Fluorescence Detection of Biological Thiols

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Fluorescence Detection of Biological Thiols

by

Yixing Guo

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science
in
Chemistry

Thesis Committee:
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Shankar B. Rananavare
Reuben H. Simoyi

Portland State University
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ABSTRACT

Glutathione (GSH) performs significant biological functions such as serving as an antioxidant. It protects cells from oxidative stress by trapping free radicals that damage DNA and RNA.\(^1\) It also has an important role in maintaining the reducing environment in cells.\(^2\) The rapid, sensitive and highly selective detection of GSH is of importance for investigating its function in disease and in disease diagnosis. We have found that in cetyl trimethylammonium bromide (CTAB) medium, a resorufin-based probe shows an instantaneous, highly selective response to GSH.

Cysteine (Cys) is involved in protein synthesis, detoxification, metabolic processes, etc.\(^3\) Abnormal levels of Cys are related to many disorders, such as impaired growth, Alzheimer’s disease and cardiovascular disease.\(^4\) Thus, the detection and quantification of Cys in physiological media is of interest. Herein, organic fluorescent indicators for the detection and quantification of Cys and GSH are presented. The probes function in human plasma. The chemical mechanisms involved are discussed.
ACKNOWLEDGEMENTS

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# TABLE OF CONTENTS

ABSTRACT....................................................................................................................................................i

ACKNOWLEDGEMENTS..........................................................................................................................ii

LIST OF FIGURES...........................................................................................................................................v

LIST OF SCHEMES.......................................................................................................................................ix

LIST OF ABBREVIATIONS.........................................................................................................................x

SECTION

1. INTRODUCTION.........................................................................................................................................1

2. EXPERIMENTAL.......................................................................................................................................11
   2.1. Instrumentation..................................................................................................................................11
   2.2. Materials..............................................................................................................................................11
   2.3. Synthesis of probe 8.........................................................................................................................12
   2.4. Synthesis of probe 5.........................................................................................................................15

3. RESULTS AND DISCUSSION..............................................................................................................18
   3.1 Selective detection of GSH with resorufin based probe.................................................................18
   3.2 Mechanism Study of GSH detection..............................................................................................22
   3.3 Selective detection of Cys using 8....................................................................................................27
   3.4 Measurement of Cys content in human plasma using 5...............................................................34

4. CONCLUSION..........................................................................................................................................36
5. REFERENCES

38
LIST OF FIGURES

Figure 1. Time-dependent fluorescence spectral changes of 4 (20 μM) with 1 equiv Cys or Hcy...............................................................4

Figure 2. Color changes of the solution of 5 (10 μM) in the presence of different biothiols (2 equiv) in 1.0 mM CTAB media buffered at pH 7.4.................................7

Figure 3. Fluorescence spectra (λex = 550 nm) of 5 (10 μM) with the addition of 1 equiv of amino acids.................................................................8

Figure 4. Time dependent fluorescence spectral of 4 (20μM) with GSH (1 equiv) in EtOH : Phosphate buffer= 8:2 (20mM,pH 7.4)..................................................9

Figure 5. Time dependent fluorescence spectral of 4 (20μM) with GSH (1 equiv) in buffered 1mM CTAB.................................................................9

Figure 6. H1NMR of 8 in DMSO-d6.........................................................13

Figure 7. 13C MNR spectrum of 8 in DMSO-d6.........................................................13

Figure 8. HRMS of 8..................................................................................14

Figure 9. 1H MNR (400 MHz) spectrum of 5 in CDCl3.................................16

Figure 10. 13C MNR spectrum of 5 in CDCl3.........................................................17

Figure 11. HRMS of 5 in CDCl3.....................................................................17
Figure 12. Color changes of the solution of 8 (10 μM) in the presence of biothiols (2 equiv) in 2.0 mM CTAB media buffered at pH 7.4.................................19

Figure 13. Fluorescence spectra (λex = 565 nm) of 8 (2.5 μM) with the addition of increasing concentrations of GSH in 2.0 mM CTAB media buffered at pH 7.4..............20

Figure 14. Kinetic curve of 8 (2.5 μM) with the addition of 2 equiv of biothiols in 2 mM CTAB media buffered at pH 7.4. λex / λem = 565/590 nm.................................21

