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Highly selective and sensitive fluorescent sensing of N-acetylcysteine: Effective discrimination of N-acetylcysteine from cysteine

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ABSTRACT

A highly selective fluorescent probe for the effective discrimination of N-acetylcysteine (NAC) from cysteine (Cys) is proposed. Probe **1** contains an N,N-diethylrhodol (**DER**) dye and a dinitrophenyl ether moiety. Upon mixing with NAC in aqueous cetyltrimethylammonium bromide (CTAB) micellar solution, **1** was thiolyzed by NAC to release **DER**, thus affording a significant increase in fluorescence emission. Whereas for Cys, it gives only a dim response at the same reaction conditions. The significant difference in reaction rates can be explained via the fact that NAC shows more hydrophobicity than Cys, therefore the Meisenheimer complex intermediate (**2a**) of its nucleophilic aromatic substitution with **1** can embed in CTAB micelles effectively, which will facilitate the formation of **2a** and hence affords an acceleration of reaction rates. The proposed method shows an excellent selectivity for NAC over Cys, homocysteine (Hcy) and other amino acids.

1. Introduction

N-acetylcysteine (NAC) is a thiol-containing antioxidant and has been used as a mucolytic agent in chronic respiratory disorders as well as treating acute paracetamol poisoning [1]. It also acts as an antioxidant by raising intracellular levels of glutathione or by the scavenging of oxidant species itself [2]. In addition, in vitro and in vivo studies indicate that NAC can protect the cells against damages promoted by free radicals [3]. Therefore, the sensitive and selective detection of NAC is very important from the biological and pharmacological stand point.

Several analytical techniques have been developed for the determination of NAC, such as high performance liquid chromatography (HPLC) [4–9], capillary electrophoresis [10], colorimetric [11–13] and electroanalytical method [14,15]. Although HPLC is widely used for detecting NAC in different samples, the method usually needs chemical derivatization, which is time-consuming and inconvenient to operate.

In recent years, several fluorescent probes and chemosensors for thiol-containing compounds have been reported. Most of them are developed based on the strong nucleophilicity of thiol group, and various mechanisms have been employed [16]. Though these probes show high sensitivity toward thiol-containing compounds, they cannot discriminate them from each other due to non-specific thiol nucleophilicity. Therefore, the direct detection of target biothiols is highly challenging due to interference from other biothiols.

In 2004, Strongin et al. introduced an aldehyde-appended fluorophore to serve as a fluorescent probe for both cysteine (Cys) and homocysteine (Hcy) [17,18]. Because both the sulfhydryl and the amino groups contribute to the sensing mechanism, it enables selectivity for Cys and Hcy over other common thiols such as glutathione (GSH). Later, research in this area has been extended and some aldehyde-containing fluorescent probes selective for Cys or Hcy have been developed [19–22]. In 2007, Wang et al. reported the first fluorescent probe which can distinguish aliphatic thiols and thiophenols by using 2,4-dinitrobenzenesulfonamide as the recognition moiety [23]. This can be attributed to the distinct pK_a values of benzenethiols ($pK_a = 6.5$) and aliphatic thiols ($pK_a = 8.5$), and to the thiolysis of dinitrophenyl ethers proceeding via nucleophilic substitution by the nucleophilic thiolate. Very recently, we developed fluorescent probes using α,β-unsaturated carbonyl as the recognition unit, which can discriminate Cys and Hcy based on their relatively different intramolecular cyclization rates [24,25]. However, so far as we know, fluorescent method that can distinguish NAC from Cys has not been realized. And this is still very challenging due to similar chemical structures of NAC and Cys (Fig. 1).

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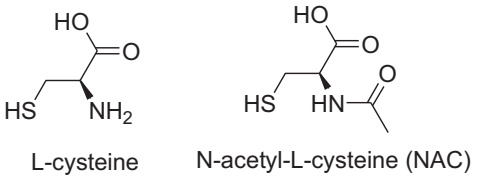


Fig. 1. Chemical structure of NAC and Cys.

