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Ratiometric fluorescence detection of cysteine and homocysteine with a BODIPY dye by mimicking the native chemical ligation†

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The selective detection of cysteine and homocysteine over glutathione and other amino acids was demonstrated with an 8-MeS-BODIPY probe by mimicking the native chemical ligation approach, which allowed the selective and ratiometric fluorescence sensing of cysteine over other biothiols at physiologically relevant concentrations and in different organs of a zebrafish.

Introduction

Biological thiols or biothiols such as glutathione (GSH), cysteine (Cys), and homocysteine (Hcy) play essential roles in maintaining the redox balance of biological systems.[1] For example, glutathione (GSH) is the major endogenous cellular antioxidant, preventing cellular damage against reactive oxygen species. Cys deficiency is associated with diseases such as haematopoiesis decrease, leukocyte loss, psoriasis, slowed growth, liver damage, skin lesions, hair depigmentation and edema.[2] Hcy is also associated with various diseases or clinical conditions, which include Alzheimer's disease, schizophrenia, end-stage renal disease, osteoporosis, and type II diabetes.[3] Elevated levels of Hcy have been recognized as an independent risk factor for cardiovascular diseases.[4] Therefore, the selective and efficient detection of these biothiols has become an important subject of current research interest.

Fluorescence microscopy with an aid of molecular probes offers an essential tool for visualizing biomolecules *in vitro* and *in vivo*.[5] Accordingly, significant efforts have been made to develop fluorescence probes for biothiols in recent years. A key issue in this endeavour is how to secure high selectivity toward a specific biothiol over competing biothiols. A number of ingenious approaches have been disclosed to address the selectivity issue in recent years, resulting in a substantial progress in this subject. For example, the selective sensing of Cys/Hcy over GSH is now feasible by taking advantage of the functional group difference between them: Cys/Hcy have amine and thiol groups nearby, whereas the two functional groups in GSH are away from each other. Therefore, in the case of Cys/Hcy, both functional groups can be favourably utilized for the reaction-based sensing approach, as exemplified by the thiazoline ring formation with aryl aldehyde type probes[6] or by conjugate addition to aryl acrylate type probes, followed by intramolecular ester aminolysis.[7]

Recently, Yang and co-workers disclosed a distinct strategy from the existing ones for the selective sensing of biothiols, which explored the nucleophilic substitution of a monochlorinated boron dipyrromethene (BODIPY) derivative by the thiol group, followed by an intramolecular sulfur-to-amine exchange reaction. This sensing scheme is of interest as it mimics the native chemical ligation (NCL), *i.e.*, the formation of the peptide bond *via* a thioester intermediate.[8] This NCL-type approach allowed them to selectively sense GSH over Cys/Hcy for the first time. During our investigation of a novel BODIPY compound, 8-MeS-BODIPY,[9] for the development of fluorescence probes,[10] we envisioned that this type of compound might be used for the ratiometric sensing of Cys through a related NCL-type conversion. Here, we report a proof-of-concept study for the ratiometric sensing of Cys using the BODIPY compound by recognizing that most of the known Cys-selective fluorescence probes are intensity-based.[11] Fluorescence signals from such intensity-based probes can be influenced by other variables such as the concentration of the probe, micro-environmental conditions or differences in the optical components. In this context, ratiometric fluorescence probes are highly desirable, as they provide built-in correction for environmental effects, and they may also increase the dynamic range of fluorescence measurement. It should be noted that only a few ratiometric fluorescence probes that selectively detect Cys have been reported so far.[12] This year, Yang and co-workers also reported NCL-type probes that selectively sense Cys/Hcy over GSH, not in the ratiometric mode but in the turn-on type

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fluorescence change.[13] Therefore, the ratiometric probes that selectively detect Cys/Hcy over GSH are still in huge demand.[14]

Results and discussion

BODIPY dyes constitute a class of excellent fluorescent dyes, which are being widely used in various research fields as labelling reagents, fluorescent switches, chemosensors, light-harvesting systems, and dye-sensitized solar cells because of their advantageous photo-physical properties, such as high photo-stability, high absorption coefficients, and high fluorescence quantum yields.[15] In this context, it is also interesting to develop BODIPY-derived sensing systems for biothiols.

