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COMMUNICATION

Highly Selective Two-Photon Imaging of Cysteine in Cancerous Cells and Tissues

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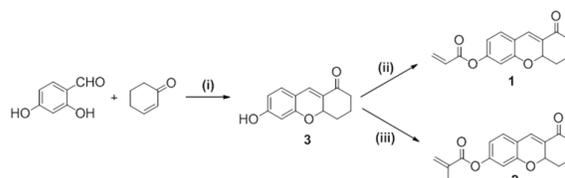
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Abnormal concentrations of Cys have been reported to be implicated in various health problems including cancer, neuropathy, and cardiomyopathy. We present a novel two-photon fluorescent probe for the specific recognition of cysteine over homocysteine and glutathione, and the bioapplication of this probe for the imaging of live cancerous cells and thick tissues.

Cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are the most important low-molecular-mass aminothiols that play crucial roles in the maintenance of biological systems with similar structures but different physiological functions.¹ Among them, Cys is an essential component of cells that plays a pivotal role in protein synthesis, cellular detoxification, and post-translational control.² It was reported that total Cys plasma levels range from 80 to 200 μM , with an average of ~ 100 μM in rats and ~ 200 μM in humans.³ Cys insufficiency is the causal factor of various medical problems, such as loss of depigmentation in hair, liver damage, growth retardation, lethargy, muscle and fat loss, skin lesions, and Alzheimer's disease.⁴ On the other hand, excessive levels of Cys could induce severe neurotoxicity and cardiovascular diseases.⁵ Thus, the detection of intracellular Cys in live cells would be a fundamental and vital approach for revealing possible pathological correlations. Therefore, in the past few years, diverse fluorescent probes for Cys have been developed based on various strategies, including Michael-type addition reaction, cyclization reaction between aminothiols and aldehyde, thiol-induced cleavage reactions, and ligand exchange.⁶ Fluorescence-based methods have several advantages such as high selectivity and sensitivity, low instrumentation cost, and a straightforward operation process.⁷ However, only a few probes are able to discriminate between Cys, Hcy, and GSH, because of their structural similarity and rapid incorporation into GSH, proteins, or Co-enzyme A. Therefore, it remains challenging to construct simple and effective fluorescent probes for the identification of Cys in physiological conditions. In

2010, Strongin *et al.* have reported a pioneering approach by which highly selective detection of Cys and Hcy is achieved by attaching an acrylate moiety as a recognition unit to the fluorophore.⁸ More recently, this strategy has been applied for the development of Cys sensors with different properties by several other groups.⁹

An additional limitation for effective tissue labeling using one-photon microscopy (OPM) is the restricted penetration depth of the probes in biological specimens. The use of two-photon excitation fluorescence (TPEF) for live imaging of tissues has remarkable advantages over OPM, including increased penetration depth, low autofluorescence levels, reduced phototoxicity, the possibility of three-dimensional imaging of living tissues, and prolonged observation times.¹⁰ TPEF imaging could be applied for the analysis of Cys distribution also in deep tissues. However, so far, there are only a limited number of reports on the development of Cys-specific two-photon probes.¹¹ Herein, we report the design and synthesis of a Cys-specific two-photon fluorescent probe (probe **1**), by incorporation of an acrylate moiety into chromene as a two-photon fluorescent analogue.¹² A reference probe (probe **2**) was also synthesized to compare the differences in steric hindrance upon addition of the acrylate moiety. The details of the synthesis of probes **1** and **2** are shown in the Electronic Supplementary Information (ESI). The chemical structures of all the compounds were verified by HRMS, ¹H NMR, and ¹³C NMR (ESI[†]).



Scheme 1 Synthesis of compounds **1** and **2**. Reagent and reaction conditions: (i) imidazole, H₂O, THF, RT; (ii) triethylamine, acryloyl chloride, THF, 0°C, 2 h; (iii) triethylamine, methacryloyl chloride, THF, 0°C, 2 h.

