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Diketopyrrolopyrrole-based ratiometric fluorescent probe for the sensitive and selective detection of cysteine over homocysteine and glutathione in living cells

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A new fluorescent probe (**DPP-AC**) based on diketopyrrolopyrrole with acrylate group was designed and synthesized for the sensitive and selective detection of biological thiols. The acrylate group of **DPP-AC** as the receptor moiety displayed obvious ratiometric changes from red to yellow upon addition of cysteine (Cys), and the change of the fluorescence ratio (I_{552}/I_{664}) was over 20 folds. The limit of detection towards Cys was calculated down to 84 nM, much lower than the normal concentration in living cells. More importantly, **DPP-AC** had outstanding selectivity towards cysteine over homocysteine (Hcy), glutathione (GSH) and other amino acids. **DPP-AC** was successfully employed to detect endogenous Cys in Hela cells and discriminate Cys from Hcy and GSH in living cells.

Introduction

Biological thiols, such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH), mainly refer to the small intracellular biomolecules containing sulfhydryl group and have strong nucleophilicity and redox reactivity.[1-3] These biothiols widespreadly exist in biological environment, and they serve significant roles in maintaining the redox homeostasis of proteins, cells, and organisms, and keeping human normal physiology function.[4-6] The abnormal contents of the thiols are closely associated with various neurodegenerative and cardiovascular diseases, such as Alzheimer`s disease, folated deficiency, various tumors, liver and skin damage, neurotoxicity, Parkinson`s disease, and so on. [7-11] For these reasons, it is of critical importance to take some scientific measures to detect these biological thiols selectively and sensitively in research and clinical treatment, which has also attracted extensive attention in recent years. [12,13]

There are many classical ways to detect these small biological thiols, such as electrochemical detection and high performance liquid chromatography. However, the development of small molecule probes for the detection of thiols under physiological conditions has attracted increasing interest because of their high sensitivity, selectivity, rapid response, and low cost.[14-17] In particular, the ratiometric fluorescent probes show better performances than traditional turn-on and turn-off fluorescent probes. Because ratio fluorescent signals with shift in emission maxima can be applied to make internal calibration of reacted and unreacted probe, so that they could be minimize the interference of the systematic errors, such as the light intensity, sample thickness and heterogeneity, dye distribution, and so on.[18-23] Therefore, the ratiometric probes are comparatively ideal fluorescent probes in biological detection and imaging.

Recently, plenty of scientific researches have been carried out to implement the detection of biological thiols. But how to discriminate these three sulfhydryl-containing amino acids (Cys, Hcy and GSH) remains a considerable problem.[24,25] The sulfhydryl groups on the side chain of these amino acids perform strong nucleophilic reactivity and redox susceptibility, which are widely applied as the recognition mechanisms, including the Michael addition, [26-29] cleavage of sulfonic ester linker, [11,30-32] cleavage of the ether linker,[33] cleavage of the disulfide group, [6,34,35] metal complex-displacement coordination, [36] and others. [12,13] As the consequence, it is fairly difficult to discriminate the thiols by these reaction mechanisms. Some impressive work has succeeded in differentiate GSH from these three thiols,[7,8,12,24,37,38] but the mechanisms of distinguishing Cys with Hcy are much less, because there are high similarities between them in structures and reaction activity, and the only difference is that Hcy has an additional methylene unit.[39] However, the reaction of conjugate addition/cyclization sequence of acrylates is one of the effective mechanisms to solve the problem. In this reaction, both the sulfhydryl and amino groups are involved in, and the intramolecular cyclization process differs in reaction rate between Cys and Hcy, meanwhile GSH cannot set off such cyclization reaction due to its overlong side chain. Acrylate group is such an ideal recognition moiety towards thiols using the addition/cyclization reaction mechanism.[40-42] Besides, acrylate group is also a strong electron-withdrawing moiety and could induce sharply bathochromic shift in fluorescence,

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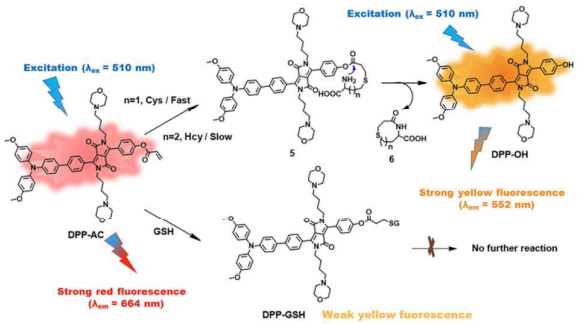
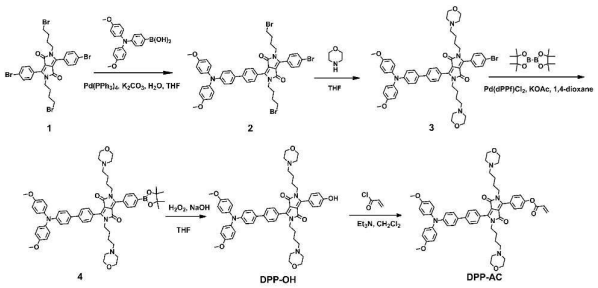


