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A Highly Selective FRET-Based Fluorescent Probe for Detection of Cysteine and Homocysteine

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Biological thiols including cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) play significant roles in redox-related biological processes.[1] Abnormal levels of Cys and Hcy are associated with a variety of human diseases such as slow growth,[2] liver damage,[2] skin lesions,[2] Alzheimer's diseases,[3a] cardiovascular diseases[3a,b] and coronary heart diseases.[3c] Thus, the development of efficient methods for the detection and quantification of biological thiols under physiological conditions is of importance in both chemistry and medicine.

Analytical methods for the detection of thiols in biological samples have been substantially advanced owing to the development of analytical techniques including high-performance liquid chromatography (HPLC),[4] capillary electrophoresis (CE),[5] electrochemical assay[6] and mass spectrometry.[7] However, laborious procedures such as sample clean-up, isolation and purification are often involved before instrumental analysis can take place. Thus, there has been a surge of interest in the development of simple and yet sensitive thiol detection methods in which the requirement of tedious sample preparation procedures and expensive instrumentation can be minimised.

Fluorescent molecular probes are advantageous because fluorescence detection is highly sensitive and has a good dynamic range. In addition, inexpensive instrumentation and simple procedures are involved.[8] The pioneering work by Strongin and co-workers on the use of an aldehyde-containing xanthene dye for fluorescent detection of Cys or Hcy[9] has stimulated considerable interest to develop new fluorescent probes for biological thiol detection.[10] However, limitations exist for the reported fluorescent thiol probes, including high background fluorescence[11] and long response times.[12] Therefore, it remains a challenging task to develop highly selective and efficient fluorescent probes for the detection of Cys and Hcy.

Along with our long-term interest in the design and synthesis of novel luminescent materials for solving biological problems, we recently explored the use of luminescent metal complexes for protein staining and cell imaging.[13] In the search for new bioconjugation reactions,[14a] we have recently developed a method for selective and reversible modification of cysteine-containing peptides by electron-deficient alkynones in aqueous medium (Scheme 1).[14b]

As depicted in Scheme 1, the thiol group of cysteine-containing peptides can be linked to electron-deficient alkynones to generate a vinyl sulfide linkage. Upon treatment with excess thiol (aromatic or aliphatic), an intermediate dithioacetal is formed. The vinyl sulfide linkage of the dithioacetal can be cleaved to regenerate free thiol through elimination of molecule **A**. Note that the vinyl sulfide linkage can only be cleaved by addition of thiols but not other nucleophiles such as amines and alcohols. We envision that this thiol-specific cleavage reaction could be employed to develop a highly selective probe for the detection of thiols.

An ideal fluorescent probe should be triggered to give a strong fluorescent signal upon selective reaction with its target analytes. With the thiol-specific cleavage reaction in hand, we set out to design and synthesise a "turn-on" fluorescent probe for thiol detection. We proposed to connect a fluorophore and a quencher through the vinyl sulfide linkage. The resulting fluorescent probe should be weakly fluorescent ("turn-off") owing to quenching through fluorescence resonance energy transfer (FRET).[15] Upon treatment

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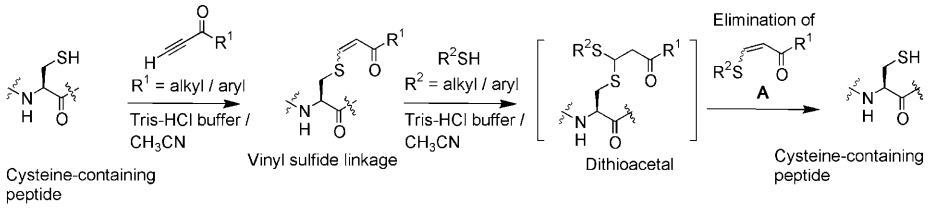
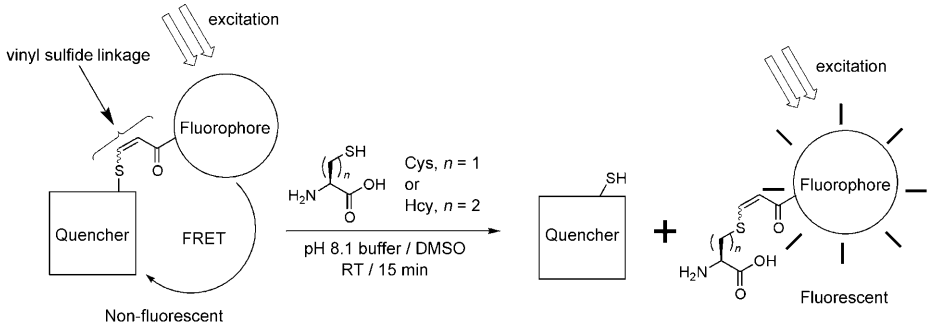