On the other hand, it was reported that cetyltrimethylammonium bromide (CTAB) can catalyze the nucleophilic aromatic substitution reaction between thiols and 1-chloro-2,4-dinitrobenzene [26–28], and the reaction rates increased with increasing the hydrophobicity of thiols [29]. This phenomenon provides a unique opportunity to develop a chemical sensing system to discriminate thiols based on differences in their hydrophobicity. Thus, we can distinguish thiol-containing compounds based on their two independent characteristics (nucleophilicity and hydrophobicity). This design strategy may lead to the double molecular recognition-based fluorescence sensing systems, which in principle, may enhance the selectivity of the probe toward the target compound over other potentially competing species as two independent molecular recognition events are involved. As a proof of concept, we utilize this finding to develop a new fluorescent sensing system for discriminating NAC from Cys.

Herein, we develop compound **1** by incorporating the 2,4-dinitrophenyl group to N,N-diethylrhodol (**DER**) fluorophore. Probe **1** shows both colorimetric and fluorescent "turn-on" response for NAC in CTAB micelles, but there is no gain for Cys under identical reaction conditions due to its low hydrophobicity compared with that of NAC. Based on this strategy, a highly selective fluorescent method for NAC detection was developed, which showed an excellent selectivity for NAC over Cys, Hcy and other amino acids.

2. Material and methods

2.1. Materials

N-Acetyl-L-cysteine (NAC) was purchased from Sinopharm Chemical reagent Co., Ltd (Shanghai, China); N,N-diethyl-*m*-aminophenol was obtained from Aladdin reagent Co. (Shanghai, China); phthalic anhydride and resorcinol were obtained from Xi'an chemical reagent factory; 1-chloro-2,4-dinitrobenzene was obtained from Shanghai Darui fine chemical Co., Ltd. Flash chromatography was performed using Qingdao Haiyang silica gel (200–300 mesh). Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents were purified and dried by standard methods prior to use. Double-distilled water was used throughout the experiments.

2.2. Instrumentation

The fluorescence spectra and relative fluorescence intensity were measured with a Shimadzu RF-5301 spectrofluorimeter with a 10 mm quartz cuvette. Unless specific noted, the excitation and emission band passes were set at 3.0/3.0 nm. The absorption spectra were measured using a Shimadzu UV-2550 spectrophotometer. High-resolution mass spectra were collected using a Bruker micrOTOF-Q II mass spectrometer (Bruker Daltonics Corp., USA) in electrospray ionization (ESI) mode. ^{1}H and ^{13}C NMR spectra were recorded on an INOVA-400 spectrometer (Varian Unity), using tetramethylsilane (TMS) as the internal standard. All pH measurements were made with a Sartorius PB-10 pH meter.