Scheme 1 Chemical structures of probe **DPP-AC** and reaction mechanism of probe **DPP-AC** with Cys, Hcy and GSH

so its introduction may cause the ratiometric fluorescence change before and after the reaction with biothiols. And a lot of works have achieved great success in this field.[43-44]

1,4-Diketo-3,6-diphenylpyrrole[3,4-c]pyrrole (DPP) and its derivatives have been extensively applied in many fields due to their exceptional thermal and photochemical stability, large extinction coefficients, and high fluorescence quantum yields in both solution and membrane.[45-47] Especially, DPP is a typical kind of electron-deficient fluorophore and has so many modification sites. When functionalized with electron-donor groups, DPP derivatives can exhibit red to near-infrared (NIR) emission and large Stokes shift, which are of great benefit to their applications in bioimaging.[48,49] However, only a few of applications of DPP were reported in the field of reaction-based fluorescent probes.[31,46,50-53]

Recently, our group have reported a red turn-on fluorescent chemodosimeter (**DPP-DNBS**) based on DPP derivative for the detection of Cys.[31] But its sensitivity and selectivity towards biothiols were not pretty satisfying for the discrimination in living cells. And the poor water-solubility caused much inconvenience to the biological applications. In order to further boost the optical performance, a new fluorescent probe based on DPP derivatives (**DPP-AC**, Scheme 1) was developed for the detection of thiols in this study. The methoxytriphenylamine group with strong electron-donating ability could enormously extend the fluorescent emission. Acrylate group was introduced as the selective reaction site and electron-withdrawing group in hope of realizing higher selective detection and ratiometric fluorescence change. Compared with 2,4-dinitrobenzenesulfonyl group, the electron-withdrawing ability of acrylate group was much weaker and might could not quench the fluorescence. And the



Scheme 2 Synthetic route of probe **DPP-AC**

morpholine groups were connected to the side chains of DPP in order to improve the water solubility, which could made the bioprobe possess better cytocompatibility.[54,55] As expected, the probe **DPP-AC** displayedhigh selectivity for detection of Cys over Hcy and GSH. And the ratiometric property made it show much lower limit of detection and more excellent anti-interference capability. Moreover, probe **DPP-AC** had been successfully used to detect endogenous Cys and discriminate Cys, Hcy and GSH in living Hela cells.

Results and discussion

Molecular design and synthesis

In order to obtain the sensitive and selective fluorescent probes with long emission wavelength for the detection of biological thiols, probe **DPP-AC** was designed and synthesized (Scheme. 2). DPP was an electron-deficient fluorophore, so the introduction of the methoxytriphenylamine group constructed a fluorescent molecule with typical donor-acceptor structure, which greatly increased the absorption and emission wavelength of the fluorophore and made the fluorescent signal obtain deeper tissue penetration and minimum interference of the auto-fluorescence in the organisms. The acrylate group had strong electron-withdrawing ability as well as the reactivity with biological thiols. So its existence might result in further red shift in fluorescence, causing the ratiometric fluorescence changes. After undergoing the

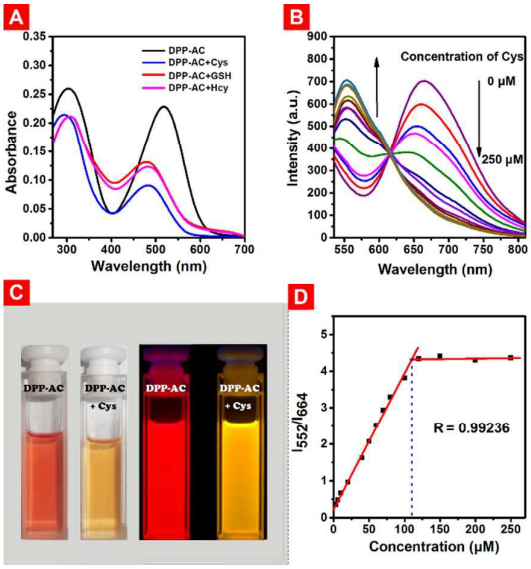


Figure 1 (A) Absorption of **DPP-AC** (10 μM) in the absence and presence of Cys, Hcy, and GSH (100 μM) in the DMSO-buffer solution. (B) Fluorescence spectra of **DPP-AC** (10 μM) reacted with various concentrations (0-250 μM) of Cys in DMSO-buffer solution. (C). Photographs of **DPP-AC** before and after the reaction with Cys, under natural light and UV radiation respectively. (D) Linear correction between the fluorescence ratio (I_{552}/I_{664}) changes and the Cys concentrations addition (0-250 μM) at 37 °C in DMSO-buffer solution (PBS: DMSO = 6: 4, v/v, pH = 7.4) at 37°C with excitation wavelength (λ_{ex}) at 510 nm.

cyclization and cleavage reaction with targets, the fluorescence would change with the leaving of the electron-withdrawing groups. Additionally, the introduction of morpholine groups would be beneficial to improve the water solubility of the molecule in some degree.

