


Cite this: *RSC Adv.*, 2018, 8, 37410Received 11th July 2018
Accepted 19th October 2018

DOI: 10.1039/c8ra05908j

rsc.li/rsc-advances

Specific and sensitive imaging of basal cysteine over homocysteine in living cells†

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Biological thiols play important roles in maintaining appropriate redox status of organisms. Accepting the challenge to differentiate structurally similar cysteine (Cys) and homocysteine (Hcy), we have successfully developed a miniature synthetic turn-on fluorescent probe based on 6-(2-benzothiazolyl)-2-naphthalenol for Cys. This probe is able to specifically react with Cys to yield its naphthalenol derivative, accompanied by remarkable green fluorescence enhancement with a detection limit of 14.8 nM. Besides, this probe displays much greater selectivity for Cys over other biological thiols, including homocysteine (Hcy) and glutathione (GSH). Practically, good cell permeability and low cytotoxicity make it suitable for monitoring basal Cys in living cells.

1. Introduction

Biological thiols including cysteine (Cys), homocysteine (Hcy) and glutathione have received a great deal of attention due to their diverse biological functions in living organisms.^{1–5} Among them, Cys is an enriched amino acid that is involved in various physiological and pathological processes. Intracellular Cys is generally generated from methionine by methionine-adenosyl-transferase, adenosyl-homocysteinase, cystathionine- β -synthase, or cystathionine- γ -lyase.⁶ At normal levels, Cys maintains the synthesis of proteins, and acts as a source of sulfide in human metabolism.^{7,8} A deficiency of Cys induces diseases such as decreased hematopoiesis, leucocyte loss, psoriasis, neurotoxicity, edema, liver damage and Parkinson's disease.^{9–11} On the other hand, excessive Cys causes rheumatoid arthritis and Alzheimer's disease.^{12–17} In order to understand the biological properties of Cys better, it is important to develop sensitive and selective methods for monitoring extracellular Cys.

As a powerful tool, fluorescence spectroscopy has been widely applied for detection of varied cellular species due to its high sensitivity, fast-response, simplicity of implementation, cost-effectiveness, real-time detection, noninvasiveness, and good compatibility with biosystems.^{18–23} For thiol-containing biomolecules fluorescence probes based on different response mechanisms have been well developed, including Michael addition,^{24–26} cyclization reaction with aldehyde,^{27–29} cleavage reaction,^{30–33} nucleophilic substitution,^{34–39} disulfide exchange

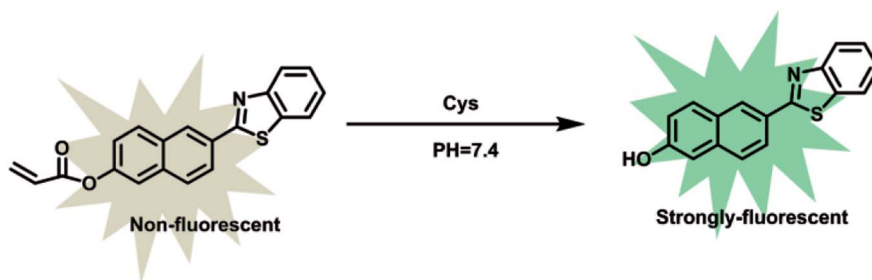
reaction^{40–42} and other mechanisms.^{43,44} To achieve better selectivity of Cys over Hcy and GSH, the Michael addition reaction is among the most widely used strategies for fluorescent sensing of Cys. Strongin and co-workers creatively developed a combination of Michael addition and cyclization reaction to improve the selectivity of Cys/Hcy over GSH.⁴⁵ Yoon and co-workers reported a fluorescent probe based on fluorescein with excellent selectivity and sensitivity for thiols, and it was successfully applied for bio imaging.⁴⁶ Sun and co-workers reported a novel fluorescent probe based on α,β -unsaturatedacyl sulfonamide to detect thiols. It reacted selectively with cysteine but not with the other natural amino acids, and subsequently applied to detect intracellular thiols.⁴⁷ Though these probes employ high sensitivity toward thiol-containing compounds, the selective detection of Cys is still challenging because of disturbance of other biological thiols, especially Hcy. This challenge arises from the structural and reactive similarity between Cys and Hcy which differ by a single methylene of their side chains. Currently claimed fluorescent probes for Cys over Hcy often utilize the different relative kinetical rates which may still cause interference from Hcy after a certain reaction time. Optimal solutions without any kinetical control are in great demand.

As our continuous interests in monitoring cellular redox status changes,^{48–51} we designed and synthesized a simple fluorescent **probe 1** to detect cellular Cys in living cells. As demonstrated in Scheme 1, **probe 1** carrying an acrylates group, which acts as a Michael addition acceptor is able to react with Cys through thioether formation and followed by cyclization to release emissive fluorophore. **Probe 1** is easily accessible by two-step synthesis method, which is weakly-fluorescent with an absolute quantum yield of 0.08 due to the photo induced electron transfer (PET) process. After the reaction with Cys, the

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c8ra05908j





Scheme 1 The reaction mechanism of probe 1 with Cys.

product emits strong green fluorescence and gives an absolute quantum yield of 0.78. Experimental results show that **probe 1** exhibits high sensitivity (the detection limit of 14.8 nM) against Cys, and high selectivity over Hcy and other biological thiols. More importantly, **probe 1** showed good cell permeability, low cytotoxicity and was successfully applied to imaging basal Cys in living cells.

2. Experimental

2.1. Materials and instrumentals

All other chemicals used in this paper were obtained from commercial suppliers and used without further purification. Silica gel (200–300 mesh, Qingdao Haiyang Chemical Co.) was used for column chromatography. NMR spectra were recorded on a Bruker Avance III at 400 MHz for ^1H NMR and at 100 MHz for ^{13}C NMR with chemical shifts reported as ppm (in $\text{DMSO}-d_6$ TMS as internal standard). Mass spectra (MS) were measured with Bruker Apex IV FTMS using electrospray ionization (ESI). Absorption spectra were recorded on a Purkinje TU-1901 spectrophotometer. Fluorescence measurements were taken on a Hitachi F-7000 fluorescence spectrometer with a 10 mm quartz cuvette. pH measurements were carried out with a pH acidometer (Mettler Toledo FE-30). Fluorescence imaging was observed under an Olympus IX81 confocal fluorescence microscope. The absolute fluorescence quantum yield values were measured using Hamamatsu Photonic Multi-Channel Analyzer PMA-12.

2.2. General procedure for analysis

Parent stock solution of fluorescent **probe 1** (1.0 mM) was prepared in dimethyl sulfoxide (DMSO). The solution of test was prepared by placing 50 μL of parent stock solution and appropriate volume of other solution into the test tube, then diluting the solution to 10 mL with the mixture of ethanol and ultrapure

water (2 : 8, v/v) containing phosphate buffered saline (PBS, 10 mM, pH = 7.4). All spectra were obtained in a quartz cuvette (path length = 1 cm).

All the solutions (10.0 mM) were prepared in deionized water. All the amino acids were purchased from Sigma-Aldrich Chemical. PBS solution was prepared with Na_2HPO_4 and KH_2PO_4 , and adjusted to pH 7.4.

2.3. Determination of the detection limit

Referring to previous papers, the detection limit was calculated based on fluorescence titration.⁵² Fluorescence titration was carried out in H_2O /ethanol solution (H_2O : ethanol = 8 : 2, (v/v), 10 mM PBS, pH = 7.4) to determine the detection limit, which was then calculated with the following equation:

$$\text{Detection limit} = 3\sigma/k$$

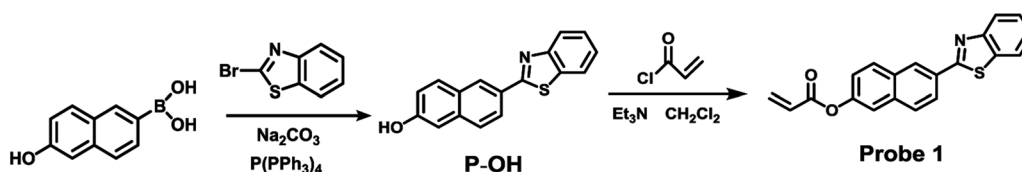
Where σ is the standard deviation of blank measurements, k is the slope between the fluorescence intensity vs. Cys concentration.

2.4. Cytotoxicity assay

HeLa cells were cultured in culture media (DMEM) in an atmosphere of 5% CO_2 and 95% air at 37 $^\circ\text{C}$. The cells were seeded into 96-well plates at a density of 3×10^3 cells per well in culture media, then 0, 5, 10 μM **probe 1** were added, respectively. Next, the cells were incubated at 37 $^\circ\text{C}$ in an atmosphere of 5% CO_2 and 95% air for 24 h. Finally, 20 μL MTT was added and cultured for another 4 h, respectively.

2.5. Cell culture and imaging

HeLa cells were grown on glass-bottom culture dishes using DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 50 $\mu\text{g mL}^{-1}$ penicillin-streptomycin in a humidified 37 $^\circ\text{C}$,



Scheme 2 Synthesis of probe 1.



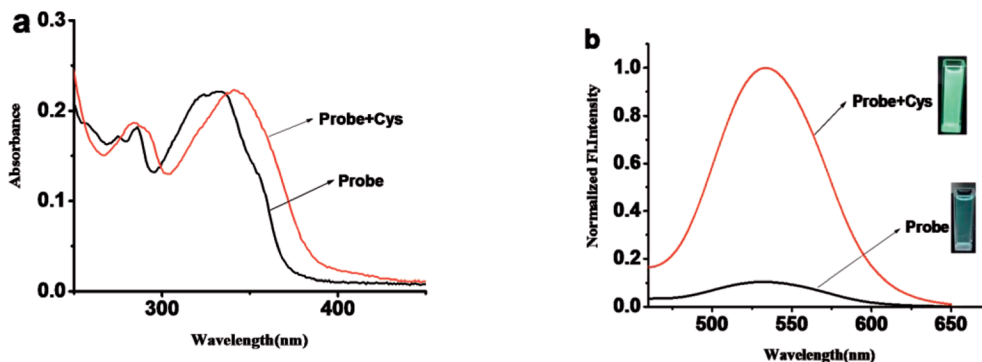


Fig. 1 (a) Absorption spectra of **probe 1** (5 μM) in the absence and presence of Cys (60 μM) in H_2O : ethanol = 8 : 2 (v/v), pH = 7.4, 10 mM PBS. (b) Fluorescence spectra of **probe 1** (5 μM) in the absence and presence of Cys (60 μM) in H_2O : ethanol = 8 : 2 (v/v), pH = 7.4, 10 mM PBS. Excitation wavelength is 400 nm, excitation and emission slit widths are 5 nm and 5 nm. Each spectrum was acquired 40 min after Cys-addition at room temperature.

5% CO_2 incubator. Before use, the adherent cells were washed three times with FBS-free DMEM. The cells were incubated with 5 μM probe in culture media for 30 min at 37 $^\circ\text{C}$ and then washed with PBS (pH 7.4) twice. Fluorescence imaging of HeLa cells was observed under an Olympus IX81 confocal fluorescence microscope, and the excitation wavelength was 405 nm.

2.6. Synthesis of fluorescent probe 1

2.6.1 Synthesis of P-OH. The synthesis route of **probe 1** was shown in Scheme 2. (6-Hydroxynaphthalen-2-yl)boronic acid (1 eq.), 2-bromobenzo[d]thiazole (1 eq.), sodium carbonate (3 eq.) and tetrakis(triphenylphosphine)palladium (0.05 eq.) were added to a 21 mL solution ($V_{\text{Methanol}} : V_{\text{Toluene}} : V_{\text{H}_2\text{O}} = 1 : 1 : 1$). The mixture was refluxed for 3 h then cooled to ambient temperature. Poured into the water, the solution was adjusted by acid and stand for 10 min, and the precipitate was obtained by filtration. The filtrate was evaporated under reduced pressure and purified by silica gel column chromatography affording **P-OH** as white solid. ^1H NMR (400 MHz, DMSO) δ 10.14 (s, 1H), 8.56 (s, 1H), 8.16 (d, $J = 7.9$ Hz, 1H), 8.12–7.99 (m, 2H), 7.86 (d, $J = 8.6$ Hz, 1H), 7.56 (t, $J = 7.6$ Hz, 1H), 7.46 (t, $J = 7.5$ Hz, 1H), 7.20 (d, $J = 13.6$ Hz, 1H).

2.6.2 Synthesis of fluorescent probe 1. To a solution of **P-OH** (1 eq.) and Et_3N (4 eq.) in 10 mL of anhydrous CH_2Cl_2 , acryloyl chloride (3 eq.) was added dropwise in an ice-bath. After stirring at 0 $^\circ\text{C}$ for 1 h, the mixture was warmed to room temperature and stirred for another 16 h. The resulting solution was diluted with CH_2Cl_2 (30 mL) and washed with H_2O (3×15 mL) and dried over anhydrous Na_2SO_4 . Solvent was removed by rotavapor. Purification by silica gel column chromatography afforded **probe 1** as yellow solid. ^1H NMR (400 MHz, DMSO) δ 8.76 (s, 1H), 8.31–8.23 (m, 2H), 8.20 (d, $J = 8.0$ Hz, 1H), 8.11 (d, $J = 8.4$ Hz, 2H), 7.87 (s, 1H), 7.59 (t, $J = 7.5$ Hz, 1H), 7.50 (t, $J = 7.5$ Hz, 2H), 6.62 (d, $J = 17.2$ Hz, 1H), 6.50 (dd, $J = 17.2$, 10.2 Hz, 1H), 6.22 (d, $J = 10.1$ Hz, 1H). ^{13}C NMR (700 MHz, DMSO) δ 167.63, 164.70, 154.10, 149.80, 135.17, 135.07, 134.51, 131.37, 131.10, 130.74, 129.25, 128.05, 127.73, 127.26, 126.16, 125.21, 123.39, 123.22, 122.93, 119.26. ESI-HRMS calcd for $\text{C}_{20}\text{H}_{13}\text{NO}_2\text{S}$ $[\text{M} + \text{H}]^+$: 332.0740, found 332.0742.

3. Results and discussion

3.1. Response of probe 1 to Cys

The absorption and fluorescence spectra of **probe 1** were measured in H_2O /ethanol solution (8 : 2, v/v, 10 mM PBS, pH =

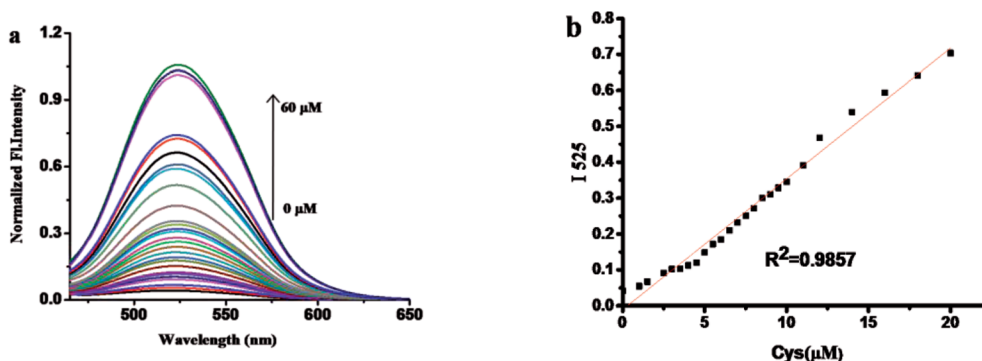


Fig. 2 (a) The fluorescence spectra of **probe 1** (5 μM) in the presence of different concentrations of Cys in H_2O : ethanol = 8 : 2 (v/v), pH = 7.4, 10 mM PBS at room temperature. (b) The plot of fluorescence intensity at 525 nm vs. Cys concentrations. Excitation wavelength is 400 nm, excitation and emission slit widths are 5 nm and 5 nm.



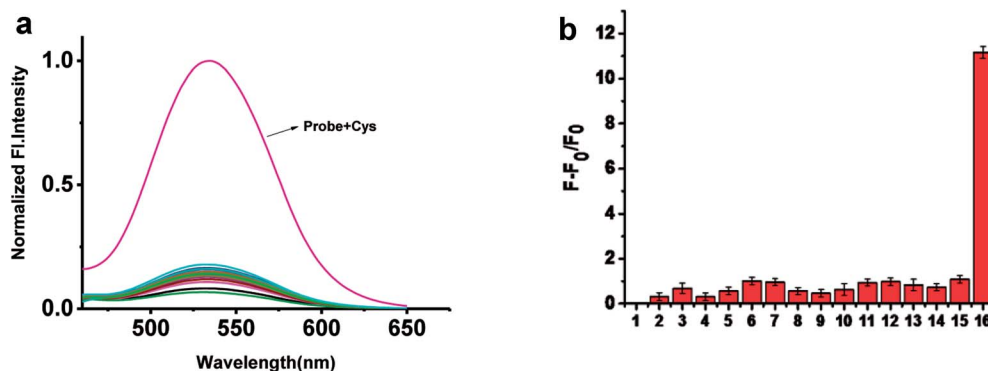
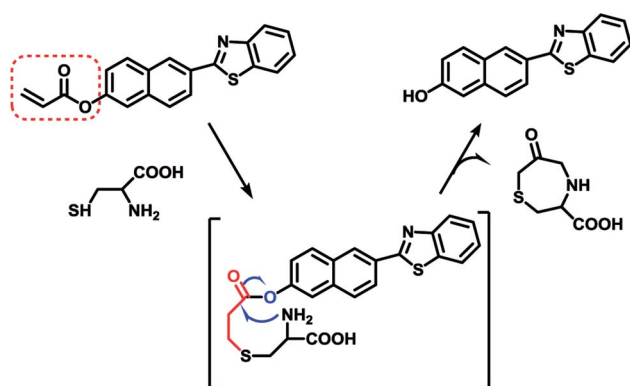


Fig. 3 (a) The fluorescence spectra of **probe 1** (5 μM) toward Cys (10 μM), Hcy, GSH and various amino acids (10 μM) in H_2O : ethanol = 8 : 2 (v/v), pH = 7.4, 10 mM PBS at room temperature: the inset shows the fluorescence of various solution under the Handhold UV Lamps (365 nm). (b) Fluorescence intensity changes at 525 nm. (1) **Probe 1** (2) L-Leu (3) Ala (4) Trp (5) L-Met (6) Val (7) Thr (8) Tyr (9) D-Met (10) Ser (11) Glu (12) Phe (13) D-Leu (14) GSH (15) Hcy (16) Cys. Excitation wavelength is 400 nm, excitation and emission slit widths are 5 nm and 5 nm. Each spectrum was acquired 40 min after various analytes addition at room temperature.



Scheme 3 Proposed response mechanism of **probe 1** to Cys.

7.4). As shown in Fig. 1, **probe 1** shows a maximum absorption at 331 nm and weak fluorescence with a relatively low quantum yield of 0.08. After the addition of Cys, the maximum absorption red shifted 13 nm to 343 nm (Fig. 1a). Meanwhile, slight blue

shift and 24-fold fluorescence enhancement at 525 nm were obtained and the solution emitted strong green fluorescence under a handhold UV lamp (Fig. 1b), just like we expected, given the absolute quantum yield of 0.78 for the final fluorophore **P-OH**.

3.2. Quantification of Cys and detection limit

The fluorescence spectra of **probe 1** with various concentrations of Cys is shown in Fig. 2. Upon gradually increasing of Cys, the fluorescence band centered at 525 nm increased subsequently. There was a good linearity between the fluorescence intensity at 525 nm and the concentrations of Cys in the range of 1 to 20 μM . The detection limit of Cys is calculated to be 14.8 nM. The results show that **probe 1** has better detection limit than those reported fluorescent probes for Cys (Table S1[†]). These above results demonstrated that **probe 1** could detect Cys quantitatively by fluorescence spectrometry methods with an excellent sensitivity (Table S1[†]).

3.3. The kinetic profile of the recognition of probe 1 for Cys

The response time of **probe 1** towards Cys is evaluated by fluorescence spectroscope. When Cys (60 μM) was added to the solution of **probe 1** (5 μM), the fluorescence intensity at 525 nm levels off after 40 min (Fig. S1[†]). The result shows that **probe 1** could completely react with Cys within 40 min, thus we chose 40 min to verify the quantification and selectivity of Cys.

3.4. The selectivity of probe 1

To confirm the selectivity of **probe 1** towards Cys, other amino acids and GSH were added into the solution of **probe 1**. As shown in Fig. 3 and S2,[†] upon addition of 10 mM GSH, and other amino acids, or cations including K^+ , Na^+ , Mg^{2+} , Al^{3+} , Fe^{2+} into 5 μM **probe 1** solution, nearly no fluorescence change were observed except Cys. Only Cys gave 12-fold enhancement of fluorescence.

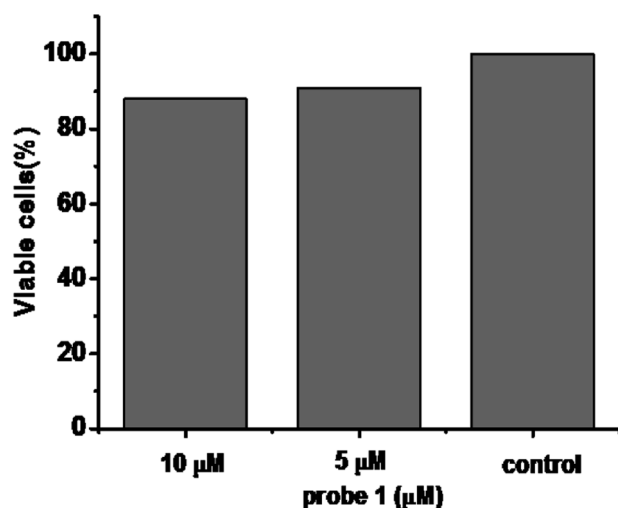


Fig. 4 Cytotoxicity assays of **probe 1** at different concentrations for HeLa cells.



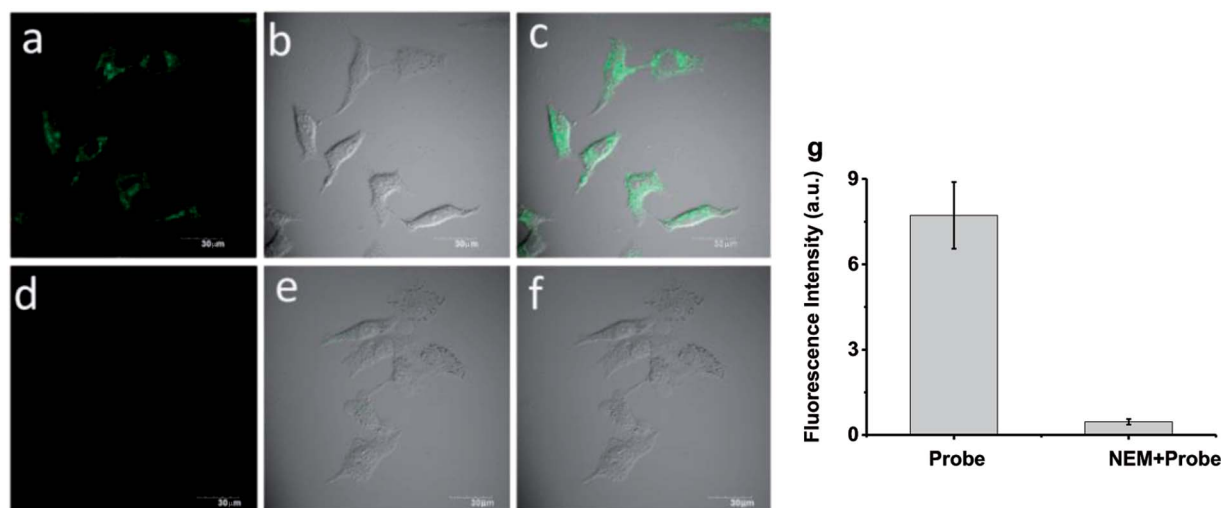


Fig. 5 Fluorescence images of Cys in living cells. (a–c) HeLa cells stained with **probe 1** (5 μM) for 30 min. (d–f) HeLa cells were treated with NEM (100 ng mL^{-1}) for 1 h before incubated with **probe 1** (5 μM) for 30 min. (a and d) Fluorescence image. (b and e) Bright field image. (c and f) Merged image. (g) Relative fluorescence intensity of **probe 1** treated cells with or without NEM management. Incubation was performed at 37 $^{\circ}\text{C}$ under a humidified atmosphere containing 5% CO_2 . Excitation wavelength is 405 nm, emission wavelength was collected from 450 to 550 nm.

3.5. Reaction mechanism

It has been well documented that the acrylate group could be used as an efficient reaction site for Cys.⁵³ The mechanism of **probe 1** responding to Cys was based on the Michael addition reaction of Cys with the acrylate group to generate the corresponding thioether, a subsequent intramolecular cyclization and further self-immolation to yield the desired lactam, as shown in Scheme 3. To gain further insight into the reaction mechanism, mass spectrometric analysis was carried out to verify the final product after reaction between **probe 1** and Cys. ESI-HRMS in Fig. S9† showed a main peak at m/z 278.0638 $[\text{M} + \text{H}]^+$ corresponding to compound **P-OH** (calculated at m/z 278.0634 $[\text{M} + \text{H}]^+$), which disclosed the proposed mechanism below.

3.6. Bioimaging applications and cytotoxicity

To further demonstrate the biological compatibility before real time imaging in living cells, we evaluated the cytotoxicity of **probe 1**. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed in HeLa cells with 0, 5, 10 μM **probe 1** for 24 h, respectively. The viability results in Fig. 4 clearly showed that **probe 1** was minimal toxic to cultured cells even at the concentration of as high as 10 μM . This illustrates **probe 1** is suitable for living cell imaging at its work concentration, 5 μM .

Inspired by the experimental results *in vitro* of the **probe 1** and encouraged by the good biocompatibility, we expected that the high sensitive probe could have a good application for imaging endogenous Cys in living cells. In order to verify our conjecture, HeLa cells were incubated with **probe 1** (5 μM) for 30 min, and a strong green fluorescence was observed as shown in Fig. 5a–c. When HeLa cells were treated with *N*-ethylmaleimide (100 ng mL^{-1}) as Cys scavenger for 1 h before they were incubated with **probe 1** (5 μM), hardly any intracellular

fluorescence was observed in Fig. 5d–f. From Fig. 5g, these results clearly demonstrated that **probe 1** was cell-permeable and could provide a good option for imaging basal Cys in living cells. Moreover, the same experiments were also tested in normal cells apart from cancer cells, like RAW 264.7 and HepG2 and similar cellular fluorescence change was observed (Fig. S3 and S4†).

4. Conclusion

In summary, we have developed a reaction based turn-on fluorescent **probe 1** conjugating with a reactive acrylate for visualization basal Cys in living cells. The synthesis of **probe 1** is simple, and the probe showed excellent selectivity toward Cys over Hcy. Different from previously reported Cys detection methods, the reactivity between Cys and Hys is significantly different, therefore kinetically disfavourability of Hcy is no longer needed to improve the selectivity. Furthermore, monitoring basal Cys effectively in living cells was easily implemented attributing to its low cytotoxic, good sensitivity and good selectivity towards Cys over Hcy and GSH. This method could become a potential tool to reveal the behaviors of Cys in living organism.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was supported by the National Nature Science Foundation of China (No. 21575015 and 21505004).



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