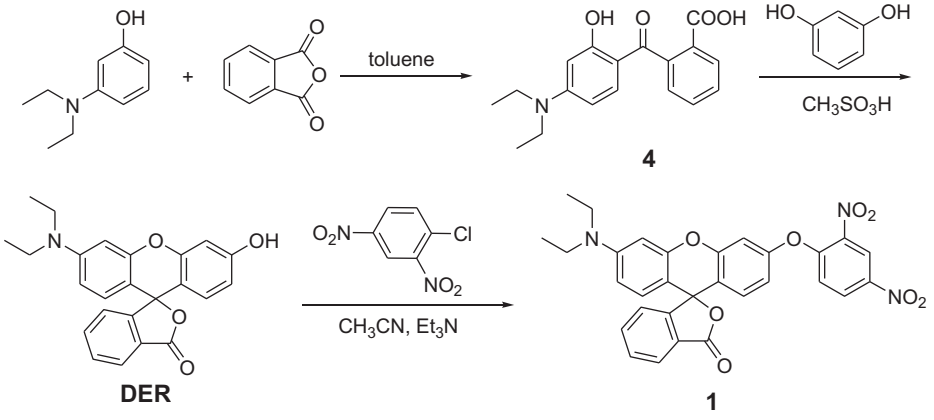
2.3. Synthesis of compound *1*

2.3.1. Preparation of *DER*

2-(4-Diethylamino-2-hydroxybenzoyl) benzoic acid (**4**) was prepared according to the literature procedures [30]. Then, a suspension of **4** (120 mg, 0.38 mmol) and m-resorcinol (42 mg, 0.38 mmol) in methanesulfonic acid (3 mL) was stirred at 90 °C for 24 h. The reaction mixture was cooled to room temperature and then poured in ice-cold water (15 mL). The precipitate was filtered and washed with brine (3 × 10 mL), then dried under vacuum. The target compound is isolated by flash column chromatography on silica gel using CH_2Cl_2:EtOAc:MeOH (20:20:3, v/v/v) for elution. Yield 51 mg, 34%. ^{1}H NMR (400 MHz, d_6-DMSO): δ 10.11 (s, 1H), 7.97 (d, 1H, J = 7.2 Hz), 7.77 (t, 1H, J = 7.0 Hz), 7.70 (t, 1H, J = 7.2 Hz), 7.26 (d, 1H, J = 7.2 Hz), 6.66 (s, 1H), 6.52–6.45 (m, 5H), 3.35 (q, 4H, J = 7.2 Hz), 1.08 (t, 6H, J = 7.2 Hz). HRMS (ESI) calc. for $C_{24}H_{20}NO_4$ $[M - H]^-$ 386.1398, found 386.1412.

2.3.2. Preparation of compound *1*

To a 25 mL flask, **DER** (0.1 g, 0.26 mmol), 1-chloro-2,4-dinitrobenzene (0.06 g, 0.31 mmol) and Et_3N (0.1 mL) in acetonitrile (10 mL) were mixed and refluxed for 6 h [31] (Scheme 1). After evaporation of the solvent, the residue was dissolved by $CHCl_3$ (50 mL). The solution was washed with brine (3 × 10 mL), dried over anhydrous Na_2SO_4. After that, the solvents were dried in vacuo



Scheme 1. Synthesis of probe **1**.

and the crude product was purified by chromatography on silica gel with $CHCl_3$-acetone (30:1, v/v) as the eluent. Yield 60 mg, 42%. ^1H NMR (400 MHz, $CDCl_3$) δ 8.86 (d, J = 2.0 Hz, 1H), 8.37 (dd, J_1 = 9.2 Hz, J_2 = 2.0 Hz, 1H), 8.03 (d, J = 7.6 Hz, 1H), 7.71 (t, J = 7.4 Hz, 1H), 7.64 (t, J = 7.4 Hz, 1H), 7.23 (d, J = 7.6 Hz, 1H), 7.17 (d, J = 9.2 Hz, 1H), 7.02 (d, J = 2.0 Hz, 1H), 6.87 (d, J = 8.8 Hz, 1H), 6.76 (dd, J_1 = 8.4 Hz, J_2 = 2.0 Hz, 1H), 6.59 (d, J = 8.8 Hz, 1H), 6.44 (s, 1H), 6.39 (d, J = 8.8 Hz, 1H), 3.37 (q, J = 6.8 Hz, 4H), 1.18 (t, J = 7.0 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.48, 155.26, 155.02, 153.27, 152.64, 152.62, 149.84, 142.06, 139.88, 135.21, 130.57, 129.96, 129.09, 128.97, 127.10, 125.17, 124.12, 122.21, 119.63, 117.96, 115.25, 108.91, 108.72, 104.49, 97.49, 83.52, 44.60, 12.55. HRMS (ESI, *m/z*) calc. for $C_{30}H_{24}N_3O_8$ $[M + H]^+$ 554.1578, found 554.1558.