Figure 15. Fluorescence spectra (λex = 565 nm) of 8 (2.5 μM) with the addition of 2 equiv of amino acids.................................................................22

Figure 16. Kinetic curve of 8 (2.5 μM) with the addition of 2 equiv of biothiols SDS buffered at pH 7.4. λex / λem = 565/590 nm.............................................23

Figure 17. Kinetic curve of 8 (2.5 μM) with the addition of 2 equiv of biothiols trition X-100 buffered at pH 7.4. λex / λem = 565/590 nm.................................24

Figure 18. Kinetic curve of 8 (2.5 μM) with the addition of 2 equiv of biothiols in BC media buffered at pH 7.4. λex / λem = 565/590 nm.................................24

Figure 19. Kinetic curve of 8 (2.5 μM) with the addition of 2 equiv of GSH in different surfactants buffered at pH 7.4. λex / λem = 565/590 nm.................................25

Figure 20. Mass spectrum of the formation of twelve member ring in CTAB........26

Figure 21. Mass spectrum of the formation of twelve member ring in CTAB........26
Figure 22. Kinetic curve of probe (2.5 μM) with the addition of 2 equiv of biothiols in DMSO/H2O=1:1 buffered at pH 7.4. λ_{ex}/λ_{em} = 565/594 nm

Figure 23. Color changes of the solution of 1 (10 μM) in the presence of biothiols (2 equiv) in buffered Triton X-100

Figure 24. Fluorescence spectra of probe (2.5 μM) upon addition of different amounts of cysteine pH 7.4 phosphate buffer solution

Figure 25. Kinetic plot of a solution containing Probe (2.5 μM) upon addition of different thiols (12.5μM) in phosphate buffer

Figure 26. Absorbance spectra of solutions containing probe (2.5 μM) upon addition of 12.5 μM of cys in phosphate buffer

Figure 27. Emission spectra of solutions containing probe (2.5 μM) in the presence of 12.5 μM cysteine in phosphate buffer (50 mM pH=7.4) λ_{ex} = 565 nm

Figure 28. Absorbance spectra of 8 (2.5 μM) with the addition of N-Acetyl Cysteine in 50mM pH=7.4 phosphate buffer for 90 min

Figure 29. Fluorescence spectra (λ_{ex} = 565 nm) of 8 (2.5 μM) with the addition of N-Acetyl Cysteine in 50mM pH=7.4 phosphate buffer for 90 min

Figure 30. HRMS of 3a

Figure 31. HRMS of formation of 3b
Figure 32. HRMS of formation of twelve membered ring.................................34

Figure 33. Fluorescence spectra ($\lambda_{ex} = 550$ nm) of 4 (10 $\mu$M) in pH 7.4 Hepes buffer in the presence of 1.0 mM CTAB at different conditions for 25 min.................................35

Figure 34. In buffered CTAB medium (50mM phosphate buffer pH=7.4)..................36

Figure 35. In buffered Triton X-100 (50mM phosphate buffer pH=7.4).....................36
LIST OF SCHEMES

Scheme 1. The mechanisms of the formation of 3-carboxy-5-oxoperhydro-1,4-thiazepines (3) from the condensation reaction of acrylates and Cys or Hcy .......................................................................................................................... 3

Scheme 2. Structures of enol and keto forms of HBT .................................................. 3

Scheme 3. Synthesis of 4 ............................................................................................... 4

Scheme 4. Proposed mechanism for sensing Hcy and Cys ........................................... 4

Scheme 5. Synthesis of 5 ............................................................................................. 5

Scheme 6. Proposed mechanism of Cys detection using 5 .......................................... 6

Scheme 7. The binding of GSH to CTAB surface ......................................................... 10

Scheme 8. Synthesis of 8 .............................................................................................. 12

Scheme 9. Synthesis of seminaphthofluorescein (SNF) ........................................... 15

Scheme 10. Proposed mechanism of Cys detection using 8 ....................................... 28
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC</td>
<td>Benzalkonium chloride</td>
</tr>
<tr>
<td>CDCl₃</td>
<td>Deuterated chloroform</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyl trimethylammonium bromide</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO-d₆</td>
<td>Deuterated dimethyl sulfoxide</td>
</tr>
<tr>
<td>EA</td>
<td>Ethyl acetate</td>
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<tr>
<td>Glutathione</td>
<td>GSH</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>Hcy</td>
<td>Homocysteine</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid)</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance Spectroscopy</td>
</tr>
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</table>
PET  Photoinduced Electron Transfer

