A water-soluble near-infrared fluorescent probe for cysteine/homocysteine and its application in live cells and mice

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Abstract

A near-infrared (NIR) and water-soluble probe was synthesized and studied for the detection of Cys/Hcy in aqueous solution, living cells and mice. The probe was composed of cyanine derivative as the NIR fluorescent reporting unit and pyrimidiny-thioether moiety as the Cys/Hcy responsive unit. Treatment with Cys/Hcy induced the formation of sulfur-substituted products, then intramolecular rearrangement reaction would occur to produce amino-substituted products and resulting in enhanced red fluorescence emissions. It could be applied to sense Cys/Hcy both in solution with the detection limit of 0.17 μM (or 0.32 μM) and in living cells. Cell imaging experiments proved that such a probe exhibited good cell penetration. In addition, the probe could detect Cys/Hcy in live mice with strong turn-on fluorescent response.

Keywords: Near-infrared (NIR), cyanine, fluorescent probe, in vivo imaging, Cys/Hcy
Introduction

In the last years, the detection and recognition of biologically important species has become a major topic of research in chemistry and biology. Biothiols, such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), are important biomarkers. They play crucial roles in many physiological processes and the abnormal level can released many diseases, such as Alzheimer’s disease, hair depigmentation, cancer, liver damage and neurodegenerative diseases.\textsuperscript{1–6} However, the concentrations of Cys, Hcy and GSH in cells are very different. As one of the most abundant non-protein thiols in cell, the content of GSH is at about millimolar levels. Nevertheless, the concentrations of Cys and Hcy are at about micromole levels.\textsuperscript{7,8} Hence, it is difficult to develop the selective and sensitive analytical method for Cys/Hcy against GSH. For various analytical methods, fluorescence sensing is attractive for its properties with high sensitivity, outstanding temporal-spatial resolution and fairly simple technical instrument.\textsuperscript{9–15} The conjunction of fluorescent probe and fluorescence imaging could provide us powerful tools for visualization of biologically relevant species.\textsuperscript{16–21}

Currently, a large quantity of fluorescent probes for detection of biothiols have been developed.\textsuperscript{22–35} In addition, a series of bioluminescence imaging probes which did not require an excitation light source for biothiols also were reported recently.\textsuperscript{36–38} However, most of them suffer from limitation of differentiation of Cys/Hcy from GSH due to the similar reactivity of sulphydryl and most of the emission wavelength of the reported fluorescent probes located in the ultraviolet or visible region and thus limited their applications for bioimaging in cells, particularly in vivo by penetration and photobleaching. Therefore, the previous reported fluorescent probes for detecting Cys/Hcy in biological samples often remained at the cell level.\textsuperscript{31–33} Besides, previous reported probes for Cys/Hcy were generally poorly water-soluble and only a few water-soluble small-molecule fluorescent probes have been screened out.\textsuperscript{34} Until now, the roles of Cys/Hcy in pathogenesis and physiology are still unclear. In view of this
problem, it is important to design and synthesize an effective fluorescent probe with red fluorescence and water solubility to monitor the accurate distribution of Cys/Hcy in cells and in mice.

Cyanine dyes have typically high absorption coefficient, and the absorption and emission wavelength locate in the NIR region. Moreover, they have been used in medicinal and biological applications based on the excellent biocompatibility and low toxicity to biosamples.39–45 The corresponding design strategies for detecting biothiols are generally based on the strong nucleophilicity of the biothiol group. Several fluorescent probes for biothiols have been constructed based on this chemical feature combined with Michael addition, cyclization reaction, displacement of coordination, cleavage reaction and others.46–50 Based on the electrophilic and biocompatible properties of pyrimidine, pyrimidinyl-thioether maybe suitable as a novel recognition unit for biothiols. To test the above hypothesis, a turn-on fluorescent probe was synthesized based on 7-nitro-2,1,3-benzoxadiazole and pyrimidinyl-thioether.46 Interestingly, this probe showed highly selective and sensitive detection for Cys/Hcy.51 However, the emission of this probe located in the visible region and limited its bioimaging in vivo.

In this paper, we report the design and synthesis of a fluorescent probe by incorporating 4,6-dimethoxy-2-pyrimidinyl-thioether and cyanine dyes (Scheme 1). This probe performs several excellent features, including (i) good water solubility and biocompatibility, (ii) remarkable fluorescent enhancement in near-infrared, (iii) high selectivity toward Cys/Hcy and strong tolerance against abundant competing biothiols inside cells and mice. This strategy holds great promise for studying biological processes associated with Cys/Hcy in complex living systems.
Results and Discussion

The spectroscopic property of Cy-S-Py

The absorption and emission responses of Cy-S-Py to Cys, Hcy and GSH were carried out in HEPES buffer (10 mM, pH 7.4). As shown in Fig. S1 (Supporting Information), free Cy-S-Py displayed a remarkable absorption at 790 nm and weak fluorescence centered at 820 nm. The maximum absorption band at 790 nm of Cy-S-Py decreased and one new band centered at 650 nm evolved after addition of Cys (Fig. S1a) (Supporting Information). Treatment of Cy-S-Py with Cys emerged a new emission band centered at 743 nm upon excitation at 650 nm and induced dramatic increase of the fluorescence intensity (Fig. S1b) (Supporting Information). Compared to Cys, the fluorescence changes of Cy-S-Py at 743 nm upon addition of Hcy displayed weaker fluorescence enhancement. In the other side, the absorption band at 790 nm decreased weakly after addition of GSH, and no significant fluorescence response was observed at 743 nm.

Fig. 1

The above results of adsorption and emission spectra showed that Cys/Hcy may react with Cy-S-Py to form sulfur-substituted products (Cy-S-Cys/Cy-S-Hcy) firstly, and very soon, intramolecular rearrangement reaction occur in order to obtain thermodynamically stable amino-substituted products by five- or six-membered cyclic transition state (Cy-N-Cys/Cy-N-Hcy), which the fluorescence emission peak at 743 nm upon excitation at 650 nm (Scheme 2). However, GSH, which lack of the adjacent amine group, cannot guide this intramolecular rearrangement reaction and do not emit in this wavelength (Scheme 2).

Scheme 2

The time-dependent kinetic studies of Cy-S-Py in the presence of Cys/Hcy were measured in HEPES buffer (10 mM, pH 7.4). As shown in Fig. 1, Cy-S-Py presented nearly non-fluorescent emission at 743 nm in the absence of Cys/Hcy. After addition of 100 equiv.
Cys/Hcy, the fluorescence intensity at 743 nm increased gradually which reached the maximum within 60 min with 103-fold and 47-fold fluorescence increment for Cys and Hcy, respectively. The results demonstrated that Cys could react more quickly with Cy-S-Py than Hcy. For Cys and Hcy, the primary amine allows further intramolecular displacement of sulfur to yield the amino-substituted product by five- of six-membered cyclic transition state and the amino-substituted products is the preferred conformation. The results showed that Cy-S-Py could detect Cys faster than Hcy in solution.

Fig. 2

We next investigated concentration-dependent responses of probe Cy-S-Py toward Cys and Hcy. The fluorescence intensity changed at 743 nm with increasing amounts of Cys and displayed a linear response to the concentration of Cys from 1 to 10 (Fig. S2a) and from 10 to 100 μM (Fig. 2) respectively. The limit of detection (LOD) for Cys was calculated to be as low as 0.33 μM based on 3σ/k. For Hcy, the linearity ranges were also from 0 to 10 μM (Fig. S2b) (Supporting Information) and 10 to 100 μM (Fig. S3) (Supporting Information) respectively, the LOD was estimated to be 0.61 μM. These results showed that Cy-S-Py was sensitive enough to image Cys/Hcy in cells. To explore the pH effect of probe Cy-S-Py toward Cys/Hcy, fluorescent intensity changes were measured at varied pH values (Fig. S4, Supporting Information). The results revealed that Cy-S-Py was beneficial for Cys/Hcy in living systems.

Selectivity and competition studies of Cy-S-Py

For the accurate detection of Cys/Hcy in complicated biological environment, the selectivity of Cy-S-Py toward Cys/Hcy is one of the most important factors. The interaction of Cy-S-Py with various analytes were studied to assess the potential application of Cy-S-Py in real biological systems. As displayed in Fig. S5 (Supporting Information), upon exposure to the analytes, only addition of Cys induced a significant fluorescence increment and Hcy ignited a slightly weaker fluorescence intensity at 743 nm. For other analytes, no obvious fluorescence
increment of Cy-S-Py could be found. Furthermore, the competition experiments were also carried out by adding Cy-S-Py to the solution of 100 equiv. of other analytes in the presence of 100 equiv. of Cys/Hcy (Fig. S6) (Supporting Information). Remarkably, the coexistent analytes had no obvious interference for the detection of Cys/Hcy. Therefore, the Cy-S-Py could be applied as a fluorescence probe to sense Cys/Hcy in live cells.

*The fluorescence imaging in live cells*

Next, the potential utility of Cy-S-Py as a probe for visualizing endogenous Cys/Hcy in living cells was evaluated. The cytotoxicity of Cy-S-Py in logarithmic phase of cell growth was detected by MTT assay before cell imaging. The MTT assays were performed as shown in Fig S7 and the concentration range of Cy-S-Py was defined as 0-25 μM. The survival rate of both HeLa cells cells were above 80% after incubation with Cy-S-Py at different concentrations (≤ 20 μM). The HeLa cells were initially incubated with Cy-S-Py, and the cells displayed bright red fluorescence, which can be ascribed to the reaction of Cys/Hcy and Cy-S-Py (Fig. 3a1). To further verify the change in the fluorescence caused by Cys/Hcy, the cells were initially treated with N-ethylmaleimide (NEM, a thiol-trapping reagent) and then incubated with Cy-S-Py. The cells exhibited much weaker fluorescence (Fig. 3a3). For SMMC- 7721 cells, similar phenomena were observed (Fig. 3b1-b4). All these results proved that the probe was promising and effective for imaging the endogenous Cys/Hcy.

*Detection of Cys/Hcy in mice*

The above results indicated that Cy-S-Py has potential for detecting Cys/Hcy in vivo. BALB/c nude female mice were anaesthetized and placed into a live animal imaging system carefully. One group were subcutaneously injected with Cy-S-Py (100 μM, 200 μL) and measured imaging intensity at different time points. As control, the other group were subcutaneously injected with NEM (20 mM, 200 μL in saline) for 30 min, followed by
subcutaneously injected with **Cy-S-Py** (100 μM, 200 μL), and were imaged at different times. As illustrated by Fig. 4, the fluorescent signal at the right rear side of the mouse enhanced gradually over time. In the control experiment, the mouse were pretreated with 200 μL of NEM (20 mM) which exhibited almost no fluorescence over time. These results confirmed that the probe **Cy-S-Py** had the real-time visualizing capability to detect and image endogenous Cys/Hcy in living mice.

Fig. 4

**Conclusions**

In summary, the successfully synthesized water-soluble probe (**Cy-S-Py**) displayed excellent selectivity and high sensitivity, with fluorescence turn-on response to Cys/Hcy. Such a cyanine-based probe contains pyrimidiny-thioether moiety as the Cys/Hcy responsive unit and responds selectively to Cys/Hcy with the detection limit of 0.17 μM and 0.32 μM, respectively. Cell imaging experiments indicated that **Cy-S-Py** possessed good cell penetration. The probe could be applied to sense Cys/Hcy in HeLa and SMMC-7721 cells. Additionally, the probe could detect Cys/Hcy in mice with strong turn-on fluorescent response. These results confirmed that **Cy-S-Py** had the real-time visualizing capability to detect and image endogenous Cys/Hcy in living mice. In addition, through above works we speculated that discrimination of Cys and should require judicious choice of recognizing group and the appropriate steric environment.

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**Supporting Information**

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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Scheme and Scheme Captions

Scheme 1  Synthetic route of Cy-S-Py.

Scheme 2  Proposed sensing mechanisms of Cy-S-Py for Cys and Hcy.

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Figure Captions

Fig. 1 Time-dependent changes (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85 and 90 min) of fluorescence spectra of **Cy-S-Py** (10 μM) upon addition of 100 equiv. Cys (a, b) and 100 equiv. Hcy (c, d) in HEPES buffer (10 mM, pH 7.4); Every data point was the mean of three measurements. The error bars represent the standard deviation. $\lambda_{ex} = 650$ nm and $\lambda_{em} = 743$ nm.

Fig. 2 Fluorescence spectra of **Cy-S-Py** (10 μM) upon addition of varied concentration (0-1000 μM) of Cys incubated for 90 min in HEPES buffer (10 mM, pH 7.4). Inset: the linear response of fluorescent intensity at 743 nm in the concentration of Cys from 0 to 100 μM. Every data point was the mean of three measurements. The error bars represent the standard deviation. $\lambda_{ex} = 650$ nm and $\lambda_{em} = 743$ nm.

Fig. 3 Confocal laser scanning microscopic images of **Cy-S-Py** responded to Cys/Hcy in living cells (a. HeLa; b. SMMC-7721). Fluorescence image of living cells incubated with **Cy-S-Py** (10 μM) for 50 min (a1, b1). Fluorescence image of living cells pretreated with NEM (5 mM, 30 min) and then incubated with **Cy-S-Py** for 50 min (a3, b3). (a2, b2) and (a4, b4) are the bright field images (excitation at 633 nm and emission at 690–740 nm).

Fig. 4 Fluorescent detection of Cys in living mice injected with Cy-S-Py. Cy-S-Py (200 μL, 100 μM) was subcutaneously injected into the mice, fluorescence images recorded at 0 min, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min in a live animal imaging system, respectively. The control group were subcutaneously injection with Cy-S-Py (100 μM, 200 μL), and were imaged at different times (excitation at 620–650 nm and emission at 690–740 nm).
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