The synthesis route of probe **DPP-AC** is shown in Scheme 2. Compound **2** was obtained through Suzuki coupling reaction between compound **1** and 4-(bis(4-methoxyphenyl)-amino)phenyl boronic acid. After introducting morpholine to the alkyl chain of DPP, compound **3** could react with bis(pinacolato)diboron under the catalysis of bis(diphenylphos-phino)ferrocene palladium dichloride. And compound **DPP-OH** was synthesized by the oxidation of compound **4** with hydrogen peroxide. Finally, with the existence of triethylamine, **DPP-OH** could react with acryloyl chloride to obtain desirable products, **DPP-AC**. All the structures of the new compounds were confirmed by ^{1}H NMR, ^{13}C NMR and MS-ESI. And the relevant spectra were shown in the Supporting Information.

Optical response of probe DPP-AC towards Cys

The absorption and time-dependent fluorescence responses of **DPP-AC** towards Cys were firstly studied in the DMSO-buffer solution (PBS: DMSO = 6: 4, v/v, pH = 7.4) at 37 °C in order to obtain more obvious experimental phenomena and clearer photographs. From the absorption spectra of **DPP-AC** (10 μM) shown in Figure 1A, the maximum absorption wavelength showed obvious blue shift about 20 nm when reacting with 10.0 equivalents (100.0 μM) of Cys, Hcy and GSH. And the addition of biothiols reduced the absorbance significantly, especially the addition of Cys. Hence, the color of the solution shallowed and turned yellow slightly, which was observed with naked eyes. Besides, we were surprised to find that the changes of fluorescence were also very obvious (Figure 1C). The solution of **DPP-AC** showed strong red fluorescent emission at 664 nm before adding Cys (Figure S1). But with the addition of Cys, the red fluorescence gradually declined, while the yellow fluorescence at 552 nm gradually increased. After 100 min, the solution showed yellow fluorescence and kept unchanged. This phenomenon might result from the effect of intramolecular charge transfer (ICT) process. The acrylate had electron-withdrawing ability, which made the fluorescence show red shifts in fluorescence by a large margin. However,

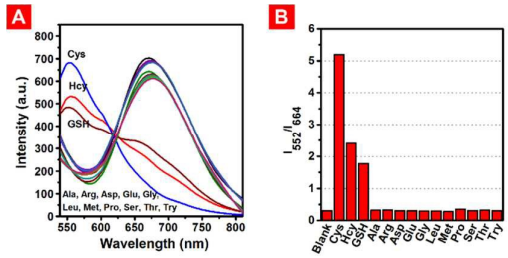


Figure 2 Fluorescent responses of **DPP-AC** (A, B) upon addition various kinds of amino acids. Fluorescent spectra (A) were acquired after the addition of various amino acids (20 μM) in DMSO-buffer solution (PBS: DMSO = 6: 4, v/v, pH = 7.4) at 37°C. Bars (B) represent the fluorescence intensity ratio I_{552}/I_{664} of DPP-AC (λ_{ex} = 510 nm).

the ICT process was cut off after the reaction of acrylate with Cys, and the fluorescence turned from red to yellow simultaneously. This kind of ratiometric fluorescent changes was of great benefit to the application in living cells, and it was also conducive to obtain lower limit of detection.

The concentration-dependent optical response towards Cys was also studied. Different concentration from 0.0 to 250.0 μM of Cys solution was added to **DPP-AC** (10 μM) solution. After 100 min, the fluorescence intensity was measured. The change in fluorescence upon titration with Cys was recorded in Figure 1B. The free **DPP-AC** exhibited the fluorescence at 664 nm. With addition of Cys, the fluorescence decreased gradually, and at the same time the fluorescence at 552 nm appeared, suggesting that a new fluorophore was built. The fluorescence intensity ratio (I_{552}/I_{664}) of **DPP-AC** showed good linear correlation with the concentrations of Cys (0.0-100.0 μM) (Figure 1D). Therefore, **DPP-AC** could also be used for quantitative detection of Cys within a certain range. The limit of detection was calculated as 84 nM, much lower than that the normal concentration (0.25-0.38 mM) in living cells. The fluorescence quantum yields of **DPP-AC** and **DPP-OH** were estimated 0.128 and 0.149 with rhodamine B in ethanol as a standard, respectively.

Selectivity towards different amino acids

When designing a probe for biological application, excellent selectivity is one of the most important properties we need take into consideration. For this purpose, the three biothiols (Cys, Hcy, GSH) and eleven other kinds of amino acids were selected to conduct contrast experiments (Figure 2), including alanine (Ala), arginine (Arg), aspartic (Asp), glutamic (Glu), glycine (Gly), leucine (Leu), methionine (Met), proline (Pro), serine (Ser), threonine (Thr), and tryptophan (Try).

As can be seen in Figure 2, all of the common amino acids without sulfhydryl groups did not cause the ratiometric change of **DPP-AC** in DMSO-buffer solution (PBS: DMSO = 6: 4, v/v, pH = 7.4). On the contrary, the addition of Cys, Hcy and GSH would induce the ratiometric changes of the fluorescence. But the fluorescence changes induced by Hcy were much less and slower than Cys. Besides, there was only a slight change in

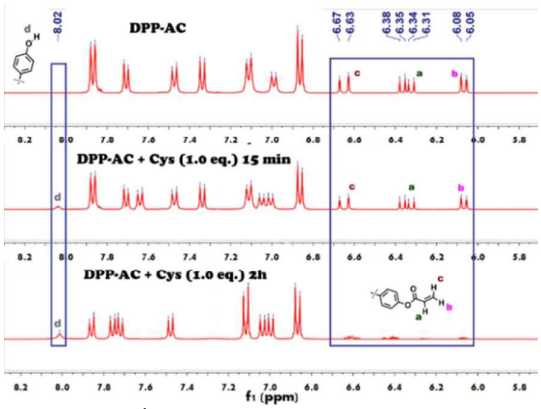


Figure 3 Segmental ^{1}H NMR spectra of **DPP-AC** (5 mM) reacted with 1.0 equivalent Cys for 0 s (without Cys), 15 min and 2h in DMSO-d_6.

fluorescence for GSH under the same conditions. This difference might result from the different relative reaction rates of intramolecular cyclization, because the cyclization reaction to form an eight-membered ring was more kinetically unfavorable than the seven-membered ring, and the overlong side chain of GSH prevent it from setting off such reactions. Based on the different reaction rate and mechanism, **DPP-AC** showed more outstanding selectivity towards Cys over Hcy, GSH and other amino acids.

Detection mechanism in sensing Cys

In order to further explain the reaction mechanism of **DPP-AC** with Cys, ^{1}H NMR and MS titrations were performed. Based on the reported conjugate addition/cyclization reaction of acrylate moiety in detecting Cys over Hcy and GSH, the carbon-carbon double bond in acrylate moiety of **DPP-AC** would disappear after the intramolecular cyclization and a small cyclic molecule would form, which definitely could bring some changes in the low field of the ^{1}H NMR (Figure 3). There were three groups of peaks ranging from 6.05 ppm to 6.67 ppm in Figure 3, corresponding to the three protons of acrylate moiety. Upon addition of Cys, they were significantly decreased and finally disappeared after 2 hours on account of the change from carbon-carbon double bond to single bond with the addition of sulfhydryl group. In the meanwhile, a new single peak gradually appeared at 8.02 ppm on account of the generation of phenolic hydroxyl group, illustrating the generation of new compound **DPP-OH**. And the MS titration was also performed to confirm the reaction mechanism (Figure S2). After reacting with Cys (10 μM) for 30 min, the reaction products were measured by the MS spectroscopy. The peak at m/z = 890.4487 and m/z = 944.4519 referred to **DPP-OH** ($[C_{57}H_{61}N_5O_8+H]^+$ calculated m/z 890.4422) and the unreacted **DPP-AC** ($[C_{54}H_{59}N_5O_7+H]^+$ calculated m/z 944.4598). Besides, the addition intermediate product (compound **5**, $[C_{61}H_{70}N_6O_{10}S+H]^+$ calculated m/z 1079.4952) and the cyclization product (compound **6**, $[C_6H_9NO_3S+H]^+$ calculated m/z 176.0311) were also found at m/z = 1079.4915 and 176.0301, respectively.

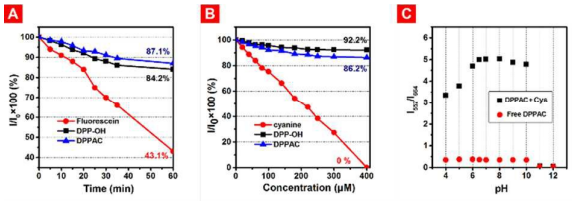


Figure 4 (A) Photostability of **DPP-AC** (blue triangles) and **DPP-OH** (black squares) compared with fluroscein isothiocyanate (red dots) under 1kW/m^2 light irradiation for 0-60 min. I_0 is the initial fluorescent intensity and I is the fluorescent intensity of the sample after the light irradiation of certain time intervals. (B) Chemostability of **DPP-AC** (blue triangles) and **DPP-OH** (black squares) compared with cyanine (red dots) after the addition with 40 equivalents of NaClO. I_0 is the initial fluorescent intensity and I is the fluorescent intensity of the sample after the addition of certain equivalents of NaClO. (C) The fluorescent intensity raito (I_{552}/I_{664}) of 10 μM **DPP-AC** and before (red dots) and after (black squares) the reaction with 100 μM Cys for 120 min at different pH values. λ_{ex} = 510 nm

