1763.
- 61 W. Lin, L. Long, L. Yuan, Z. Cao, B. Chen and W. Tan, *Org. Lett.*, 2008, **10**, 5577–5580.
- 62 D. Zhang, M. Zhang, Z. Liu, M. Yu, F. Li, T. Yi and C. Huang, *Tetrahedron Lett.*, 2006, **47**, 7093–7096.
- 63 M. Zhang, M. Li, Q. Zhao, F. Li, D. Zhang, J. Zhang, T. Yi and C. Huang, *Tetrahedron Lett.*, 2007, **48**, 2329–2333.
- 64 X. Zhang, X. Ren, Q. H. Xu, K. P. Loh and Z. K. Chen, *Org. Lett.*, 2009, **11**, 1257–1260.
- 65 L. Duan, Y. Xu, X. Qian, F. Wang, J. Liu and T. Cheng, *Tetrahedron Lett.*, 2008, **49**, 6624–6627.
- 66 P. Wang, J. Liu, X. Lv, Y. Liu, Y. Zhao and W. Guo, *Org. Lett.*, 2012, **14**, 520–523.
- 67 O. Rusin, N. N. St. Luce, R. A. Agbaria, J. O. Escobedo, S. Jiang, I. M. Warner and R. M. Strongin, *J. Am. Chem. Soc.*, 2004, **126**, 438–439.
- 68 W. Wang, O. Rusin, X. Xu, K. K. Kim, J. O. Escobedo, S. O. Fakayode, K. A. Fletcher, M. Lowry, C. M. Schowalter, C. M. Lawrence, F. R. Fronczek, I. M. Warner and R. M. Strongin, *J. Am. Chem. Soc.*, 2005, **127**, 15949–15958.
- 69 S. Lim, J. O. Escobedo, M. Lowry, X. Xu and R. Strongin, *Chem. Commun.*, 2010, **46**, 5707–5709.
- 70 H. Li, J. Fan, J. Wang, M. Tian, J. Du, S. Sun, P. Sun and X. Peng, *Chem. Commun.*, 2009, 5904–5906.
- 71 K. Huang, H. Yang, Z. Zhou, H. Chen, F. Li, T. Yi and C. Huang, *Inorg. Chim. Acta*, 2009, **362**, 2577–2580.
- 72 H. Chen, Q. Zhao, Y. Wu, F. Li, H. Yang, T. Yi and C. Huang, *Inorg. Chem.*, 2007, **46**, 11075–11081.
- 73 Y. Ma, S. Liu, H. Yang, Y. Wu, C. Yang, X. Liu, Q. Zhao, H. Wu, J. Liang, F. Li and W. Huang, *J. Mater. Chem.*, 2011, **21**, 18974–18982.
- 74 X. Liu, N. Xi, S. Liu, Y. Ma, H. Yang, H. Li, J. He, Q. Zhao, F. Li and W. Huang, *J. Mater. Chem.*, 2012, **22**, 7894–7901.

- 75 L. Xiong, Q. Zhao, H. Chen, Y. Wu, Z. Dong, Z. Zhou and F. Li, *Inorg. Chem.*, 2010, **49**, 6402–6408.
- 76 R. Zhang, X. Yu, Z. Ye, G. Wang, W. Zhang and J. Yuan, *Inorg. Chem.*, 2010, **49**, 7898–7903.
- 77 H. Chen, X. Li, Y. Wu, W. Gao and R. Bai, *Dalton Trans.*, 2012, **41**, 13292–13297.
- 78 H. Hu, J. Fan, H. Li, K. Song, S. Wang, G. Chen and X. Peng, *Org. Biomol. Chem.*, 2011, **9**, 980–983.
- 79 T. K. Kim, D. N. Lee and H. J. Kim, *Tetrahedron Lett.*, 2008, **49**, 4879–4881.
- 80 K. S. Lee, T. K. Kim, J. H. Lee, H. J. Kim and J. I. Hong, *Chem. Commun.*, 2008, 6173–6175.
- 81 Y. Yue, Y. Guo, J. Xua and S. Shao, *New J. Chem.*, 2011, **35**, 61–64.
- 82 Z. Yang, N. Zhao, Y. Sun, F. Miao, Y. Liu, X. Liu, Y. Zhang, W. Ai, G. Song, X. Shen, X. Yu, J. Sun and W. Wong, *Chem. Commun.*, 2012, **48**, 3442–3444.
- 83 J. Mei, J. Tong, J. Wang, A. Qin, J. Sun and B. Tang, *J. Mater. Chem.*, 2012, **22**, 17063–17070.
