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Orchestration of dual cyclization processes and dual quenching mechanisms for enhanced selectivity and drastic fluorescence turn-on detection of cysteine

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Hongjuan Tong,[a,b] Jianhong Zhao,[a,b] Xiangmin Li,[b] Yajun Zhang,[b] Shengnan Ma,[b] Kaiyan Lou*[b] and Wei Wang*[b,c]

We reported a new approach to achieve enhanced selectivity with drastic turn-on fluorescence response for detection of Cys through dual intramolecular cyclization processes and dual PET and ICT quenching mechanisms by incorporation of an acrylate and a maleimide group onto two opposite sides of a single coumarin fluorophore.

Low molecular weight aminothiols, such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), play different important roles in biological systems.[1-6] Among these bioaminothiols, Cys is a precursor for peptides and proteins as well as for GSH, acetyl coenzyme A, and taurine. The levels of these aminothiols including Cys are highly regulated under normal physiological conditions, while altered levels often indicate different disease states.[7-10] Therefore, methods for detection these aminothiol's levels with high selectivity under complex biological context are greatly needed for studying their specific physiological and pathological roles. So far, high performance liquid chromatography (HPLC),[11] capillary electrophoresis,[12] electrochemical assay,[13] UV-vis spectroscopy,[14] and fluorescence spectroscopy[15] have been employed in thiol detections. Among them, fluorescence sensing has attract most attention recently because of its high sensitivity, ease of use, and real-time imaging capabilities.[16-18] Many fluorescent probes for Cys, Hcy and GSH have been devised using various specific reaction and recognition groups.[15, 19-21] To achieve selectivity, different cyclization processes were incorporated in the probe design, which take advantage of reaction kinetic differences in the formation of different sizes of rings when the thiol and amine groups in Cys, Hcy, and GSH undergo cascade nucleophilic reactions with a single recognition group. Such reactive groups involving a cyclization process include aldehyde,[11, 22] acrylate,[23] thioester,[24] maleimide,[25] or electron-deficient aromatic halide[26-28] (Scheme S1, ESI†). Although good to excellent selectivity could generally be achieved between Cys/Hcy and GSH, only modest selectivity could be realized between Cys and Hcy due to their minute structural difference by just one methylene group. The selectivity was thus limited by the reaction kinetic differences in the formation of x-membered ring for Cys and (x+1)-membered ring for Hcy (x=5, 6, or 7 for recognition groups shown in Scheme S1, ESI†) and difficult to improve for a single recognition group.

Here we reported a new approach to improve the selectivity of Cys detection over Hcy by a synergetic use of two different recognition groups with different intramolecular cyclization processes. To demonstrate the feasibility of this approach, we chose the acrylate group and the maleimide group in our probe design as both the cascade reaction and sensing mechanisms for the two groups toward aminothiols were well-studied (Scheme 1). Acrylate group reacting with Cys/Hcy follows a thiol-Michael addition and cyclization-deprotection cascade process.[23] The cyclization is 7-*exo-trig* for Cys and 8-*exo-trig* for Hcy according to Baldwin's nomenclature.[29] While for the maleimide group, a thiol-Michael addition and transcyclization cascade reaction happens for Cys and Hcy in 6-*exo-trig* and 7-*exo-trig*, respectively.[25] We expected that the combined use of the two different cyclization would give a better selectivity for Cys over Hcy. For GSH, the cyclization step is kinetically unfavorable as it would involve the formation of either 11- or 12-membered ring. So the reaction products stay as the thiol-Michael adducts (Scheme S2, ESI†). In terms of fluorescence sensing mechanism, for the maleimide group, it is a well-established quenching group through photo-induced electron transfer (PET) mechanism due to its low-lying empty π* orbital of the double bond.[30, 31] The fast thiol-Michael addition removes the PET effect, resulting in a quick and usually drastic turn-on