3. Results and discussion

3.1. Development of probe **1** and its sensing mechanism toward NAC

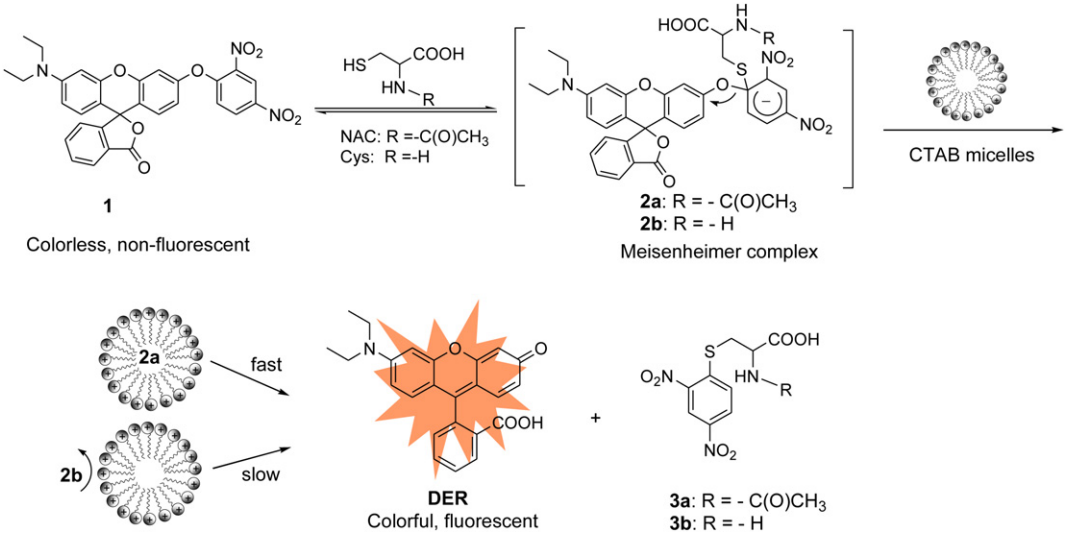
It was reported that nucleophilic aromatic substitution reaction between 1-chloro-2,4-dinitrobenzene and thiols in CTAB micelles can generate the corresponding 2,4-dinitrophenyl thioether, and the reaction rates increased with increasing hydrophobicity of the nucleophile [29]. On the other hand, NAC shows more hydrophobicity than Cys as an acetyl group is attached to the nitrogen atom. Therefore, we hypothesized that this chemical principle could be used for picking out NAC from Cys due to their significant differences in hydrophobic density.

Based on the above mechanism, we constructed probe **1** for NAC sensing in aqueous CTAB micellar solution. In probe **1**, 2,4-dinitrophenyl group is selected not only because it is crucial for activating the nucleophilic aromatic substitution reaction but also serve as an efficient quencher in the designed fluorescent probe **1** [32]. **DER**, a hybrid structure of fluorescein and rhodamine B, is selected as the fluorophore because it is known to be highly fluorescent in aqueous solution and are more resistant to photo bleaching in comparison with that of fluorescein [33].

Probe **1** was conveniently synthesized via the condensation of **DER** with 1-chloro-2,4-dinitrobenzene in CH_3CN, and its structure was confirmed by ^1H NMR, ^{13}C NMR, HRMS spectra (ESI, Figs. S7–S9). ^{13}C NMR data shows a prominent peak at 83.12 ppm, indicating that probe **1** is present in its colorless, non-fluorescent spirolactone form [34]. Upon mixing in CTAB medium, the nucleophilic substitutions reaction between probe **1** and NAC are thought to occur, forming the Meisenheimer complex intermediate (**2a**). Finally, the fluorophore **DER** leaves and forms **3a** (Scheme 2). The formation of **DER** and **3a** were confirmed by mass spectrometric analysis of the mixture of **1** with NAC. The prominent peaks at m/z 386.1393 and 328.0242, corresponding to [**DER** − H]$^-$ (calc. 386.1398 for $C_{24}H_{20}NO_4$) and [**3a** − H]$^-$ (calc. 328.0245 for $C_{11}H_{10}N_3O_7S$) were shown in the HRMS data (ESI, Fig. S10). The rate-limiting step of above S_NAr reaction has been proposed at the δ-bond formation in the Meisenheimer complex [27]. The polar head of CTAB is positively charged and it can stabilize the negatively charged **2a**. This would be expected to lower the energy of the activated complex and hence affords an acceleration of reaction rates. However, Cys is less hydrophobic and its corresponding Meisenheimer complex (**2b**) cannot embed in CTAB micelles effectively, which will decrease the reaction rate significantly due to destabilization of the transition state. Another possible explanation is that Cys is present in the zwitterionic form in neutral buffer solutions (the pK_a values of its −COOH and $-NH_3^+$ are 1.96 and 10.29, respectively) [35]. Therefore, the positively charged amino group of **2b** will hamper its embedding in CTAB micelles. In the case of **2a**, however, it is negative and can enter the positively charged CTAB micelles readily. Based on the above mechanism, NAC can be discriminated from Cys.