A simple BODIPY compound, 8-MeS-BODIPY, was prepared from pyrrole in three steps by following the literature procedure.[9] We envisioned that the methylthio group might be readily exchanged with other thiols. In the case of Cys and Hcy, the corresponding thiol adduct could further undergo intramolecular displacement by the nearby amino group to give the corresponding amino-BODIPY compounds. This NCL-mimicking transformation would be favoured with Cys/Hcy, not with GSH, thereby offering a means for the selective detection of Cys/Hcy over GSH. Furthermore, substitution of the methylthio group with the amino group would result in changes in the absorption and emission wavelengths of the dye, thus enabling the ratiometric detection of Cys/Hcy. This is indeed the case. A further elaboration of the BODIPY moiety to longer emitting derivatives would provide us versatile imaging probes later.

Sensing properties

The absorption spectra of probe **1** (10 μM) were taken before and after treatment with typical biothiols (1.0 mM) in pH 7.4 HEPES buffer containing 1% CH_3CN for 20 min at 25 °C (Fig. 1a). Upon treatment of the probe with Cys or Hcy, the strong absorption band from the probe (λ_{abs} = 483 nm) disappeared, while a new peak in the shorter wavelength (λ_{abs} = 400 nm) appeared. The hypsochromic shift supports the sensing mechanism that involves the displacement of the 8-methylthio group with the amino group of the biothiols. The aminoBODIPYs have the absorption maxima around 400 nm.[16] In contrast, upon treatment of the probe with GSH, the absorption peak from the probe at 483 nm disappeared, while a new peak at the longer wavelength (λ_{abs} = 515 nm) appeared. This bathochromic shift suggests that the sensing product is the GSH adduct corresponding to the reaction intermediate in the sensing Scheme 1; the small bathochromic shift is probably caused by the structural change. To obtain a supporting evidence for the thiol adduct intermediate in the case of GSH, we treated the probe with *N*-acetyl-cysteine (NAC) where thiol is the only reactive group; the NAC–probe adduct again exhibited a bathochromic shift (Fig. S1†) comparable to the case of GSH, supporting that the GSH adduct is the thiol adduct intermediate.

Similarly, the emission spectral changes of the probe in the presence of biothiols were monitored by exciting at 400 nm under the same conditions as mentioned above (Fig. 1b). Upon treatment of the probe with Cys or Hcy, the emission peak of the probe (λ_{em} = 524 nm) disappeared, while a new peak in the shorter wavelength (λ_{em} = 467 nm) appeared. Again, there was no such hypsochromic shift in the case of GSH; instead, a strong peak near the probe's emission peak appeared. The emission spectral changes also correspond to the absorption spectral changes. The different emission spectral changes between Cys/Hcy and GSH correspond to the distinct emission colour changes between them; green-to-blue in the case of Cys/Hcy but no change in the case of GSH. The GSH-adduct emitted a little brighter fluorescence than the probe itself.

Ratiometric response at biological concentrations

Although probe **1** responded to both Cys and Hcy with a comparable reactivity at 0.1 mM concentration, the ratiometric response of the probe at their physiological concentrations was found to be highly selective toward Cys owing to the significantly different cellular concentrations of the biothiols (0.1–0.25 mM Cys, 10–12 μM Hcy, and 5–10 mM GSH).[17] The ratiometric fluorescence change of the probe in the presence of each biothiol at a given physiological concentration (0.1 mM Cys, 10 μM Hcy, and 10 mM GSH) shows a minor change in the case of Hcy (Fig. 2). As shown in Fig. 2a, the intensity increase in the blue emission band from that of the probe caused by Hcy is considerably smaller compared with that caused by Cys (Fig. 2b). Therefore, probe **1** may be used for the ratiometric sensing of Cys over Hcy in biological systems. GSH caused almost no fluorescence change even at a much higher concentration, as GSH underwent the intramolecular displacement very slowly (Fig. S2†).