[a] *H. Tong and J. Zhao contributed equally to this work.*
[b] *Shanghai Key Laboratory of New Drug Design, Shanghai Key Laboratory of Chemical Biology, School of Pharmacy, and State Key Laboratory of Bioengineering Reactor, East China University of Science & Technology, 130 Meilong Road, Shanghai 200237, China.*
[c] *Department of Chemistry and Chemical Biology, University of New Mexico, Albuquerque, NM 87131-0001, USA.*
* *To whom correspondence should be addressed.*
K. Lou, Email: kylou@ecust.edu.cn; W. Wang, E-mail: wwang@unm.edu.
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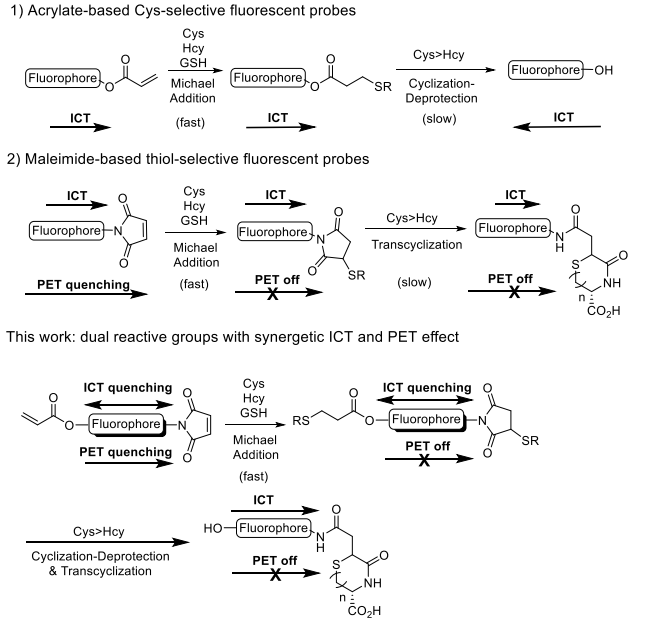
fluorescence response.[32] Since this step only involves thiol group, thus it is not selective for Cys, Hcy, and GSH. The slow transcyclization step may change fluorescence emission but generally not as drastic as the thiol-Michael step. Therefore, maleimide-based fluorescent probes are generally not selective among different thiols. In contrast, fluorescence sensing mechanism of acrylate-based fluorescent probes are mainly based on the change of internal charge transfer (ICT) at the excited state due to the change of substituent from the electron-withdrawing ester to the electron-donating hydroxyl group in the cyclization-deprotection step.[23] Therefore, the acrylate based fluorescent probes were generally selective for Cys/Hcy over GSH (Scheme 1, Scheme S2, ESI†). Based on the above reaction and sensing mechanisms, we envisioned that incorporation of the acrylate and maleimide groups onto a fluorophore would result in a fluorescent probe adopting dual cyclization processes to achieve increased kinetic differences for improved selectivity of Cys over Hcy. In order to fully translate reaction kinetic differences of dual cyclizations to fluorescence signal readout for high selectivity, the electron-withdrawing acrylate and the maleimide group have to be put at the two ends of a fluorophore for efficient ICT quenching and allows drastic fluorescence turn-on response only after dual cyclization processes (Scheme 1).



Scheme 1 Schematic illustration of synergetic PET and ICT effect of the maleimide and acrylate groups in a single fluorophore.

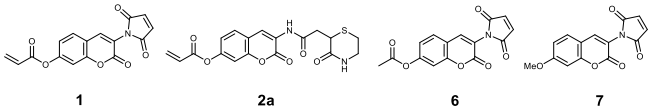


Fig. 1 Structure of fluorescent probe **1** and the reference fluorescent probes.

To test our hypothesis, we designed the target probe **1** and three reference probes **2a**, **6**, and **7** using 3-amino-7-hydroxycoumarin (**4**) as the model fluorophore (Fig. 1).

Probe **1** was conveniently synthesized from 3-amino-7-hydroxycoumarin (**4**)[33] in two steps with high yield (Scheme S3, ESI†). The coumarin **4** first reacted with maleic anhydride to form the maleic amide acid **5**. Then the acid **5** was stirred with excess acryloyl chloride in the presence of triethylamine. The hydroxyl group was protected as an acrylate group, and the maleic amide acid was simultaneously converted to a maleimide group. When acyl chloride was used instead of acryloyl chloride, the reference probe **6** was obtained (Scheme S5, ESI†). Similarly the reference probe **7** was synthesized from 3-amino-7-methoxy-coumarin (**8**)[34] (Scheme S6, ESI†). Structures of these probes were firmly confirmed by ^{1}HNMR, ^{13}CNMR, and HRMS (see ESI†).

With the probe **1** in hand, we first tried model reaction of probe **1** with excess amount of cysteine methyl ester (Cys-OMe) in a mixed solvent of DMSO and water. An inseparable mixture of two diastereomers in a ratio about 3:2 were isolated based on their ^{1}HNMR spectrum. The double bond absorption peaks of the acrylate (δ 6.60, 6.45, 6.23) and the maleimide group (δ 7.32) were disappeared, indicating that both groups were reacted (Fig. S13, ESI†). HPLC-MS studies of probe **1** with 40 equiv. of Cys-OMe confirmed the removal of the acrylate group and addition of 1 equiv. of Cys-OMe in the final product. However, whether the transcyclization happened after thiol-Michael addition of the maleimide group was difficult to confirm. We then turned to the model reaction with cysteamine. When probe **1** reacted with 1 equiv. of cysteamine in THF for 5 min, compound **2a** was isolated in 64% yield, in which the acrylate group was retained while the maleimide group was converted to 6-membered thiomorpholinone ring through thiol-Michael addition and transcyclization cascade reaction. The formation of 6-membered ring was confirmed by 2D-NOESY spectrum (Fig. S18, ESI†). The compound **2a** was used as a reference probe only containing an acrylate group. When 2 equiv. of Cys were used, product **3a** was isolated in 84% yield. Similarly, its 6-membered ring was confirmed by 2D-NOESY spectrum (Fig. S17, ESI†). The second equiv. of cysteamine reacted with the acrylate group and deprotected it through thiol-Michael addition and cyclization-deprotection cascade sequence. The model reactions so far supported that both reactive groups in probe **1** should follow established mechanisms when reacted with 2-aminothiols, including cysteamine, Cys-OMe, and Cys.

We then studied fluorescence response of probe **1** with Cys. Initial fluorescence titration of probe **1** (1 μM) towards increasing amount of Cys showed a concentration-dependent fluorescence turn-on response toward addition of Cys. A minimum 40 equiv. Cys was required to achieve a maximum fluorescence turn-on response in 30 min (Fig. S1, ESI†). Time-dependent fluorescence emission spectra of the probe **1** (1 μM) with 40 μM Cys verified maximum fluorescence response was achieved in 30 min (Fig. 2c). The probe to Cys ratio of 40 and incubation time of 30 min were selected for further photophysical studies. Under this condition, the probe **1** gave turn-on fluorescence at 476 nm with very low fluorescence background (Fig. 2a). The maximum fluorescence excitation and emission wavelength were 340 nm and 476 nm, respectively

(Fig. S3, ESI†). The normalized fluorescence spectrum of probe **1** incubation with 40 equiv. Cys for 30 min almost overlapped with that of product **3a** isolated from the model reaction of probe **1** with 2 equiv. of cysteamine (Fig. S4, ESI†), suggesting the reaction of probe **1** with Cys follows the same mechanism with cysteamine. The detection limit of probe **1** was determined to be 13.7 nM at S/N=3 at low Cys concentrations (0 to 5 μM), (Fig. S5, ESI†).

