Supporting Information

for

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

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1. Apparatus and Materials

Unless stated otherwise, the solvents were dried by standard procedures. All chemicals were obtained from commercial suppliers and used without further purification. 4-hydroxybenzaldehyde was obtained from Sinopharm. Chemical Regent Co. Ltd. N-ethylmaleimide (NEM) was purchased from Alfa Aesar Chemical Company. Cysteine (Cys), homocysteine (Hcy), glutathione (GSH), NaH and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company. Cy-7.Cl was synthesized in our laboratory [28]. Sartorius ultrapure water (18.2 MΩ cm) was used throughout the analytical experiments.

Fluorescence measurements were performed on a FLS-920 Edinburgh fluorescence spectrophotometer with a Xenon lamp and a 1.0 cm quartz cells at the slits of 4.0/4.0 nm. All pH measurements were performed with a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode. 1H and 13C NMR spectra were recorded on Bruker 300-MHz spectrometer. The fluorescence images of cells were taken using a LTE confocal laser scanning microscope (Germany Leica Co., Ltd.) with an objective lens (×40). Absorbance was measured in a TRITURUS microplate reader in the MTT assay.

2. Synthesis and Characterization of Cy-O-CHO

Synthesis of Cy.7.Cl

2,3,3-Trimethyl-1-ethyl-3H-indolium, inner salt (a): 2,3,3-trimethylindolenine (10 mL, 0.062 mol) and iodoethane (14.5 g, 0.093 mol) were mixed and heated under reflux for 24 h. Then the reaction mixture was cooled to room temperature and filtered. The precipitate was washed with ethyl acetate (3×10 mL). The pink crystals (15.2 g, yield 91.8%) were obtained.

2-Chloro-1-formyl-3-hydroxymethylene cyclohexene (b): Forty milliliters of dimethylformamide previously mixed with 40 mL of methylene chloride was chilled in an ice bath, and then 37 mL of phosphorus oxychloride dissolved in 35 mL of methylene chloride was added dropwise with stirring, followed by 10 g of cyclohexanone. The solution was refluxed for 3 h, cooled, poured onto 200 g of ice,
and allowed to stand overnight. The yellow solid was crystallized from a small volume of acetone cooled with dry ice, to give 13 g (73.9%) with the melting point of 130–132 °C. Elemental analysis (%) calculated for C₈H₉ClO₂ (found): C 55.7 (55.3), H 5.3 (5.5), Cl 20.5 (20.5).

Cy.7.Cl: Compound (a) (3.66 g, 11.66 mmol), compound (b) (0.96 g, 5.44 mmol) and sodium acetate (0.47 g) were dissolved in acetic anhydride (30 mL). The mixture was stirred at room temperature for 2 h. Then the solvents were removed under vacuum. The residue was washed with ether to give 3.2 g of pure product with yield of 91%. ¹H NMR (CDCl₃, 300 MHz): δ1.47 (t, 6H), 1.72-1.78 (t, 12H), 1.99 (m, 2H), 2.79 (t, 4H), 4.26-4.28 (t, 4H), 6.23-6.30 (d, 2H), 7.17-7.41 (d, 8H), 8.21-8.38 (d, 2H).

Synthesis of Cy-O-CHO

4-Hydroxybenzaldehyde (146.5 mg, 1.2 mmol) and NaH (28 mg, 1.2 mmol) were dissolved in 10 mL of anhydrous DMF. After stirred at room temperature for 15 minutes, Cy.7.Cl (255.6 mg, 0.4 mmol) was slowly added to the reaction flask. The reaction was stirred under an argon atmosphere for 4 hours at room temperature until all of these starting material appeared to be consumed by thin layer chromatography (TLC). Then the solvent was removed under reduced pressure, and the crude product was purified by silica gel chromatography with ethyl acetate/methanol (10:1 v/v) to give Cy-O-CHO as a green solid (185.8 mg, 57%). HR-MS (m/z): 579.3469 ([M– I]⁺). ¹H NMR (300 MHz, d₆-DMSO, 298 K) δ(ppm): 9.91(s, 1H), 8.00(d, J = 6 Hz, 3H), 7.73(d, J = 15 Hz, 3H), 7.50(d, J = 9 Hz, 3H), 7.73-7.38(m, 8H), 7.18(s, 1H), 6.22(d, J = 12 Hz, 2H), 4.18(s, 5H), 1.93(s, 4H), 1.64(s, 2H), 1.23(s, 31H), 0.91(t, J = 6 Hz, 3H). ¹³C NMR (75 MHz, d₆-DMSO, 298 K) δ(ppm): 171.7, 164.0, 162.8, 161.6, 141.9, 141.5, 140.6, 133.0, 131.9, 131.4, 129.0, 125.3, 122.9, 121.3, 115.7, 111.5, 100.7, 65.4, 49.0,36.3, 27.5, 24.2, 13.9. IR: 3417, 1637 cm⁻¹.

