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A highly sensitive near-infrared fluorescent probe for cysteine and homocysteine in living cells†

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A near-infrared fluorescent probe (Cy–O–CHO) for the detection of endogenous Cys/Hcy in living cells was designed and synthesized. Cy–O–CHO exhibited high sensitivity and good selectivity to Cys/Hcy under physiological conditions with a detection limit of 7.9 nM for Cys.

Cysteine (Cys) and homocysteine (Hcy) are considered to be related to a wide range of physiological processes.[1–3] The deficiency of Cys can cause slowed growth, hair depigmentation, edema, lethargy, liver damage, muscle and fat loss, skin lesions, and weakness.[4] Elevated Hcy in the blood is a well-known risk factor for a number of diseases, such as Alzheimer's,[5] cardiovascular diseases[6] and osteoporosis.[7] However, the mechanisms underlying their deleterious influences have not yet been elucidated[8] due to lack of an available method to measure Cys and Hcy quantitatively in living cells, especially in tissues.

In recent years, a wide variety of sensing mechanisms have been used in the design of Cys/Hcy fluorescent probes, such as Michael addition,[9] cleavage of sulfonamide and sulfonate ester,[10] metal complexes-displace coordination,[11] and cleavage of disulfide.[12] However, these methods were usually hampered by interference from structurally related thiols, especially glutathione (GSH), which is the most abundant intracellular nonprotein thiol.[13]

In order to develop highly selective methods for Cys/Hcy, Strongin and coworkers[14,15] first constructed two xanthene-based fluorescent probes for selective detection of Cys and Hcy based on the cyclization reaction of aldehydes with Cys/Hcy. However, these two probes exhibited fluorescence quenching towards Cys/Hcy, and all experiments were carried out under alkaline conditions (pH 9.5). Subsequently, based on the same principle, some probes for Cys/Hcy under physiological pH conditions[16] were developed. Unfortunately, most of them operated in pure organic solvents or solutions with a high level of organic solvents, such as DMF,[17] DMSO,[18] acetonitrile,[19] and ethanol.[20] Although a few probes have been reported and successfully applied to intracellular Cys/Hcy imaging,[21–23] the detection wavelengths of these probes are located in the visible range, in which the detection would be interfered with cell autofluorescence. In a word, all of the abovementioned fluorescent recognitions of Cys/Hcy were realized under non-physiological conditions or performed in the visible wavelength range, which made them not ideal candidates for cell-imaging applications. Besides, these probes are also not suitable for deep tissue imaging.

As it is known, near-infrared (NIR) dyes, wavelengths of which are located in the 650–900 nm range, have the unique advantage of tracing molecular activity *in vivo* because their NIR photons can penetrate relatively deeply into tissues with a low auto-fluorescence background. Moreover, the NIR radiation shows less damage to biological samples.[24] Therefore, it is vital to develop a highly sensitive NIR fluorescent probe for detecting Cys/Hcy under physiological conditions. In 2012, Park and Yoon's group[25] reported a ratiometric fluorescent probe CyAC for detecting Cys, and the probe has been successfully applied to image Cys in living cells. With the addition of Cys, the fluorescence intensity at 780 nm (λ_{ex} = 720 nm) decreased significantly. In contrast, a new fluorescence emission peak at 570 nm (λ_{ex} = 520 nm) was observed, which corresponds to the reaction product CyAK. And in their cell imaging experiments, the excitation and emission wavelengths of detection were 560 nm and 590 nm, respectively.

To date, there is no genuine NIR probe for direct detection of endogenous Cys/Hcy *in vivo*. Herein, a highly sensitive Cy7-based probe (Cy–O–CHO) for the detection of endogenous Cys/Hcy was designed and synthesized based on the unique cyclization between Cys (or Hcy) and aldehydes. Heptamethine cyanine dye (Cy) with a high molar absorption coefficient and NIR emission was selected as the fluorophore. Cy–O–CHO was prepared by the reaction of Cy.7.Cl with 4-hydroxybenzaldehyde in the presence of sodium hydride and purified using standard

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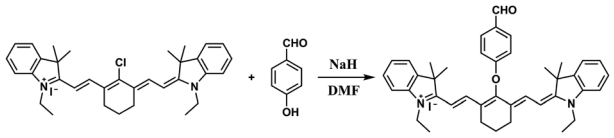

Scheme 1 Synthesis of Cy–O–CHO.

column chromatography (Scheme 1). The structure of Cy–O–CHO was fully characterized by HR-MS, ^{1}H NMR, ^{13}C NMR and IR (ESI,† Fig. S7a–d).