3.2. Spectral characteristics

The absorption spectra of **1** and **DER** in pH 7.4 Tris-HCl buffer solution were recorded (ESI, Fig. S1-a), and it was observed that **DER** has a strong absorption band centered at 521 nm. However, when its hydroxyl group was converted into the corresponding 2,4-dinitrophenoxy group, it turns to be colorless and shows almost no absorption at visible spectra range (400–650 nm), indicating that probe **1** exists in the spirolactone form predominantly in neutral buffer solution, which is valuable for constructing probes through protection and deprotection of phenolic hydroxyl group by using “turn-on” colorimetric and fluorimetric techniques [36]. Furthermore, the fluorescence emission spectra of **1** and **DER** were also compared in Fig. S1-b. It can be seen that incorporation of a dinitrophenyl group in the **DER** fluorophore leads to essentially non-fluorescent dinitrophenyl ether **1**.



Scheme 2. Proposed mechanism of discrimination of NAC from Cys when using **1** in CTAB micelles.

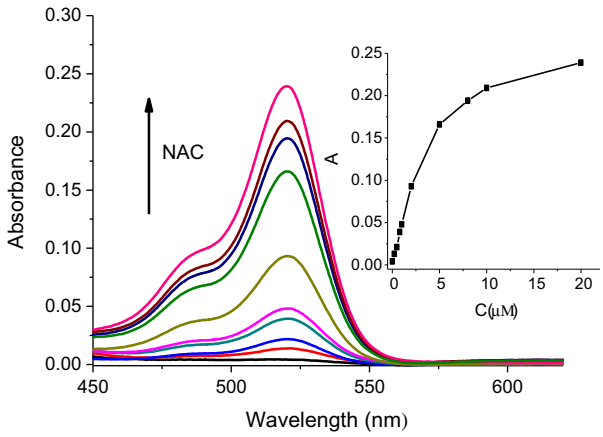


Fig. 2. Absorption spectra of **1** (10 μM) with the addition of increasing concentrations of NAC (0–20 μM) in 1.5 mM CTAB media buffered at pH 7.4 (Tris-HCl buffer, 10 mM) for 40 min. The inset figure shows the plot of the absorbance at 521 nm as a function of NAC concentration.

3.3. UV–Vis and fluorescence spectra of *1* titrated with NAC

The sensing response of probe **1** toward NAC was examined with absorption and fluorescence spectroscopy at room temperature (25 °C), respectively. Upon mixing with NAC in aqueous CTAB micellar solution buffered at pH 7.4, a dramatic change of color was observed owing to the thiolysis of probe **1** to give the parent dye **DER** (ESI, Fig. S2). Addition of an increasing amount of NAC to the solution of **1** in CTAB media elicited a gradual increase of the absorption peak at about 521 nm (Fig. 2). It can also be observed that absorbance at 521 nm increased with increasing NAC concentration, indicating that colorimetric method might be used for NAC assay (ESI, Fig. S3). Furthermore, the changes in the fluorescence spectra of **1** in the presence of NAC were shown in Fig. 3. It was observed that the free probe is essentially non-fluorescent; however, the introduction of NAC caused a dramatic increase in fluorescence emission. The fluorescence intensity at 545 nm increases linearly with increasing NAC concentration in the range 0.2–2.0 μM (ESI, Fig. S4). Furthermore, as low as 20 nM of NAC can be readily detected when using 0.2 μM of probe **1** (ESI, Fig. S5), indicating the sensitivity of the proposed method is remarkably high.