3. General Procedure for Cys Detection

60 µL the Cy-O-CHO stock solution (0.1 mM), 1 mL PBS buffer solution (0.1 M, pH 7.4) and certain amounts of Cys or Hcy standard solution were sequentially added to each tube at room temperature. Then the reaction solutions were diluted to 3 mL
with ultrapure water, mixed thoroughly and then incubated at 37 °C for 2 h. The fluorescence spectra were obtained in 778 nm by use of the maximal excitation wavelength at 730 nm.

4. Cell Cultures and Confocal imaging

HepG2 cells were maintained following the protocols provided by the American Type Tissue Culture 80 Collection. Cells were first grown in a circular petri dish (60 mm) using RPMI 1640 medium with 10 % fetal bovine serum (FBS), NaHCO₃ (2 g / L), and 1 % antibiotics (penicillin /streptomycin, 100U / mL), and were maintained in a humidified incubator at 37 °C, in 5 % CO₂ / 95% air. One day before imaging, cells were passed and plated on 18-mm glass coverslips in culture dish. In addition, small heated imaging chambers placed onto the microscope stage to closed-system chambers with temperature controlled and humidified CO₂ atmosphere were used in order to culture cells for real-time imaging. Confocal imaging Fluorescence imaging studies were performed with a TCS SP5 confocal laser scanning microscope (Germany Leica Co., Ltd.) with an objective lens (× 40). A solution of 2 µM Cy-O-CHO in culture solution was prepared before confocal imaging. Excitation of probe-loaded cells at 633 nm was carried out with a He-Ne laser, and emission was collected using a META detector between 700 and 800 nm. Prior to imaging, the medium was removed. Cell imaging was carried out after washing cells with PBS (pH 7.4, 40 mM) for three times.

5. The pH Dependence of the Probe Reaction

To be useful in biological applications, it is necessary for a probe to function over a suitable pH range and particularly at physiological pH values. Hence, we examined the influence of pH (6.5–8.6) on the fluorescence intensities of the probe itself and the probe plus Cys (Figure S1). The fluorescence response of the probe toward Cys was pH dependent, and the maximal signal was observed in the pH range of 7.0-7.6. The results showed that the probe was suitable for biological application.
Fig. S1. The fluorescence intensities at 778 nm of Cy-O-CHO (2.0 μM) before and after (red line) addition of Cys (2.0 μM) at various pH values

6. Effect of probe concentration and buffer concentration

Fig. S2. (a) Effect of probe concentration (Cys concentration: 2.0 μM; PBS: pH 7.4, 40 mM). (b) Effect of buffer concentration (probe concentration: 2.0 μM; Cys concentration: 2.0 μM). The excitation wavelength was 730 nm.

7. Detecting mechanism for probe Cy-O-CHO toward Hcy

Fig. S3: Proposed recognition mechanism of probe Cy-O-CHO toward Hcy
8. Fluorescence Quantum Yield ($\Phi_F$) of Cy-O-CHO and Cy-O-Th

For measurement of the quantum yield of Cy-O-CHO, the solution of the probe was adjusted to an absorbance of ~ 0.05. The emission spectra were recorded using a maximum excitation wavelength and the integrated areas of the fluorescence-corrected spectra were measured. Relative fluorescence quantum yield ($\Phi_F = 0.036$) of Cy-O-CHO was obtained by comparing the area under the emission spectrum of the test samples with that of a solution of IR-786 in methanol ($\Phi_F = 0.159$). $\Phi_F$ (0.089) of Cy-T-Th was obtained by the same method.

9. Photo-bleaching Experiments

Photo-bleaching is an irreversible photochemical inversion of fluorescent molecules into a non-fluorescent state. The photo-stability of Cy-O-CHO was investigated by time-sequential scanning of the living cells incubated with 2.0 μM Cy-O-CHO. After 500 s of continuous irradiation with a 633 nm laser, no obvious changes were observed in fluorescence brightness of Cy-O-CHO (Figure S4a). In order to quantitatively determine the photo-bleaching rate, we chose three regions and calculated the average intensity to obtain a curve with scanning time. Figure S4b showed that the fluorescence intensities after 500 s of time-sequential scanning were about 90% of the initial value. These data indicated that the probe is highly resistant to photo-bleaching.