Scheme 1. Electron-deficient alkynones as cleavable reagents for modification of cysteine-containing peptides in aqueous medium.

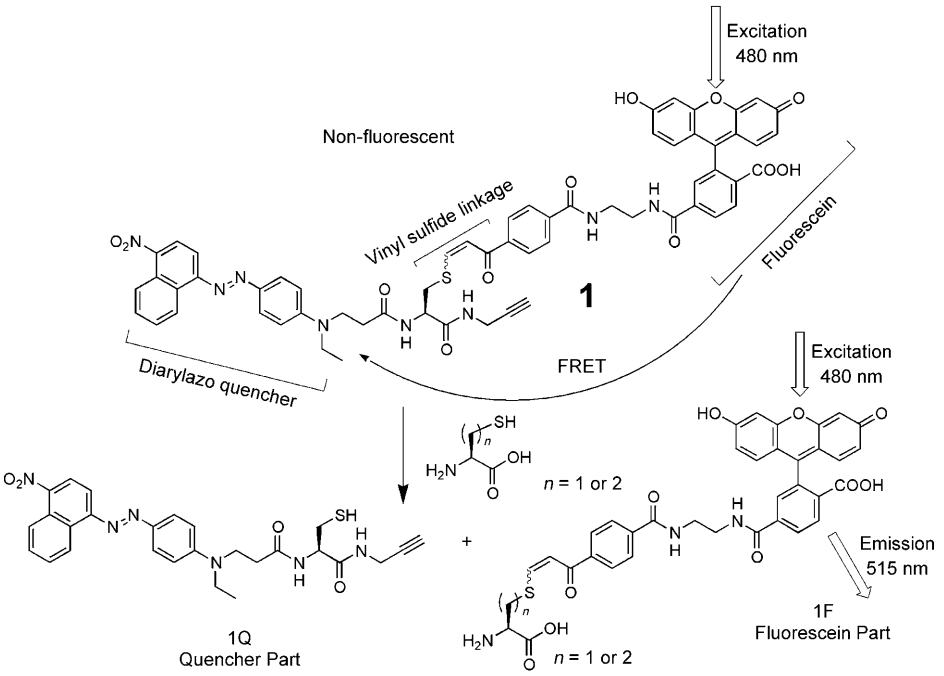
with thiols, the quencher unit is disconnected from the probe through cleavage of the vinyl sulfide linkage. As a result, the fluorescent probe is turned-on to give a strong fluorescent signal (Scheme 2).

The turn-on mechanism of the FRET-based fluorescent probe was designed on the basis of a highly selective conjugate 1,4-addition of thiols to an α,β-unsaturated vinyl sulfide linkage followed by elimination. We envision that the rate of this conjugate 1,4-addition is dependent on the steric bulk of thiol nucleophiles. It is expected that this fluorescent probe is able to differentiate thiols of different steric bulk. Hcy, which has the least bulky thiol group, would give higher fluorescent signal than Cys, whereas no significant signal would be obtained for sterically bulky GSH. A ketocoumarin-based thiol detection probe featuring conjugate 1,4-addition of thiols to α,β-unsaturated ketones based on an intramolecular charge system (ICT) has recently been reported.[16] However, the reported probe displays little selectivity among different thiol-containing compounds. Herein, we report the development of a highly selective turn-on fluorescent probe **1** for detection of Cys and Hcy by connecting a diarylazo quencher and a fluorescein fluorophore through the vinyl sulfide linkage.[17]

Scheme 3 depicts the working principle of **1** as a fluorescent sensor for Cys and Hcy. Probe **1** was prepared by connecting a diarylazo quencher and a fluorescein fluorophore through the vinyl sulfide linkage (see the Supporting Information). Fluorescein absorbs at 480 nm and emits at 515 nm (green-coloured fluorescence),[15a] whereas the diarylazo quencher exhibits maximum light absorption at 515 nm.[17] Therefore, probe **1** is non-fluorescent because there is significant spectral overlap between fluorescein emission and quencher absorption spectra, that is, excitation at 480 nm, and results in limited emission at 515 nm. Because FRET is distance dependent (effective within 100 Å), cleavage of **1** by thiols separates the fluorescein from the quencher. Therefore, the quencher (**1Q**) could no longer effectively quench the emission from the fluorophore (**1F**). As a result, a strong emission at 515 nm from **1F** was detected.



Scheme 2. Turn-on fluorescence can be achieved after removal of the quencher by the thiol-assisted cleavage of the vinyl sulfide linkage.



Scheme 3. Working principle of **1** as a fluorescent sensor in response to cysteine and homocysteine.

At first, the time course of the reaction between probe **1** and Hcy was studied by monitoring the fluorescence intensity of the reaction mixture at 515 nm (Figure 1). A solution of **1** (5 μM) in phosphate-buffered saline (PBS buffer, pH 8.1) is weakly emissive ($\Phi_F = 0.03$ in NaOH (0.1 M), see the Supporting Information). The low background fluorescence reveals that fluorescein is effectively quenched by the diarylazo quencher. Upon treatment with Hcy (0.2 mM) in pH 8.1 PBS buffer/DMSO (9:1) at room temperature, a more than 20-fold increase in the fluorescence intensity at 515 nm was detected within the first 5 min. This abrupt increase in the emission intensity signified a high initial reaction rate in the cleavage of the vinyl sulfide linkage of **1** by the nucleophilic Hcy. After 15 min the increase in emission intensity levelled off. Therefore, an assay time of 15 min was selected in the evaluation of the selectivity and sensitivity of **1** towards detection of thiols in subsequent studies. Up to a 30-fold increase in the fluorescence intensity was observed when **1** (5 μM) was treated with Hcy (0.2 mM) in a molar ratio of 1:40. Up to a 20-fold increase in the fluorescence intensity was observed when **1** (5 μM) was treated with Cys (0.2 mM) in a molar ratio of 1:40. Figure 2 shows the fluorescence emission of **1** when treated with Hcy or Cys.

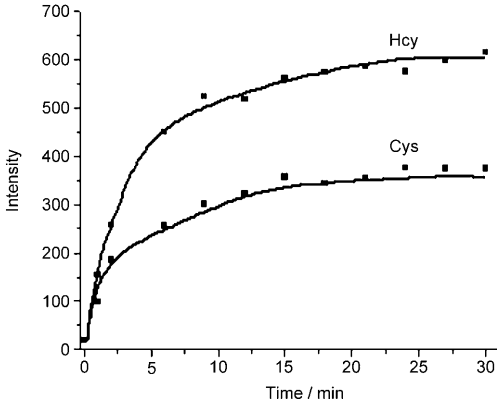


Figure 1. Respective time course of the reaction between **1** and homocysteine and cysteine in a molar ratio of 1:40.

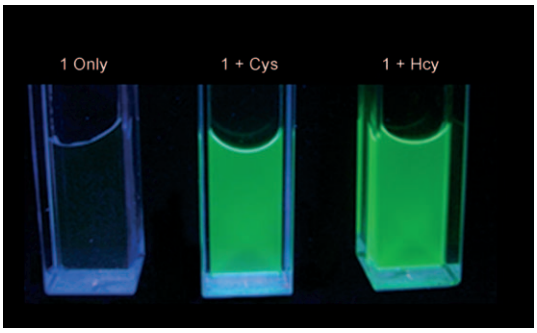
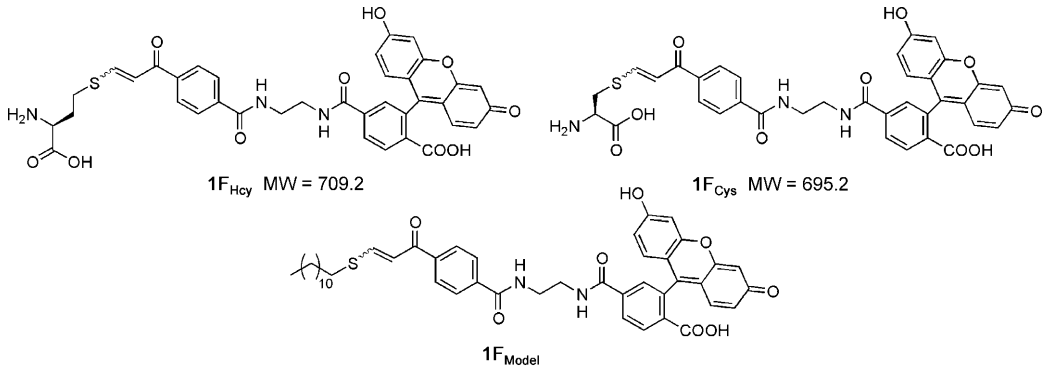


Figure 2. A photograph showing the fluorescence emission of **1** when treated with Cys or Hcy.

ESIMS analysis of the reaction mixture containing **1** (5 μM) and Hcy (0.2 mM) revealed three signals. In addition to the signal from unreacted **1** ($m/z = 1106.4$), the other two signals ($m/z = 532.2$ and 709.2) are consistent with the formulation of the quencher part **1Q** and the fluorescein part $\mathbf{1F}_{Hcy}$, respectively. Similarly, three signals were found in the ESIMS analysis of the reaction mixture of **1** (5 μM) and Cys (0.2 mM). The three species were unreacted **1** ($m/z = 1106.4$), the detached quencher **1Q** ($m/z = 532.2$) and the fluorescein part $\mathbf{1F}_{Cys}$ ($m/z = 695.2$).

To study the fluorescent properties of the fluorescent parts ($\mathbf{1F}_{Hcy}$ and $\mathbf{1F}_{Cys}$), a model compound $\mathbf{1F}_{model}$ was constructed (Scheme 4, see the Supporting Information). $\mathbf{1F}_{model}$ was found to be strongly fluorescent, with $\Phi_F = 0.74$ in NaOH (0.1 M; see the Supporting Information). To demonstrate the linearity of the response of **1** towards Hcy, **1** (5 μM) was treated with Hcy at different concentrations in pH 8.1 PBS buffer/DMSO (9:1), the corresponding emission intensity at 515 nm was measured after 15 min. When the emission intensity of **1** at 515 nm was plotted against Hcy concentration, a calibration curve (Figure 3) revealing a good linear relationship (R value = 0.99) in the Hcy concentration range of 0–0.3 mM was obtained. Moreover, the addition of Hcy to the solution resulted in up to a 40-fold increase in emission intensity at 515 nm when the concentration ratio of [Hcy]/[**1**] increased from 0 to 60.

Up to a 30-fold increase in emission intensity at 515 nm was obtained when the concentration ratio of [Cys]/[**1**] in-



Scheme 4. Structures of the fluorescent parts $\mathbf{1F}_{Hcy}$, $\mathbf{1F}_{Cys}$ and $\mathbf{1F}_{Model}$.

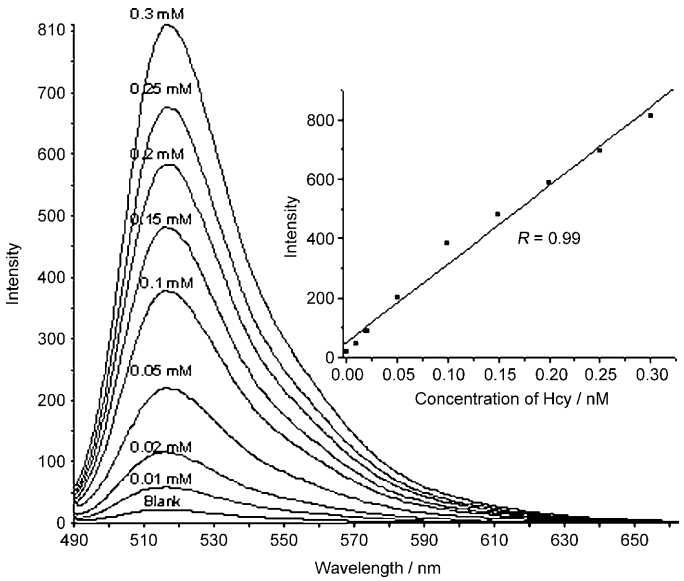


Figure 3. Emission spectral traces of **1** (5 μM) in PBS buffer with increasing [Hcy]/[**1**] ratio (from 0 to 60) at room temperature. Each spectrum was acquired 15 min after Hcy addition.