Upon excitation at 730 nm, Cy–O–CHO displayed faint fluorescence at 778 nm and a low fluorescence quantum yield (Φ_F) of 0.036 in PBS buffer (40 mM, pH 7.4). Fig. 1a showed the fluorescence responses of Cy–O–CHO to different concentrations of Cys. The emission intensities at 778 nm increased significantly with the increasing concentration of Cys. A marked enhancement in the fluorescence quantum yield (from 0.036 to 0.089) upon addition of Cys to Cy–O–CHO was observed. These results should be ascribed to the Cys-triggered conversion of the aldehyde group to the thiazoline derivative, which resulted in the fluorescence recovery of the cyanine dye.

When more than 2.0 μM (1 equiv.) of Cys was added, the enhancement of the fluorescence intensity reached a maximum without further variation (Fig. 1b), which indicated that Cy–O–CHO could detect Cys quantitatively. The emission intensity of 778 nm showed a good linear relationship with Cys concentrations (0.03 to 2.0 μM). The regression equation was $F = 1043.59 + 101.83 \times [\mathrm{Cys}]$ (10^{-7} M) with a linear coefficient of 0.9941 and a detection limit of 7.9×10^{-9} M, respectively. To the best of our knowledge, the detection limit is lower than those of the formerly reported fluorescence probes (ESI,† Table S1).

To validate the recognition mechanism of Cy–O–CHO to Cys, high resolution mass spectrometry experiments have been carried out (Fig. 2). The probe showed a characteristic peak of *m*/*z* at 597.3464. For the mixture of Cy–O–CHO and Cys, a new peak of *m*/*z* at 750.3573 was observed, which corresponded to the cyclization product (Cy–O–Th). The result confirmed the sensing mechanism of the probe toward Cys, which resulted in the formation of the thiazoline derivative. The recognition mechanism toward Hcy was also confirmed by the HR-MS method (ESI,† Fig. S3).

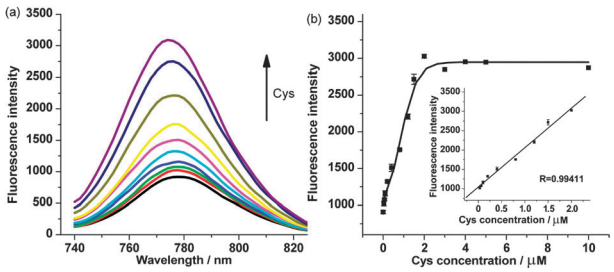

Fig. 1 (a) Fluorescence responses of 2 μM Cy–O–CHO to different concentrations of Cys (0, 0.03, 0.06, 0.1, 0.2, 0.4, 0.8, 1.2, 1.5, 2.0 μM). The curves were acquired 2 h after the addition of Cys in 40 mM PBS buffer (pH 7.4) at 37 °C. (b) Response of the probe fluorescence intensity to Cys concentration. The curve was plotted with the fluorescence intensity at 778 nm with Cys concentration. Inset: a linear correlation between emission intensities at 778 nm and concentrations of Cys (λ_{ex} = 730 nm).

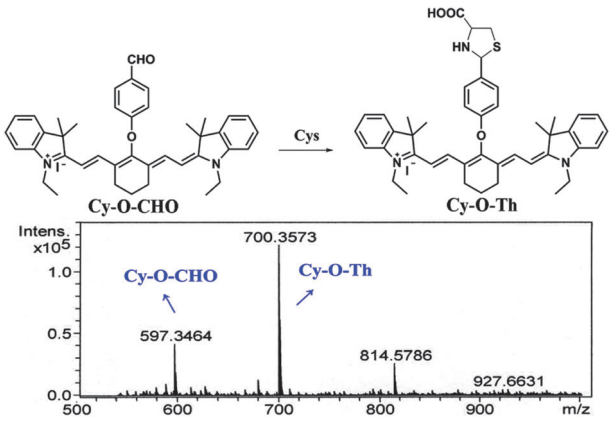

Fig. 2 Proposed recognition mechanism of Cy–O–CHO toward Cys.

The selectivity of Cy–O–CHO toward Cys/Hcy was testified. As can be seen in Fig. 3, no visible changes in the spectra were observed upon the addition of 20 equiv. of other amino acids. Notably, 10 mM of GSH did not interfere with the fluorescence (Fig. 3a). The results verified that Cy–O–CHO was highly selective toward Cys/Hcy over GSH and other amino acids. Considering the possible interactions of metal ions, the responses of Cy–O–CHO to a series of metal ions were also tested. Fig. 3b showed that no significant changes in the fluorescence were observed in the presence of metal ions, such as K^+, Zn^{2+}, and Cu^{2+} (400 μM, 200 equiv.); Cd^{2+}, Mn^{2+}, Ca^{2+}, and Al^{3+} (800 μM, 400 equiv.); Mg^{2+} (2 mM, 1000 equiv); Na^+ (3.2 mM, 1600 equiv.). Therefore, Cy–O–CHO could be used as a highly selective NIR fluorescent sensor for Cys/Hcy.

