CHEMISTRY
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Accepted Article

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To be cited as: *Chem. Asian J.* 10.1002/asia.201701733

Link to VoR: http://dx.doi.org/10.1002/asia.201701733

A Journal of

ACES Asian Chemical Editorial Society

A sister journal of Angewandte Chemie and Chemistry – A European Journal

WILEY-VCH

10.1002/asia.201701733

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Bifunctional fluorescent probe for sequential sensing thiols and primary aliphatic amines in distinct fluorescence channels

Meiyu Huang,[a] Lingliang Long,*[a] Ning Wang,[a] Xiangqi Yuan,[a] Siyu Cao,[a] Aihua Gong,[b] and Kun Wang[a]

Abstract: Thiols and primary aliphatic amines (PAA) are ubiquitous and extremely important species in biological system. They have performed significant interplaying roles in complex biological events. A single fluorescent probe differentiating both thiol and PAA can contribute to understanding the intrinsic inter-relationship of thiols and PAA in biological processes. Herein, we rationally constructed the first fluorescent probe that can respond to thiols and PAA in different fluorescence channels. The probe exhibited high selectivity and sensitivity to thiols and PAA. In addition, it displayed sequential sensing ability when the thiols and PAA were coexisted. The application experiment indicated that the probe can be used for sensing thiols and PAA in human blood serum. Moreover, the fluorescence imaging of endogenous thiols and PAA as well as antihypertensive drugs captopril and amlodipine in living cells were successfully conducted.

Introduction

Biological thiols such as cysteine (Cys), homocysteine (Hcy), glutathione (GSH), and protein thiols play significant roles in cellular growth and maintaining redox homeostasis.[1] Abnormal levels of cellular thiols have been implicated in a number of health conditions, including cancer, neurodegenerative disorders, alzheimer's disease, and cardiovascular diseases.[2] On the other hand, biological PAA such as dopamine, serotonin, noradrenaline, and histamine act as neurotransmitter, performing crucial roles in interneuronal chemical communication.[3] Spermidine, spermine, putrescine, and cadaverine are associated with protein synthesis, regulation of cell proliferation, and modulation of gene expression.[4] PAA group is also the key functional group for amino acids, which are the basic unit of proteins. In addition, biological PAA are biomarkers of many diseases. For instance, a high plasma level of putrescine and spermidine is related to breast, colon, and skin cancers.[5]

[a] M. Huang, L. Long, N. Wang, X. Yuan, S. Cao, and K. Wang
Scientific Research Academy & School of Chemistry and Chemical Engineering
Jiangsu University
Zhenjiang, Jiangsu 212013, P. R. China.
Email: longlingliang@163.com
[b] A. Gong
School of Medicine
Jiangsu University
Zhenjiang, Jiangsu 212013, P. R. China.

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Particularly, the thiols and PAA exert interplaying roles in complex biological events. For example, both thiols and PAA in residues of protein are used as robustly nucleophilic groups for protein conjugation and cross-linking.[6] Thiol and PAA also play interrelated roles in the microbicidal process of hypochlorous acid (HOCl), which is generated in neutrophils.[7] Nitrosylations of protein thiols and PAA have been shown to confer nitric oxide (NO)-like biological activities and to regulate protein functions.[8] Both thiols and PAA residues in peptide are involved in peptide ligation reaction.[9] Protein thiols and protein PAA have been found to participate in the nucleophilic reaction with 4-hydroxy-2-nonenal (4HNE), that is a major product of the lipid peroxidation process. Moreover, recent studies implicate that nucleophilic reactions of thiols and PAA with 4HNE are related to the pathogenesis of several diseases, including atherosclerosis and alzheimer's disease.[10]

To better understand the important biological functions of thiol and PAA, especially dissect their complex inter-relationship, indicators that are capable of displaying signals to thiols and PAA in distinct signal channels are highly desirable. A high-performance liquid chromatography (HPLC)-based method has been reported to detect thiols and PAA.[11] However, the HPLC method is unsuitable for tracking the distribution and concentration of thiol and PAA in living cell in non-destructive manner. By contrast, fluorescent probe offers an attractive technique to study biomolecules of interest in a noninvasive manner with high spatial and temporal resolution.[12] Recently, numerous of excellent fluorescent probes for thiols[13] or PAA[14] have been constructed, and these probes have been widely applied for imaging thiols or PAA in biological system. While promising, the currently reported fluorescent probes only specific responded to one analyte (thiol or PAA). To our best knowledge, none of them are able to respond to both thiols and PAA in different signal channels. This issue may be partially addressed by using combined two fluorescent probes (one thiol probe and one PAA probe). Nevertheless, for intracellular imaging, the combination of two fluorescent probes will lead to several problems such as cross-talk, a larger invasive effect, as well as different localization and photobleaching rates of the individual probes, which makes the sensing results complicated.[15] Therefore, to gain deeper insight into biological functions of thiols and PAA in living system, the exploration of a single fluorescent probe responding to both thiols and PAA in different signal channels is in great demand.

To this end, we present the rational design, synthesis, spectral properties, and living cell imaging studies of fluorescent probe **1** (Figure 1a), the first single fluorescent probe that can respond to thiol and PAA in two different fluorescence channels. Specifically, the probe itself exhibited fluorescence in the red channel. After treatment with thiols and

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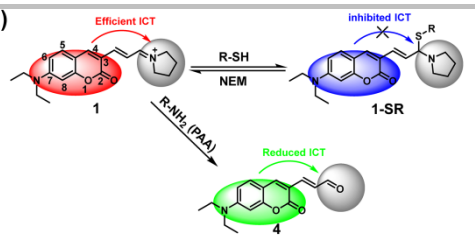
(a)



(b)

Adding	Blue channel	Green channel	Red channel
Free	●	●	●
Thiols	●	●	●
PAA	●	●	●
Thiols + PAA	●	●	●
(i) Thiols + PAA; (ii) NEM.	●	●	●

Figure 1. (a) The sensing reaction of probe **1** with thiol and PAA; (b) the fluorescence response of probe **1** in different fluorescence channels by adding thiols, PAA, thiols + PAA, and (i) thiols + PAA; (ii) NEM.

PAA respectively, probe **1** exhibited its response in blue fluorescence channel and green fluorescence channel correspondingly (Figure 1b). Moreover, in the presence of both thiols and PAA, probe **1** can sequentially respond to thiols and PAA. Probe **1** has been successfully applied for quantitative detecting thiols and PAA in human blood serum. The fluorescence imaging experiments indicated that probe **1** can be used to sequentially detect endogenous thiols and PAA as well as antihypertensive drugs captopril and amlodipine in living cells.

Results and Discussion

Design of the probe. 7-diethylamino-coumarin dye is a commonly used fluorophore for developing fluorescent probes. Moreover, its fluorescence wavelength is highly regulated by the intramolecular charge transfer (ICT) process from the 7-diethylamino-coumarin dye to the substituent linked to the 3-position of the coumarin ring.[16] Thus, a single fluorescent probe responding to thiol and PAA in different wavelength channels can be intriguingly constructed by regulating the ICT efficiency in a platform of 7-diethylamino-coumarin dye. With these considerations in mind, compound **1** was judiciously designed. In probe **1**, the 7-diethylamino-coumarin dye was used as fluorophore, while the electron deficient 1-methylenepyrrolidinium moiety was employed as specific recognition unit for both thiols and PAA. In addition, the 1-methylenepyrrolidinium moiety is essentially conjugated to 3-position of 7-diethylamino-coumarin ring through an ethylene linker. Thus, efficient ICT process from 7-diethylamino-coumarin to 1-methylenepyrrolidinium moiety will occur in probe **1**. However, after reaction with thiol and PAA respectively, the 1-methylenepyrrolidinium in probe **1** will be transformed into two different functional groups with varied electron withdrawing ability. Thus, in the two sensing reaction products, the ICT efficiencies from the 7-diethylamino-coumarin to the 3-position substituents should be different. Consequently, upon treatment with thiol and PAA correspondingly, probe **1** will display fluorescence response in two different wavelength channels. Notably, the strategy of recognition unit conjugated to the 3-position of 7-diethylamino-coumarin through an ethylene linker has also been employed for the construction of other fluorescent probes.[17] However, in probe **1**, the 1-methylenepyrrolidinium group was firstly used as recognition unit for both thiols and PAA. Thus, probe **1** is apparently new and different from these reported probes.

Optical Responses to thiols and PAA. Probe **1** was readily synthesized (Scheme S1). Then, we proceeded to examine the optical response of probe **1** to thiols and PAA. The examinations were performed in 20 mM potassium phosphate buffer / DMF (1 : 3 v/v, pH 7.4) solution at room temperature. As shown in Figure S1a, probe **1** itself featured a prominent fluorescence in the red channel centered at 598 nm (Φ_f = 0.136), which displayed 138 nm red-shift relative to that of 7-diethylamino-coumarin **2**. The large red-shifted fluorescence is apparently ascribed to the efficient ICT process from 7-diethylamino-coumarin to 3-position substituent 1-methylenepyrrolidinium. However, upon adding increasing amount (0-240μM) of 2-mercaptoethanol (ME, a typical thiol compound), the fluorescence in the red channel decreased gradually (Figure 2c), while the fluorescence in the blue channel centered at 467 nm (Φ_f = 0.491) enhanced dramatically (Figure 2a). Thereby, upon treatment with thiols, the fluorescence wavelength of probe **1** showed a large blue shift of 131 nm, implying that the ICT process from the 7-diethylamino-coumarin moiety to the 3-position substituent was almost completely inhibited. Meanwhile, no fluorescence was observed in the green channel (Figure 2b). Thus, probe **1** could be employed to detect thiols in the blue fluorescence channel. Notably, the fluorescence intensity of probe **1** in the blue channel showed linear calibration curve to the concentration of thiols (Figure S2). Thus, probe **1** can be potentially used to quantitatively detect thiols. In addition, the detection limit of probe **1** response to the ME in the blue channel was determined to be 1.80×10^{-8} M (Figure S3).

PAA is the key functional group in most of the amino acids. Therefore, the amino acid glycine (Gly) was utilized as a PAA example to investigate optical response of probe **1** to PAA. After treatment with increasing concentration of Gly (0-300μM), the fluorescence in red channel (centred at 598 nm) decreased (Figure 2f). Concurrently, a new fluorescence in the green channel (centred at 523 nm, Φ_f = 0.341) enhanced rapidly (Figure 2e). After addition of PAA, the fluorescence wavelength of probe **1** exhibited a relative small blue shift of 75 nm, denoting that the ICT process was partially reduced, but not completely inhibited. In the meantime, no fluorescence in blue channel was observed (Figure 2d). Therefore, probe **1** was able to detect PAA in the green channel. The fluorescence intensity of probe **1** at 523 nm was linear to the concentration of Gly (Figure S4), and the detection limits was determined to be 3.08×10^{-8} M (Figure S5).

Then, the responses of probe **1** in the presence of both thiols and PAA were explored. The amino acids cysteine (Cys) contains both thiol and PAA functional groups. Thus, the Cys was utilized to study the response of probe **1** to both thiols and PAA. Upon addition of Cys (0-240μM), the fluorescence in the red channel gradually decreased (Figure 2i), as expected. However, dramatic fluorescence enhancement was only observed in the blue channel (Figure 2g). By contrast, no visible

fluorescence enhancement was noticed in the green channel (Figure 2h). These results suggested that probe **1** preferentially responded to thiols in spite of the presence of both thiols and PAA.

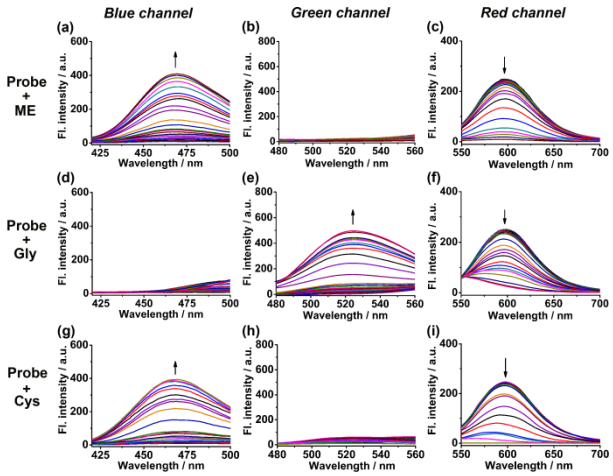


Figure 2. The fluorescence spectra of probe **1** (5 μM) upon addition of (a-c) ME (0-240 μM), (d-f) Gly (0-300 μM), and (g-i) Cys (0-240μM) in 20 mM potassium phosphate buffer / DMF (v/v 1 : 3, pH 7.4) solution. Excitation at 400 nm for the (a), (d), and (g); 466 nm for (b), (e), and (h); and 514 nm for (c), (f), and (i).

The absorption spectra response of probe **1** to thiols and PAA were investigated. Probe **1** exhibited main absorption centered at 516 nm. However, upon addition of ME or Gly, the absorption spectra showed blue shift to 396 nm (Figure S6a) and 448 nm (Figure S6b), respectively. The absorption spectra of probe **1** with thiol displayed a larger blue shift than that with PAA, consistent with the fact that the ICT efficiency in probe **1** with thiol was almost inhibited, while the ICT efficiency in probe **1** with PAA was partially reduced. The absorption spectra of probe **1** to Cys exhibited similar response to ME (Figure S6c). Additionally, the responses of probe **1** to thiols and PAA at different pH solution were explored. Probe **1** can function well at physiological pH condition for both thiols (Figure S7) and PAA (Figure S8). Moreover, the effect of DMF volume percentage in aqueous solution has been investigated (Figure S9, S10). The results indicated that probe **1** can be used for sensing thiols and PAA even in aqueous solutions containing only 20% DMF.

Kinetic studies. In order to clarify the reasons of the preferential response to thiols, the kinetic characteristics of probe **1** reacting with ME or Gly were studied by fluorescence spectroscopy. As shown in Figure 3, probe **1** exhibited quick response to both thiol and PAA. However, the response of probe **1** to thiol is more rapid than that to PAA. After addition of thiol or PAA, the fluorescence response can complete within 1 and 5 minutes correspondingly. In addition, the pseudo-first-order rate constant (k') of probe **1** reacting with ME and Gly was determined to be 3.94427 (Figure S11) and 0.57999 min^{-1} (Figure S12), respectively. Thus, in the presence of both thiols and PAA, the probe **1** mainly responds to thiols is probably due to that the sensing reaction rate for probe **1** with thiols is much faster than PAA.

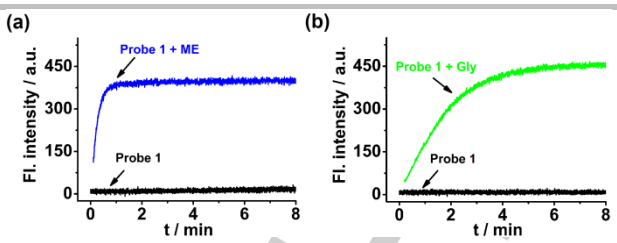


Figure 3. Time dependent fluorescence intensity changes of probe **1** (5 μM) in the absence and presence of (a) 300μM ME (λ_{em} = 467 nm; λ_{ex} = 400 nm) and (b) 300μM Gly (λ_{em} = 523 nm; λ_{ex} = 466 nm).

Reversible studies. The reversibility of the sensing reactions was studied. The ME treated probe **1** was further incubated with *N*-ethylmaleimide (NEM, a thiol reactive reagent).[18] Obviously, the fluorescence in the blue channel was decreased (Figure 4a), but in the red channel was recovered (Figure 4c). This suggested that the sensing reaction product of probe **1** with thiol was converted back to probe **1** when the thiols in solution were eliminated by NEM, which was further confirmed by ^{1}H NMR spectra (Figure S13). Therefore, the sensing reaction of probe **1** with thiols was reversible. Base on this finding, we prudently anticipated that probe **1** can be utilized to sense PAA after probe **1** reacting with thiols. To demonstrate this, the Cys (containing both thiol and PAA) treated probe **1** was further incubated with NEM. The fluorescence in the blue channel was decreased (Figure 4d). However, the fluorescence in the red channel was not recovered (Figure 4f). By contrast, pronounced fluorescence was observed in the green channel (Figure 4e). Apparently, the fluorescence in green channel was originated from the reaction product of the recovered probe **1** with PAA group in Cys. These demonstrated that probe **1** can sequentially sense thiols and PAA in an environment containing both thiols and PAA. Additionally, the reversibility for the sensing reaction of probe **1** with PAA was also examined, and the sensing reaction of probe **1** with PAA is irreversible (Figure S14).

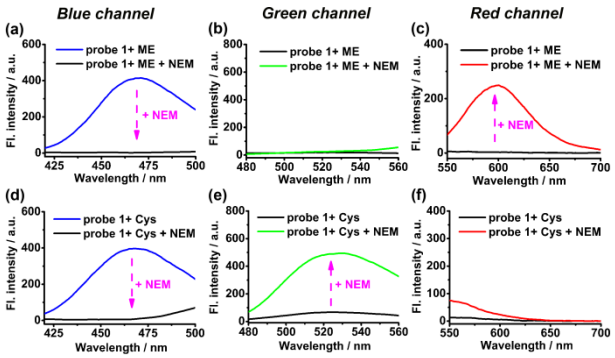


Figure 4. The fluorescence spectra of ME (240 μM) (a-c) and Cys (240 μM) (d-f) treated probe **1** (5 μM) upon further incubated with NEM (1 mM). Excitation at 400 nm for (a) and (d); 466 nm for (b) and (e); and 514 nm for (c) and (f).

Selectivity studies. To assess the specific nature of probe **1** toward thiols and PAA, the fluorescence response of probe **1** to various relevant species were examined by monitoring the fluorescence intensity at 467 nm (blue channel) and 523 nm (green channel), respectively. As shown in Figure 5a and Figure

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S15a, the addition of 300 μM non-thiol-containing amino acids (Gly, Met, Lys, Phe, Trp, His, Ala, Pro, Tyr, Ser, Arg, Gln), n-Butylamine, Ethylenediamine, Aniline, o-Phenylenediamine, Adenine, Guanine, Di-n-butylamine, Piperazine, Morpholine, Triethylamine, Benzenethiol, Resorcinol, Acetic acid, cations (Li^+, Na^+, K^+, Mg^{2+}, Al^{3+}, Zn^{2+}, Fe^{3+}, Fe^{2+}, Cu^{2+}, Hg^{2+}, Ag^+, Ni^+), and anions (Cl^-, I^-, ClO_4^-, SO_4^{2-}, NO_3^-, SO_3^{2-}, HSO_3^-, CO_3^{2-}, HCO_3^-) to the solution of probe **1** (5 μM) caused no noticeable changes in the fluorescence intensity at 467 nm. The only prominent fluorescence enhancement appeared when the thiols (ME, Cys, Hcy, and GSH) were introduced.

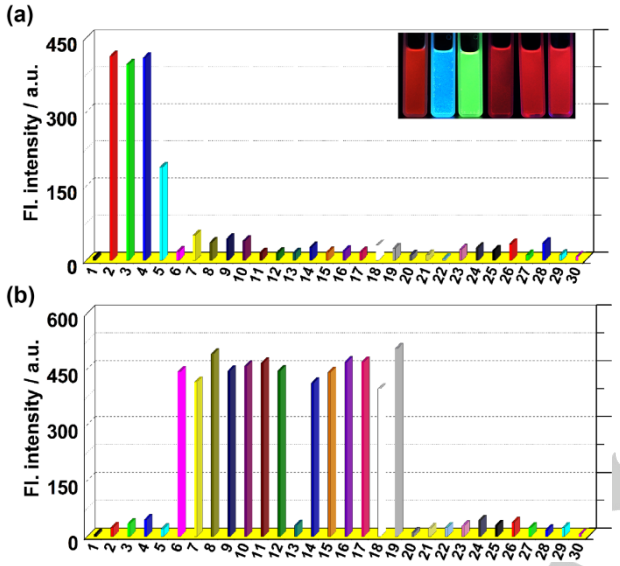

Figure 5. Fluorescence intensity of probe **1** (5 μM) at (a) 467 nm (λ_{ex} = 400 nm) and (b) 523 nm (λ_{ex} = 466 nm) before and after the addition of various species (300 μM). (1) blank; (2) ME; (3) Cys; (4) Hcy; (5) GSH; (6) Gly; (7) Met; (8) Lys; (9) Phe; (10) Trp; (11) His; (12) Ala; (13) Pro; (14) Tyr; (15) Ser; (16) Arg; (17) Gln; (18) n-Butylamine; (19) Ethylenediamine; (20) Aniline; (21) o-Phenylenediamine; (22) Adenine; (23) Guanine; (24) Di-n-butylamine; (25) Piperazine; (26) Morpholine; (27) Triethylamine; (28) Benzenethiol; (29) Resorcinol; (30) Acetic acid. Inset of (a): visual fluorescence color changes of probe **1** (5 μM) in the presence of various species (300 μM), the photo was taken under illumination of a handheld UV lamp, from left to right: blank, ME, Gly, acetic acid, Aniline, adenine.

The fluorescence response of probe **1** to various species at 523 nm was displayed in Figure 5b and Figure S15b. Pronounced fluorescence enhancement at 523 nm was merely observed by addition of molecules with PAA functional group, such as non-thiol-containing amino acids (Gly, Met, Lys, Phe, Trp, His, Ala, Tyr, Ser, Arg, Gln), n-Butylamine, and ethylenediamine. As described above, no fluorescence response at 523nm for Cys, Hcy, and GSH is due to the preferential sensing reaction of probe **1** with thiol group in these molecules. Interestingly, the aromatic amines (Aniline, o-Phenylenediamine, Adenine, Guanine), secondary amines (Di-n-butylamine, Piperazine, Morpholine), and tertiary amine (Triethylamine) also gave no fluorescence response at 523 nm, indicating that probe **1** can distinguish PAA from aromatic amines, secondary amines, and tertiary amines. It should be mentioned that the amino acid Pro also gave no fluorescence response at 523 nm. This is because the Pro molecular structure only contains a secondary amine. These results corroborated that probe **1** had selective response toward thiols and PAA in blue channel and green channel, correspondingly. In addition, the probe **1** can be conveniently used to distinguish thiols and PAA by "naked-eyes" (Figure 5a, inset).

Sensing reaction products. The sensing reaction products of probe **1** with thiols and PAA were inspected. Excess ME was added to the solution of probe **1** in d_6-DMSO and the solution was used for ^{1}H NMR spectra study. The results indicated that the sensing reaction of probe **1** with ME was the nucleophilic addition reaction of the thiol to C=N$^+$ double bond in probe **1**, which afforded the adduct compound **1**-ME (Figure S16, Scheme S2). The reaction product of probe **1** with ME was also confirmed by the ESI-MS spectra (Figure S17). Then, the sensing reaction product of probe **1** with Gly was demonstrated to be compound **4** according to the ^{1}H NMR spectra (Figure S18), ESI-MS spectra (Figure S19), absorption spectra and fluorescence spectra (Figure S20). On the basis of the sensing reaction product, a plausible sensing reaction mechanism of probe **1** with Gly was proposed in Scheme S3. It is worth noting that compound **4** contains an aldehyde group which has been reported to display fluorescence response to thiols.[17j, 19] Thus, compound **4** would be possible to further react with thiols, interfering the sensing process. However, in our experiments, the thiols were eliminated by NEM before the sequential detection of PAA. Consequently, no thiols will participate in the reaction with compound **4**. Additionally, probe **1** responding to thiol and PAA can complete within 5 min, whereas the reported fluorescent probes with aldehyde group responding to thiols require more than 50 min.[17j, 19] No pronounced fluorescence changes were observed after compound **4** incubating with Cys within 5 min (Figure S21). Accordingly, under the testing condition, the resulting compound **4** will not be interfered by thiols.

Theoretical calculation. According to the optical response of probe **1** to thiol and PAA, the ICT efficiency from the 7-diethylamino-coumarin moiety to the 3-position substituent was partially reduced upon treating with PAA, and was almost inhibited upon treating with thiol. To verify this, time-dependent density functional theory (TD-DFT) calculations were conducted using the Gaussian 09 program. The calculated electron distributions in HOMO and LUMO of probe **1** as well as its sensing reaction products **4** (probe **1** with PAA) and **1**-ME (probe **1** with thiol) are shown in Figure S22. Apparently, the HOMO-LUMO transition in probe **1** was the electron redistribution from the 7-diethylamino-coumarin moiety to the 3-postion substituent 1-methylenepyrrolidinium. Thus, the ICT process in probe **1** should be efficient. Similarly, the HOMO-LUMO transition in compound **4** was the charger transfer from the 7-diethylamino-coumarin moiety to the 3-postion substituent aldehyde group. However, the ICT efficiency in compound **4** was reduced relative to that in probe **1**. For compound **1**-ME, the ICT process from the 7-diethylamino-coumarin moiety to the newly formed 3-postion substituent pyrrolidine was completely inhibited. Furthermore, owing to the pyrrolidine is a good electron donating group, chargers even transfer from the pyrrolidine group back to 7-diethylamino-coumarin moiety. The variation of ICT processes was also studied by means of charge transfer parameter f_{CT} (Table S1).[20] At the ground state geometry, the f_{CT} values for **1**, **4**, and **1**-ME was calculated to be 0.300719, 0.251691, and -0.05898, respectively. Thus, compared with that of probe **1**, the ICT process from the 7-diethylamino-coumarin dye to the 3-

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position substituent in compound **4** was partially reduced, and in compound **1**-ME was completely inhibited. It is known that the decrease of ICT process from the 7-diethylamino-coumarin moiety to the 3-position substituent makes the fluorescence wavelength blue shift.[16] Therefore, compound **4** showed blue shift fluorescence in the green channel while compound **1**-ME displayed larger blue shift fluorescence in the blue channel have been theoretically revealed.