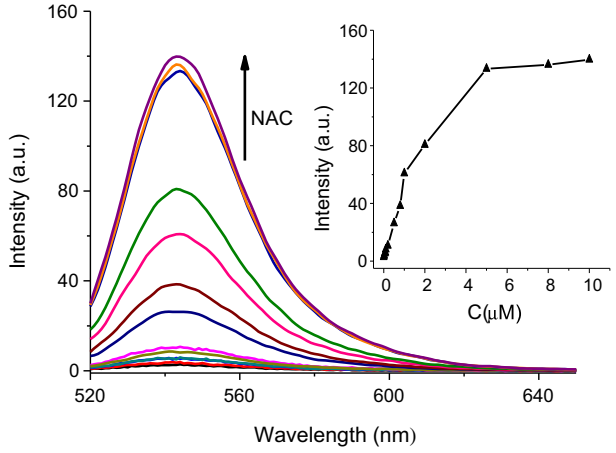


Fig. 3. Fluorescence spectra of probe **1** (2.0 μM) upon addition of increasing amount of NAC (0–10 μM) in CTAB media (1.5 mM) buffered at pH 7.4 (Tris-HCl buffer, 10 mM) for 40 min. The inset figure shows the plot of the fluorescence intensity at 545 nm as a function of NAC concentration. Data were acquired at 25 °C with excitation at λ_{ex} = 510 nm. Slit: 1.5 nm/1.5 nm.

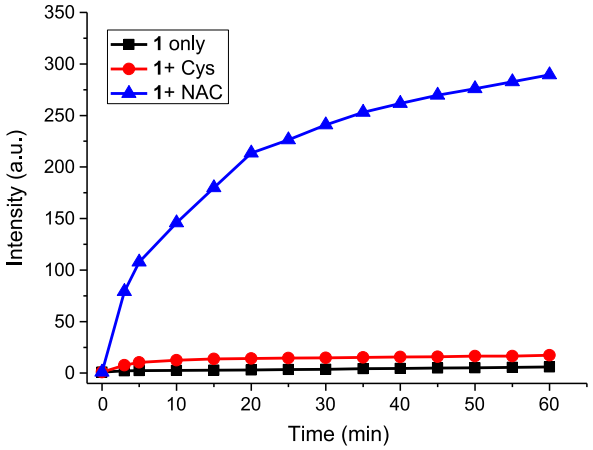


Fig. 4. Time-dependent fluorescence intensity changes of **1** (2.0 μM) upon addition of 1 equiv of NAC or Cys in 1.5 mM CTAB media buffered at pH 7.4 (Tris-HCl buffer, 10 mM). Data were acquired at 25 °C with excitation and emission at $\lambda_{ex}/\lambda_{em}$ = 520/545 nm. Slit: 1.5 nm/3 nm.

3.4. Kinetic studies

For better understanding of the proposed fluorogenic reaction, the time-dependent fluorescence intensity changes of probe **1** with NAC or Cys in CTAB micelles were studied and the results were shown in Fig. 4. Upon addition of NAC, the solution of probe **1** showed an initial fast, followed by a gradual increase in fluorescent intensity, however, Cys exhibited no significant changes in emission intensity at the same conditions. In this work, an assay time of 40 min was selected for the evaluation of the selectivity and sensitivity of **1** toward NAC.

3.5. Effect of surfactants

In order to investigate which kind of surfactant functions well in the present fluorogenic sensing system. Some organized media, including cationic (CTAB, Hexadecylpyridinium bromide (CPDB), Dodecyltrimethylammonium bromide (DTAB)), anionic (Sodium dodecyl benzene sulfonate (SDBS), Sodium dodecyl sulfate (SDS)) and non-ionic surfactants (Span 80, Tween 80) were tested and the results are shown in Table 1. It was observed that cationic surfactants show an increase in fluorescence emission, and CTAB gives the most prominent fluorescence increase; whereas other surfactants show no effect for the proposed reaction. These results can be explained by the fact that the proposed fluorogenic reaction involves a build up of negative charge in the transition state, which can be stabilized by the positive electrostatic field. Therefore, CTAB is used in the present sensing system.