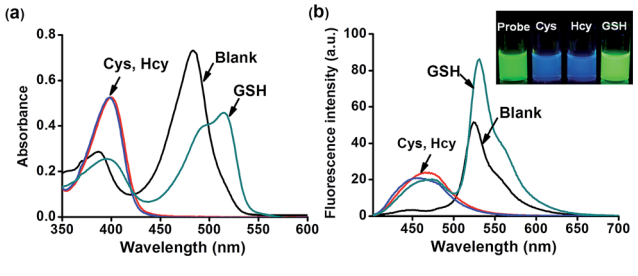
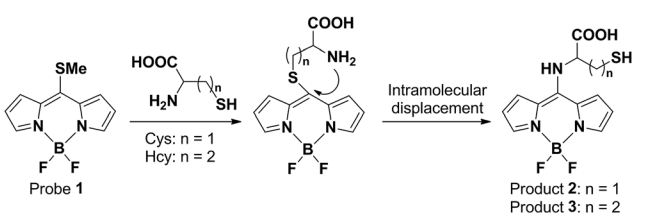


Fig. 1 (a) Absorption and (b) emission spectra of probe **1** (10 μM) taken 20 min after addition of 1.0 mM of Cys, Hcy or GSH in HEPES buffer (pH 7.4, containing 1% CH_3CN) at 25 °C under excitation at 400 nm (inset: fluorescence colour change of the probe upon addition of the biothiols).



Scheme 1 Proposed mechanism for the reaction of probe **1** with Cys/Hcy.

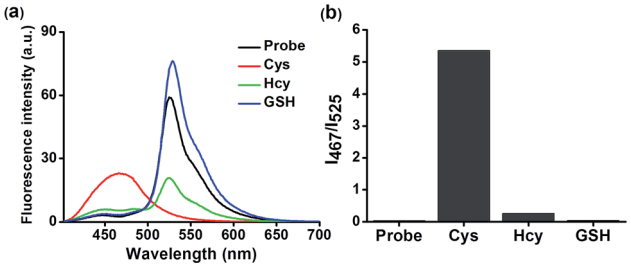


Fig. 2 Emission spectral changes of probe **1** (10 μM) upon treatment of Cys (0.1 mM), Hcy (10 μM) and GSH (10 mM) at their physiological concentrations in HEPES buffer (10 mM, pH 7.4, containing 1% CH_3CN). The spectra were acquired 20 min after the addition of analyte at 25 °C under excitation at 400 nm. (b) Ratiometric spectral changes given by the ratio of the peak height at 467 nm over that at 525 nm, I_{467}/I_{525}.

The time-course of fluorescence change of probe **1** in the presence of Cys or Hcy at 0.1 mM level was monitored at 25 °C in HEPES buffer (pH 7.4, containing 1% CH_3CN) under excitation at 400 nm. The ratiometric fluorescence changes, I_{467}/I_{524} and I_{457}/I_{525} for Cys and Hcy, respectively, indicate that the signal saturation was reached within 20 min (10 min in the case of Hcy) under the given conditions (Fig. 3 and S3†). The same titrations of the probe with Cys or Hcy at 1.0 mM level resulted in the signal saturation within 2 min (Fig. S4†). Thus, the NCL approach using probe **1** is faster than the reported NCL-based probes.[18]

Fluorescence titration

The fluorescence titration of probe **1** (10 μM) with Cys (0–4.0 mM) in HEPES buffer (10 mM, pH 7.4) at 25 °C was carried out. The results in Fig. 4 show a gradual decrease of the emission peak at 525 nm, while a progressive increase of a new fluorescence peak at 465 nm, with an isosbestic point at 502 nm. The height ratio of the two peaks (I_{465}/I_{525}) shows a drastic variation from 0.048 in the absence of Cys to 5.771 in the presence of Cys (4.0 mM), corresponding to a 120-fold enhancement (Fig. 4). Similar ratiometric behaviour was observed with Hcy (Fig. S5†). Such a huge ratiometric change is a highly desirable property of a ratiometric probe, as it governs the sensitivity and the dynamic range of the probe.[19] Furthermore, the emission ratios

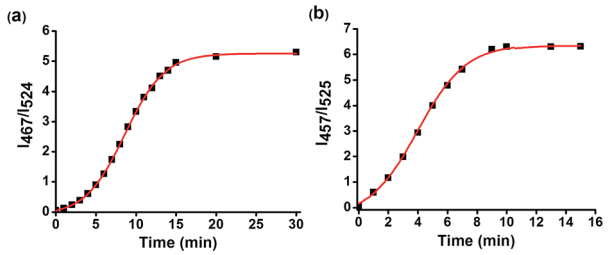


Fig. 3 Time-dependent fluorescence spectra of probe **1** (10 μM) with 0.1 mM of (a) Cys and (b) Hcy. Each spectrum was acquired in HEPES buffer (10 mM, pH 7.4, containing 1% CH_3CN) at 25 °C under excitation at 400 nm.

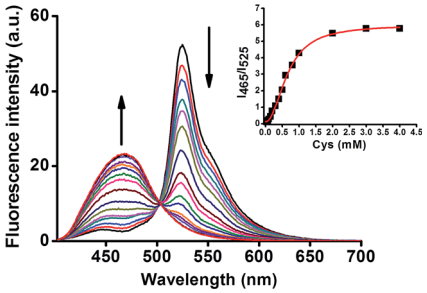


Fig. 4 Fluorescence spectra of probe **1** (10 μM) upon the addition of increased concentrations of Cys. Each spectrum was acquired in HEPES buffer (10 mM, pH 7.4, containing 1% CH_3CN) at 25 °C under excitation at 400 nm.

(I_{465}/I_{525}) were linearly proportional to the concentration of Cys in a large dynamic range (0–1.0 mM), from which the detection limit ($S/N = 3$) was estimated to be 0.80 μM. Thus, the probe allows quantitative determination of Cys at much lower concentrations than its biological level (Fig. S6†).

Selectivity/competition assay

The analyte selectivity of probe **1** was evaluated toward various amino acids (Cys, Hcy, GSH, Val, Ser, Phe, Glu, Thr, Leu, Ala, Trp, His, and Arg), glucose, and a reactive oxygen species (hydrogen peroxide) by following the absorption and emission spectral changes at 10 μM of the probe and 1.0 mM of analyte. All of the analytes, except Cys and Hcy, caused no appreciable change from that of the probe alone (Fig. 5a and S7†). These results support that the NCL reaction is the sensing mechanism of Cys and Hcy by the probe.

The high selectivity of probe **1** toward Cys and Hcy was confirmed by the competition assay. Thus, the fluorescence response of the probe toward Cys was checked in the presence

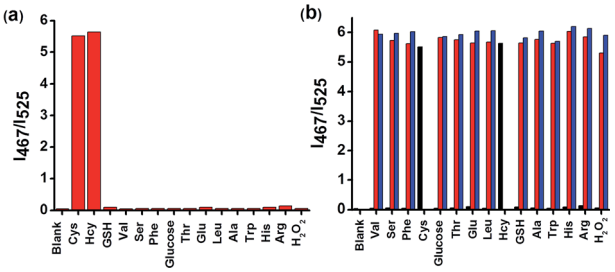


Fig. 5 (a) Fluorescence response of probe **1** (10 μM) toward various analytes (1.0 mM, Cys, Hcy, GSH, Val, Ser, Phe, glucose, Glu, Thr, Leu, Ala, Trp, His, Arg, and H_2O_2), expressed by the peak height ratio (I_{467}/I_{525}) of the emission spectra taken in HEPES buffer (10 mM, pH 7.4, containing 1% CH_3CN) after 20 min of mixing. Excitation wavelength is 400 nm. (b) Fluorescence response of probe **1** (10 μM) toward various analytes at 1.0 mM (from the left: blank (probe only), Val, Ser, Phe, Cys, glucose, Thr, Glu, Leu, Hcy, GSH, Ala, Trp, His, Arg, or H_2O_2), expressed by the peak height ratio (I_{467}/I_{525}) of the emission spectra taken in HEPES buffer (10 mM, pH 7.4, containing 1% CH_3CN) after 20 min of mixing: (probe + analyte) → black bar; (probe + analyte + Cys) → red bar; (probe + analyte + Hcy) → blue bar. Excitation wavelength is 400 nm.

of each of the other analytes under the same conditions as mentioned above; no appreciable change was observed in all the cases, except Hcy (Fig. 5b).

The competition assay toward Hcy also gave no appreciable change in all the cases, except Cys. These results indicate that probe **1** can be used for the selective detection of Cys and Hcy even in the presence of other competing species.

pH dependence

The optimal pH range for the fluorescence sensing of Cys and Hcy with probe **1** was found to be pH 7 or higher (Fig. S8†). At pH 6 or lower, there was a negligible fluorescence change owing to the reduced nucleophilicity of the mercapto ($pK_a = 8.37$) and amino ($pK_a = 10.70$) groups under acidic conditions.

Sensing mechanism

The sensing mechanism was investigated by monitoring the reaction progress, as well as by isolating the product (Fig. S9†). In the presence of Cys, the signal from the methylthio protons (–SMe) of probe **1** at 3.07 ppm disappeared, and two new peaks appeared at 3.15–3.20 ppm and 4.37–4.41 ppm, supporting that the isolated product is compound **2**. In the presence of Hcy, similarly, the methylthio protons disappeared, and new signals appeared at 2.94–3.01 ppm and 4.45–4.50 ppm. Furthermore, the isolated products **2** and **3** have identical absorption spectra with those of probe **1** titrated with Cys and Hcy, respectively (Fig. S10†). In the case of GSH, its reaction product with the probe gave a very complex ^{1}H NMR spectrum, making it difficult to draw any conclusion. Therefore, the ^{1}H NMR spectrum of the NAC–probe adduct (a thiol adduct, Fig. S11†) was compared with those of Cys– or Hcy–probe adducts, which clearly supports that the latter adducts are the amine adducts.

Bioimaging application

Finally, probe **1** was applied to the bioimaging of biothiols in a living species, zebrafish. Three sets of imaging experiments were carried out for different zebrafish incubated with (A) probe only, (B) NEM followed by the probe, and (C) NAC followed by the probe, where NEM (*N*-ethylmaleimide) and NAC (*N*-acetylcysteine) are a thiol-reactive agent[20] and a membrane-permeable precursor of Cys, respectively.[21] For example, for the experimental set (B), a 4-month-old zebrafish was incubated with probe **1** (10 μM) for 30 min, followed by NEM (1.0 mM) for 30 min, and then it was dissected to isolate tissues of different organs for imaging by fluorescence confocal microscopy. The imaging results are summarized in Fig. 6.

As shown in the solution study mentioned above, several organs emit green fluorescence, in addition to the expected blue emission from the NCL type reaction between the probe and Cys/Hcy present in the organs. We suspect that the green emission mostly results from the remaining probe after the NCL type conversion, although the possibility of biothiol adducts other than with Cys/Hcy cannot be excluded. If the endogenous concentration of Hcy in zebrafish is not much deviated from that in human cells, the blue emission is likely to represent Cys mostly, because the probe showed high selectivity over Hcy under biological concentrations. The probe will be consumed by the higher concentration level of Cys compared to that of Hcy. The experimental set (A) shows stronger blue emission from the eye and gill compared to other organs, suggesting a higher level of Cys in those organs.