- 84 J. Mei, Y. Wang, J. Tong, J. Wang, A. Qin, J. Sun and B. Tang, *Chem.–Eur. J.*, 2013, **19**, 613–620.
- 85 P. Das, A. K. Mandal, N. B. Chandar, M. Baidya, H. B. Bhatt, B. Ganguly, S. K. Ghosh and A. Das, *Chem.–Eur. J.*, 2012, **18**, 15382–15393.
- 86 O. García-Beltrán, N. Mena, E. G. Pérez, B. K. Cassels, M. T. Nuñez, F. Werlinger, D. Zavala, M. E. Aliaga and P. Pavez, *Tetrahedron Lett.*, 2011, **52**, 6606–6609.
- 87 Y. Sun, M. Chen, J. Liu, X. Lv, J. Li and W. Guo, *Chem. Commun.*, 2011, **47**, 11029–11031.
- 88 W. Lin, L. Yuan, Z. Cao, Y. Feng and L. Long, *Chem.–Eur. J.*, 2009, **15**, 5096–5103.
- 89 L. Yi, H. Li, L. Sun, L. Liu, C. Zhang and Z. Xi, *Angew. Chem., Int. Ed.*, 2009, **48**, 4034–4037.
- 90 S. Lim, S. Lee, S. Park and H. Kim, *Tetrahedron Lett.*, 2011, **52**, 3902–3904.
- 91 H. S. Jung, K. C. Ko, G. Kim, A. Lee, Y. Na, C. Kang, J. Y. Lee and J. S. Kim, *Org. Lett.*, 2011, **13**, 1498–1501.
- 92 H. J. Ha, D. H. Yoon, S. Park and H. J. Kim, *Tetrahedron*, 2011, **67**, 7759–7762.
- 93 L. Yuan, W. Lin and Y. Yang, *Chem. Commun.*, 2011, **47**, 6275–6277.
- 94 H. Kwon, K. Lee and H. Kim, *Chem. Commun.*, 2011, **47**, 1773–1775.
- 95 A. Y. Cho and K. Choi, *Chem. Lett.*, 2012, **41**, 1611–1612.
- 96 X. D. Liu, R. Sun, Y. Xu, Y. J. Xu, J. F. Ge and J. M. Lu, *Sens. Actuators, B*, 2013, **178**, 525–531.
- 97 A. Lim and H. Kim, *Tetrahedron Lett.*, 2011, **52**, 3189–3190.
- 98 P. Yang and N. Aykin, *J. Chromatogr., B: Biomed. Appl.*, 2001, **753**, 287–292.
- 99 S. Mare, S. Penugonda and N. Ercal, *Biomed. Chromatogr.*, 2005, **19**, 80–86.
- 100 D. Kand, A. M. Kalle, S. J. Varmaa and P. Talukdar, *Chem. Commun.*, 2012, **48**, 2722–2724.
- 101 S. Girouard, M. H. Houle, A. Grandbois, J. Keillor and S. W. Michnick, *J. Am. Chem. Soc.*, 2005, **127**, 559–565.
- 102 J. Guy, K. Caron, S. Dufresne, S. W. Michnick, W. G. Skene and J. W. Keillor, *J. Am. Chem. Soc.*, 2007, **129**, 11969–11977.
- 103 M. E. Langmuir, J. R. Yang, A. M. Moussa, R. Laura and K. A. Lecompte, *Tetrahedron Lett.*, 1995, **36**, 3989–3992.
- 104 T. O. Sippel, *J. Histochem. Cytochem.*, 1981, **29**, 314–316.
- 105 T. O. Sippel, *J. Histochem. Cytochem.*, 1981, **29**, 1377–1381.
- 106 A. P. deSilva, H. Q. N. Gunaratne and T. Gunnlaugsson, *Tetrahedron Lett.*, 1998, **39**, 5077–5080.
- 107 J. E. T. Corrie, *J. Chem. Soc., Perkin Trans. 1*, 1994, 2975–2982.
- 108 T. Matsumoto, Y. Urano, T. Shoda, H. Kojima and T. Nagano, *Org. Lett.*, 2007, **9**, 3375–3377.
- 109 Q. Lin, C. Bao, S. Cheng, Y. Yang, W. Ji and L. Zhu, *J. Am. Chem. Soc.*, 2012, **134**, 5052–5055.
- 110 B. K. McMahon and T. Gunnlaugsson, *J. Am. Chem. Soc.*, 2012, **134**, 10725–10728.
- 111 X. Tang, W. Liu, J. Wu, W. Zhao, H. Zhang and P. Wang, *Tetrahedron Lett.*, 2011, **52**, 5136–5139.