Monitoring thiol and PAA levels in human blood serum. Due to thiol and PAA playing important interrelated roles in biological system, it is urgent to establish an effective method to sense thiol and PAA in biological samples. Thus, probe **1** was employed to sequentially detect thiols and PAA levels in human blood serum. The commercially available human blood serum was firstly treated with a reducing agent, triphenyl-phosphine, to reduce the oxidized disulfide to free thiols.[21] Then, 0.5 mL of the reduced serum samples were added to a solution of probe **1** (5 μM) at room temperature. Clearly, probe **1** displayed visible fluorescence response to the serum thiols in blue channel (Figure 6). According to the fluorescence intensity in blue channel and the equation in Figure S2, the level of thiols in human blood serum was determined to be 308.8μM, which is in agreement with the results in literature.[22] Next, the serum treated probe **1** solution was further incubated with NEM (1 mM). Pronounced fluorescence response to serum PAA in the green channel was observed. Together with the equation in Figure S4, the PAA level in serum was determined to be 3150.8μM. Thus, probe **1** can be used to sequentially detect thiols and PAA in human blood serum.

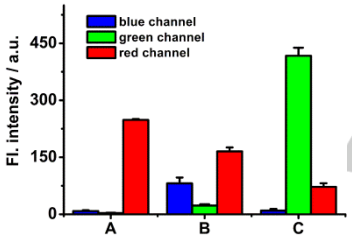

Figure 6. Detection of thiols and PAA in human blood serum (blue channel: λ_{em} = 467 nm; λ_{ex} = 400 nm; green channel: λ_{em} = 523 nm; λ_{ex} = 466 nm; red channel: λ_{em} = 598 nm; λ_{ex} = 514 nm). Sample A: probe **1** (5 μM); sample B: probe **1** (5 μM) treated with reduced serum (0.5 mL); sample C: probe **1** treated with reduced serum (0.5 mL), then further treated with NEM (1 mM).

Fluorescence imaging of thiols and PAA in live cells. The application of probe **1** for imaging thiols and PAA in living cells was also conducted. Firstly, probe **1** was employed to sequentially image endogenous thiols and PAA in living cells. The HepG2 cells were directly stained with probe **1** (5 μM) for 15 min, and then the cells were used for fluorescence imaging by a laser confocal scanning microscope. Obviously, intense fluorescence was observed in blue channel (Figure 7b), but almost no fluorescence was observed in green channel (Figure 7c) and red channel (Figure 7d). These indicated that probe **1** sensed the endogenous thiols in living cells. Next, the cells were further incubated with NEM (1 mM). The fluorescence in the blue channel quenched (Figure 7f) while in the green channel enhanced (Figure 7g). Thus, the single probe **1** can sequentially sense endogenous thiols and PAA in living cells.

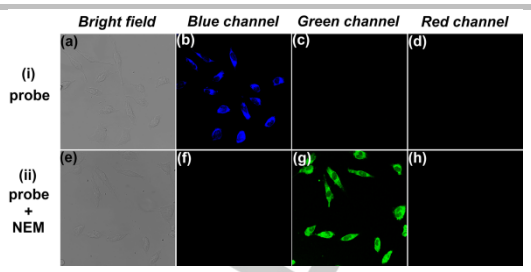

Figure 7. Fluorescence imaging endogenous thiols and PAA in HepG2 cells. (i) The HepG2 cells were stained with probe **1** (5 μM) for 15 min; (ii) the cells were firstly stained with probe **1** (5 μM) for 15 min, and then the cells were further incubated with NEM (1 mM) for 30 mins.

Captopril and amlodipine are well-known antihypertensive drugs. In their molecular structures, captopril contains a thiol group and amlodipine contains a PAA group. Moreover, captopril and amlodipine have been reported for combination antihypertensive therapy.[23] Monitoring the levels of captopril and amlodipine in biological system may help to know the combination therapy effect. Thus, probe **1** was applied for sequentially tracking the captopril and amlodipine levels in living cells. The SMMC-7721 cells were firstly pretreated with 1 mM methyl isothiocyanate (MITC, a thiol and amine reactive reagent)[24] to consume the free thiols and PAA within the cells. Then the cells were stained with probe **1** (5 μM) for 15 min. It was found that intense intracellular fluorescence was observed in the red channel (Figure 8d), but almost no fluorescence was observed in the blue channel (Figure 8b) and green channel (Figure 8c), indicating that probe **1** did not respond to other non-thiols and non-PAA species in the living cells. Subsequently, the cells were supplemented with both captopril (500 μM) and amlodipine (500 μM). It gave intense fluorescence in blue channel (Figure 8f) but essentially no fluorescence in green channel (Figure 8g) and red channel (Figure 8h), implying that probe **1** specific responded to the thiol group in captopril at this stage. Next, the cells continued to be treated with NEM (1 mM). Strong fluorescence was observed in green channel (Figure 8k), but no fluorescence was noticed in blue channel (Figure 8j) and red channel (Figure 8l). These results indicated that the probe **1** can sequentially tracking captopril and amlodipine levels in living cells.

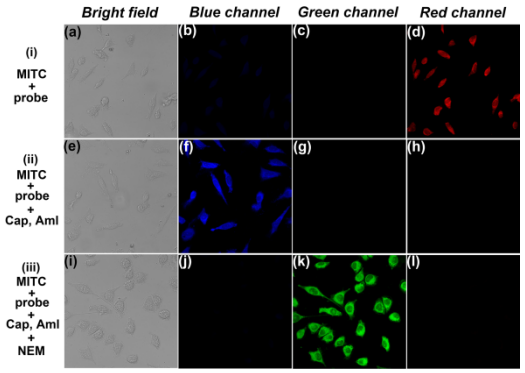

Figure 8. Fluorescence imaging captopril and amlodipine levels in SMMC-7721 cells. (i) The cells were pretreated with MITC (1 mM) for 15 min, and then stained with probe **1** (5 μM) for 15 min; (ii) The cells were pretreated with MITC (1 mM) for 15 min, and then stained with probe **1** (5 μM) for 15 min, subsequently with captopril (Cap) (500 μM) and amlodipine (Aml) (500 μM) for

15 min; (iii) The cells were pretreated with MITC (1 mM) for 15 min, and then stained with probe **1** (5 μM) for 15 min, subsequently with captopril (500 μM) and amlodipine (500 μM) for 15 min, and finally with NEM (1 mM) for 30 min.

Conclusions

In this work, we rationally constructed the first fluorescent probe, compound **1**, for sensing thiols and PAA in distinct fluorescence channels. In the absence of thiols and PAA, probe **1** itself displayed fluorescence in the red channel. However, upon addition of thiols and PAA separately, probe **1** displayed obviously fluorescence enhancement in the blue fluorescence channel and green fluorescence channel correspondingly. Moreover, in the presence of both thiols and PAA, probe **1** can sequentially sense thiols and PAA. The optical experiments indicated that probe **1** exhibited highly specific response to thiols and PAA. Other species even including aromatic amines, secondary amines, and tertiary amine gave no fluorescence response. TD-DFT calculation denoted that the response of probe **1** to thiols and PAA in different fluorescence channels is due to the variation of ICT efficiency after the sensing reaction of probe **1** with thiols and PAA. The practical utility of the probe **1** for sequential sensing thiols and PAA in human blood serum has been successfully conducted. In addition, the fluorescence imaging experiment showed that probe **1** can be applied for sequentially sensing endogenous thiols and PAA in living cells. Moreover, sequential tracking the levels of antihypertensive drugs captopril and amlodipine in living cells also has been performed. We believe that the novel fluorescent probe developed in this work will be widely used for dissecting the complex inter-relationship of thiols and PAA in biological system.

Experimental Section

Reagents. 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. Twice-distilled water was used throughout all experiments. The solutions of various relevant species were prepared from 2-mercaptoethanol (ME), Cys, Hcy, GSH, Gly, Met, Lys, Phe, Trp, His, Ala, Pro, Tyr, Ser, Arg, Gln, n-Butylamine, Ethylenediamine, Aniline, o-Phenylenediamine, Adenine, Guanine, Di-n-butylamine, Piperazine, Morpholine, Triethylamine, Benzenethiol, Resorcinol, Acetic acid, LiCl, NaCl, KCl, $MgCl_2{\cdot}6H_2O$, $Al(NO_3)_3{\cdot}9H_2O$, $Zn(NO_3)_2{\cdot}6H_2O$, $Fe(NO_3)_3{\cdot}9H_2O$, $FeSO_4{\cdot}7H_2O$, $CuCl_2{\cdot}2H_2O$, $HgCl_2$, $AgNO_3$, $NiCl_2{\cdot}6H_2O$, NaI, $LiClO_4{\cdot}3H_2O$, Na_2SO_4, $NaNO_3$, Na_2SO_3, $NaHSO_3$, Na_2CO_3, Na HCO_3. TLC analyses were performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200-300), both of which were obtained from the Qingdao Ocean Chemicals.

Apparatus. Melting points of compounds were measured on a Beijing Taike XT-4 microscopy melting point apparatus, and all melting points were uncorrected. Mass spectra were recorded on a LXQ Spectrometer (Thermo Scientific) operating on ESI. ^1H and ^{13}C-NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz and 100 MHz respectively. Elemental (C, H, N) analysis were carried out using Flash EA 1112 analyzer. Electronic absorption spectra were obtained on a SHIMADZU UV-2450 spectrometer. Fluorescence spectra were measured on a Photon Technology International (PTI) Quantamaster fluorometer with 2 nm excitation and emission slit widths. Confocal fluorescence microcopy imaging experiments were performed on a Leica TCS SP5 II laser confocal scanning microscope. The pH measurements were performed with a pH-3c digital pH-meter (Shanghai ShengCi Device Works, Shanghai, China) with a combined glass-calomel electrode.

Synthesis. The general synthetic procedures for probe **1** were displayed in Scheme S1. In brief, treatment of 7-diethylaminocoumarin **2** with DMF and $POCl_3$ afforded the 7-diethylaminocoumarin-3-aldehyde **3,** which was further converted to the intermediate **4**. Then, the probe **1** was readily prepared by condensation of **4** with pyrrolidine. The details for the preparation of the compounds and their NMR and ESI-MS spectra can be found in the Supporting Information.

Preparation of the test solution. The stock solution of probe **1** (5×10^{-5} M) was prepared in DMF, and the stock solution of various relevant testing species (1×10^{-3} M) was prepared by dissolving an appropriate amount of testing species in water or in DMF. The test solution of the probe **1** (5 μM) in 20 mM potassium phosphate buffer / DMF (1 : 3 v/v, pH 7.4) was prepared by placing 0.5 mL of the probe **1** stock solution, 3.25 mL DMF and an appropriate aliquot of each testing species stock into a 5 mL volumetric flask, and then diluting the solution to 5 mL with 20 mM potassium phosphate buffer (pH 7.4). The resulting solution was shaken well and incubated at room temperature for 5 min before recording the spectra. After the sensing reaction of probe **1** with thiols, the sequential sensing PAA was conducted by further adding NEM to the testing solution, and then incubating for another 30 min before recording the spectra.

Determination of thiols and PAA in human blood serum. For sensing biological thiols in human blood serum, the serum was firstly treated with a reducing agent, triphenylphosphine, to reduce all the oxidized disulfide to free thiols. Briefly, 2 mL human blood serum sample was diluted with 1 mL distilled water, then treated with 1 mL triphenylphosphine solution in DMF (1.5×10^{-3} M) for 30 min at room temperature. After filtration, the reduced serum sample (0.5 mL) was added into a 5 mL volumetric flask with a solution of 0.5 mL probe **1** stock solution (5×10^{-5} M) and 3.125 mL DMF. And then, the solution was diluted to 5 mL with 20 mM potassium phosphate buffer (pH 7.4). The solution was shaken well and incubated at room temperature for 5 min before recording the spectra. For sequential sensing PAA in human blood serum, NEM (1 mM) was further added into the above serum treated probe **1** solution. After incubation for 30 min, the fluorescence emission spectra were recorded.

Cell culture and fluorescence imaging. HepG2 cells and SMMC-7721 cells were seeded in a 24-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum for 24 h.

For sequential fluorescence imaging of endogenous thiols and PAA, the HepG2 cells were directly stained with probe **1** (5 μM) in the culture medium for 15 min at 37°C. After washing with PBS three times to remove the remaining probe, the fluorescence images in the red channel, green channel, and blue channel were acquired on a Leica TCS SP5 II laser confocal scanning microscope with an objective lens (×40). Red Channel was set at 575-650 nm with excitation at 514 nm. Green Channel was set at 510-540 nm with excitation at 458 nm. Blue Channel was set at 450-480 nm with excitation at 405 nm. Subsequently, the above cells were further treated with NEM (1 mM) for 30 min. After washing with PBS three times, the fluorescence images were acquired again in the red channel, green channel, and blue channel.

For sequential fluorescence imaging of antihypertensive drugs captopril and amlodipine in living cells, the SMMC-7721 cells were pretreated with 1 mM MITC to consume the free thiols and PAA within the cells. After washing with PBS three times, the cells were stained with probe **1** (5 μM) for 15 min. Fluorescence images were acquired in the red channel, green channel, and blue channel for imaging the fluorescence of probe **1** in living cells. Then, the cells were further treated with captopril (500 μM) and amlodipine (500 μM) for 15 min. The fluorescent images were acquired in the red channel, green channel, and blue channel for imaging the level of captopril in living cells. Subsequently, the above cells were

further treated with NEM (1 mM) for 30 min. After washing with PBS three times, the fluorescence images were acquired again in the red channel, green channel, and blue channel for imaging the level of amlodipine in living cells.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (21202063), the Natural Science Foundation of Jiangsu Province (BK2012281), and the Research Foundation of Jiangsu University (11JDG078).

Keywords: Fluorescence spectroscopy • Sensors • Dyes/Pigments • Thiols • Primary aliphatic amine

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10.1002/asia.201701733

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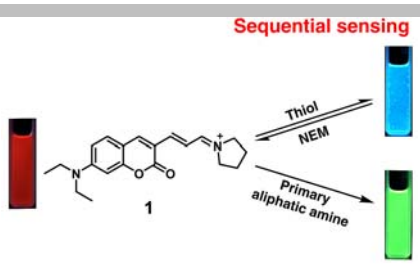
FULL PAPER

Herein, we present the rational design, synthesis, spectral properties, and living cell imaging studies of fluorescent probe **1**, the first single fluorescent probe that can respond to thiol and primary aliphatic amines in two different fluorescence channels.



Meiyu Huang,[a] Lingliang Long,[a] Ning Wang,[a] Xiangqi Yuan,[a] Siyu Cao,[a] Aihua Gong,[b] and Kun Wang[a]*

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Bifunctional fluorescent probe for sequential sensing thiols and primary aliphatic amines in distinct fluorescence channels