Stability

Stability is another important indicator to evaluate the property of the fluorescent probe. For this purpose, the experiments on photostability, chemostability and pH stability were performed (Figure 4). As shown in Figure 4A, after the strong light irradiation of 1 kW/m2 (a 300 W xenon lamp as a white exited source which can simulate the solar spectrum) for over 60 min, the fluorescence intensity of fluorescein isothiocyanate declined dramatically, even more than 50%. On the contrary, the intensity of **DPP-AC** and its reaction product **DPP-OH** decreased no more than 16% under the same condition, demonstrating pretty good photostability.

NaClO was selected to detect the chemostability of the probes (Figure 4B). After titration with 40 equivalents of NaClO, the fluorescence of **DPP-AC**, and **DPP-OH** both remained almost unchanged and showed good chemostability. However, as a comparison, the pentamethine cyanine dye had already become non-fluorescence because of its poor chemostability towards oxidizing agents.

The effect of pH was also studied. The information in Figure 4C demonstrated that **DPP-AC** could react sensitively with Cys in the range of pH 6-10, and the fluorescence changes were extremely obvious in this pH range. In acidic condition (pH < 6), the reaction rate and the extent of fluorescence changes became slightly lower on account of the lower reactivity at low pH values. And if the pH value is too high (pH > 10), the molecular structures of **DPP-AC** would be completely damaged and the fluorescence was disappeared. In consequence, this probe showed fairly good stability and fluorescence response towards Cys in the physiological pH range and could be employed for the detection in living cells.

Intracellular detection

Probe **DPP-AC** were applied to detect cellular biothiols in living Hela cells (Figure 5). The bioimaging was observed with confocal laser scanning microscopy (CLSM). The Hela cells were firstlyincubated with 10 μM **DPP-AC** for 20 min at 37 °C. There was strong fluorescence observed in green channel (532.8 nm – 572.8 nm) and weak fluorescence observed in red channel (642.8 nm – 682.8 nm) under the fluorescence microscope. We speculated that **DPP-AC** had already reacted with endogenous Cys in living cells, because the limit of detection of the probe was much lower than the concentration of endogenous Cys. Thus the control experiment was then performed to identify the explanation. The Hela cells were firstly pretreated with 5 mM N-ethylmalemide (NEM) for 60 min to remove the endogenous thiol species in cells, followed by the incubation with 10 μM **DPP-AC** for 20 min at 37 °C. Just as expected, little fluorescence could be observed in the green channel, while the fluorescence in red channel became a lot stronger than Figure 5B. Meanwhile, another control experiment was continued. The pre-incubating cells with NEM were firstly stained with **DPP-AC** (10 μM), then Cys (50 μM) was added and incubated for 20 min. We found that

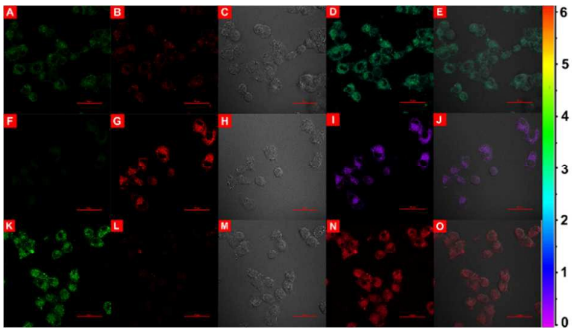


Figure 5 Confocal laser scan images of Hela cells. Cell images were obtained using an excitation wavelength of 510 nm. (A-E) Images of Hela cells incubated with **DPP-AC** (10 μM) at for 20 min at 37 °C. (F-J) Images of Hela pretreated by NEM (5 μM) for 60 min, and then incubated with **DPP-AC** (10 μM) for 20 min at 37 °C. (K-O) Images of Hela cells stained with **DPP-AC** (10 μM) for 20 min at 37 °C, and then further treated with Cys (50 μM) for 20 min at 37 °C. (A, F, K) green channel (λ_{ex} = 510.0 nm, λ_{em} = 532.8 nm – 572.8 nm). (B, G, L) red channel (642.8 nm – 682.8 nm). (C, H, M) bright field image (D, I, N) pseudocolored ratiometric images (F_{green}/F_{red}) generated from green channel to red channel (E, J, O) overlay of the ratio images and bright field images. Note: the ratiometric images were obtained by the image analysis software, Image-Pro. Scale bar = 50 μm.

the fluorescence in green channel become strong again, and the fluorescence in the red channel became much weaker simultaneously. This results showed that the fluorescence changes were indeed caused by the endogenous Cys in living cells, and the probe **DPP-AC** had the ability to detect endogenous Cys in living cells due to its much lower LOD than the concentration of Cys in Hela cells. And it was more visual to analyze the changes from the intensity ratio signals of the green/red channels. Moreover, the favorable overlay of fluorescent image and bright field image illustrated the probe distributed in the cytoplasm homogeneously.

The similar experiments were also conducted for Hcy and GSH. The pre-incubating cells with NEM were firstly stained with **DPP-AC** (10 μM), then Cys, Hcy, and GSH (50 μM) was added respectively and then incubated for 20 min. As shown in Figure 6, compared with the other two biothiols, the change of fluorescence intensity ratio was much more obvious when the cells treated with Cys. And the ratiometric fluorescence signals successfully helped us discriminate Cys from Hcy and GSH in living Hela cells.

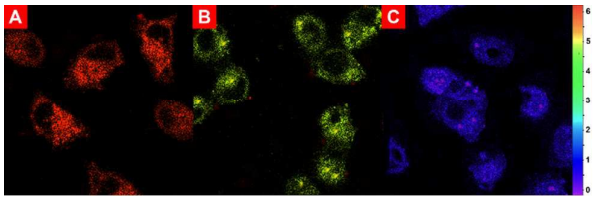


Figure 6 Pseudocolored ratiometric images (F_{green}/F_{red}) of Hela cells incubated with **DPP-AC** (10 μM) for 20 min, and then treated with 50 μM three different biothiols (Cys, Hcy and GSH) for 20 min at 37. °C. Cell images were obtained using an excitation wavelength of 510 nm. (A) Cys (B) Hcy (C) GSH. Note: the ratiometric images were obtained by the image analysis software, Image-Pro. Scale bar = 50 μm.

Conclusions

In this study, a new fluorescent probe (**DPP-AC**) based on diketopyrrolopyrrole with acrylate group was developed with a convenient synthetic route for the detection of biothiols. Depending on the strong electron-withdrawing ability and specific cyclization and cleavage reaction of acrylate moiety with Cys, **DPP-AC** displayed impressive ratiometric fluorescence change towards Cys, which was beneficial to rectify the system errors and improve the signal-noise ratio. The change of the fluorescence ratio (I_{552}/I_{664}) was over 20 folds, and the limit of detection was calculated as 84 nM, much lower than the concentration in living cells. Both ^{1}H NMR and MS titrations were performed to explain the cyclization and cleavage reaction mechanism of detecting Cys. Besides, **DPP-AC** showed perfect sensitivity and selectivity towards Cys over Hcy, GSH and other various amino acids, which solved a perplexing problem in biothiol probe design. More importantly, the ratiometric probe was successfully employed to detect endogenous Cys in living Hela cells, and the fluorescence ratio signals were conducive to the quantitative analysis of Cys. This performance made the probe possess the potential of monitor and analyze the concentrations of biothiols in cells to facilitate clinical diagnosis. This research paved a significant way for designing DPP-based ratiometric fluorescent probes with effective sensing capability and high biocompatibility for biological applications.

Experimental section

Synthesis of compound 2

Compound **1** (400 mg, 0.56 mmol), $Pd(PPh_3)_4$ (24 mg, 0.04 mmol) and 4-(bis(4-methoxyphenyl)amino)phenyl boronic acid (196 mg, 0.56 mmol) were dissolved in THF (15 mL). Under the protection of argon atmosphere, 5 mL aqueous potassium carbonate solution (2 M) was added. The reaction mixture was then stirred at 40 °C for 3 hours before being allowed to cool to room temperature. The reaction mixture was extracted by dichloromethane (3 × 15 mL) and then the organic layer was separated and washed with water and brine. After drying over Na2SO4 and removal of the solvent under reduced pressure, the residue was purified over silica gel using dichloromethane/petroleum ether (3:1, v/v) as the eluent to obtain the pure compound 2 (210 mg, 0.22 mmol, 39% yeild). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.88 (d, *J* = 8.0 Hz, 2H), 7.87-4.69 (m, 6H), 7.48 (d, *J* = 8.0 Hz, 2H), 7.11 (dd, *J* = 8.0 Hz, 4H), 6.99 (d, *J* = 8.0 Hz, 2H), 6.87 (d, *J* = 8.0 Hz, 2H), 3.87-3.78 (m, 10H), 3.37-3.34 (m, 4H), 1.87-1.77 (m, 8H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 162.94, 160.31, 156.20, 150.81, 149.02, 146.28, 143.59, 140.48, 130.93, 129.12, 127.59, 126.98, 126.71, 125.71, 120.09, 116.55, 114.81, 109.76, 108.37, 55.52, 53.44, 32.82, 29.81, 27.92. HRMS (ESI, m/z): $[M+H]^+$ calcd. for $C_{46}H_{43}N_3Br_3O_4^+$ 938.0804, found 938.0814.

Synthesis of compound 3

Compound **2** (200 mg, 0.21 mmol) and morpholine (75 mg, 0.85 mmol) were dissolved in 15 mL THF. The reaction mixture

was then stirred under argon atmosphere overnight at room temperature. After removing the solvent under reduce pressure, the residue was purified over silica gel using ethyl acetate/dichloromethane (1/20, v/v) as the eluent to obtain the pure compound **3** (146 mg, 0.15 mmol, 72% yeild). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): .7.84 (d, *J* = 8.0 Hz, 2H), 7.74 (d, *J* = 8.0 Hz, 2H), 7.69 (d, *J* = 8.0 Hz, 2H), 7.45 (d, *J* = 8.0 Hz, 2H), 7.11 (dd, *J* = 8.0 Hz, 4H), 6.97 (d, *J* = 8.0 Hz, 2H), 6.86 (dd, *J* = 8.0 Hz, 4H), 3.89-3.81 (m, 10H), 3.71-3.66 (m, 8H), 2.40-2.31 (m, 12H), 1.68-1.67 (m, 4H), 1.50-1.49 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 162.27, 161.43, 153.30, 140.18, 139.48, 133.58, 133.17, 132.87, 131.21, 130.69, 128.41, 127.87, 127.61, 125.09, 121.20, 115.89, 91.92, 68.48, 58.73, 57.48, 54.48, 45.86, 28.51, 27.28. HRMS (ESI, m/z): $[M+H]^+$ calcd. for $C_{54}H_{59}BrN_5O_6^+$ 952.3649, found 952.3626.

Synthesis of compound 4

A mixture of compound **3** (200 mg, 0.21 mmol), bis(pinacolato) diboron (216 mg, 0,85 mmol), potassium acetate (84 mg, 0.86 mmol) and 1,1`-bis(diphenylphosphino)ferrocene palladium dichloride (91 mg, 0.11 mmol) was dissolved in 15 mL 1,4-dioxane which was used as the solvent. The reaction mixture was stirred and reflexed for 4 hours under the protection of argon atmosphere. After cooling to the room temperature, the reaction solution was extracted with dichloromethane (3 × 15 mL) and then the combined organic layer was dried with anhydrous Na_2SO_4. After removing the organic solvent under reduced pressure, the residue was purified over silica gel using ethyl acetate/dichloromethane (1/15, v/v) as the eluent. After dried in vacuo, the Compound **4** was obtained as the red solid (90 mg, 0.08 mmol, 41% yeild). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.97 (d, *J* = 8.0 Hz, 2H), 7.88 (d, *J* = 8.0 Hz, 2H), 7.77 (d, *J* = 8.0 Hz, 2H), 7.72 (d, *J* = 8.0 Hz, 2H), 7.47 (d, *J* = 8.0 Hz, 2H), 7.11 (dd, *J* = 8.0 Hz, 4H), 6.99 (d, *J* = 8.0 Hz, 2H), 6.86 (d, *J* = 8.0 Hz, 2H), 3.86-3.79 (m, 10H), 3.36-3.33 (m, 8H), 3.32-3.30 (m, 4H), 2.51-2.47 (m, 8H), 1.83-1.74 (m, 8H), 1.37-1.26 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 162.86, 162.03, 153.90, 147.61, 144.53, 140.78, 140.07, 135.91, 135.46, 134.18, 131.29, 131.25, 130.70, 129.01, 128.47, 128.21, 125.68, 121.79, 116.48, 100.33, 82.14, 59.33, 58.08, 55.07, 46.45, 29.10, 27.88, 26.62. HRMS (ESI, m/z): $[M+H]^+$ calcd. for $C_{60}H_{71}BrN_5O_8^+$ 1000.5396, found 1000.5390.