- 112 N. Zhao, Y. Wu, L. Shi, Q. Lin and Z. Chen, *Dalton Trans.*, 2010, **39**, 8288–8295.
- 113 H. Wang, G. Zhou, H. Gai and X. Chen, *Chem. Commun.*, 2012, **48**, 8341–8343.
- 114 X. Yang, Y. Guo and R. M. Strongin, *Org. Biomol. Chem.*, 2012, **10**, 2739–2741.
- 115 Z. Guo, S. Nam, S. Park and J. Yoon, *Chem. Sci.*, 2012, **3**, 2760–2765.
- 116 V. Hong, A. A. Kislukhin and M. G. Finn, *J. Am. Chem. Soc.*, 2009, **131**, 9986–9994.
- 117 W. Wei, X. Liang, G. Hua, Y. Guo and S. Shao, *Tetrahedron Lett.*, 2011, **52**, 1422–1425.
- 118 H. Zhang, P. Wang, Y. Yang and H. Sun, *Chem. Commun.*, 2012, **48**, 10672–10674.
- 119 K. Zacharisa, P. D. Tzanavaras and D. G. Themelis, *J. Pharm. Biomed. Anal.*, 2009, **50**, 384–391.
- 120 Y. Zeng, G. Zhang and D. Zhang, *Anal. Chim. Acta*, 2008, **627**, 254–257.
- 121 Y. Zeng, G. Zhang, D. Zhang and D. Zhu, *Tetrahedron Lett.*, 2008, **49**, 7391–7394.
- 122 R. B. Smith, C. Canton, N. S. Lawrence, C. Livingstone and J. Davis, *New J. Chem.*, 2006, **30**, 1718–1724.
- 123 S. Gracheva, C. Livingstone and J. Davis, *Anal. Chem.*, 2004, **76**, 3833–3836.
- 124 G. Hignett, S. Threlfell, A. J. Wain, N. S. Lawrence, S. J. Wilkins, J. Davis, R. G. Compton and M. F. Cardosi, *Analyst*, 2001, **126**, 353–357.
- 125 Z. Yan, S. Guang, H. Xu and X. Liu, *Analyst*, 2011, **136**, 1916–1921.
- 126 J. V. Ros-lis, B. García, D. Jiménez, R. Martínez-Mañez, F. Sancenón, J. Soto, F. Gonzalvo and M. C. Valldecabres, *J. Am. Chem. Soc.*, 2004, **126**, 4064–4065.
- 127 X. Zhang, C. Li, X. Cheng, X. Wang and B. Zhang, *Sens. Actuators, B*, 2008, **129**, 152–157.
- 128 J. V. Ros-Lis, M. D. Marcos, R. Martínez-Mañez, K. Rurack and J. Soto, *Angew. Chem., Int. Ed.*, 2005, **44**, 4405–4407.

- 129 H. S. Hewage and E. V. Anslyn, *J. Am. Chem. Soc.*, 2009, **131**, 13099–13106.
- 130 J. V. Ros-Lis, R. Casasús, M. Comes, C. Coll, M. D. Marcos, R. Martínez-Mañez, F. Sancenón, J. Soto, P. Amorós, J. El Haskouri, N. Garró and K. Rurack, *Chem.–Eur. J.*, 2008, **14**, 8267–8278.
- 131 E. Climent, M. D. Marcos, R. Martínez-Mañez, F. Sancenón, J. Soto, K. Rurack and P. Amorós, *Angew. Chem., Int. Ed.*, 2009, **48**, 8519–8522.
- 132 S. Huang, K. Ting and K. Wang, *Anal. Chim. Acta*, 2008, **620**, 120–126.
- 133 F. J. Huo, Y. Q. Sun, J. Su, J. B. Chao, H. J. Zhi and C. X. Yin, *Org. Lett.*, 2009, **11**, 4918–4921.
- 134 F. J. Huo, Y. Q. Sun, J. Su, Y. T. Yang, C. X. Yin and J. B. Chao, *Org. Lett.*, 2010, **12**, 4756–4759.
- 135 Y. T. Yang, F. J. Huo, C. X. Yin, A. M. Zheng, J. B. Chao, Y. Q. Li, Z. X. Nie, R. Martínez-Mañez and D. S. Liu, *Biosens. Bioelectron.*, 2013, **47**, 300–306.
- 136 X. Chen, S. Ko, M. J. Kim, I. Shin and J. Yoon, *Chem. Commun.*, 2010, **46**, 2751–2753.
- 137 K. Lewicki, S. Marchand, L. Matouba, J. Lulek, J. Coulon and P. Leroy, *Talanta*, 2006, **70**, 876–882.