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Graphical Abstract

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A dicyanoisophorone-based turn-on fluorescence probe, for highly selective and sensitive detection of cysteine was designed based on nucleophilic substitution mechanism. The detection limit toward cysteine is calculated to be as low as 0.70 μM.

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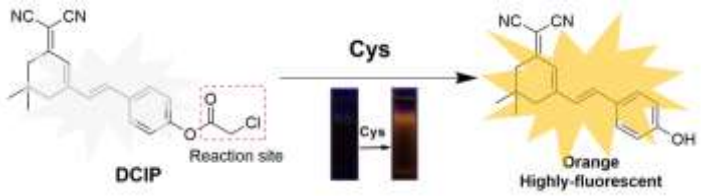

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ABSTRACT

In this work, a dicyanoisophorone-based turn-on fluorescent probe, **DCIP**, for highly selective and sensitive detection of cysteine was designed based on nucleophilic substitution mechanism. Moreover, compared with typical cysteine probes, **DCIP** showed great selectivity and sensitivity for cysteine over other amino acids including the similar structured homocysteine (Hcy) and glutathione (GSH). Further, the detection limit toward cysteine was calculated to be as low as 0.70 μM. In addition, the utility of **DCIP** as a bioanalytical molecular tool was demonstrated by fluorescence imaging of biothiols in living cells.

1. Introduction

Recently, much effort has been devoted to developing fluorescent probes for small biothiols because they have various important biochemical functions and play pivotal roles in the physiological environment. [1-3] Compared with other amino acids, Cysteine (Cys) deficiency is associated with many syndromes, such as slowed growth in children, hair depigmentation, edema protein synthesis, lethargy, liver damage, muscle and fat loss, skin lesions, weakness, detoxification and metabolism.[2-5] Furthermore, the whole cellular Cys concentration is closely related to the high risk of various tumor progressions.[6-8] Therefore, it is of great significance to establish efficient, sensitive and selective methods for detection of cysteine.

Some conventional techniques for the detection of cysteine using different methods such as electrochemical methods, high performance liquid chromatography, mass spectrometry, luminescent chemosensors, colorimetric detection, Gold Nanorods and inductively coupled plasma emission spectrometry.[9-12] Compared with them, fluorescence method is an ideal strategy in biological and environmental sciences as it is low-cost, real-time detection, simplicity for implementation and suitable for bioimaging.[13-17] In recent years, a great number of fluorescent probes for Cys detection have been developed based on different mechanisms,[18-37] such as cyclization with aldehydes,[18-20] the Michael addition reaction,[21-23] the addition-cyclization with acrylates,[24-27] the native chemical ligation reaction,[28,29] the aromatic substitution-rearrangement reaction[30-32] and others. However, many probes still have the following shortcomings: low efficiency, complex structure, complex synthetic work or even need complicated and specialized equipment. Therefore, a new strategy for the sensitive detection of biothiols was expected to be explored. To the best of our knowledge, sensors based on a nucleophilic substitution mechanism usually show excellent selectivity.

With this in mind, we rationally designed a derivative dicyanoisophorone dye, 2-(3-(4-hydroxystyryl)-5, 5-dimethylcyclohex-2-enylidene) malononitrile (Compound **2**) featured a long wavelength emission. Thus a dicyanoisophorone dye involved a chloracetyl group was designed for the detection of Cys using nucleophilic substitution and subsequent intramolecular cyclization. The synthetic route of probe **DCIP** is shown in Scheme 1. This probe exhibited a longer wavelength emission with a remarkable stokes shift than comparable excellent Cys probes such as benzothiazole based and coumarin based probes.[10,38] This probe possess several merits such as simple structure, easy synthesizing from cheap commercially raw materials with high yield, high sensitivity and selectivity for Cys, and provides a rapid fluorescence detection process for Cys. What is more important is that this probe exhibited excellent sensing properties, showing high sensitivity and selectivity for Cys with remarkable enhancement of fluorescence around 590 nm and a large Stokes shift (λ_{em}-λ_{abs}= 176 nm). A large stokes shift can avoid the overlap of the absorption spectrum and

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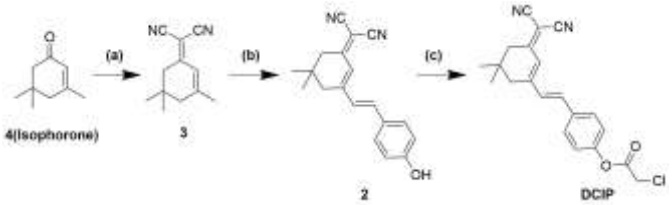
emission spectrum, which is highly demanded for fluorescence probes as it prevents the self-absorption or inner filter effect to increase the signal to noise ratio for fluorescence imaging.[39] Furthermore, probe **DCIP** can be successfully used in fluorescence imaging of intracellular Cys in living cells with low cytotoxicity.



Scheme 1. Synthetic route of **DCIP**. Reagents and conditions: (a) ethanol, malononitrile, piperidine, AcOH, reflux 7 h, 77%; (b) p-hydroxybenzaldehyde, acetonitrile, piperidine, reflux 5 h, 85%; (c) chloroacetyl chloride, acetonitrile, Et_3N, reflux 6 h, 90%.

2. Experimental

The synthetic route to the desired probe **DCIP** is showed in Scheme 1. The intermediate **3**, intermediate **2** and final product **DCIP** were confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS. The detailed information can be seen in **ESI**.

3. Spectral properties

The spectral properties and the response of **DCIP** to cysteine were subsequently investigated in DMSO/H_2O (1:1, v/v) solution. As shown in the Fig.1, probe **DCIP** exhibits an absorption peak with maximum at 397 nm and red shift to 430 nm with an isosbestic point at around 414 nm after adding 6 equiv of Cys into the solution of probe **DCIP** (10 μM). Due to an enhanced ICT efficiency, in the fluorescence spectra, an emission peak at around 590 nm increased significantly.

4. Selectivity

The selectivity of **DCIP** towards Cys and other amino acids such as Ala, Arg, Asn, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Trp, Tyr, Val, GSH and Hcy were investigated by fluorescence spectra.

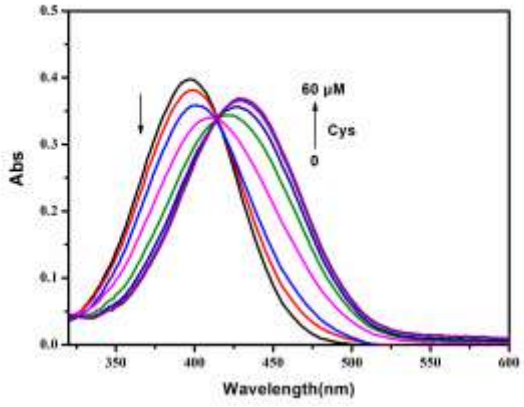


Fig.1 UV–vis absorption spectral changes of probe **DCIP** (10 μM) upon addition of Cys (60 μM).

As shown in Fig. 2a, only the addition of cysteine made such an obvious signal changes in the fluoresce spectra of **DCIP**. In contrast, even the addition of 10 equiv of other analytes made almost negligible changes in the spectra. Although Hcy displayed slight fluorescence enhancement, it is far less than that was caused by Cys even though Hcy was used with higher concentration. These results show that the probe **DCIP** has highest selectivity for Cys. In addition, to study the influence of other amino acids on the addition of Cys to **DCIP**, the competitive experiments in the presence of other various analytes have been conducted. As shown in the Fig. 2b, there are no responses together with the various analytes. Upon addition of Cys to the mixture solution, a significant change in fluorescence intensity has been observed. Clearly, all these results confirm that probe **DCIP** has high selectivity for Cys over even high concentration of other analytes. This result implied that the conjugated double bond and the dicyano-vinyl group in **DCIP** were inactivated to strong oxidant and nucleophiles, and the chloracetyl group was highly reactive to biothiols over other analytes.

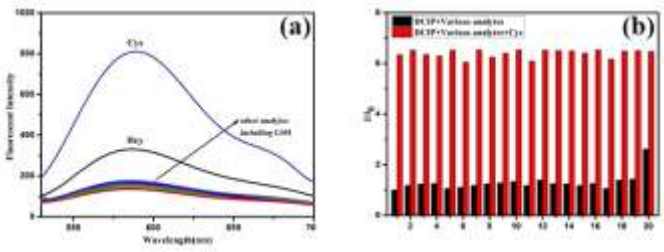


Fig.2 (a). Fluorescence responses of probe **DCIP** (10 μM) to various analytes. Ex = 414 nm, slit: 10 nm/10 nm; (b). Fluorescent intensity changes of probe **DCIP** (10 μM) at 590 nm to various amino acids (the black bars) in the presence of various representative analytes (100 μM) each in a DMSO/H_2O (v/v= 1/1) solution. Analytes 1–21: 1.None, 2.Ala, 3.Arg, 4.Asn, 5.Asp, 6.Glu, 7.Gly, 8.His, 9.Ile, 10.Leu, 11.Lys, 12.Met, 13.Phe, 14.Pro, 15.Ser , 16.Trp, 17.Tyr, 18.Val, 19.GSH, 20.Hcy.

5. Titration

The sensing ability of **DCIP** was investigated by fluorescence titration experiments in DMSO/H_2O (1:1, v/v) solution. As shown in Fig.3a, in the absence of Cys, **DCIP** exhibited negligible fluorescence as the chloracetyl group efficiently quenched the fluorescence by blocking the ICT process of the fluorophore. With the increase of Cys concentration, the emission band centered at 590 nm enhanced progressively and show good linear correlation with the concentration of cysteine in the range of 0-30 μM and the slope k was calculated to be 15.93953 (Fig. 3b). Based on ten times independent measurements for the 590 nm value of Cys-free **DCIP** solution (10μM), the standard deviation (S.D.) of our UV-vis spectrophotometer was calculated to be 3.69752. According to the equation LOD=3×S.D./k,[40,41] the limit of detection for Cys by fluorescence spectral titration was determined to be 0.70 μM. This result indicated that **DCIP** is highly sensitive to cysteine, and it can detect low concentration of cysteine.

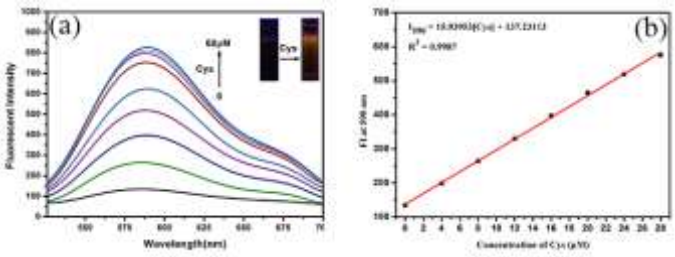
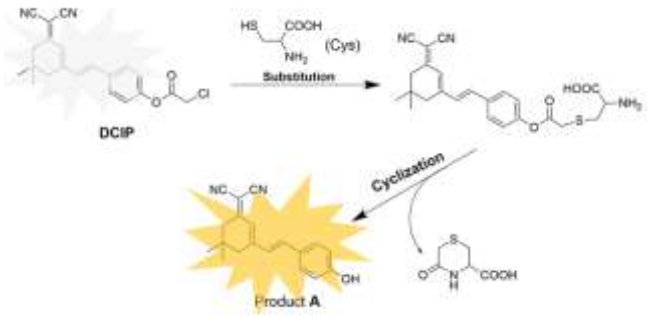


Fig 3. (a) Fluorescence titration of probe **DCIP** (10 μM) upon addition of Cys (0–60 μM). (b) Stand curve of fluorescence at 590 nm versus Cys concentration (from 0 to 30 μM). Ex = 414 nm, slit: 10 nm/10 nm;