PPh$_3$  Triphenylphophine oxide

SDS  Sodium dodecyl sulfate

SNF  Seminaphthofluorescein

TEA  Triethylamine

UV-Vis  Ultraviolet-visible spectroscopy

H$_2$O  Water
SECTION 1

INTRODUCTION

The detection of bioactive thiols is of great current interest. Our program focuses on developing convenient methods for the detection of small biological molecules. This thesis describes new sensing methods for detecting GSH and Cys.

1.1 Research goals

My research goal is to detect biologically relevant amino acids under physiological conditions, which mainly include (i) uncovering the differences in the organic chemistry of biological thiols; (ii) understanding the fundamental organic chemistry relevant to the involvement of Cys and GSH in disease; (iii) creating simple new methods which will lead to selective, straightforward detection of Cys and GSH.

The approach involves synthetic fluorescent organic sensing agents. Understanding the detection mechanism, quenching and generation of fluorescence are the important aspects of the work. There have been many probes for biological thiols. However, indicators that are selective for specific biothiols, especially GSH, are relatively rare and more research is necessary. Several recent papers, for instance, describe GSH selectivity; however, the indicators display significant responses to Cys and related nucleophilic thiols. A promising GSH-selective probe for selective intracellular imaging applications has been developed by Shao et al.
Innovative nanoparticle or polymeric indicators for GSH also exhibit high selectivity, however, they either have not been used successfully to date in biological media, are based on toxic CdSe\textsuperscript{12} or require the handling of highly toxic mercury salts to function.\textsuperscript{13-14}

1.2 Simultaneous Fluorescence Detection of Cysteine and Homocysteine

The condensation of certain acrylates with Cys results in the formation of substituted 1,4-thiazepines.\textsuperscript{15} The mechanism involves conjugate addition to the acrylates (such as 1\textsubscript{a}) to generate thioethers (2\textsubscript{a}), which undergo an intramolecular cyclization to yield 3\textsubscript{a} as illustrated in Scheme 1.1. In the case of Hcy, the corresponding thioether 2\textsubscript{b} should be readily generated.\textsuperscript{16} However, the intramolecular cyclization reaction to form an eight member ring should be kinetically less favorable compared to the formation of 3\textsubscript{a}.\textsuperscript{17} Therefore, we designed a probe to discriminate Cys and Hcy based on a new sensing mechanism: the difference in the relative rates of intramolecular cyclizations.

It is well known that 2-(2'\textprime-hydroxyphenyl)benzothiazole (HBT) exhibits dual emission bands due to an excited-state intramolecular photon-transfer (ESIPT) process via enol and keto tautomeric forms.\textsuperscript{18-19}
Scheme 1. The mechanism of the formation of 3-carboxy-5-oxoperhydro-1,4-thiazepines (3) from the condensation reaction of acrylates and Cys or Hcy.

Scheme 2. Structures of enol and keto forms of HBT.

We hypothesized that the synthesis of an acrylate derivative of 2-(2’-hydroxy -3’-methoxyphenyl)benzothiazole (HMBT) 4 (Scheme 3) may allow the selective differentiation of Cys and Hcy based on different reaction rates as shown in Scheme 4. The sensing mechanism of the HMBT acrylate probe combines PET and ESIPT. Figure 1 shows the response of 4 to Cys and Hcy at different wavelengths.20

Scheme 4. Proposed mechanism for sensing Hcy and Cys.

Figure 1. Time-dependent fluorescence spectral changes of 4 (20 μM) with 1 equiv Cys (a) or Hcy (c) in EtOH:phosphate buffer (20 mM, pH 7.4, 2:8 v/v); (b) and (d)
are time-dependent fluorescence intensity changes of 4 (20 μM) in the presence of 1 equiv Cys and Hcy, respectively. \( \lambda_{ex} = 304 \text{ nm.} \)

1.3 Highly selective detection of Cys using SNF probe

Due to the low quantum yield\(^{21}\) and short excitation wavelength (304 nm) of HMBT which may limit its application in biological samples, we synthesized the SNF probe (Scheme 5) for the long wavelength, highly selective detection of Cys.\(^{22}\)

Scheme 5. Synthesis of 5.