creased from 0 to 60. Yet, treatment of up to 40 equivalents of GSH (a cysteine-containing peptide bearing three amino acids) with **1** did not result in emission enhancement. These results indicated that **1** is responsive to the steric hindrance of the thiol species. The intensity of the emission signal is in the order Hcy > Cys ≫ GSH. This is consistent with the difference in reaction rate of the thiols with different steric bulk towards conjugate 1,4-addition.[18] Note that probe **1** is able to differentiate between the subtle steric hindrance difference between Hcy and Cys (Figure 4). As expected, probe **1** gave no response to sterically bulky cysteine-containing biomolecules including peptide STSSSCNLSK (SK-10), bovine serum albumin (BSA) and human serum albumin (HSA; Figure 4). This is of importance for probe **1** to selectively detect Cys and Hcy in a complex biological matrix.

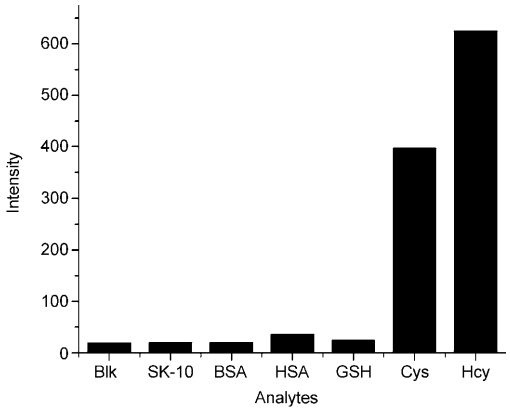


Figure 4. Enhancement of emission intensity of **1** by SK-10, BSA, HSA, GSH, Cys and Hcy.

To examine the selectivity of the FRET-based probe, **1** was treated with various biologically relevant analytes including amino acids, glucose, metal ions, a reactive oxygen species and a reducing agent. Amino acids (Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val), metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{3+} and Zn^{2+}), a reducing agent (nicotinamide adenine dinucleotide (NADH)) and glucose did not result in any emission enhancement, even at 40:1 molar ratio of the analyte versus **1**. The results demonstrated that **1** is selective to thiol species among the various biologically relevant analytes. H_2O_2 (a strong oxidising agent) gave a weak fluorescent signal upon reaction with **1**. A possible explanation may be due to oxidative degradation of the diarylazo quencher[19] (Figure 5)

In addition, we applied probe **1** to fluorescent sensing of Cys/Hcy in commercial human blood plasma. The conversion of disulfides to free thiols was conducted by treatment of the plasma sample with a reducing agent, triphenylphosphine, according to a literature procedure.[9b] Aliquots of commercial human blood plasma (0, 50, 70, 100, 150 or 200 μL) were added to a solution of probe **1** (5 μM) in PBS