6. Sensing mechanism

It is noteworthy that the optical spectral changes of **DCIP** toward Cys occur immediately (Fig. S14). Such rapid response indicates that **DCIP** is very sensitive to Cys, which is very suitable for a real-time detection. To investigate the mechanism, the reaction of **DCIP** with Cys was carried out in DMSO/H_2O (1:1, v/v) solution. After stirring at room temperature for 1 min, the product **A** was separated by column chromatography and characterized by TLC and ^{1}H NMR analysis with the reference compound **2** (Fig. S11–Fig. S12). The reaction mixture of probe **DCIP** and Cys was also investigated by the HRMS analysis, in which the peak of product **A** (Fig. S9) can be assigned to compound **2** (Fig. S7), and the peak at *m/z* 160.0063 can be assigned to 5-oxothiomorpholine-3-carboxylic acid - H^+ (Fig. S10). These data are in good agreement with the proposed sensing mechanism shown in Scheme 2.



Scheme 2. Sensing mechanism of **DCIP** based on nucleophilic substitution mechanism.

7. Cell imaging

To demonstrate the potential bioapplications of the probe, the potential of **DCIP** for imaging Cys in living cells was investigated. A significant bright fluorescence was found when HeLa cells were incubated with probe **DCIP** (Fig. 4a-c). The result indicates that probe **DCIP** is capable of permeating into cells and reacting with cysteine to generate fluorescence. When the HeLa cells were pretreated with N-ethylmaleimide (NEM), a well-known thiolblocking agent for the depletion of intracellular thiol species, there was almost no fluorescence. To evaluate cytotoxicity of the probe, a CCK8 assay in HeLa cells with different concentrations of **DCIP** (5, 10, 20, 50 μM) was performed. The results showed that the viability of HeLa cells was more than 90% when they were incubated with reasonably high concentration of **DCIP** for 12 h (Fig. S15). These results suggested that **DCIP** possessed low cytotoxicity and clearly demonstrated that probe **DCIP** either has good membrane permeability or can be used as a new fluorescent probe for imaging Cys in living cells.

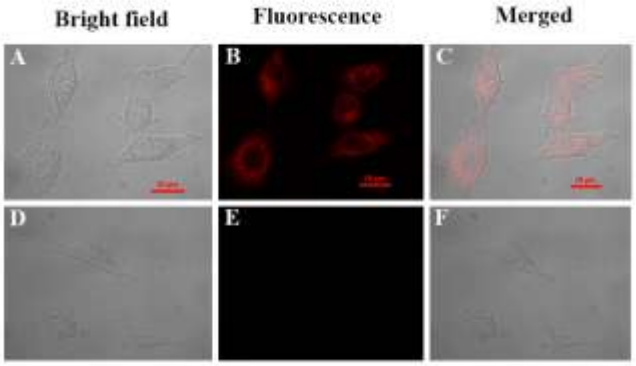


Fig 4. The upside: confocal microscopy images of Hela cells incubated with probe **DCIP** (10 μM) for 15 min; (a) bright field image, (b) fluorescence image, (c) merged image of (a) and (b). The downside: confocal microscopy images of Hela cells incubated with NEM (5 mM) for 20 min and then incubated with probe **DCIP** (10 μM) for 15 min; (d) bright field image, (e) fluorescence image, (f) merged image of (d) and (e).

Conclusion

In summary, a new fluorescent probe **DCIP** for Cys was designed and readily synthesized. The probe **DCIP** exhibits a rapid and high selective and sensitive fluorescence turn-on detection process for Cys. Moreover, the probe **DCIP** showed a low detection limit (0.70 μM) and a large Stokes shift (176 nm) toward Cys. Additionally, the results of bioimaging in living cells suggest that the probe **DCIP** could be applied for sensing intracellular Cys with low cytotoxicity and satisfactory cell membrane permeability. Therefore, the probe **DCIP** has a great potential for application in detection of Cys.

Acknowlegements

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Supplementary data

^{1}H NMR, ^{13}C NMR, and MS of compound **2** and **DCIP** are available in **ESI**.

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