![Fig. S4. Test of photostability of Cy-O-CHO (2 μM). Confocal fluorescence images (0 ~500 s) were achieved by means of time-sequential scanning of the probe-loaded HepG2 cells for 15 min (a). Normalized fluorescence intensity of the three selected regions of (a) from 0 to 500 s (b).](image)
## 10. Comparison of fluorescent probes for the determination of Cys

<table>
<thead>
<tr>
<th>Dye</th>
<th>Detection Wavelength ((\lambda_{em} \lambda_{ex}))</th>
<th>Detection media</th>
<th>Sample</th>
<th>Limit of Detection (mol L(^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Styryl-BODIPY</td>
<td>590 / 520</td>
<td>MeOH / H(_2)O ((4 : 1, \text{v/v}))</td>
<td>SGC-H446 cells</td>
<td>7.2 \times 10^{-6}</td>
<td>1</td>
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<tr>
<td>([\text{Ru}(\text{CHO-bpy})_3]^2\text{PF}_6)](_2)</td>
<td>635 / 485</td>
<td>DMSO</td>
<td>No application</td>
<td>1.4 \times 10^{-6}</td>
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<tr>
<td>Coumarin</td>
<td>557 / 435</td>
<td>CH(_3)CN / PBS ((1 : 9, \text{v/v})), pH 7.4</td>
<td>MCF-7 cells</td>
<td>7.5 \times 10^{-7}</td>
<td>3</td>
</tr>
<tr>
<td>7-Dimethylamino-1, 4-benzoxazin-2-one</td>
<td>560 / 430</td>
<td>CH(_3)CN / HEPES ((3 : 7, \text{v/v})), pH 7.4</td>
<td>Osteoblasts cells</td>
<td>6.8 \times 10^{-7}</td>
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<tr>
<td>BODIPY</td>
<td>564 / 528</td>
<td>CH(_3)CN / HEPES ((2 : 8, \text{v/v})), pH 7.4</td>
<td>HeLa cells</td>
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<tr>
<td>Coumarin334</td>
<td>512 / 490</td>
<td>DMSO / PBS ((1 : 9, \text{v/v})), pH 7.4</td>
<td>HepG2 cells</td>
<td>10^{-7} M</td>
<td>6</td>
</tr>
<tr>
<td>NRFTP</td>
<td>510 / 470</td>
<td>CH(_3)CN / PBS ((10 : 9, \text{v/v})), pH 7.4</td>
<td>HeLa cells</td>
<td>8.2 \times 10^{-8}</td>
<td>7</td>
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<tr>
<td>Rhodamine-6G</td>
<td>552 / 500</td>
<td>EtOH / PBS ((3:7, \text{v/v})), pH 7.0</td>
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<tr>
<td>BODIPY</td>
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<td>DMSO / HEPES ((1 : 1, \text{v/v})), pH 7.8</td>
<td>Human serum</td>
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<td>9</td>
</tr>
<tr>
<td>PBI-Hg</td>
<td>532 / 484</td>
<td>DMF / H(_2)O ((9 : 1, \text{v/v}))</td>
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<td>10</td>
</tr>
<tr>
<td>Fluorescein</td>
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<td>PBS, pH 7.4</td>
<td>No application</td>
<td>3.9 \times 10^{-9}</td>
<td>11</td>
</tr>
<tr>
<td>Cyanine</td>
<td>778 / 730</td>
<td>PBS, pH 7.4</td>
<td>HepG2 cells</td>
<td>7.9 \times 10^{-9} ([a])</td>
<td>This work</td>
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</tbody>
</table>

Table S1: Comparison of fluorescent probes for the determination of Cys. \([a]\) Limit of Detection(LOD) = 3\(\Delta S/K\). \(\Delta S\) is the standard deviation by performing 11 parallel measurements of blank sample; K is the slope of linear curve.

### 11. MTT Assay

3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HepG2 cells (106 cell mL\(^{-1}\)) were dispersed within replicate 96-well microtiter plates to a total volume of 200 mL well\(^{-1}\). Plates were maintained at 37°C in a 5% CO\(_2\) / 95% air incubator for 5 hours. HepG2 cells were then incubated for 24 hours with different probe concentrations of 2\times 10^{-4}, 10^{-4}, 5\times 10^{-5}, 1\times 10^{-5}, 5\times 10^{-6} and 10^{-6} M,
respectively. MTT (Sigma) solution (5.0 mg mL\(^{-1}\) in PBS) was then added to each well. After 4 h, the remaining MTT solution was removed and DMSO (150 µL) was added to each well to dissolve the formazan crystals. The absorbance was measured at 490 nm in a TRITURUS microplate reader.

Fig. S6. MTT assay of HepG2 cells in the presence of different concentrations of Cy-O-CHO

12. HR-MS, \(^1\)H-NMR, \(^{13}\)C-NMR and IR Spectra of Cy-O-CHO

Fig. S7. (a) HR-MS spectra of the Cy-O-CHO
Fig. S7: (b) $^1$H NMR of the Cy-O-CHO probe

Fig. S7: (c) $^{13}$C NMR of the Cy-O-CHO probe
Figure S7: (d) IR of the Cy-O-CHO probe

Fig. S7 (a) HR-MS spectra of the Cy-O-CHO; (b) ^1H NMR of the Cy-O-CHO probe; (c) ^13C NMR of the Cy-O-CHO probe; (d) IR of the Cy-O-CHO probe

References: