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Communication

# A Fluorescent Probe for Thiol Based on Strong Nucleophilic Attack of Sulfhydryl and Its bioimaging

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**A fluorescent probe has been synthesized and characterized as a selective and sensitive sensor for thiols detection. The probe exhibits a rapid response and high sensitivity to thiol in HEPES: DMF = 1:1 (V/V pH = 7.0) solution. The practical utility of the probe was demonstrated by its application to the detection of thiol in live cells.**

## 1. Introduction

Biothiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are important part of many proteins and small molecules, and play an important role in cellular antioxidant systems. It is considered that a large number of biological phenomena depend on these thiols containing mercapto group. For example, deficiency of Cys will result in many syndromes such as slow growth in children, hair depigmentation, edema, lethargy, liver damage, loss of muscle and fat, skin lesions, and weakness.<sup>[1-4]</sup> Furthermore, homocysteine is a risk factor for Alzheimer's and cardiovascular diseases.<sup>[5,6]</sup> GSH, which is the most abundant intracellular non-proteinogenic thiol, plays a pivotal role in many diseases such as liver damage, leukocyte loss, cancer, AIDS and neurodegenerative diseases.<sup>[7,8]</sup> Therefore, the rapid, selective and sensitive detection of thiols in biological samples has attracted a great deal of attention.

Many instrumental methods for the detection of thiol levels have been reported, e.g., HPLC,<sup>[9]</sup> capillary electrophoresis<sup>[10]</sup>, MS<sup>[11,12]</sup>, electrochemical methods,<sup>[13,14]</sup> and fluorescent methods.<sup>[15,16]</sup> Among all kinds of detection methods, fluorescent detection techniques have been developing dramatically in this last two decades due to its high sensitivity, selectivity, simplicity and low detection limit. In the past few years, various fluorescent probes for thiols based on different mechanisms have been exploited, including Michael addition,<sup>[17-23]</sup> cyclization reaction with aldehyde,<sup>[24-31]</sup> cleavage reaction by thiols<sup>[32-36]</sup> and others.<sup>[37-43]</sup> Kand<sup>[44]</sup> et al. reported a colorimetric probe for detection of thiols based on intramolecular charge transfer, but no fluorescence response which limit its application of imaging. The luminescent probe for Cys/Hcy reported by Zhang<sup>[26]</sup> showed a remarkable luminescence enhancement with a large blue-shift of the maximum emission wavelength from 720 to 635 nm, whereas detection limits are 1.41  $\mu$ M for Cys and 1.19  $\mu$ M for Hcy, respectively. So, there is still plenty of room for improvement in terms of selectivity, sensitivity, and performance with a different interaction mechanism.

In this work, a fluorescent probe has been synthesized and characterized for its application as a selective and sensitive sensor for thiols detection. The investigation showed that the changes of the probe on absorbance spectra and emission spectra after

reaction with thiols could be used for the detection of thiols.

## 2. Experimental

### 2.1. Materials

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Sigma-Aldrich (St. Louis, MO). Sodium hydroxide solution (0.1 mol/L) was added to aqueous HEPES (10 mmol/L) to adjust the pH to 7.0. Amino acids were purchased from Shanghai Experiment Reagent Co., Ltd (Shanghai, China). All other chemicals used were of analytical grade.

### 2.2. Preparation and characterization of probe

The synthesis of probe is summarized in Scheme 1. Addition a mixture of 1,4-Phthalaldehyde (134.13mg, 1mmol) and 1,2,3,3-tetramethyl-3H-indolium iodide (602 mg, 2 mmol) in EtOH (20 mL), then adding dropwise 200  $\mu$ l pyridine into it as catalyst. The mixture solution was heated under reflux for 4h. The clear solution obtained was concentrated and left to cool, and then filtered to obtain the dark solid. <sup>1</sup>H NMR (DMSO, 300 MHz):  $\delta$  8.49 (s, 2H), 8.42 (s, 4H), 7.96 (d, 2H,  $J$  = 6 Hz), 7.90 (d, 2H,  $J$  = 4.8 Hz), 7.84 (s, 2H), 7.69 (s, 4H), 4.24 (s, 6H), 1.84 (s, 12H), <sup>13</sup>C NMR (DMSO, 75 MHz):  $\delta$  183.16, 152.26, 145.36, 143.30, 139.63, 132.26, 131.33, 130.56, 124.41, 117.04, 116.77, 53.92, 36.46, 26.59; Elemental analysis (calcd. %) for C<sub>32</sub>H<sub>34</sub>I<sub>2</sub>N<sub>2</sub>: C, 54.87, H, 4.89, I, 36.24 N, 4.00, Found: C, 54.35, H, 4.97, N, 3.79. ESI-MS m/z: [probe - 2I + CH<sub>3</sub>OH]<sup>+</sup> Calcd for C<sub>33</sub>H<sub>37</sub>N<sub>2</sub>O 477.29, Found 477.08 (Fig. S1, ESI<sup>+</sup>).

## 3. Results and discussion

### 3.1. Selectivity over thiols

In order to examine the selectivity of probe 1, the optical responses of probe 1 to various natural amino acids, including L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan and L-valine, as well as biothiols (Cys, Hcy, and GSH) were tested under the same conditions. As shown in Fig. 1a, probe 1 displayed significant fluorescence quenching not only towards Cys, but also towards Hcy and GSH, which indicates that probe 1 can be used to detect these three biothiols simultaneously. In contrast, addition of other analytes showed almost no noticeable change of the fluorescence signal. Fig. 1b shows that fluorescence optical density at 545 nm when various amino acids are added and fluorescence color changes. Furthermore, these three biothiols could induce a change on UV-vis spectra, while other amino acids couldn't (Fig. 2). For other

thiols, such as ME (2-mercaptoethanol) and MPA (Mercaptopropionic Acid), probe showed similar fluorescence responses (Fig. S2, ESI†). Furthermore, competitive experiments showed that colorimetric and fluorescent detection of biothiols can be carried out by probe 1 in the presence of various amino acids.

### 3.2. UV-vis and fluorescence spectra of detecting Hcy

The titration experiment of the probe for thiols was also carried out. Fig. 3a shows the change in the UV-vis spectra when Hcy was added to HEPES: DMF = 1:1 (V/V pH = 7.0) solution containing probe (5  $\mu\text{mol/L}$ ). With increasing Hcy concentration, the absorption peak at 445 nm gradually decreased and a new peak appeared at 292 nm. A well-defined isosbestic point was noted at 400 nm, which may indicate the formation of single new species. The changes in the fluorescence spectra of probe 1 ( $\Phi = 0.11$ ) (15  $\mu\text{mol/L}$ ) in the absence or presence of Hcy (0–480  $\mu\text{mol/L}$ ) in HEPES: DMF = 1:1 (V/V pH=7.0) are displayed in Fig. 3b. As it can be seen the addition of Hcy caused fluorescence quenching at 545 nm in the fluorescence spectra ( $\Phi = 0.03$ ). Upon addition of Cys and GSH to probe, respectively, similar phenomena both on UV-vis spectra and fluorescence spectra can be observed (Fig. S3, ESI†).

### 3.3. Detection limit of probe for Hcy

To investigate the detection limit of probe for Hcy, probe (15  $\mu\text{mol/L}$ ) was treated with various concentrations of Hcy (0–480  $\mu\text{mol/L}$ ) and the relative emission intensity at 545 nm was plotted as a function of the Hcy concentration (Fig. 4). The emission intensity of probe was linearly proportional to Hcy concentrations. The detection limit, based on the definition by IUPAC ( $C_{\text{DL}} = 3 \text{ Sb/m}$ ),<sup>[45]</sup> was found to be 0.035  $\mu\text{mol/L}$ . Similarly, the detection limits were estimated to be 0.053  $\mu\text{mol/L}$  for Cys and about 0.052  $\mu\text{mol/L}$  for GSH (Fig. S4, ESI†). Probe shows a high sensitivity towards the thiol which is comparable to other reported thiol chemosensors (Table 1).<sup>[26,46–48]</sup>

### 3.4. Time-dependence in the detection process of Hcy

Time-dependent modulations in the fluorescence spectra of probe were monitored in the presence of 10 eq. of thiols. The kinetic study showed that the fluorescence signal for the system decreases gradually with increasing reaction time and the reaction was complete within 20 s for Hcy, Cys and within 5 min for GSH, indicating that probe reacts rapidly with thiols under the experimental conditions (Fig. S5, ESI†). This unprecedented fast response could provide the possibility of quantitative detection without any pretreatment of samples.

### 3.5. pH dependence

The effect of pH on the fluorescence properties of the system was also investigated. Fig. S6 showed the fluorescence intensity obtained for the free probe and probe-Hcy in different pH values. Over the pH 2.0–6.0, the fluorescence emission of probe 1 is strong, but Hcy induced a small fluorescence change for probe. It is evident that the fluorescence signal of the probe decreased sharply from pH 7.0 to 9.0 and even to no fluorescence in the pH values from 12.0 to 13.0. When pH value exceeds 6.0, the fluorescence change of probe solution containing Hcy becomes obvious. Therefore, the pH 7.0 is effective for this probe and was used for further studies.

### 3.6. Proposed mechanism

To explore the detection mechanism, 2-mercaptoethanol (ME) was selected as the thiol species for the mechanism study based on previously reported methods.<sup>[6]</sup> The reaction between probe 1

and ME was monitored by <sup>1</sup>H NMR (Fig. S7). Upon addition of ME in solution of probe, one of C=C protons in the <sup>1</sup>H NMR of probe shift to high field ( $\delta$  5.27) due to the interrupt of conjugate system. Protons of methyl connected with N atom in the <sup>1</sup>H NMR split into two peaks ( $\delta$  4.13, 3.12), and also protons of methyl connected with C atom split into two peaks ( $\delta$  1.78, 1.36). These changes indicate the reaction of ME with the C=N double bond (Scheme 2). Mass spectral analysis for its adduct showed corroborative evidence for the product (probe-ME) at 522.92 m/z (calcd 523.28 for [probe+ME-I<sup>+</sup>]) (Fig. S8, ESI†). Therefore, the nucleophilic attack of thiol on the C=N double bond between the two main molecular moieties would interrupt the conjugation, which would contribute to the blue shift in the absorbance spectra, and quenching to a certain extent in the emission spectra. It is noted that some nucleophilic reagents such as CN<sup>-</sup> and HS<sup>-</sup> did not response for probe, which was attributed to the sulfhydryl having more strong nucleophilicity than others.

### 3.7. Cellular Imaging

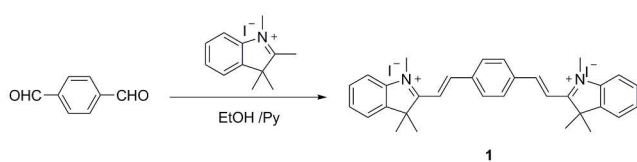
In order to further demonstrate the permeability and real time monitoring thiols in living cells, confocal microscopy experiments were carried out. As shown in Fig. 5A, under selective excitation at 450 nm, HepG2 cells incubated with probe 1 (15  $\mu\text{mol/L}$ ), a certain yellow fluorescence was exhibited inside the cells. In a control experiment, when cells were pretreated with N-ethylmaleimide (NEM, 20  $\mu\text{mol/L}$ , 30 min, a trapping reagent of thiol species), a little stronger fluorescence intensity was observed (Fig. 5B). In a further experiment it was found that HepG2 cells displayed almost no fluorescence (Fig. 5C) when the cells were first incubated with 15  $\mu\text{mol/L}$  of probe for 30 min at 37°C and then incubated with Hcy (200  $\mu\text{mol/L}$ ), indicating the specific detection of thiols by 1. Confocal fluorescence images in HepG2 cells incubated with probe 1 and GSH also carried out. (Fig. S9, ESI†) These results suggest that probe 1 can enter cells and make fluorescent labeling.

### 4. Conclusions

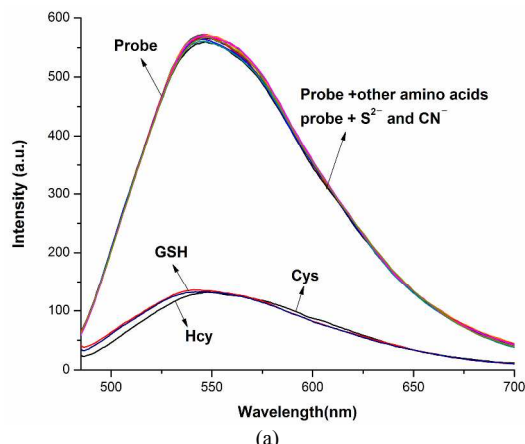
In summary, we have developed a fluorescent probe for the detection of Cys, Hcy and GSH over other natural amino acids in aqueous solution. The probe displayed a dramatic change in fluorescence intensity selectively for Cys, Hcy and GSH over other natural amino acids in HEPES buffer (10 mmol/L, pH 7.0). In combination with photochemical properties, convenient synthetic procedure, water solubility and neutral nature of the compound under physiological conditions allow this probe to act as an effective tool for mark Cys, Hcy and GSH within living cells. We believe that this effort has shown the utility of a new strategy to design bio-thiols selective probes.

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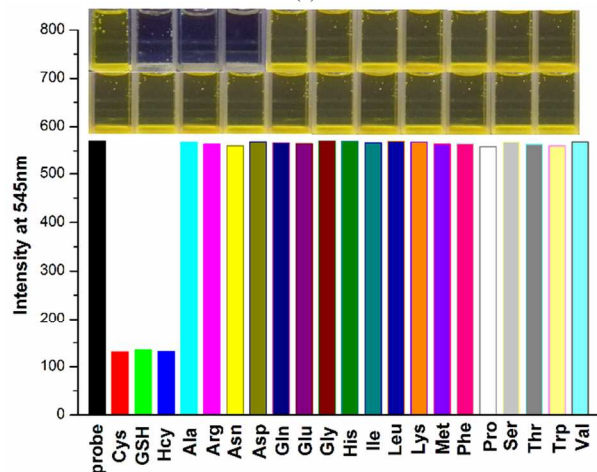
### Inserting Graphics



Scheme 1 The synthesis of the probe.

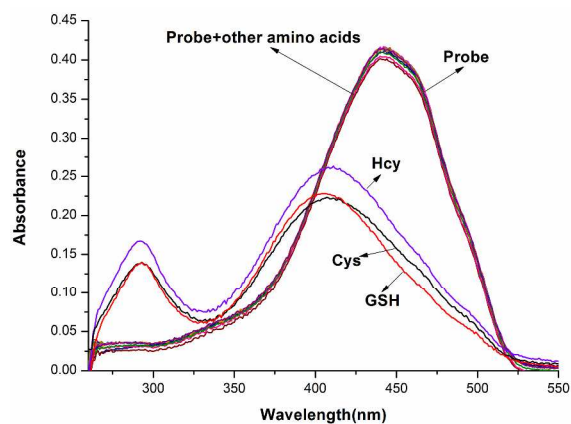


(a)

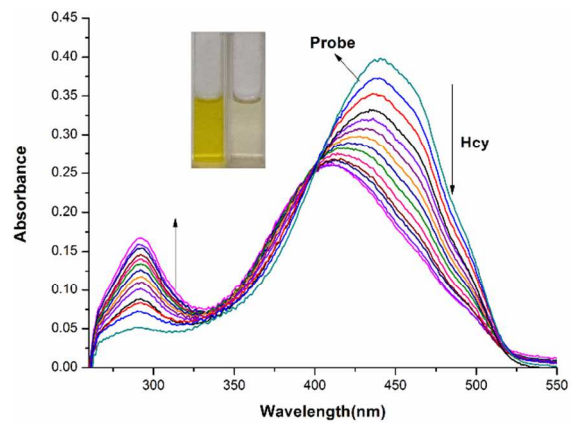


(b)

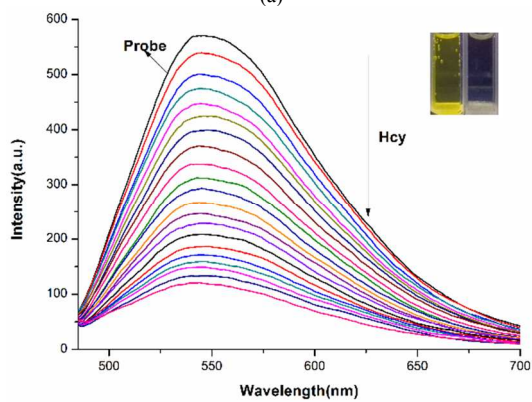
**Fig. 1** (a) Fluorescence emission spectra of the probe (15  $\mu\text{mol/L}$ ) in HEPES: DMF = 1:1 (V/V pH=7.0) in the presence of 1000  $\mu\text{M}$  Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Val and Hcy (480  $\mu\text{mol/L}$ ), GSH (280  $\mu\text{mol/L}$ ), Cys (700  $\mu\text{mol/L}$ ). (b) Optical density graph of the probe (15  $\mu\text{mol/L}$ ) at 545 nm upon the addition of several analytes (1000  $\mu\text{mol/L}$ ) for 5 min in HEPES: DMF = 1:1 (V/V pH=7.0) ( $\lambda_{\text{ex}}$ =450 nm, slit: 5/5 nm). Inset: a color change photograph for thiol and other amino acids under illumination with a 365 nm UV lamp.



**Fig. 2** The absorption spectra of the probe (5  $\mu\text{mol/L}$ ) in the presence of HEPES: DMF = 1:1 (V/V pH=7.0) in the presence of 1000  $\mu\text{mol/L}$  Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Val and Hcy (190  $\mu\text{mol/L}$ ), GSH (90  $\mu\text{mol/L}$ ), Cys (375  $\mu\text{mol/L}$ ).



(a)



(b)

**Fig. 3** (a) UV-vis spectra of probe (5  $\mu\text{mol/L}$ ) in HEPES: DMF = 1:1 (V/V pH=7.0) upon addition of various concentrations of Hcy (0–190  $\mu\text{mol/L}$ ); (b) Fluorescence spectra of the probe (15  $\mu\text{mol/L}$ ) in the presence of various concentrations of Hcy (0–480  $\mu\text{mol/L}$ ) in HEPES: DMF = 1:1 (V/V pH=7.0) ( $\lambda_{\text{ex}}$ =455nm, slit: 5 nm/5 nm).

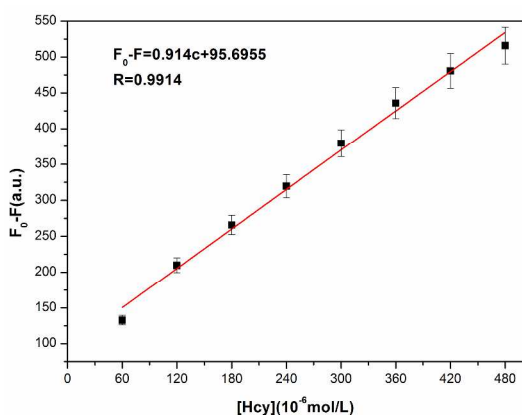


Fig. 4. The linearity of the relative fluorescence intensity versus Hcy concentration.

Method	Analyte	Signal output	Solvent	Detection limit
Ref.[26]	Hcy	Fluorescence	CH <sub>3</sub> CN/H <sub>2</sub> O	0.12 μM
Ref.[46]	Hcy	Fluorescence	DMSO/H <sub>2</sub> O	1.19 μM
Ref.[47]	Hcy	Fluorescence	DMSO/H <sub>2</sub> O	1.96 μM
Ref.[48]	Hcy	Fluorescence	CH <sub>3</sub> CN/H <sub>2</sub> O	0.13 μM
This work	Hcy	Fluorescence	DMF/H <sub>2</sub> O	0.035 μM

Table 1 A comparison table about the detection limits for Hcy.



Scheme 2. The mechanism of chemosensor.

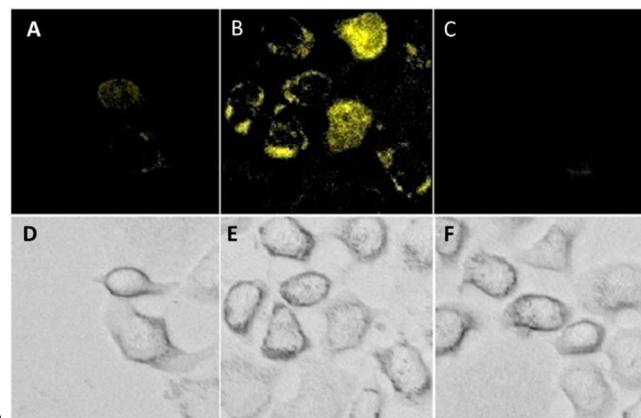


Fig.5. Confocal fluorescence images of HepG2 cells: (a) Fluorescence image of HepG2 cells with adding probe (15 μmol/L) and its bright field image (d); (b) Fluorescence image of HepG2 cells incubated with 15 μmol/L probe after pretreated with NEM (20 μmol/L) for 30 min at 37°C and its bright field image (e). (c) Fluorescence image of HepG2 cells incubated with 15 μmol/L probe and 200 μmol/L Hcy for 30 min at 37°C and its bright field image (f).

## Notes and references

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## Abstract Graphic

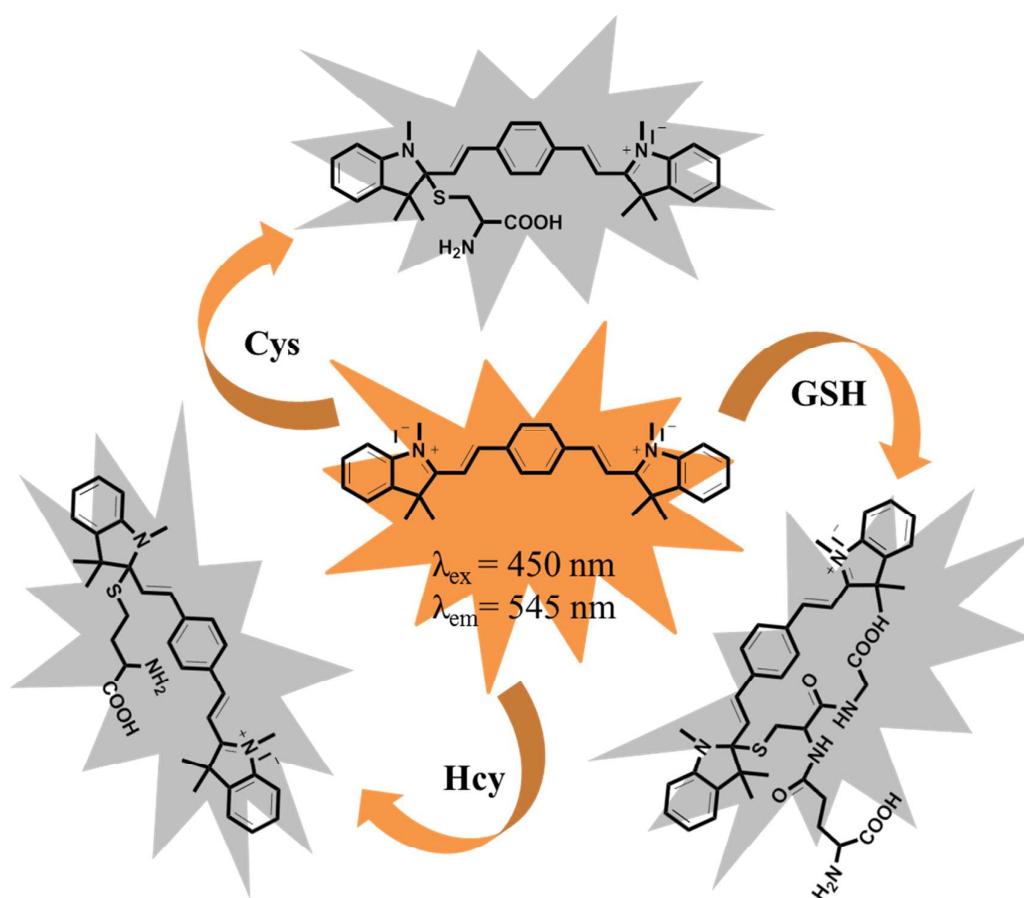
### The Title:

A Fluorescent Probe for Thiol Based on Strong Nucleophilic Attack of Sulfhydryl and Its bioimaging

### List of Authors:

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The statement:



The sensor displayed selectivity for copper ions, as evidenced by a green fluorescence to colorless change, was characterized using fluorescence spectroscopy and its potential application to bioimaging was also illustrated.