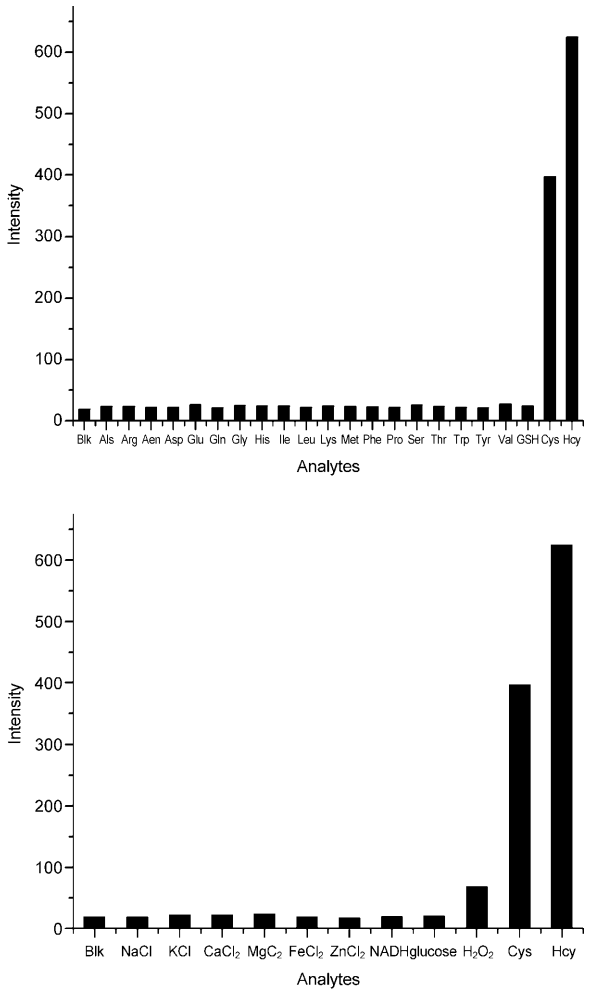


Figure 5. Enhancement of emission intensity of **1** by different analytes.

buffer solution (pH 8.1) at room temperature. As shown in Figure 6, an increase in the volume of the reduced blood plasma leads to a corresponding linear enhancement in the fluorescence intensity, revealing that the probe is able to detect thiols in the plasma sample.

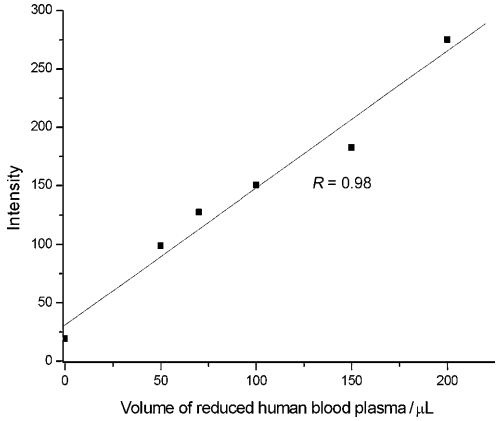


Figure 6. Calibration curve obtained by treating **1** (5 μM) with different amounts of commercial human blood plasma (0, 50, 70, 100, 150 or 200 μL).

For quantitative measurement, a standard addition method with Hcy as the standard was employed to estimate the unknown concentration of thiols in the commercial human blood plasma. The total content of thiols in the plasma was found to be 0.31 mM (see the Supporting Information), which is well within the range of reported thiol concentrations from normal human blood plasma.[20]

We have employed probe **1** to detect Cys and Hcy in cells (MKN-45 and SK-HEP-1 cells) by using confocal laser scanning microscopy. As shown in Figures 7a and 8a, a significant fluorescence enhancement was observed after incubating the fixed cells with a solution of **1** (10 μM) in ethanol/PBS (7:3 v/v, pH 7.4) for 10 min at 25 °C. The brightfield images of MKN-45 and SK-HEP-1 cells were also recorded. The overlay of fluorescence and brightfield images (Figures 7c and 8c) revealed that fluorescence signals with different intensities were localised in different compartments of the cells. As a control, no fluorescence was observed for cells pre-treated with *N*-methylmaleimide (a thiol-reactive compound) for 2 h (see the Supporting Information), revealing the specificity of probe **1** towards Cys/Hcy.

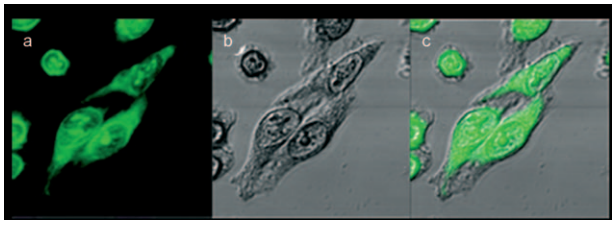


Figure 7. Fluorescence and brightfield images of fixed MKN-45 cells: a) fluorescence image of cells incubated with **1** (10 μM) for 10 min at room temperature; b) brightfield image of cells shown in panel a; c) the overlay of panels a and b.

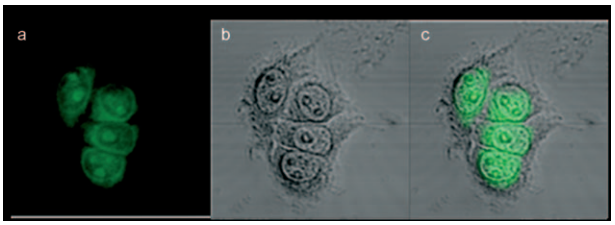


Figure 8. Fluorescence and brightfield images of fixed SK-HEP-1 cells: a) fluorescence image of cells incubated with **1** (10 μM) for 10 min at room temperature; b) brightfield image of cells shown in panel a; c) the overlay of panels a and b.

