Supporting Information for

Preparation of a two-photon fluorescent probe with a large turn-on signal for imaging hypochlorous acid in living tissues

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# Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials and instruments</td>
<td>S3</td>
</tr>
<tr>
<td>Culture and preparation of Hela cells</td>
<td>S3</td>
</tr>
<tr>
<td>Cytotoxicity assay</td>
<td>S3</td>
</tr>
<tr>
<td>Synthesis</td>
<td>S4</td>
</tr>
<tr>
<td>Figure S1</td>
<td>S5</td>
</tr>
<tr>
<td>Figure S2</td>
<td>S6</td>
</tr>
<tr>
<td>Figure S3</td>
<td>S6</td>
</tr>
<tr>
<td>Figure S4</td>
<td>S7</td>
</tr>
<tr>
<td>Figure S5</td>
<td>S7</td>
</tr>
<tr>
<td>Figure S6</td>
<td>S8</td>
</tr>
<tr>
<td>Figure S7</td>
<td>S8</td>
</tr>
<tr>
<td>Figure S8</td>
<td>S9</td>
</tr>
<tr>
<td>Spectral characterization</td>
<td>S10-S11</td>
</tr>
</tbody>
</table>
Materials and instruments

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments; Mass spectrometric analyses were measured on a Finnigan MAT 95 XP spectrometer; High resolution mass spectrometric (HRMS) analyses were measured on an Agilent 1100 HPLC/MSD spectrometer; NMR spectra were recorded on an AVANCE III 400 MHz Digital NMR Spectrometer, using TMS as an internal standard; Electronic absorption spectra were obtained on a Shimadzu UV-2700 power spectrometer; Photoluminescent spectra were recorded with a HITACHI F4600 fluorescence spectrophotometer with a 1 cm standard quartz cell; The fluorescence imaging of cells was performed with a Nikon A1MP confocal microscope; TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals.

Culture and preparation of HeLa cells

HeLa cells were cultured in DMEM (Dulbecco’s modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO₂ and 95% air at 37 °C. Before the experiments, seed the HeLa cells in 35-mm glass-bottomed dishes at a density of 2×10⁶ cells per dish in 2 mL of culture medium and incubate them inside an incubator containing 5% CO₂ and 95% air at 37 °C. Incubate the cells for 24 h. Cells will attach to the glass surface during this time.

Cytotoxicity assay

In vitro cytotoxicity was measured using the colorimetric methyl thiazolyl tetrazolium (MTT) assay on HeLa cells. Cells were seeded into the 36-well tissue culture plate in the presence of 100 μL Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C and 5% CO₂ atmosphere for overnight and then incubated for 24 h in the presence of TP-HA at different
concentrations (0, 1, 5, 10, 20, 30 μM). Then cells were washed with PBS buffer and 100 μL supplemented DMEM medium was added. Subsequently, 10 μL MTT (5 mg/mL) was added to each well and incubated for 4 h. Violet formazan was dissolved in 100 μL sodium dodecyl sulfate solution in the water-DMF mixture. Absorbance of the solution was measured at 570 nm using a microplate reader. The cell viability was determined by assuming 100% cell viability for cells without TP-HA.

Synthesis

Scheme S1 Synthesis of probe TP-HA

Scheme S2 The speculated reaction mechanism of probe TP-HA

Synthesis of compound 1

Compound GCTPOC was prepared according to the reported reference. A mixture of 2-hydroxy salicylaldehyde (2.76 g, 20 mmol, 2.0 eq), 2-cyclohexen-1-one (2.8 g, 30 mmol, 3.0 eq) and triethylene diamine (Dabco 5.6 g, 50 mmol, 5.0 eq) in 1,4-dioxane/water = 1 solution was refluxed for 40 h at 100 °C under a nitrogen atmosphere. After completion of the reaction, the mixture was then cooled to room temperature, extracted with ethyl acetate, dried over
anhydrous sodium sulfate, and concentrated in vacuo. The resulting residue was purified by column chromatography on silica gel (petroleum ether to methanol/dichloromethane = 1/20, v/v) to afford the compound **GCTPOC** as a yellow powder (530 mg, yield: 12%).