The experimental set (B) shows much weaker blue emission from all the organs, except the fin, compared with those in the experimental set (A). As NEM "quenching" biothiols, very little fluorescence is detected from most of the organs. When an exogenous source of Cys (NAC, 500 μM) was pre-incubated, in

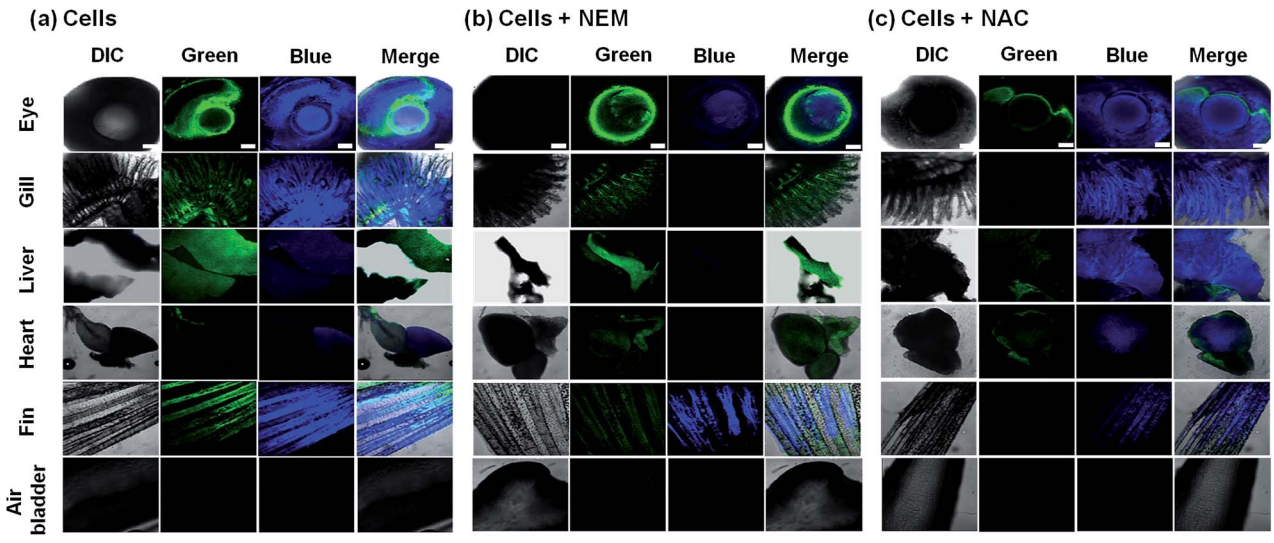


Fig. 6 DIC (bright field) and confocal fluorescence microscopic images of zebrafish organs. (a) Zebrafish was treated with 10 μM of probe **1** for 30 min. (b) Zebrafish was pre-incubated with 1.0 mM NEM for 30 min, and then treated with 10 μM of the probe for 30 min. (c) Zebrafish was pre-incubated with 500 μM NAC for 30 min, and then treated with 10 μM of the probe for 30 min. Scale bar: 200 μm. Experimental details are described in the ESI.†

experimental set (C), a blue emission that is much brighter than the green emission was observed from all the organs. The highly reduced green emission supports the fact that the higher concentration of Cys consumed most of the probe, and thus little unreacted probe molecules remained. It should be noted that the relative emission images in the three sets of experiments should not be compared with each other, because a different zebrafish was used for each experimental set.

The imaging results demonstrate that probe **1** has potential for the ratiometric fluorescence imaging of biothiols in living systems.

Conclusions

The native chemical ligation has emerged as a promising strategy for the selective sensing of biothiols, which is a challenging subject in the molecular imaging field. A new type of the native chemical ligation approach toward a ratiometric sensing system for cysteine and homocysteine is disclosed here. As a proof-of-concept, a simple BODIPY compound was chosen and its sensing characteristics were evaluated toward potentially competing analytes by spectrophotometry and confocal fluorescence microscopy. The probe, an 8-MeS-BODIPY, was found to selectively sense cysteine and homocysteine over glutathione and other amino acids, exhibiting a ratiometric fluorescence response through a chemical conversion that mimics the native chemical ligation. Under physiologically relevant concentrations of biothiols, the ratiometric fluorescence response to homocysteine became minor. The probe was thus applied to the imaging of biothiols in different organs of zebrafish, using confocal fluorescence microscopy. The approach demonstrated here, which mimics the native chemical ligation, also provides a novel route for the bioconjugation of cysteine and homocysteine with related BODIPY dyes. Studies in this direction are underway, and the results will be reported soon.

Acknowledgements

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