In summary, a turn-on fluorescent probe **1** for the detection of thiols on the basis of conjugate addition of thiols to vinyl sulfide linkages followed by elimination was synthesised. Probe **1** features high sensitivity and excellent selectivity for Cys/Hcy over other amino acids, GSH, BSA and HSA under physiological conditions. In addition, the probe also features fast signal response times and a good linearity range for quantification. We have demonstrated that **1** can be used as an effective sensing probe for Cys/Hcy in human blood plasma and in fixed cells by confocal laser scanning microscopy.

Experimental Section

General: Chemicals purchased from commercial sources were used without further purification. Electronic absorption spectra were recorded with a HP Agilent 8453 UV/Vis spectrophotometer. The emission spectra were measured on a PerkinElmer LS 55 fluorescence spectrometer. TLC analyses were performed on silica-gel plates and flash column chromatography was conducted over silica gel 60 (230–400 mesh ASTM) with ethyl acetate/*n*-hexane or methanol/dichloromethane as the eluent. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX-300 or DPX-400 spectrometer. Chemical shifts (ppm) are referenced to TMS. Mass spectra were measured by using a hybrid QTOF mass spectrometer (QSTAR-XL system, ABI, USA) and Finnigan MAT 95 or LCQ mass spectrometer.

Spectral measurements: The amino acids (Cys, Hcy, Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val), GSH, metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{3+} and Zn^{2+}), reactive oxygen species (hydrogen peroxide), reducing agent (NADH), glucose, peptide STSSSCNLSK (SK-10), BSA and HSA stock solutions were prepared in doubly distilled water. Probe **1** was dissolved in DMSO at room temperature to afford the probe stock solution (0.1 mM). The probe stock solution (20 μL) and the analyte stock solution (8 μL) were added to a solvent mixture of DMSO (20 μL) and PBS buffer (pH 8.1, 352 μL). The resulting solution was shaken well. After 15 min, the emission spectra were recorded. Unless otherwise stated, for all measurements the excitation wavelength was 480 nm and the excitation and emission slit widths were 5 nm. The thiol assay in human blood plasma typically requires the reduction of disulfides to free thiols. This can be accomplished by using an excess of triphenylphosphine at room temperature for 30 min. Aliquots of the human blood plasma after reduction were then directly added to probe **1** (5 μM) and diluted to 400 μL with pH 8.1 PBS buffer solution, and the emission at 515 nm was recorded. The unknown amount of thiols in human blood plasma sample was estimated by using the standard addition method with Hcy as the standard.

Cell culture: MKN-45 and SK-HEP-1 cells were maintained in DMEM (Dulbecco's modified eagle's medium) and MEM (minimum essential medium), respectively, which were both supplemented with fetal bovine serum (10% v/v) and penicillin/streptomycin (100 units mL^{-1}). The cells (1×10^4) were seeded to the eight-well chamber slide (9×9 mm each) and allowed to adhere for 24 h at 37°C, 95% air and 5% CO_2.

Fluorescence imaging: Confocal fluorescence imaging was performed with a Zeiss LSM510 META laser scanning microscope with a 40× objective lens. Green fluorescence was excited at 488 nm with an argon laser and emission was collected by a 515–565 nm band pass filter. The differential interference contrast (DIC) and the fluorescence was captured, digitised and processed to generate the pseudo-colour images by using Zeiss LSM Image Examiner software. For fixed cell imaging, immediately before the experiments cells were washed with the PBS buffer and then incubated with **1** (10 μM) in ethanol/PBS solution (7:3 v/v, pH 7.4) for 10 min at 25°C. Fluorescence imaging was then carried out after washing the cells with the PBS buffer. For the control experiment, the cells were pre-treated with a solution of *N*-methylmaleimide (50 μM) in an ethanol/PBS solution (70:30, v/v, pH 7.4) for 2 h in the incubator, and then incubated with **1** (10 μM) in an ethanol/PBS solution (70:30 v/v, pH 7.4) for 10 min at 25°C.

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Keywords: bioimaging · fluorescence · FRET · quenchers · thiols

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