The spectroscopic properties of probe **1** and **2** were characterized under simulated physiological conditions. As shown in Fig. 1a, in the absence of Cys, solutions of probe **1** and **2** exhibit a similar UV/Vis spectrum with two absorption peaks at 302 and 358 nm. As expected, when Cys (100 μM) was added to the solution of probe **1**, the intensities of the absorption peaks at 302 and 358 nm rapidly reduced (15 min), and a concomitant red-shifted broad absorption peak was

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detected at 398 nm with a shoulder peak at 444 nm. The excitation and emission spectra indicated that excitation of probe **1** at 444 nm gives the best fluorescent response towards Cys at 509 nm (*ca.* 22-fold) (Fig. S1, ESI[†]). However, probe **2** presented only a negligible change in fluorescence and absorption spectra upon addition of Cys.

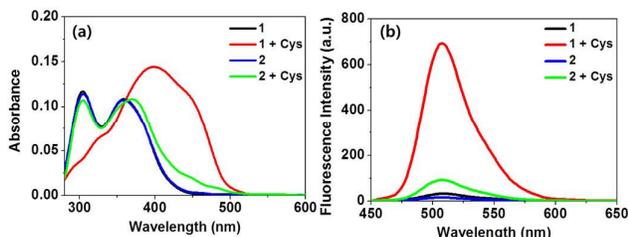
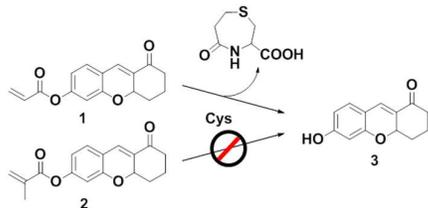


Fig. 1 (a) UV/Vis and (b) fluorescence spectra of probes **1** and **2** (10 μ M) in the presence and absence of Cys (100 μ M). The spectra were recorded 15 min after addition of analyte in ethanol-phosphate buffer (10 mM, pH 7.4, 2:8 v/v) at 37°C after excitation at 444 nm.

This could be explained by the mechanism shown in Scheme 2. The fluorescence response of probe **1** in the presence of Cys involves the nucleophilic addition of Cys to acrylate and the subsequent rapid intramolecular cyclization induces the release of free chromene fluorophore, leading to enhanced fluorescence. In the case of probe **2**, after nucleophilic addition of Cys to acrylate moiety, the extra methyl induced much stronger steric hindrance, inhibiting further intramolecular cyclization.



Scheme 2 Proposed reaction mechanisms of probes **1** and **2** with Cys.

In addition, the fluorescence response of probe **1** to Cys under different pH was examined. As shown in Fig. S2 (ESI[†]), the fluorescence spectra of probe **1** in the absence of Cys show no significant changes in wide pH ranges (pH 6-9). In the presence of Cys, fluorescent changes were detectable in the pH range of 6.5-9, and the best responses were obtained for a pH between 7.4 and 9. These results indicate that probe **1** could be applied for Cys detection at a physiologically relevant pH.

Using fluorescence titration assays, probe **1** (10 μ M) was treated with different concentrations of Cys (Fig. 2a). The fluorescence intensity at 509 nm gradually increased with increasing amounts of Cys after 15 min incubation. The change of fluorescence (I_{509}) was plotted as a function of the Cys concentration. There was a good linearity between fluorescence intensity (I_{509}) and Cys concentration in the range of 0 to 20 μ M and the detection limit was calculated to be 53.1 nM (Fig. S3, ESI[†]), which is sufficient for the detection of Cys in cells and in human blood.³

Furthermore, the selectivity for Cys over other thiol-containing amino acids (Hcy and GSH) was examined by comparing the kinetic reaction rates of probe **1** (10 μ M) with

Cys (20 μ M), Hcy (20 μ M), and GSH (100 μ M). As shown in Fig. 2b, the reaction of the probe with Cys was faster than with either Hcy or GSH, resulting in a remarkable fluorescence enhancement after 15 min for Cys, whereas there is only a slight increase in fluorescence for Hcy and GSH. These results indicate that probe **1** was able to selectively and rapidly detect Cys over Hcy and GSH. In order to assess the selectivity of probe **1** towards Cys, changes in the fluorescence intensity (I_{509}) were also monitored for probe **1** upon exposure to various biologically relevant analytes in aqueous medium. Except Cys, none of the other tested amino acids (Val, Tyr, Thr, Tau, Ser, Pro, Phe, Met, Lys, Leu, Ile, His, Gly, Gluc, Glu, Gln, Asp, Asn, Arg, Ala, Trp) or biological metal ions (K(I), Na(I), Ca(II), Cu(II), Fe(II), Fe(III), Mg(II), Zn(II)) caused a notable change in fluorescence even for very high concentrations (1 mM) (Fig. S4, ESI[†]). It is worth noting that probe **1** could be of value for the highly selective detection of Cys in biological applications.