Table 1
Effect of surfactant on the fluorescence sensing behavior of **1** toward NAC.[a]

Surfactants	*C* (M)	F_0	F	F/F_0
SDBS	0.14	9.2	10.1	1.09
SDS	0.081	6.8	8.1	1.19
Span 80	0.04 g L^{-1}	1.4	1.9	1.35
Tween 80	0.05 g L^{-1}	1.8	2.3	1.28
DTAB	0.016	3.8	98.6	20.54
CPDB	2.4×10^{-3}	3.2	42.0	13.12
CTAB	9.3×10^{-4}	15.4	826.2	53.64
–	–	1.2	2.0	1.67

[a] Where F and F_0 are the fluorescence signal of the system in the presence and absence of NAC (2.0 μM), respectively. The fluorogenic reaction was carried out in different surfactant media buffered at pH 7.4 (Tris-HCl buffer, 10 mM) for 40 min. The CMC of all surfactants were used for the fluorogenic reaction.

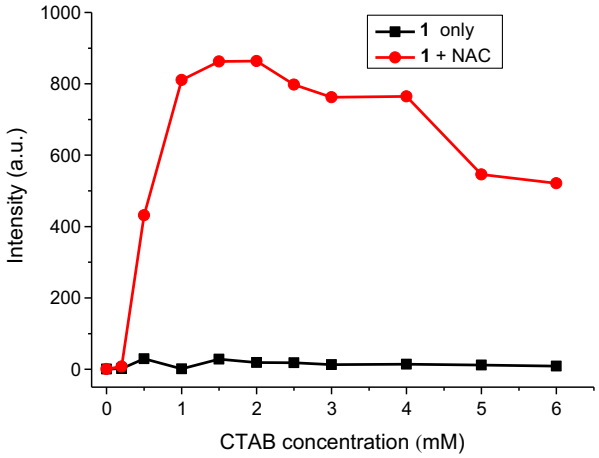


Fig. 5. Effect of CTAB concentration on the fluorogenic reaction of **1** (2.0 μM) with NAC (2.0 μM) in pH 7.4 Tris-HCl buffer. Reaction time, 40 min. Data were acquired 40 min after addition of NAC at 25 °C with excitation and emission at $\lambda_{ex}/\lambda_{em}$ = 520/545 nm.

The effect of CTAB concentration on the proposed fluorogenic reaction was studied and the result is shown in Fig. 5. It can be observed that the fluorescence signal increased with increasing CTAB concentration in the range 0–1.0 mM and remained almost constant when CTAB concentration was in the range of 1.0–4.0 mM, and thereafter decreased. Thus, CTAB concentration was selected at 1.5 mM for further experiment. The optimal CTAB concentration is close to the critical micelle concentration (CMC) of CTAB in a pure aqueous solution (CMC = 0.93 mmol L^{-1}) [37], indicating that micellar medium of CTAB plays a key role in the present fluorogenic reaction.

3.6. Selectivity studies

The selectivity of probe **1** for NAC was investigated by screening its response to other biologically relevant analytes under physiological pH conditions. As shown in Fig. 6, only Cys, Hcy, cysteamine

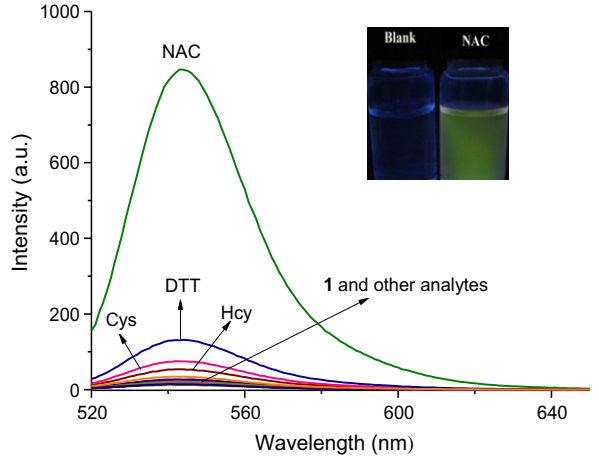


Fig. 6. Fluorescence spectra of **1** (2.0 μM) with the addition of different kinds of analytes (Na^+, K^+, Ca^{2+}, Zn^{2+}, Mg^{2+}, Al^{3+}, Cu^{2+}, Fe^{3+}, F^-, Br^-, I^-, tryptophan, glutamic acid, arginine, valine, leucine, aspartic acid, citric acid, glucose, sucrose, ascorbic acid, urea, starch, Hcy, Cys, cysteamine and DTT) in CTAB media (1.5 mM) buffered at pH 7.4 (Tris-HCl buffer, 10 mM) for 40 min NAC, Cys, Hcy, cysteamine and DTT were 2.0 μM; Na^+, K^+, Ca^{2+} and urea were 200 μM. Other analytes, 20 μM. Data were acquired at 25 °C with excitation at λ_{ex} = 510 nm. Inset: Color changes of the solution of **1** after addition of NAC under irradiation at 365 nm using UV lamp.