Synthesis of DPP-OH

Compound **4** (100 mg, 0.10 mmol) was dissolved in 15mL THF and stirred under an argon atmosphere. Keeping the reactor in ice bath, 2 drops of hydrogen peroxide solution (30%) and 0.1 mL sodium hydroxide solution (1 M) were slowly added into the reaction mixture. The color of the solution get darker. After stirred for 20 min, diluted hydrochloric acid was added dropwise to the solution until the pH value was adjusted to 6.0. After removing the solvent under reduced pressure, the residue was purified over silica gel using ethanol/dichloromethane (1/20, v/v) as the eluent to obtain pure compound **DPP-OH** as a deep red solid (80 mg, 0.09 mmol, 90% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.12 (s, 1H), 7.84 (d, *J* = 8.0 Hz, 2H), 7.5 (d, *J* = 8.0 Hz, 2H), 7.69 (dd, *J* = 8.0 Hz, 4H),7.46 (d, *J* = 8.0 Hz, 2H), 7.12 (dd, *J* = 8.0 Hz, 4H), 6.99 (dd, *J* = 8.0 Hz, 4H), 6.87 (d, *J* = 8.0 Hz, 2H), 3.88-3.70 (m, 10H), 3.69-3.68 (m, 8H), 2.48-2.38 (m, 12H), 1.68-1.67 (m, 4H), 1.53-1.48 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 160.86, 160.03, 159.29, 142.53, 138.78, 138.07, 133.46, 132.18, 129.54, 129.29, 127.01, 126.47, 126.21, 125.47, 123.68, 119.79, 114.96, 114.48, 89.60, 67.08, 57.33, 56.08, 53.07, 44.45, 27.10, 25.88. HRMS (ESI, m/z): $[M+H]^+$ calcd. for $C_{54}H_{60}N_5O_7^+$ 890.4493, found 890.4445.

Synthesis of DPP-AC

A mixture of **DPP-OH** (100 mg, 0.11 mmol) and acrylyl chloride (31 mg, 0.33 mmol) was dissolved in 10 mL anhydrous dichloromethane. Under argon atmosphere, 0.1 mL trithylamine was added to the solution at 0 °C. The reaction mixture was then warmed to room temperature and stirred overnight. The organic solvent was then removed in vacuo. And the residue was purified over silica gel using petroleum ether/dichloromethane as eluent (1/20, v/v) to obtain the red solid (51 mg, 0.05 mmol, 51% yeild). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.87 (dd, *J* = 8.0 Hz, 4H), 7.71 (d, *J* = 8.0 Hz, 2H), 7.47 (d, *J* = 8.0 Hz, 2H), 7.34 (d, *J* = 8.0 Hz, 2H), 7.11 (dd, *J* = 8.0 Hz, 2H), 6.99 (d, *J* = 8.0 Hz, 1H), 6.88 (m, 1H), 6.85 (d, *J* = 8.0 Hz, 1H), 3.86-3.81 (m, 10H), 3.68-3.63 (m, 8H), 3.66-3.63 (m, 4H), 2.53-2.49 (m, 8H), 1.82-1.78 (m, 8H). 13C NMR (100 MHz, CDCl3) δ (ppm): 126.84, 162.41, 156.25, 155.86, 154.84, 149.15, 140.41, 131.27, 130.74, 129.26, 129.00, 127.61, 127.03, 126.69, 122.75, 122.24, 120.56, 119.99, 114.82, 110.25, 109.35, 55.52, 53.46, 32.95, 32.78, 29.78, 29.68, 27.99. HRMS (ESI, m/z): $[M+H]^+$ calcd. for $C_{57}H_{62}N_5O_8^+$ 944.4598, found 944.4524.

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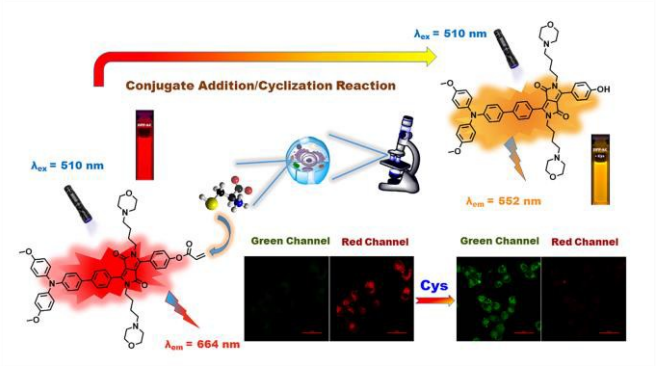
Graphical Abstract

Diketopyrrolopyrrole-based ratiometric fluorescent probe for the sensitive and selective detection of cysteine over homocysteine and glutathione in living cells



Xiao Zhang, Yandi Hang, Weisong Qu, Yongchao Yan, Ping Zhao and Jianli Hua**

A new fluorescent probe (**DPP-AC**) based on diketopyrrolopyrrole with acrylate group was designed and synthesized for the sensitive and selective detection of biological thiols. The acrylate group of **DPP-AC** as the receptor moiety displayed obvious ratiometric changes from red to yellow upon addition of cysteine (Cys), and the change of the fluorescence ratio (I_{552}/I_{664}) was over 20 folds. The limit of detection towards Cys was calculated down to 84 nM, much lower than the normal concentration in living cells. More importantly, **DPP-AC** had outstanding selectivity towards cysteine over homocysteine (Hcy), glutathione (GSH) and other amino acids. **DPP-AC** was successfully employed to detect endogenous Cys in Hela cells and discriminate Cys from Hcy and GSH in living cells.