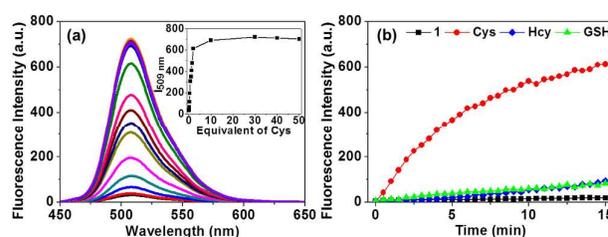


Fig. 2 (a) One-photon fluorescence spectra of probe **1** (10 μ M) in the presence of increasing concentrations of Cys and upon excitation at 444 nm. Inset: Change in fluorescence intensity at 509 nm as a function of Cys concentration. (b) Time-dependent fluorescence response of probe **1** (10 μ M) for Cys (20 μ M), Hcy (20 μ M), and GSH (100 μ M). The spectra were recorded 15 min after addition of analyte in ethanol-phosphate buffer (10 mM, pH 7.4, 2:8 v/v) at 37°C.

To confirm the potential use of probe **1** for TPEF imaging, we also tested its fluorescent response using two-photon microscopy (TPM) with 740 nm excitation (Fig. S5, ESI[†]).¹² Addition of Cys (100 μ M) to the probe **1** solution significantly enhanced fluorescence (*ca.* 13-fold), which is consistent with the results using OPM. Thus, probe **1** could be used for fluorescence imaging of Cys in living species by both OPM and TPM.

Considering these desirable properties, a series of cell-based experiments were achieved to study the capacity of probe **1** to selectively image Cys in living cells. As shown in Fig. 3a, probe **1** (10 μ M) showed good cell-permeability and low cytotoxicity up to 50 μ M (Fig. S6, ESI[†]) when incubated with live HepG2 cells (1 h). Furthermore, the bright green fluorescence revealed the intracellular Cys distribution in live HepG2 cells. Green emission was significantly reduced upon depletion of intracellular Cys levels by *N*-ethylmaleimide (NEM, a thiol specific inhibitor) and increased upon intracellular Cys accumulation induced by incubation of the cells in culture medium without glucose (Fig. 3b, 3c, and S7, ESI[†]).¹³ These results confirm that the detected fluorescence changes in the cells are caused by the reaction of probe **1** with intracellular Cys. We also carried out imaging studies with live HepG2 cells by TPM (Fig. S8, ESI[†]). TPEF images were recorded under excitation at 740 nm. These results indicate that probe **1** can track endogenous Cys level with high sensitivity and selectivity using both OPM and TPM.

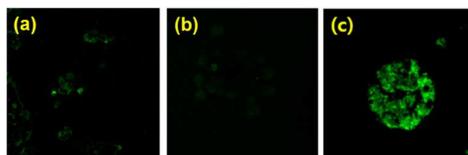


Fig. 3 OPM images of HepG2 cells using probe **1**. (a) Cells were incubated with probe **1** (10 μ M) for 1 h. (b) Cells were incubated with 500 μ M NEM for 30 min, washed, and then treated with probe **1** (10 μ M) for 1 h. (c) Cells were incubated in glucose-free medium for 2 h, and then treated with probe **1** (10 μ M) for 1 h. Magnification: 400 \times .

Consequently, the practical utility of probe **1** for intracellular Cys detection was investigated using different carcinoma cell lines. The probe was able to detect Cys in all tested cells and the confocal images are presented in Fig. 4 and S9 (ESI[†]). The average fluorescent intensities were measured and compared with previously reported data for each of the cell lines (Fig. S9, ESI[†]). Among them, HeLa cells showed the highest fluorescent response. The observed differences in fluorescence corresponded to the differences in Cys levels previously reported in literature.¹⁴ Thus, probe **1** can accurately detect Cys variation in different carcinoma cell lines.

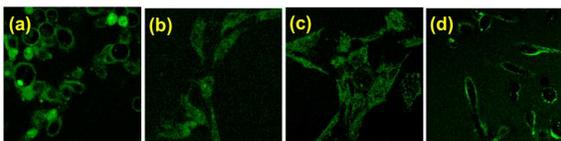


Fig. 4 OPM images of probe **1** in four carcinoma cell lines (HeLa, Hep3B, Huh7, and MDA-MB-231). Cells were incubated with probe **1** (10 μ M) for 1 h. Magnification: 400 \times .

Encouraged by its remarkable features, we applied probe **1** for the visualization of endogenous Cys in various cryopreserved mouse tissues. As shown in Fig. 5a, snap-frozen tissues from different organs (liver, spleen, kidney, heart, and testis) as well as tumor tissues of wild-type and xenograft mice were stained with 10 μ M probe **1** for 2 h. With both OPM and TPM, strong fluorescence was observed in tumor tissues (Fig. 5a) and liver tissues (Fig. 5b and S10a, ESI[†]), which were both known to express excess amounts of Cys.^{3a,15} Our data (Fig. 5 and S10, ESI[†]) is in accordance with the results of a previous study that showed that Cys concentration range from 10 to 100 μ M in various rat tissues and that the highest concentrations are found in the liver.¹⁶ Furthermore, the fluorescence intensity detected by OPM and TPM in tumor tissues and tissues from different organs (liver, heart, spleen, and testis) are comparable to the detected fluorescence intensities for intracellular Cys, even though it is known that common TPM has a lower spatial resolution.¹⁷ Therefore, we applied probe **1** to disclose the natural distribution of Cys in cancer tissue in live conditions by three-dimensional (3D) TPM images, which has deeper tissue penetration but low phototoxicity. Serial Z-sectioned two-photon images (30 sections) were acquired after staining cancer tissue with probe **1**. The 3D TPM images showed a good contrast between bright and dim parts at a depth range of 10 - 60 μ m (Fig. 5k).

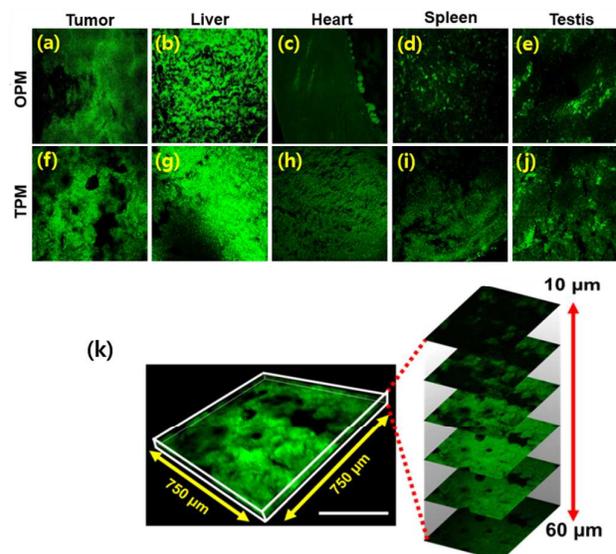


Fig. 5 (a-e) OPM and (f-j) TPM images of cryosectioned xenograft mouse tumors and organs (liver, heart, spleen, and testis) were stained with 10 μ M probe **1** for 2 h. Magnification: 100 \times . (k) (left) 3D TPM image of tumor labeled with probe **1** (10 μ M) at a depth of 10 - 60 μ m with magnification at 20 \times . (right) Serial Z-sections of TPM images of tumor tissue at different depths. The images shown are representative images out of 30 sectional images obtained at a depth of 10 - 60 μ m. TPEF images were recorded after excitation at 740 nm and the corresponding emissions were collected at 450 - 600 nm. Scale bar represents 300 μ m.

Thiol expression from cancer cells has been reported previously but is poorly characterized in living cancerous cells or deep inside of intact organs. Here, we have developed a new two-photon fluorescent probe for the detection of Cys in cancerous biospecimens with high selectivity, high sensitivity, and low toxicity. Probe **1** was able to monitor intracellular Cys alterations in different living carcinoma cells and in cryosectioned tumor tissue up to a depth of 60 μ m. Because of its deep tissue penetration and low photo-cytotoxicity, TPM using probe **1** could reveal the Cys distribution in living tissues without interference from other biologically relevant species. Consequentially, this novel fluorescent probe could aid as an efficient tool for intracellular Cys recognition, which could be useful in various biological and clinical applications, for example in cancer diagnosis.

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<Graphical abstract>