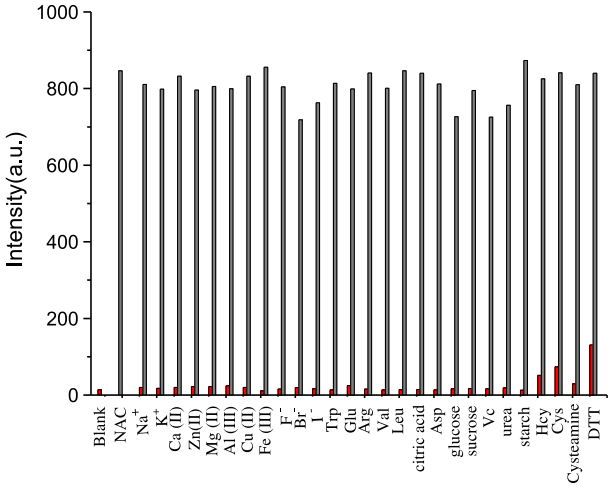


Fig. 7. Fluorescence response of **1** (2.0 μM) in the presence of NAC(2.0 μM) and/or various analytes in CTAB media (1.5 mM) buffered at pH 7.4 (Tris-HCl buffer, 10 mM) for 40 min. Gray bars represent the addition of other amino acids or metal ions to the solution of **1**. Red bars represent the subsequent addition of NAC to the solution. Cys, Hcy, cysteamine and DTT are 1 equiv of NAC, Na^+, K^+, Ca^{2+} and urea are 100 equiv of NAC, other analytes are 10 equiv of NAC. Data were acquired at 25 °C with excitation and emission at $\lambda_{ex}/\lambda_{em}$ = 520/545 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and dithiothreitol (DTT) promote small fluorescence intensity changes, while other metal ions (Na^+, K^+, Ca^{2+}, Zn^{2+}, Mg^{2+}, Al^{3+}, Cu^{2+}, Fe^{3+}), amino acids (tryptophan, glutamic acid, arginine, valine, leucine, aspartic acid), anions (F^-, Br^-, I^-), citric acid, glucose, sucrose, ascorbic acid, urea and starch showed no significant changes in fluorescent emission under identical conditions, indicating that the selectivity of **1** toward NAC over other biologically relevant analytes is remarkably high. Furthermore, to examine whether probe **1** could still retain its sensing response to biothiols in some biological systems, competitive experiments were carried out by treating probe **1** with NAC in the presence of some biologically relevant analytes. As displayed in Fig. 7, all the biologically relevant analytes tested have virtually no influence on the fluorescence detection of NAC. Thus, we confirm that probe **1** offers a good selectivity for sensing NAC in CTAB micelles, even with the involvement of some thiol-containing amino acids.

4. Conclusion

In summary, we have present a new strategy for distinguishing NAC from Cys based on their different reaction rates with probe **1** in CTAB micelles. The method employs the thiolysis of **1** by NAC in CTAB medium to release the free **DER** and thus results in a dual chromo- and fluorogenic response. The selectivity of the present system can be explained via the fact that NAC is more hydrophobic than Cys, thus its Meisenheimer complex (**2a**) can enter the positively charged CTAB medium effectively, which will contribute to the transition stabilization and hence affords an acceleration of reaction rates. The investigation demonstrates that discrimination of thiols can be realized based on their nucleophilicity and hydrophobicity, which provides a new ideal for rational developing probes for other structurally similar molecules. And this research work is still under way in our laboratory.

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Appendix A. Supplementary material

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dyepig.2012.10.014.

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