Organic & Biomolecular Chemistry

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *Org. Biomol. Chem.*, 2021, **19**, 873

A novel near-infrared fluorescent probe based on isophorone for the bioassay of endogenous cysteine†

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Received 2nd December 2020,
Accepted 21st December 2020

DOI: 10.1039/d0ob02405h

rsc.li/obc

A dicyanoisophorone/acrylate-combined probe (**DDP**) was synthesized and designed as a near-infrared (NIR) fluorescent sensor for the rapid identification of Cys over Hcy and GSH in aqueous solution with a large Stokes shift (143 nm). The detection limit of Cys was 1.23 μM, which was lower than that of the intracellular Cys concentration. **DDP** was cell membrane-permeable and had been successfully applied to the detection of intracellular Cys in HeLa cells. The detection mechanism was determined by ^{1}H NMR titration, MS and DFT calculations.

Introduction

Biothiols, such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), play significant roles in redox balance, biological catalysis, metal complexes and post-translational modifications.[1–3] Cys is a precursor of GSH, acetyl-Co-A and taurine, and a source of sulfides in iron-sulfur clusters. It is not only an essential amino acid in the process of protein synthesis, but also plays a role in the protein spatial structure.[4,5] A lack of Cys is associated with many syndromes, such as neurotoxicity, child growth retardation, skin damage, liver injury, baragnosis fat loss, psoriasis, leukocyte loss, *etc*.[6–10] Hcy disorder is a risk factor for various diseases including cardiovascular disease and Alzheimer's disease.[11] GSH consists of glutamate, cysteine and glycine and is the most abundant (1–10 mM) and representative nonprotein mercaptans in cells.[12] Therefore, it is of great significance to observe the distribution of biothiols in the life system for the study of cellular function and the early diagnosis of some biothiol related diseases.[13]

As biothiol detection methods, high performance liquid chromatography (HPLC), electrochemical mass spectrometry (ECMS), and capillary electrophoresis (CE) have many disadvantages, such as high cost and complexity, tissue or cell damage, and cumbersome operation.[14–17] Compared with the above methods, fluorescence detection is simple and sensitive and can be used for the analytes in cells.[18–20] In recent years, some fluorescent probes as chemodosimeters have been reported for the detection of biothiols. Their sensing mechanism included Michael addition, nucleophilic substitution reactions,[7,21–25] and so on. But most of them are not near-infrared (NIR) probes, which had some advantages of weak background luminescence, long wavelength, high cell permeability,[26,27] *etc*. As a result, some NIR fluorescent probes were designed for the detection of Cys.[26,28] However, it is still a challenge to distinguish Cys over Hcy and GSH using a NIR probe.

As part of our continuing research of fluorescent probes[29–35] for biothiol detection, herein we report a colorimetric chemodosimeter **DDP** ((*E*)-2-(2-(3-(dicyanomethylene)-5,5-dimethylcyclohex-1-en-1-yl) vinyl)-5-(diethylamino)phenyl acetate) as a NIR fluorescent probe to detect Cys in aqueous solution. **DDP** demonstrated a long emission wavelength with good cell penetration and it was an effective tool with high selectivity and sensitivity to identify intracellular Cys from other amino acids including Hcy and GSH. Furthermore, **DDP** can be used for bioimaging in HeLa cells.

Results and discussion

Design and synthesis of the probe

As shown in Scheme 1, the designed probe **DDP** was synthesized by Knoevenagel condensation-acylation steps (see the Experimental section for details), then characterized using ^{1}H, ^{13}C NMR and ESI-MS spectra (Fig. S1–S3, ESI†). **DDP** was therefore constructed based on a dicyanoisophorone skeleton

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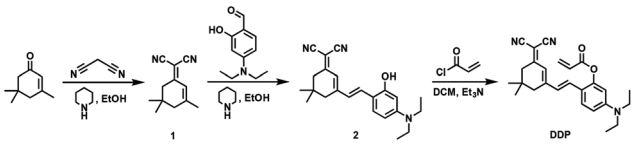
†Electronic supplementary information (ESI) available: Spectral data, copies of ^{1}H/^{13}C NMR, ESI-MS and other materials. See DOI: 10.1039/d0ob02405h



Scheme 1 Synthesis of the probe **DDP**.

as a NIR fluorescence group and an acryloyl moiety as a recognition group.

Spectroscopic responses of DDP

In order to determine the spectroscopic responses of **DDP**, the effects of solvents and pH on the probe were studied (Fig. S4 and S5, ESI†). DMSO : PBS = 4 : 1 (v/v) at pH 7.4 was selected as experimental conditions. As shown in Fig. 1a, **DDP** had an obvious absorption peak at about 525 nm in UV-vis spectra. With the addition of 200.0 μM of Cys, a new absorption peak appeared around 550 nm. For Hcy and GSH, the absorption peak was around 535 nm and 530 nm, respectively. The color of the solution changed from pink to purple only after the addition of Cys. In fluorescence spectra, **DDP** showed an emission peak at 693 nm (Fig. 1b). The Stokes shift for **DDP** was as high as 143 nm, which was higher than those of many fluorescent probes for Cys.[36–38] After the addition of Cys, the fluorescence was enhanced obviously. For Hcy and GSH, the fluorescence had almost no change. These results indicated that **DDP** can be used for the identification of Cys over Hcy and GSH in aqueous solution.

In order to evaluate the selectivity of **DDP**, various amino acids (Pro, Ala, Thr, Phe, His, Ser, Asn, Asp, Lys, and Arg) were added to the solution of **DDP**, and their UV-Vis absorption spectra and fluorescence spectra were measured. As shown in Fig. 2a, compared with other amino acids, only Cys had an obvious absorption peak at 550 nm, and only Cys showed an increase in fluorescence intensity (Fig. 2b). All competitive amino acids almost did not interfere with the detection of Cys (Fig. S6, ESI†). Therefore, **DDP** demonstrated high specificity for Cys, and competitive amino acids had almost no effect on the detection.

The sensitivity of **DDP** to the detection of Cys was studied by fluorescence titration. As shown in Fig. 3, in the absence of Cys, **DDP** showed fluorescence at 693 nm. With the increase of the concentration of Cys, the emission gradually increased. The linear relationship between fluorescence intensity and Cys concentration was good in a range of 3.0–16.0 μM. Based on the LOD = 3 σ/s, where s is the slope of the curve equation and

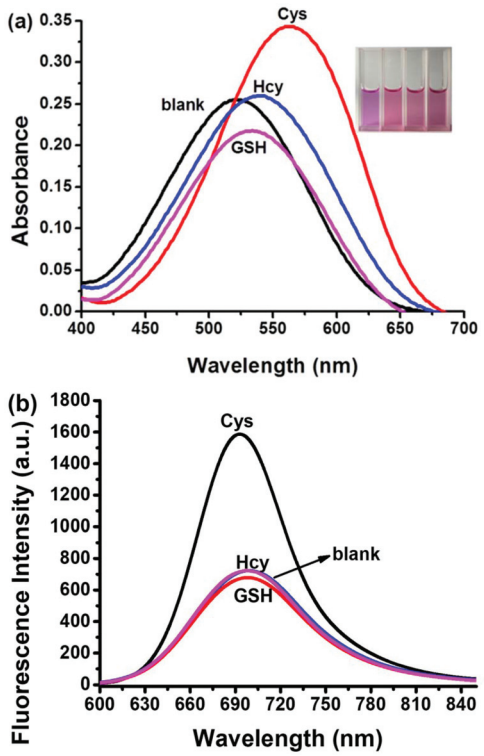


Fig. 1 (a) Absorption spectra of **DDP** (10.0 μM) in DMSO : PBS = 4 : 1 (v/v) solution with Cys, Hcy and GSH (200.0 μM) for 0.5 h at pH 7.4. Inset: The photograph of **DDP** with biothiols under visible light. From left to right: Cys, blank, Hcy, and GSH. (b) Fluorescence spectral changes of the **DDP** (10.0 μM) after the addition of Cys, Hcy and GSH (200.0 μM) in DMSO : PBS = 4 : 1 (v/v) solution for 0.5 h at pH 7.4 (λ_{ex} = 550 nm).

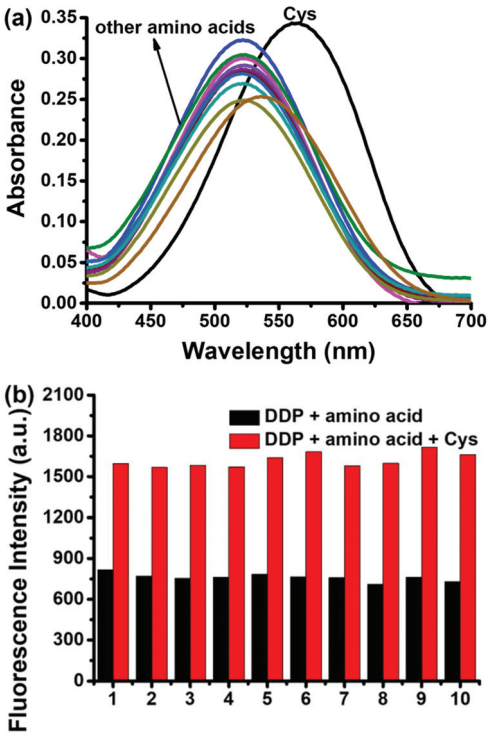


Fig. 2 (a) Absorption spectra of **DDP** (10.0 μM) in the presence of Cys (200.0 μM), and other amino acids (200.0 μM) in DMSO : PBS = 4 : 1 (v/v) solution at pH 7.4. (b) Fluorescence response of **DDP** (10.0 μM) to other amino acids (the black bars), and **DDP** + Cys in the presence of other amino acids (the red bars) in DMSO : PBS = 4 : 1 (v/v) solution at pH 7.4. From 1 to 10: Pro, Ala, Thr, Phe, His, Ser, Asn, Asp, Lys, and Arg (λ_{ex} = 550 nm).

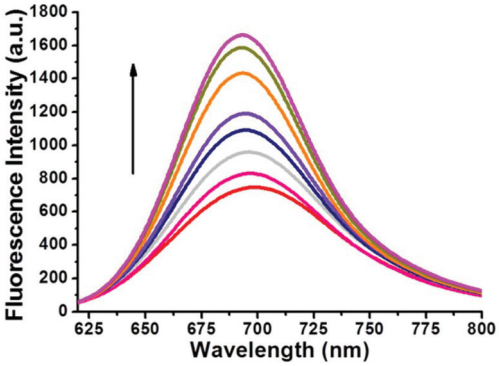


Fig. 3 Fluorescence spectra of **DDP** (10.0 μM) after the addition of Cys (0–200.0 μM) at pH 7.4 (λ_{ex} = 550 nm).

σ is the standard deviation, the detection limit was determined to be 1.29 μM (Fig. S7, ESI†). The fluorescence quantum yield (Φ) of the solution was enhanced from 0.139 to 0.193. Furthermore, with the increase of Cys concentration, the color of the **DDP** solution also gradually changed from pink to purple, which can be identified with the naked eye (Fig. S8, ESI†). These results showed that **DDP** was highly sensitive to Cys and could detect low concentration of Cys in aqueous solution. As shown in Fig. S9 (ESI†), after the addition of Cys, the reaction was completed within 10 minutes. The pseudo-first order reaction constant (k_{obs}) was the slope of the linear equation, which can be calculated as 0.383 s^{-1}. But for Hcy or GSH, the corresponding reaction was very slow. These results indicated that **DDP** can be used for the identification of Cys over Hcy and GSH by dynamic control.

Rationalization of the recognition of Cys

In order to explore the reaction mechanism, the reaction of **DDP** and Cys was monitored by ^{1}H NMR. As shown in Fig. 4, with the addition of Cys to **DDP** in DMSO-d_6, the original signals H_a, H_b and H_c of the acrylate group disappeared and the signals of compound **2** were observed which was in accordance with the corresponding data in ref. 39. The mixture of Cys and **DDP** was also characterized by ESI-MS spectrometry.

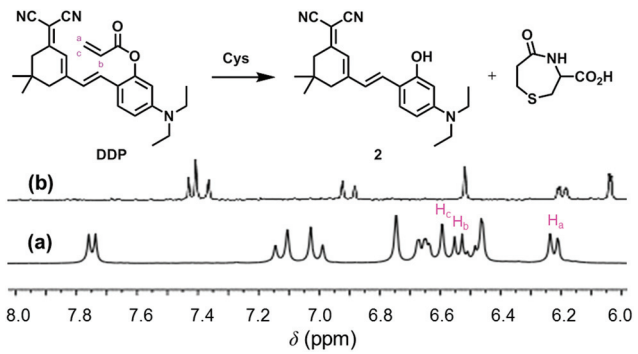


Fig. 4 Partial ^{1}H NMR spectra of (a) **DDP** and (b) **DDP** + Cys in DMSO-d_6.

As shown in Fig. S10 (ESI†), a peak at 362.1821 (m/z) was observed, which corresponded to that of the eventual product **2**. The above results showed that this recognition event could release a seven- or eight-membered lactam with Cys or Hcy. However, the dynamic performance of the formation of the seven-membered ring was much better than that of the eight-membered ring. Therefore, when the reaction between Cys and **DDP** finished, almost no reaction of Hcy with **DDP** was observed. For GSH, the analogous intramolecular cyclization reaction did not occur.

Computational studies

In order to further study the fluorescence properties of compound **2** and probe **DDP**, the density functional theory (DFT) analysis was carried out at the B3LYP/6-311G level using Gaussian 09 software. As shown in Fig. 5, electrons of compound **2** were mostly distributed over the whole dicyanoisophorone skeleton at both HOMO and LUMO levels, indicating that **2** had a strong NIR fluorescence emission. For **DDP**, its electrons were actually located on the dicyanoisophorone skeleton only at the HOMO level. At the LUMO level, however, the electrons were partially transferred to the acryloyl ester group, implying that an ICT process occurred and the fluorescence intensity of **DDP** was weaker than that of **2**. These results were in good agreement with fluorescence enhancement during the detection process.

Bioimaging in living cells

Finally, **DDP** was applied to the fluorescence imaging of endogenous Cys in HeLa cells. As shown in Fig. 6b, when **DDP** (10.0 μM) was incubated with HeLa cells at 37 °C, red fluorescence appeared, indicating that **DDP** had cell membrane permeability and endogenous Cys could be detected by confocal fluorescence imaging. In a control experiment,

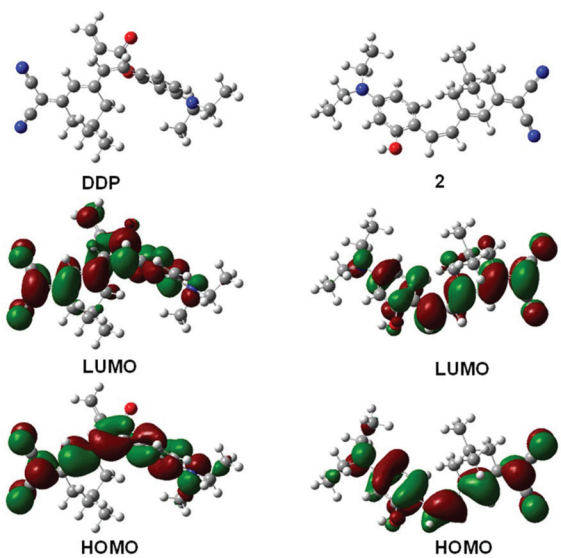


Fig. 5 Optimized structures, energy levels, and molecular orbital plots of **DDP** and **2**.

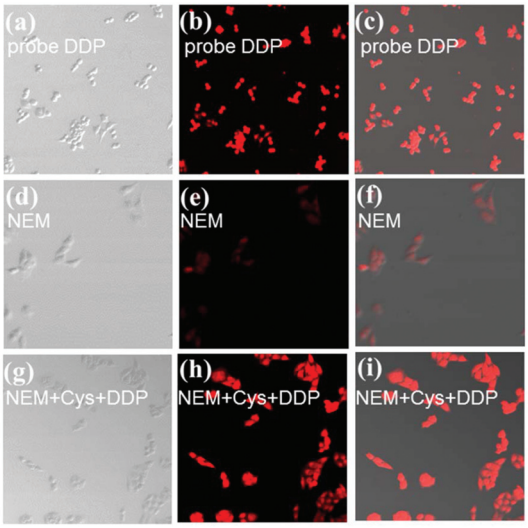


Fig. 6 HeLa cell imaging: (a)–(c) bright field, red channel and merged imaging of **DDP** (10.0 μM) with HeLa cells for 30 min; (d)–(f) bright field, red channel and merged imaging of HeLa cells incubated with NEM (500.0 μM) for 30 min, and then incubated with **DDP** (10.0 μM) for 40 min; and (g)–(i) bright field, red channel and merged imaging of HeLa cells incubated with NEM (500.0 μM) for 30 min, then incubated with **DDP** (10.0 μM) for 40 minutes, and then incubated with Cys (200.0 μM) for 30 minutes. Emission was collected at the red channel.

N-ethylmaleimide (NEM, 500.0 μM) was used as a thiol-blocking reagent to incubate HeLa cells, and then **DDP** (10.0 μM) was added. As shown in Fig. 6e, red fluorescence was significantly reduced. But after adding 200.0 μM of Cys to the system, strong red fluorescence was observed again (Fig. 6h). These results indicated that **DDP** could penetrate the cell membrane and enter the cell, and it could successfully detect the intracellular and exogenous Cys.

Conclusions

A near-infrared fluorescent probe **DDP** based on the dicyanoisophorone skeleton with an acryloyl group as the recognition moiety was synthesized. Its maximum emission wavelength was 693 nm, and the Stokes shift was 143 nm. When **DDP** was used to detect Cys, this probe showed small fluorescence interference, high sensitivity and selectivity. **DDP** was also a good colorimetric probe due to its clear color changes in the aqueous solution, which can be identified with the naked eye. More importantly, **DDP** demonstrated good membrane permeability and can be used for biological imaging of endogenous Cys in HeLa cells.

Experimental

Details of chemicals and materials, spectral measurements, live cell imaging, and characterization of the probe **DDP** can be found in the ESI.†

As shown in Scheme 1, Compound **2** was synthesized from compound **1**, according to the reported method.[40] To a 50 mL, single-necked, round-bottom flask were added **2** (99.8 mg, 0.24 mmol), dichloromethane (10.0 mL) and triethylamine (80.0 μL). The whole system was placed at −8 °C and acryloyl chloride (40.0 μL) was added. After 10 minutes, the mixture was warmed to room temperature and stirred overnight. The mixture was then extracted with dichloromethane (2 × 30.0 mL) and washed with water (10.0 mL) and brine (10.0 mL). The organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography (DCM/EtOAc = 5 : 1) on silica gel to afford **DDP** (86.2 mg, 75%) as a black solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.75 (d, J = 8.8 Hz, 1H), 7.12 (d, J = 16.0 Hz, 1H), 7.00 (d, J = 16.0 Hz, 1H), 6.74 (s, 1H), 6.68–6.42 (m, 4H), 6.21 (dd, J = 10.0, 2.0 Hz, 1H), 3.40 (q, J = 7.2 Hz, 4H), 2.56 (s, 2H), 2.40 (s, 2H), 1.11 (t, J = 6.8 Hz, 6H), 0.98 (s, 6H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6): δ = 169.8, 164.0, 156.5, 150.7, 149.6, 133.7, 131.1, 128.6, 127.5, 125.2, 120.8, 114.5, 114.3, 113.4, 109.8, 104.7, 73.7, 43.8 (2C), 42.2, 37.9, 31.5, 27.3 (2C), 12.4 (2C) ppm. ESI-MS: m/z 416.2067 $[M + H]^+$.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

This work was supported by the NNSFC (no. 21572091 and 21772078), and the Fundamental Research Funds for the Central Universities (no. 2682019CX70, 2682019CX71 and 2682020CX55) by the MoE of China. We also thank the Science and Technology Department of Sichuan Province (2020JDRC0021). We would like to thank the Analytical and Testing Center of Southwest Jiaotong University for the laser confocal microscopy test.

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