**Synthesis of compound TP-HA**

Compound **GCTPOC** (108 mg, 0.5 mmol, 1.0 eq), dimethylaminothiocarbonyl chloride (250 mg, 2.0 mmol, 4.0 eq) and DIEA (100 μL, 3.0 eq) were dissolved in 3 mL of dry methylene chloride. The reaction was carried out at room temperature for 10 h. After completion of the reaction, the mixture was diluted with methylene chloride, washed with water and brine, dried over sodium sulfate, and concentrated in vacuo. The resulting residue was purified by column chromatography on silica gel (petroleum ether to ethyl acetate/petroleum ether = 1/10, v/v) to afford the compound **TP-HA** as a light yellow powder (65 mg, yield: 43%).

\[ ^1H\text{ NMR (400 MHz, CDCl}_3\text{)} \delta 7.44 (d, J = 2.2 Hz, 1H), 7.25 (d, J = 8.3 Hz, 1H), 6.70 (dd, J = 8.2, 2.2 Hz, 1H), 6.64 (d, J = 2.0 Hz, 1H), 5.07 (ddd, J = 10.8, 6.0, 2.3 Hz, 1H), 3.47 (s, 3H), 3.35 (s, 3H), 2.64 – 2.57 (m, 1H), 2.53 – 2.46 (m, 1H), 2.46 – 2.35 (m, 1H), 2.15 – 2.07 (m, 1H), 2.07 – 1.96 (m, 1H), 1.78 – 1.66 (m, 1H). \]

\[ ^13C\text{ NMR (101 MHz, CDCl}_3\text{)} \delta 197.36 (s), 186.93 (s), 156.54 (s), 156.46 (s), 130.91 (s), 130.08 (s), 129.79 (s), 119.98 (s), 116.87 (s), 111.09 (s), 74.80 (s), 43.28 (s), 38.84 (s), 38.79 (s), 29.63 (s), 17.97 (s). \]

HRMS (ESI): m/z calculated for C\text{16}H\text{18}NO\text{3}S 304.1007 [M+H]^+, found: 304.1014.

**Figure S1** (A) The color changes of the probe **TP-HA** in PBS solution (10 μM) before and after added HOCl (20 μM) and (B) The fluorescence changes of the probe **TP-HA** in PBS solution (10 μM) before and after added HOCl (20 μM) with 365 nm ultraviolet light.
Figure S2 The linear response between the fluorescence intensity ratio $I/I_0$ at 515 nm of the probe TP-HA (10 µM) and the concentration hypochlorous acid in the PBS solvent. ($\lambda_{ex}=440$ nm)

Figure S3 The emission intensity changes at 515 nm of compound TP-HA in different pH PBS buffer, containing 1% DMF as a cosolvent. ($\lambda_{ex}=440$ nm)
Figure S4 (A) The fluorescence spectrum of GCTPOC in different pH PBS buffer, containing 1% DMF as a co-solvent and (B) The emission intensity changes at 515 nm of GCTPOC in PBS buffer with different pH values. ($\lambda_{ex} = 440$ nm)

Figure S5 The fluorescence intensities at 515 nm of TP-HA (10 μM) in the presence of HOCl (20 μM) at room temperature (25 °C) for continuously monitored at time intervals periods (0-10 min) in PBS.
**Figure S6** Fluorescence intensities of TP-HA (10 μM) treated with various species (HOCl:20 μM, others:400 μM) in PBS.

**Figure S7** Cytotoxicity assays of TP-HA at different concentrations for HeLa cells
**Figure S8** Two-photon fluorescence images of a fresh rat liver slice incubated with 30 μM TP-HA *in the absence* of HOCl at the depths of approximately 0~200 μm. Excitation at 780 nm.
Figure S9 $^1$H-NMR (CDCl$_3$) spectrum of TP-HA.

Figure S10 $^{13}$C-NMR (CDCl$_3$) spectrum of TP-HA.
Figure S11 HRMS (ESI) spectrum of TP-HA.

Figure S12 HRMS (ESI) spectrum of the reaction products of TP-HA with HOCl.