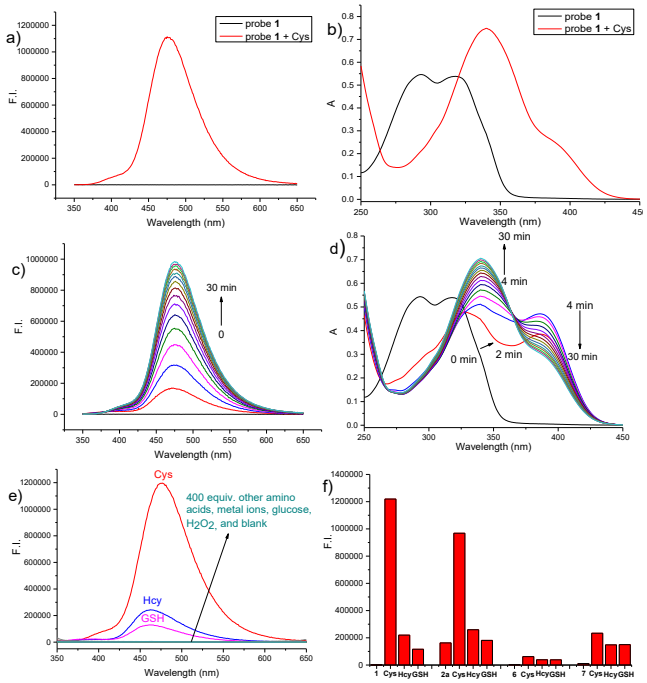


Fig. 2 a) The fluorescence emission spectra of probe **1** (1 μM) in the absence and presence of 40 μM Cys in PBS buffer. (λ_{ex} = 340 nm, incubation time = 30 min); b) Absorption spectra of probe **1** (50 μM) in the absence and presence of 2 mM Cys in PBS buffer (incubation time = 30 min); c) time-dependent fluorescence emission spectra of 1 μM probe **1** upon addition of 40 μM Cys in PBS buffer (λ_{ex} = 340 nm, measured in every two minutes); d) time-dependent absorption spectra of probe **1** (50 μM) upon addition of Cys (2 mM); e) Fluorescence spectra of probe **1** (1 μM) (λ_{ex} = 340 nm) toward various species in PBS buffer (10 mM, pH 7.4) including 40 equiv. aminothiols (Cys, Hcy, GSH), 400 equiv. different amino acids (Gly, Lys, Arg, His, Tyr, AsP, Thr, Trp, Glu, Pro), 400 equiv. metal ions (K^+, Ca^{2+}, Na^+, Mg^{2+}, Zn^{2+}, Fe^{3+}), 400 equiv. H_2O_2, and 400 equiv. glucose. The control group was the probe **1** (1 μM) alone; f) Comparision of fluorescence intensity at 476 nm (λ_{ex} = 340 nm) of 1 μM probe **1**, reference probe **2a**, **6**, and **7** toward 40 equiv. of Cys, Hcy, and GSH. (All measurements were taken in 10 mM PBS buffer solution at pH = 7.4)

More reaction details was revealed from UV-vis studies. Probe **1** showed two maximum absorption bands at 293 and 318 nm. After reaction with 40 equiv. Cys for 30 min, the maximum absorption band red-shifted to 340 nm (Fig. 2b). Time-dependent absorption spectra of probe **1** clearly showed an isobestic point at about 370 nm after 4 min, indicating the formation of an intermediate (Fig. 2d). From comparison with the time-dependent UV-vis spectra of probe **1** with 40 equiv. *N*-acetyl cysteine (NAC) (Fig. S6, ESI†) and the reference probe **2a** with 40 equiv. of NAC at extended time (Fig. S7, ESI†), it was deduced at the time point of 4 min, both the recognition groups were likely converted to the thiol-Michael adducts but not yet underwent cyclization (Fig. S8, ESI†). The structure of the intermediate **2** and the proposed reaction mechanism was shown in Scheme S7 (see ESI†).