The sensing mechanism couples the conjugate addition/cyclization mechanism to a xanthene dye spirolactone-opening reaction as illustrated in Scheme 6.
Interestingly, upon mixing Cys in colorless solution of 5 in 1.0 mM cetyltrimethylammonium bromide media (CTAB) buffered at pH 7.4, both a pink color and strong fluorescence appeared gradually, while solutions other amino acids and biothiols, such as Hcy and GSH afforded no obvious changes under the same conditions. This interesting feature indicates that 5 can serve as a selective “naked-eye” dosimeter for Cys.

Scheme 6. Proposed mechanism of Cys detection using 5.
Figure 2. Color changes of the solution of 5 (10 μM) in the presence of different biothiols (2 equiv) in 1.0 mM CTAB media buffered at pH 7.4 (Hepes buffer, 20 mM) after 25 min.

To evaluate the selectivity of 5 for Cys, changes in the fluorescence intensity of 5 caused by other analytes were also measured. Figure 4 shows the fluorescence spectra of the solution of 5 after the addition of these analytes (25 min). It was observed that other amino acids and biothiols promote almost no fluorescence intensity changes at the same conditions, demonstrating that the selectivity of 5 for Cys over other analytes is excellent.
Figure 3. Fluorescence spectra ($\lambda_{ex} = 550$ nm) of 5 (10 $\mu$M) with the addition of 1 equiv of Cys, Hcy, GSH, leucine, proline, arginine, histidine, valine, methionine, threonine, glutamine, alanine, aspartic acid, norleucine, isoleucine, lysine, tryptophan, tyrosine, phenylalanine, cystine and homocystine for 25 min in 1 mM CTAB buffered at pH 7.4 (Hepes buffer, 20 mM).

1.4 The role of surfactants

In CTAB media, GSH reacts significant faster with the HMBT acrylate probe (compare Figures 4 and 5.) to form the conjugate addition product.\textsuperscript{23} In fact, the conjugate addition is faster than both Cys and Hcy. However, in order to achieve GSH selectivity, one needs a method to catalyze large (12-membered) ring
formation as compared to 7- and 8-membered ring closure as in the cases of Cys and Hcy.

It has been reported that CTAB can catalyze large ring (8-14 atom) formation. In CTAB medium, the order of preference for ring closure of medium membered rings vs. large membered rings can be reversed.²⁴ It is also known that GSH binds the CTAB micelle surface²⁵ due to favorable electrostatic interactions between carboxylate and ammonium moieties (Scheme 7). We thus reasoned that a more planar dye such as 8 may bind more tightly in the micelle²⁶ thereby facilitating large ring closure and selective detection of GSH.

**Figure 4.** Time dependent fluorescence spectral of 4 (20μM) with GSH (1 equiv) in EtOH : Phosphate buffer= 8:2 (20mM,pH 7.4).

**Figure 5.** Time dependent fluorescence spectral of 4 (20μM) with GSH (1 equiv) in buffered 1mM CTAB (Phosphate buffer (20mM,pH 7.4).
Scheme 7. The binding of GSH to CTAB surface.$^{25}$

Structure of resorufin acrylate probe.
SECTION 2

EXPERIMENTAL

2.1 Instrumentation

$^1$H-NMR and $^{13}$C-NMR spectra were recorded on a Bruker AMX-400 NMR spectrometer, using TMS as an internal standard. ESI-HRMS (high resolution mass spectrometry) spectra were obtained on a Thermo Electron LTQ Orbitrap hybrid mass spectrometer. UV-visible spectra were collected on a Cary 50 UV-Vis spectrophotometer; Fluorescence spectra were collected on a Cary Eclipse (Varian, Inc.) fluorescence spectrophotometer with slit widths were set at 5 nm for both excitation and emission, respectively. Unless specifically noted, the high voltage of the fluorescence spectrophotometer was set at 600 V for SNF and 500 V for resorufin-based probe. The pH measurements were carried out with an Orion 410A pH meter.

2.2 Materials

In all experiments enantiomerically pure natural amino acids were used except for Hcy which was used as the racemate. Cetyl trimethylammonium bromide (CTAB), Triton X-100, sodium dodecyl sulfate (SDS), hydrochloric acid (HCl), sodium hydroxide (NaOH), N-acetyl-L-cysteine ethanol, methanol, sodium sulfate (Na$_2$SO$_4$), dichloromethane (DCM), triethylamine (TEA), ethyl acetate (EA), acetone, sodium phosphate (NaHPO$_4$), disodium phosphate (Na$_2$HPO$_4$), (4-(2-hydroxyethyl)-1-
pierazinethanesulfonic acid) (HEPES), triphenylphospine (PPh₃), resorufin, SNF, deuterated DMSO, deuterated chloroform, human plasma, etc., were commercially available and used without further purification.

2.3 Synthesis of probe 8


To a solution of resorufin (220mg) and Et₃N (1.5 equiv) in 15ml of anhydrous CH₂Cl₂, acryloyl chloride (2.0 eq, mixed with 5 mL of CH₂Cl₂) was added dropwise at 0°C. After stirring at this temperature for 60 min, the resulting mixture was allowed to warm to rt and stirred overnight. The mixture was then diluted with CH₂Cl₂ (20 mL), washed with water (10 mL ×3) and dried over anhydrous Na₂SO₄. The solvent was removed by evaporation to get product as orange solids (172mg 64% yield). Probe 8 is fully characterized by H¹NMR, C¹³ NMR and HRMS. (Figures 6-8)
Figure 6. $^1$H NMR of 8 in DMSO-d6.

Figure 7. $^{13}$C MNR spectrum of 8 in DMSO-d6.
Figure 8. HRMS of 8.
2.4 Synthesis of probe 5

SNF was synthesized in two steps according to the method reported by Lippard et al.\textsuperscript{27} \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400MHz), δ (ppm): 8.44 (d, 1H, \(J = 9.2\) Hz), 8.04 (d, 1H, \(J = 7.2\) Hz), 7.74 (m, 2H), 7.30 (d, 1H, \(J = 9.2\) Hz), 7.26 (dd, 1H, \(J_1 = 2.4\) Hz, \(J_2 = 9.2\) Hz), 7.19 (d, 1H, \(J = 7.6\) Hz), 7.12 (s, 1H), 6.62 (m, 2H), 6.51 (d, 1H, \(J = 8.8\) Hz), 2.51 (s, 3H). ESI-FTMS \(m/z = 395.0923\) [M-H]\textsuperscript{-}, calc. 395.0919 for C\textsubscript{25}H\textsubscript{15}O\textsubscript{5}.

Synthesis of 5

To a solution of SNF (120 mg, 0.30 mmol) and Et\textsubscript{3}N (2 equiv) in 10 mL of anhydrous CH\textsubscript{2}Cl\textsubscript{2}, acryloyl chloride (2.5 eq, mixed with 5 mL of CH\textsubscript{2}Cl\textsubscript{2}) was added dropwise at 0°C. After stirring at this temperature for 90 min, the resulting mixture was allowed to warm to rt and stirred overnight. The mixture was then diluted with CH\textsubscript{2}Cl\textsubscript{2} (25
mL), washed with water (12 mL ×3) and dried over anhydrous Na₂SO₄. The solvent was removed by evaporation to get crude product as yellow solids, which was then purified by flash column chromatography (silica gel, CHCl₃/EtAC 100:6) to get 4 as light yellow solids (99 mg, 65% yield). ¹H NMR (CDCl₃, 400MHz), δ (ppm): 8.58 (d, 1H, J = 9.2 Hz), 8.07 (d, 1H, J = 8.0 Hz), 7.66 (m, 3H), 7.46 (m, 2H), 7.16 (d, 1H, J = 6.4 Hz), 6.83 (m, 2H), 6.76 (d, 1H, J = 8.4 Hz), 6.70 (br s, 1H), 6.66 (br s, 1H), 6.41 (dd, 1H, J₁ = 1.6 Hz, J₂ = 10.4 Hz), 6.37 (dd, 1H, J₁ = 1.6 Hz, J₂ = 10.4 Hz), 6.08 (m, 2H), 2.51 (s, 3H). ¹³C NMR (CDCl₃, 100 MHz), δ 169.52, 164.57, 164.05, 153.67, 150.31, 150.15, 149.84, 146.93, 135.38, 135.27, 133.49, 133.22, 130.09, 127.90, 127.49, 125.31, 124.83, 124.28, 123.96, 123.60, 122.09, 121.99, 119.45, 118.88, 118.15, 116.49, 112.42, 82.92, 9.87. ESI-FTMS m/z = 505.1308 [M+H]+, calc. 505.1287 for C₃₁H₂₁O₇.