To access the feasibility of Cys/Hcy detection in living cells, the confocal imaging of intracellular Cys/Hcy was performed. In previous reports, additional stimulation[25] or exogenous Cys[26] was necessary for cell imaging because of the low sensitivity of probes. In the present study, Cy–O–CHO with the detection limit of 7.9 nM was successfully used for bioimaging of endogenous Cys/Hcy in living cells. In order to prove that the probe was specific to the intracellular Cys/Hcy, a control experiment was

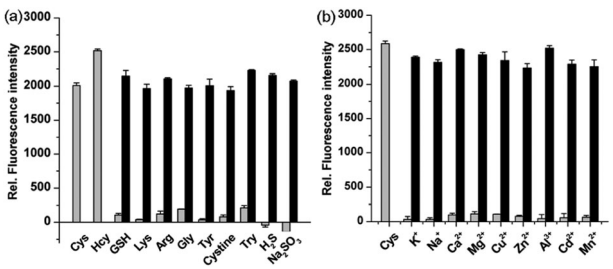

Fig. 3 Fluorescence responses of Cy–O–CHO (2.0 μM) to various analytes in PBS (pH 7.4, 40 mM). Light gray bars represent the addition of analytes, black bars represent the subsequent addition of 2.0 μM Cys to the mixture. (a) Relative fluorescence intensity ($F - F_0$) of Cy–O–CHO upon addition of 20 equiv. of various amino acids, 5000 equiv. of GSH or 1 equiv. of H_2S and Na_2SO_3. (b) Relative fluorescence intensity ($F - F_0$) of Cy–O–CHO upon addition of K^+ (400 μM), Na^+ (3200 μM), Ca^{2+} (800 μM), Mg^{2+} (2000 μM), Cu^{2+} (400 μM), Zn^{2+} (400 μM), Al^{3+} (800 μM), Cd^{2+} (800 μM), and Mn^{2+} (800 μM). The data were acquired 2 h after the addition of the analytes in 40 mM PBS buffer (pH 7.4) at 37 °C ($\lambda_{ex}/\lambda_{em}$ = 730/778 nm).

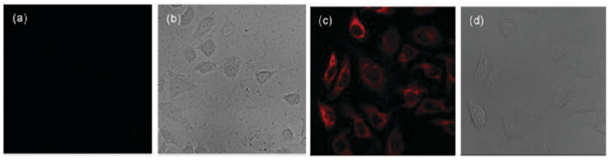

Fig. 4 Bright-field and fluorescence images of living HepG2 cells. (a) Fluorescence and (b) bright-field image of cells pretreated with 1 mM NEM for 30 min, and then incubated with 2.0 μM probe for 15 min. (c) Fluorescence and (d) bright-field image of cells incubated with 2.0 μM probe for 15 min.

performed by removing intracellular thiols using 1 mM *N*-methylmaleimide (NEM, a thiol-blocking reagent) before incubation of the cells with Cy–O–CHO. A marked fluorescence quenching was observed (Fig. 4a). Living HepG2 cells were incubated with Cy–O–CHO (2 μM) in PBS buffer solution (pH 7.4) for 15 min at 37 °C and then washed thrice with PBS buffer solution. The bright-red fluorescence image corresponding to the adduct of Cy–O–CHO and Cys/Hcy was obtained (Fig. 4c). These results revealed that the probe could indeed react with intracellular Cys/Hcy to produce fluorescence, which further confirmed that Cy–O–CHO can detect and image intracellular endogenous Cys/Hcy, which is promising.

In summary, we have developed a Cy7-based fluorescent probe (Cy–O–CHO) for detecting Cys/Hcy in living cells. The probe ($\lambda_{ex/em}$ = 730/780 nm) has good water solubility, membrane permeability, and little cytotoxicity. Cy–O–CHO featured excellent sensitivity and high selectivity to Cys/Hcy over other amino acids and thiols, especially GSH, under physiological conditions. Importantly, the detection limit of the probe is in the nanomolar range (7.9 nM), which is lower than those of the reported probes. Furthermore, Cy–O–CHO has been successfully used to image endogenous Cys/Hcy in living HepG2 cells. We anticipate that this probe will be of great benefit to biomedical researchers for studying the physiological and pathological functions of Cys/Hcy in biological systems.

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