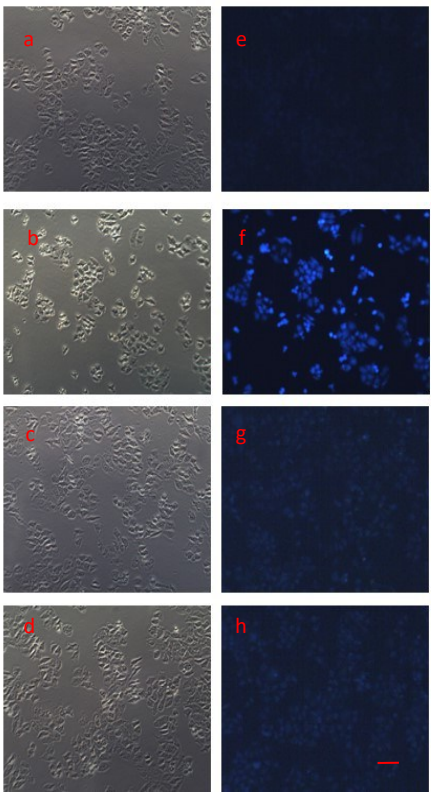


Fig. 3 Fluorescence images (e-h) collected at 430-495 nm (blue to cyan-blue, λ_{ex} = 400 nm) and the corresponding bright field view (a-d) of HeLa cells after different treatment. a) and e) were pretreated with 1 mM *N*-ethylmaleimide (NEM) before incubation with the probe **1** for 1 h. (b, f), (c, g), and (d, h) were first pretreated with 1 mM NEM 1 h, then pretreated with 500 μM Cys, Hcy, and GSH for 30 min respectively, finally incubated with 20 μM probe **1** for 1 h (scale bar = 100 μM).

We then investigated selectivity of the probe **1** for Cys detection over Hcy, GSH, other amino acids, metal ions, hydrogen peroxide, and glucose (Fig. 2e and Fig. S10, ESI†). For various species tested so far, probe **1** showed good to excellent selectivity for Cys. Notably, probe **1** showed enhanced selectivity for Cys than both the reference probe **2a** and **7** (Fig. 2f) with only one recognition group. The ratios of fluorescence intensity at 476 nm of the fluorescent probe in response to Cys versus to Hcy were increased from 3.7, and 1.6 for the probe **2a** and **7**, respectively, to 5.5 for the probe **1**. Moreover, probe **1** gave much lowered fluorescence background and a drastic fluorescence turn-on (> 930-fold) response for Cys, while the fluorescence turn-on ratio was only 5.9-fold for the reference probe **2a**. Compared with the probe **1**, the reference probe **6** had similarly low fluorescence background from dual PET and ICT quenching mechanisms, but gave a much lowered fluorescence turn-on response, indicating the importance of the removal of acrylate group and associated ICT quenching effect for the observed drastic fluorescence turn-on response of probe **1**. The dual quenching mechanisms and drastic fluorescence

turn-on response after dual cyclization process was also supported by the measured fluorescence quantum yields of the probe **1**, reference probe **2a**, and product **3a** as 0.003, 0.149, and 0.809, respectively (Fig. S9, ESI†).

The selectivity of probe **1** for detection of Cys over Hcy and GSH was further investigated by cell imaging studies. Before cell studies, pH-dependent studies (Fig. S11, ESI†) showed probe **1** was suitable for use at biological pHs from 4-8, while MTS assay demonstrated that the probe **1** did not have significant toxicity up to 100 μM to HeLa cells (Fig. S14). Then, HeLa cells were incubated with with *N*-ethylmaleimide (NEM, 1 mM) for 1 h to quench intracelluar total thiols, and then treated with Cys (0.5 mM), Hcy (0.5 mM), and GSH (0.5 mM), respectively, for 30 min to increase the specific thiol level inside cells, and further incubated with probe **1** (20 μM in PBS containing 0.2% DMSO) at 37 °C for 1 h before cells were imaged using a fluorescence microscope at the blue channel (430-495 nm). As the control group, cells with only NEM treatement and incubation with probe **1** were also imaged. The results were shown in Fig. 3, cells pretreated with NEM (1 mM) gave almost no blue fluorescence (Fig. 3e), while Cys-incubated cells (Fig. 3f) exhibits strong blue emissions. In contrast, the cells from Hcy (Fig. 3g) and GSH (Fig. 3h) treated groups both gave very low fluorescence signals. The above cell imaging studies clearly demonstrated that probe **1** is capable of selective detection and imaging of Cys over Hcy and GSH in cells.

In summary, we presented here a new approach to achieve enhanced selectivity for detection of Cys over Hcy and GSH through dual cyclization processes and dual PET and ICT quenching mchanism. This approach was demonstrated by incorporation of an acrylate and a maleimide group onto two opposite sides of a single coumarin fluorophore, 3-amino-7-hydroxycoumarin, and the obtained fluorescent probe **1** gave drastically improved fluorescence turn-on response and enhanced selectivity for detection of Cys over Hcy and GSH than using either the acrylate or the maleimide group alone. We also demonstrated the capability of the probe for selective turn-on fluorescence detection of Cys over Hcy and GSH in cell imagings.

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