Figure 9. ¹H MNR (400 MHz) spectrum of 5 in CDCl₃.
Figure 10. $^{13}$C MNR spectrum of 5 in CDCl$_3$.

Figure 11. HRMS of 5 in CDCl$_3$. 
3.1 Selective detection of GSH with resorufin based probe (8)

Methods for the direct detection of GSH are hampered due to interference from common, structurally related biological thiols such as Cys and Hcy. In this project, we developed highly selective methods for the detection of GSH using inexpensive, available materials. The mechanism involved in the detection helps understanding of the unique chemistry of GSH and may also help us to understand the role of GSH in disease.

Equivalent amounts of GSH, Cys and Hcy are added to 4 ml solutions of resorufin-acrylate probe (10 μM) in phosphate buffer (50 mM, pH=7.4), respectively. The final concentration of probe is 10μM, the thiols concentration are 20μM. A strong pink color formed in the GSH containing solution immediately upon addition of CTAB (Figure 12). Other amino acids, such as Hcy and Cys, did not show obvious changes under the same conditions. This interesting feature indicates that 8 can serve as a selective visual inspection dosimeter for GSH.
Figure 12. Color changes of the solution of 8 (10 μM) in the presence of biothiols (2 equiv) in 2.0 mM CTAB media buffered at pH 7.4 (Phosphate buffer, 50 mM), the picture was taken immediately upon the addition of CTAB.

The sensitivity of 8 was studied by fluorescence changes as a function of GSH concentrations as shown in Figure 13. The fluorescence enhancement displayed a linear relationship with the concentration of GSH, coefficient = 0.994. The GSH concentration was varied from 0.2 μM-6μM, within the range of GSH in human plasma.28
Figure 13. Fluorescence spectra ($\lambda_{ex} = 565$ nm) of 8 (2.5 $\mu$M) with the addition of increasing concentrations of GSH in 2.0 mM CTAB media buffered at pH 7.4 (Phosphate buffer, 50 mM). The inset Figure shows the changes in fluorescence intensity at 587 nm as a function of GSH concentration. The spectral was taken immediately upon the addition of CTAB.

The time course of the reaction between 8 and GSH is shown in Figure 14. It can be seen that the fluorescence of the reaction with GSH reaches a high intensity immediately; however, for Hcy and Cys, there is no significant fluorescence enhancement during this period.
To evaluate the selectivity of 8 towards GSH, control experiments using other amino acids were done and the fluorescence changes were measured. It is obvious that other amino acids barely produce fluorescence enhancement as compared to GSH, demonstrating that the selectivity of 8 for GSH over other amino acids is excellent (Figure 15). To further test the selectivity of 8 toward GSH, competition experiments were conducted with excess of other amino acids added to GSH, and no significant fluorescence intensity difference was observed in comparison to solutions containing GSH only.
**Figure 15.** Fluorescence spectra ($\lambda_{ex} = 565$ nm) of 8 (2.5 μM) with the addition of 2 equiv of Cys, Hcy, GSH, leucine, proline, arginine, histidine, valine, methionine, threonine, glutamine, alanine, aspartic acid, norleucine, isoleucine, lysine, tryptophan, tyrosine, phenylalanine, cystine and homocystine in 2 mM CTAB buffered at pH 7.4 (Phosphate buffer, 50mM). Emission spectra is immediately monitored after the addition of thiols and other amino acids.

3.2 Mechanism Study of GSH detection

Experiments were carried out to understand the mechanism involved in GSH detection. First, different surfactants, such as sodium dodecyl sulfate (SDS), benzalkonium chloride (BC) and Triton X-100 were employed. It was found that only in the presence of cationic surfactants (such as CTAB and BC), can 8 afford
selective detection of GSH. In SDS or Triton X-100, 8 shows excellent selectivity towards Cys instead of GSH (Figures 16 and 17). This result strongly suggests the role of favorable Coulombic attractions between cationic micelles and GSH (Figures 18 and 19).

Figure 16. Kinetic curve of 8 (2.5 μM) with the addition of 2 equiv of biothiols SDS buffered at pH 7.4. $\lambda_{ex}/\lambda_{em} = 565/590$ nm.
Figure 17. Kinetic curve of 8 (2.5 μM) with the addition of 2 equiv of biothiols trition X-100 buffered at pH 7.4. $\lambda_{\text{ex}} / \lambda_{\text{em}} = 565/590$ nm.

Figure 18. Kinetic curve of 8 (2.5 μM) with the addition of 2 equiv of biothiols in BC media buffered at pH 7.4. $\lambda_{\text{ex}} / \lambda_{\text{em}} = 565/590$ nm.
Figure 19. Kinetic curve of 8 (2.5 μM) with the addition of 2 equiv of GSH in different surfactants buffered at pH 7.4. \( \lambda_{ex} / \lambda_{em} = 565/590 \) nm.

The formation of a 12 membered ring and free dye resorufin in CTAB medium was confirmed by a major peak at m/z 360.08 in the MS. (Figures 20 and 21). Moreover, since surfactants mimic organic environments, we also studied GSH detection in DMSO:buffer (1:1) and found that the resorufin acrylate probe does show selectivity for GSH as shown in Figure 22.
Figure 20. Mass spectral of the formation of twelve member ring in CTAB micelle.

Figure 21. Mass spectral of the formation of twelve member ring in CTAB micelle.
**Figure 22.** Kinetic curve of probe (2.5 μM) with the addition of 2 equiv of biothiols in DMSO/H2O=1:1 buffered at pH 7.4. $\lambda_{\text{ex}} / \lambda_{\text{em}} = 565/594$ nm.

3.3 Selective detection of Cys using 8

In buffer without a cationic surfactant, as expected, 8 shows excellent selectivity towards Cys due to the reported kinetically favored seven member ring formation. Upon mixing Cys with 8, the conjugate addition product 8a is formed, which then undergoes a rapid cyclization reaction to produce 3a while releasing the free resorufin (Scheme 10) and significant fluorescence enhancement can be observed. In the case of Hcy, because it has an additional methylene group in its side chain, a kinetically less favored 8-membered ring would form. As for GSH, 1,4-addition of
thiols to the α,β-unsaturated carbonyl moieties of 8 can occur readily; however, the ensuing intramolecular cyclization similar to that of Cys cannot proceed without the presence of surfactant.


Equivalent amounts of GSH, Cys and Hcy are added to 4ml solutions of resorufin-based probe (10μM) in phosphate buffer (50mM, pH=7.4), respectively. The final concentration of probe is 10μM, the thiols concentration are 20μM. A strong pink color formed in the Cys containing solution after about 10mins. While for other amino acids, such as Hcy and GSH did not show obvious change at the same condition. This interesting feature indicates that 8 can also serve as a selective “naked-eye” dosimeter for Cys (Figure 23).
Figure 23. Color changes of the solution of 1 (10 μM) in the presence of biothiols (2 equiv) in buffered Triton X-100 (50mM phosphate buffer, pH 7.4) the picture was taken 10 minutes after the addition of thiols.

The fluorescence spectra of the solution from the reaction between 8 and Cys is monitored as shown in Figure 19. It can be observed that the fluorescence intensity at 587nm increases with an increasing concentration of Cys within human plasma cysteine range. The observed fluorescence intensity has a good linear relationship with Cys concentration with an R value of 0.995. As low as 0.2 μM of Cys can be readily detected.
Figure 24. Fluorescence spectra of probe (2.5 μM) upon addition of different amounts of cysteine pH 7.4 phosphate buffer solution. Reaction time, 60min.

In addition, the time course of the fluorescence assay is shown in Figure 25. It can be seen that the fluorescence of the reaction with Cys increases with time and reaches a plateau after about 90 min, whereas for Hcy and GSH, the rates are significantly slower.
Figure 25. Kinetic plot of a solution containing Probe (2.5 µM) upon addition of different thiols (12.5µM) in phosphate buffer (50 mM pH=7.4).

To prove the necessity of the presence of a free NH$_2$ control experiments using N-acetyl cysteine have been performed (Figures 26-29). The results indicate that the free NH$_2$ group is required as a nucleophile for the intramolecular cyclization and addition/elimination reaction to occur.
**Figure 26.** Absorbance spectra of solutions containing probe (2.5 μM) upon addition of 12.5 μM of cys in phosphate buffer (50 mM pH=7.4).

**Figure 27.** Emission spectra of solutions containing probe (2.5 μM) in the presence of 12.5 μM cysteine in phosphate buffer (50 mM pH=7.4) λ<sub>ex</sub> = 565 nm.

**Figure 28.** Absorbance spectra of 8 (2.5 μM) with the addition of N-Acetyl Cysteine in 50mM pH=7.4 phosphate buffer for 90mins.

**Figure 29.** Fluorescence spectra (λ<sub>ex</sub> = 565 nm) of 8 (2.5 μM) with the addition of N-Acetyl Cysteine in 50mM pH=7.4 phosphate buffer for 90min.
Finally, the reaction products between 8 and thiols was studied using HRMS (Figures 30-32).

Figure 30. HRMS of 3a.

Figure 31. HRMS of formation of 3b.
Figure 32. HRMS of formation of twelve membered ring.

3.4 Measurement of Cys content in human plasma using 5

As an application, 5 was used for the quantitative measurement of Cys content in human plasma sample. 0.5 mL of human plasma was reduced using HCl (0.2 M, 40 μL) in the presence of triphenylphosphine (0.1 M, 80 μL) as catalyst for 15 min at rt.\textsuperscript{11} Proteins present in the sample after reduction were precipitated by the addition of acetonitrile (0.5 mL), followed by centrifugation (4000 rpm) of the sample for 20 min. The supernatant liquid was then added to a solution of 5 (10 μM) in pH 7.4 Hepes buffer solution (0.1 M, 5 mL) in the presence of 1.0 mM CTAB. As shown in Figure 32, the fluorescence emission shows a significant increase with the
addition of reduced plasma. However, the fluorescence emission of the solution of 5 showed no obvious increase when controls triphenylphosphine alone or deproteinized plasma (without reducing agent) were added, respectively proving that the fluorescent increment of the solution of 5 is indeed involved in the Cys. The amount of Cys in the plasma sample was determined by the standard addition method to be 172.8 ± 8.7 μM (n = 3), which is well within the reported Cys concentration range (135.8 – 266.5 μM) for human plasma samples from the healthy individuals. These results prove that the proposed dosimeter may be useful for the quantitative detection of Cys in biological samples.

![Fluorescence spectra](image)

**Figure 33.** Fluorescence spectra (λ<sub>ex</sub> = 550 nm) of 4 (10 μM) in pH 7.4 Hepes buffer in the presence of 1.0 mM CTAB at different conditions for 25 min.
SECTION 4

CONCLUSION

Probe 8 is a new organic fluorescent probe for the highly selective, sensitive detection of two significant biological thiols: GSH and Cys as shown in Figures 33 and 34.

**Figure 34.** In buffered CTAB medium (50mM phosphate buffer pH=7.4).

**Figure 35.** In buffered Triton X-100 medium (50mM phosphate buffer pH=7.4).

For GSH detection, this new developed fluorescent probe 8 embodies a new sensing mechanism which couples conjugate addition reaction and a micelle-catalyzed large member ring formation/elimination sequence. A new class of fluorescent probes for GSH may be developed through this new mechanism. Due to the lack of fluorescence...
probes for the highly selective detection of GSH, the discovery of 8 is highly significant.

For Cys detection, probe 8 also exhibits excellent selectivity. Resorufin is firstly quenched through the protection of free hydroxyl group. Upon conjugate addition of thiols, due to the kinetic favored seven membered ring formation with Cys, a rapid elimination happens, leading to the recovery of free hydroxyl group which accounts for the fluorescent enhancement.
REFERENCES


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