SUPPORTING INFORMATION

Highly Sensitive and Rapid Detection of Hypochlorous Acid in Aqueous Media and its Bioimaging in Live Cells and Zebrafish using ESIPT-Driven Mycophenolic Acid-Based Fluorescent Probe

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**General Information:**

*Reagents:* All chemicals used for experimental procedures were purchased from commercial suppliers like Sigma–Aldrich, Tokyo Chemical Industry, etc. Unless noted, all the chemicals used were of pure analytical reagent grade and used, as such, without any further purification or other treatment. Solvents such as dichloromethane, and N, N–dimethylformamide (DMF) were distilled from calcium hydride. All reaction conditions were performed by well–oven–dried apparatus components while being held under an N₂ blanket. When performing experiments, all air and moisture–sensitive compounds were introduced via syringe. Performance and progress of the reactions were observed by thin-layer chromatography (TLC). All components were purified by flash column chromatography by using E. Merck silica gel 60 (230–400 mesh ASTM).

* Instruments:* All mentioned below Nuclear magnetic resonance (NMR) spectra were measured on an Agilent–NMR– 400 MHz spectrometer. ESI–mass spectrometry was recorded on a BRUKER micro OTOF–QII. A Time–of–Flight mass spectrometer was operated at a resolution of 20,000. The fluorescence spectra were measured and recorded using a Shimadzu RF–5301pc spectrofluorophotometer and the absorption spectra were collected using a JASCO V–530 spectrophotometer.

**Experimental**

**Generation of Different ROS and RNS Species**

**Generation of OCl⁻:** HOCl solutions were prepared by using commercially available NaOCl bleach; its concentration was determined by measuring the absorbance at 209 nm (molar extinction coefficient = 350 M⁻¹ cm⁻¹).

**Generation of H₂O₂:** Hydrogen peroxide (H₂O₂) solution was directly prepared by commercially available 30% H₂O₂ solution; its concentration was determined by measuring the absorbance at 240 nm with a molar extinction coefficient of 43.6 M⁻¹ cm⁻¹.

**Generation of tBuOOH:** The tert–butyl hydroperoxide solutions were formed through dilution with deionized water and stirred well before use.

**Generation of O₂⁻:** Potassium superoxide (KO₂) was dissolved in DMSO and stirred well before use¹,². The solution was used immediately.

**Generation of •OH:** Hydroxyl radical (•OH) was produced by the Fenton-type reaction *in situ*. Ferrous chloride (FeCl₂) was added to 10 equiv. of hydrogen peroxide to help generate the hydroxyl radical.

**Generation of peroxynitrite (ONOO⁻):** To a precooled mixture of NaNO₂ and H₂O₂, hydrochloric acid followed by NaOH was added quickly. This stock solution was placed at temperatures lower than –18 °C.
The solution was always brought to ambient temperature and used directly. To determine the concentration of the stock solution, it was dissolved in 0.10 M NaOH and measured the absorbance at 302 nm with a molar extinction coefficient of 1670 M$^{-1}$cm$^{-1}$.

**Generation of NO•**: Nitric oxide was produced from SNP (sodium nitroferricyanide (III) dehydrate). All experiments were executed under anaerobic conditions. Initially, the deionized water was degassed well with argon for about 60 min; then finally, SNP was mixed into degassed and deionized water under an argon blanket and further stirred (well) for about 30 min at ambient temperature. Before the reaction of NO• with the molecular probe, this analyte solution was also degassed well with argon.

**Determination of the detection limit**

The detection limit (LOD) was calculated based on fluorescence titrations. To achieve the standard deviation ($\sigma$) of the blank measurement, fluorescence emission spectra of the probe Myco-OCl were measured three times in the absence of $\cdot$OCl. Specifically, to obtain the value of the slope (k), the fluorescence intensity at 522 nm was plotted as a concentration of $\cdot$OCl. Finally, LOD was calculated with the following equation:

$$\text{Detection limit} = 3\sigma/k$$

Where $\sigma$ is the standard deviation of 10 blank measurements, k is the slope of the calibration curve and 3 is used as a calibration of signal–to–noise ratio.

All data were taken in PBS (10 mM) buffer solution of pH = 7.4 and incubated for 1 min.

**Determination of the Quantum Yield**

$$\Phi_1 = \frac{\Phi_B I_B A_B \lambda_{exB} \eta_B}{I_B A_B \lambda_{ex1} \eta_B}$$

Where $\Phi$ is the fluorescence quantum yield

I is the integrated area under the corrected emission spectral signal

A is the absorbance at the excitation wavelength

$\lambda_{ex}$ is the excitation wavelength

$\eta$ is the refractive index of the solution

The subscripts 1 and B refer to the unknown and the standard, respectively.

We chose the fluorescein standard which is present with 0.10 M NaOH as standard additive. Under these conditions, the quantum yield of this species is 0.95.
Table S1. The summary of previously reported fluorescent probes for the detection of HOCl.

<table>
<thead>
<tr>
<th>Probes</th>
<th>Strokes Shift (nm)</th>
<th>LOD</th>
<th>Response time</th>
<th>Solvent</th>
<th>Bio-Application</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Probes Image" /></td>
<td>105</td>
<td>97.33 nm</td>
<td>&lt; 5 s</td>
<td>PBS</td>
<td>Cells Zebrafish</td>
<td>This work</td>
</tr>
<tr>
<td><img src="image2" alt="Probes Image" /></td>
<td>170</td>
<td>0.47 μM</td>
<td>4 s</td>
<td>MeOH: water 1: 4 v/v</td>
<td>Cells Mice</td>
<td>Chem. Sci. 2019, 10, 3715-3722.</td>
</tr>
<tr>
<td><img src="image3" alt="Probes Image" /></td>
<td>140</td>
<td>164 nm</td>
<td>5 min</td>
<td>50% DMSO/10 mM PBS (7.4)</td>
<td>Cells</td>
<td>Talanta 2019, 196, 352-356.</td>
</tr>
<tr>
<td><img src="image5" alt="Probes Image" /></td>
<td>&lt; 30 nm</td>
<td>31.7 nm</td>
<td>&lt; 30 s</td>
<td>1% DMF/10 mM PBS(7.4)</td>
<td>Cells C. elegans</td>
<td>Dyes Pigm. 2020, 177, 108508.</td>
</tr>
<tr>
<td>Compound</td>
<td>Concentration</td>
<td>Time</td>
<td>Condition</td>
<td>Cell Type</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
<td>------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td><img src="image1" alt="Compound 1" /></td>
<td>46</td>
<td>72 nM</td>
<td>10 min</td>
<td>10 mM PBS(7.4)</td>
<td>Cells</td>
<td>Talanta 2020, 215, 120892</td>
</tr>
<tr>
<td><img src="image2" alt="Compound 2" /></td>
<td>109</td>
<td>120 nM</td>
<td>&lt; 3 min</td>
<td>1.0% DMSO</td>
<td>Cells</td>
<td>Spectrochim. Acta. Part A: Mol. Biomol. Spectrosc. 229, 117992, (2020)</td>
</tr>
<tr>
<td><img src="image3" alt="Compound 3" /></td>
<td>125</td>
<td>16.6 nm</td>
<td>Within seconds</td>
<td>50% EtOH</td>
<td>Cells</td>
<td>J. Am. Chem. Soc. 2015, 137, 5930.</td>
</tr>
<tr>
<td><img src="image4" alt="Compound 4" /></td>
<td>155</td>
<td>0.17 μM</td>
<td>100 s</td>
<td>30% EtOH/PBS</td>
<td>Cells</td>
<td>Sens. Actuators B 2019, 287, 453-458.</td>
</tr>
<tr>
<td><img src="image5" alt="Compound 5" /></td>
<td>37</td>
<td>10.8 pM</td>
<td>&lt; 5 s</td>
<td>PBS (0.01 M, pH = 7.4)</td>
<td>Cells</td>
<td>Biosens. Bioelectron. 2018, 107, 218–223.</td>
</tr>
<tr>
<td><img src="image6" alt="Compound 6" /></td>
<td>20</td>
<td>20 nM</td>
<td>20 s</td>
<td>PBS (pH = 7.4)</td>
<td>Cells</td>
<td>Sens. Actuators B 2020, 307, 127652.</td>
</tr>
<tr>
<td><img src="image7" alt="Compound 7" /></td>
<td>300</td>
<td>0.19 μM</td>
<td>25 s</td>
<td>DMF: PBS (5:5, v/v; pH = 7.4)</td>
<td>Cells</td>
<td>Sens. Actuators B 2018, 255, 666–671.</td>
</tr>
</tbody>
</table>
1. Synthesis

![Chemical Structure](image)

**Myco-OH**  
**Myco-OCI (76%)**

**Synthesis of Myco-OCI.**

The Myco-OH was prepared by literature methods.\(^{21}\)

Myco-OH (62.5 mg, 0.2 mmol) was dissolved in anhydrous DCM (5 ml) in the N\(_2\) environment and stirred for 5 minutes. Then, this solution was treated with triethylamine (56 \(\mu\)L, 0.6 mmol) and stirred again for 15 minutes to afford a clear solution. After that, dimethylthiocarbamoyl chloride (37 mg, 3.00 mmol) was added slowly at 0\(^\circ\)C. This mixture was stirred for 16 h at room temperature. The solvent was evaporated and the residue was purified by silica gel flash chromatography using n-hexane/EtOAc (16:1) as an eluent to afford Myco-OCI as a white solid (97 mg, 76% yield). \(^1\)H NMR (400 MHz, DMSO\(_d_6\)): \(\delta = 7.85\) (s, 1H, H\(_6\)), 3.38 (s, 3H, H\(_2\)), 3.37 (s, 3H, H\(_{13}\)), 2.98 (s, 3H, H\(_{12}\)), 2.67 (s, 3H, H\(_{10}\)). \(^1^3\)C NMR (100 MHz, DMSO–d\(_6\)) \(\delta\): 185.52 (C\(_{11}\)), 167.77 (C\(_3\)), 164.98 (C\(_1\)), 147.60 (C\(_7\)), 135.05 (C\(_9\)), 133.63 (C\(_8\)), 131.91 (C\(_5\)), 130.73 (C\(_4\)), 123.39 (C\(_6\)), 43.49 (C\(_{12}\)), 39.31 (C\(_{13}\)), 24.26 (C\(_2\)), 16.84 (C\(_{10}\)). HRMS (ESI): calcd for C\(_{13}\)H\(_{13}\)BrN\(_2\)O\(_3\)S + Na: 380.9832; found: m/z 380.9706 (M + Na)\(^+\).

**Proposed sensing mechanism:**

![Proposed Sensing Mechanism](image)

Fig. S1. Proposed sensing mechanism of HOCl with probe Myco-OCI\(^i\).

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\(^{21}\) Reference to literature method for the synthesis of Myco-OH.

\(^i\) Reference to proposed mechanism for the sensing of HOCl with Myco-OCI.
Figure S2. Normalize Emission spectra for the comparison between the probe Myco-OCl (red line) in response to HOCl (5 Eq.) and the fluorophore Myco-OH (black line).

Fig. S3. $^1$H NMR spectrum of the Myco-OCl in DMSO–d$_6$ (brown line), $^1$H NMR spectrum of the Myco-OH in DMSO–d$_6$ (green line), and $^1$H NMR spectrum of the Myco-OCl + HOCl (5 Eq.) (Blue line) in DMSO–d$_6$ and D$_2$O.
Fig. S4. HR–MS (ESI) spectrum of Myco-OC1 + HOCl.
Fig. S5. (top) $^1$H and (bottom) $^{13}$C NMR spectrum of Myco-OCl.
Fig. S6. (top) COSY spectrum and (bottom) HSQC NMR spectrum of Myco-OC1.

Fig. S7. HMBC spectrum of Myco-OC1.
Fig. S8. $^1$H NMR spectrum of Myco-OH.

Fig. S9. HR–MS (ESI) spectrum of Myco-OCl.
Photophysical study:

Figure S10: (A) Normalized UV- absorption spectra of Myco-OCI (10.0 µM) in absence (black) and presence (red) of HOCl (5 eq.) (B) Emission spectra of Myco-OCI (10.0 µM) in absence (black) and presence (red) of HOCl (0–50 µM) in the solution of PBS (10 mM, 7.4 pH) (λ_{ex} = 417 nm, λ_{em} = 522 nm; slit width = 5 nm/5 nm at RT).

Figure S11: The time course of the fluorescence with the probe is shown supporting the photostability of Myco-OCI (λ_{ex} = 417 nm) without adding HOCl.
Fig. S12. Interference study of various ROS and bio thiols (100 equiv.) with Myco-OCI (10 µM) and HOCl (5 equiv) (1 to 14: = blank, HOCl, O$_2^-$, ONOO$, ^\cdot$BuOOH, $^\cdot$BuOOH, OH, NaHS, GSH, Cys, Hcy, ascorbic acid, and tyrosine respectively). ($\lambda_{ex}$ = 417 nm and $\lambda_{em}$ = 522 nm, slit width 5 nm / 5 nm). (Error bars are the average of three experiments).

Fig. S13. Interference study of various metals and anions (100 equiv.) with Myco-OCI (10 µM) and HOCl (5 equiv) (1 to 22: = blank, HOCl, K$^+$, Na$^+$, Ca$^{2+}$, Mg$^{2+}$, Pb$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, Fe$^{2+}$, Fe$^{3+}$, Mn$^{2+}$, Cd$^{2+}$, Al$^{3+}$, Co$^{2+}$, Cr$^{3+}$, SO$_4^{2-}$, S$_2$O$_3^{2-}$, S$_2$O$_5^{2-}$, and SO$_3^{2-}$ respectively ($\lambda_{ex}$ = 417 nm and $\lambda_{em}$ = 522 nm, slit width 5 nm / 5 nm). (Error bars are the average of three experiments).
Cell imaging study:

![Bar graph showing fluorescent intensities of A549 cells upon treatment with Myco-OCI, Myco-OCI + NaOCl, Myco-OCI + PMA, and Myco-OCI + ABAH + PMA.](image)

**Fig. S14.** Bar graphs showing the relative fluorescent intensities of the confocal microscopic images of A549 cells upon treatment with Myco-OCI (10 μM), Myco-OCI + NaOCl (100 μM), Myco-OCI + PMA (1 μg mL⁻¹), and Myco-OCI + ABAH (250 μM) + PMA (1 μg mL⁻¹ μM).

Zebrafish imaging study:

**Animal Care (zebrafish):**

Wild Type adult zebrafish were housed at 14/10 hour light/dark cycle at 28.5°C. Adult wild type and Tg(huc:egfp) were mated and embryos were collected and raised in E3 medium (5mM NaCl, 0.17mM KCl, 0.33mM CaCl₂, 0.33mM MgSO₄, 10-5% Methylene Blue) at 28.5°C. Proper developmental stages were observed until experiments were conducted. All fish were obtained from the Zebrafish Center for Disease Modeling, Chungnam National University, Republic of Korea. All experiments were conducted according to the guidelines approved by the Animal Ethics Committee of Chungnam National University (CNU-0866).

**Chemical Treatment:**

To identify the lethality of the Myco-OCI probe, 6dpf zebrafish were used. Four different concentrations (10, 20, 40, and 80μM) were prepared with 0.1% DMSO. Control (0.1% DMSO) was prepared separately. 6 dpf zebrafish larvae were exposed to control and four different concentrations in 24 well-plates and lethality was calculated using Kruskal Wallis One Way ANOVA post-test. Similarly, Tg(huc:egfp)
transgenic zebrafish lines were also exposed to four concentrations (10, 20, 30, 40 μM) to identify any abnormalities in neuronal development.

**Determination of morphological abnormalities:**
Post 24-hour exposure, 7dpf zebrafish were analyzed for morphological abnormalities. We checked heartbeat rate, eye movement, eye size, pigment formation, pericardial edema, swim bladder development, and overall morphological defects. Similarly, Tg(huc:egfp) transgenic zebrafish were used to determine neuronal development in larval zebrafish. Each larva was then anesthetized using Tricaine (MS-222) and used for imaging.

**Animal Model Imaging:**
7dpf zebrafish after verification for lethality was imaged under the MZ16 Brightfield microscope (Leica Biosystems, Wetzlar, Germany). 6dpf zebrafish fluorescent imaging was done with Logos Biosystems (Aligned Genetics, Republic of Korea).

**Fig. S15.** (A) Investigation of morphological abnormalities of zebrafish larvae at 6dpf after 24h exposure to various concentrations (10, 20, 40, 80µM) of **Myco-OCl probe**. (B) Effects of **Myco-OCl** probe with different concentrations (10 µM, 20 µM, 40 µM, and 80 µM) on development of central nervous system in zebrafish larvae at 6 dpf. Fluorescent neurons are monitored in live transgenic zebrafish, Tg(huc:gfp). The midbrain region is marked with red arrowheads. Signals in the intestine, yolk, and lens are due to autofluorescence.
References:


