Mitochondria-targeted colorimetric and fluorescent probes for hypochlorite and their applications for \textit{in vivo} imaging

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Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental section</td>
<td>S2-4</td>
</tr>
<tr>
<td>Figure S1</td>
<td>S5</td>
</tr>
<tr>
<td>Figure S2</td>
<td>S5</td>
</tr>
<tr>
<td>Figure S3</td>
<td>S6</td>
</tr>
<tr>
<td>Figure S4</td>
<td>S6</td>
</tr>
<tr>
<td>Figure S5</td>
<td>S7</td>
</tr>
<tr>
<td>Figure S6</td>
<td>S7</td>
</tr>
<tr>
<td>Figure S7</td>
<td>S8</td>
</tr>
<tr>
<td>Figure S8</td>
<td>S8</td>
</tr>
<tr>
<td>Scheme S2</td>
<td>S9</td>
</tr>
<tr>
<td>Figure S9</td>
<td>S9</td>
</tr>
<tr>
<td>Figure S10</td>
<td>S9</td>
</tr>
<tr>
<td>NMR and MS copies of various compounds</td>
<td>S10 -13</td>
</tr>
</tbody>
</table>
Experimental Section

General remarks for experimental

$^1$H NMR, $^{13}$C NMR spectra were measured on a Bruker AM400 NMR spectrometer. Proton Chemical shifts of NMR spectra were given in ppm relative to internals reference TMS (1H, 0.00 ppm). ESI-MS and HRMS spectral data were recorded on a Finnigan LCQDECA and a BrukerDaltonics Bio TOF mass spectrometer, respectively. 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. Fluorescence emission spectra were obtained using FluoroMax-4 Spectrofluorophotometer (HORIBA JobinYvon) at 298 K. Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. All the solvents were dried according to the standard methods prior to use. All of the solvents were either HPLC or spectroscopic grade in the optical spectroscopic studies.

Fluorescence analysis. Probes Rh-TPP and Rh-Py were prepared in DMSO at a concentration of 2 mM. All UV/Vis and fluorescence titration experiments were performed using 5 $\mu$M Rh-TPP or Rh-Py in PBS buffer solution (pH 7.4, 10 mM) with varying concentrations of analytes at room temperature. The time dependences of the response of Rh-TPP or Rh-Py (5$\mu$M) to NaClO (50 $\mu$M) were determined by mixing the two reactants in PBS buffer solution (pH 7.4).

Imaging of cells. Hela cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% Antibiotic-Antimyocytic at 37°C in a 5% CO$_2$/95% air incubator. For fluorescence imaging, cells (4×10$^3$/well) were passed on a 6-well plate and incubated for 24h. Immediately before the staining experiment, cells were washed twice with PBS, incubated with 5 $\mu$M Rh-TPP or Rh-Py for 30 min at 37 °C. Then confocal fluorescent images were captured with an excitation light at 543 nm. Then, 100 $\mu$M NaClO was added and incubated for another 10 min and was imaged.

Co-localization imaging of cells. Hela cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% Antibiotic-Antimyocytic at 37°C in a 5% CO$_2$/95% air incubator. For fluorescence imaging, cells (4×10$^3$/well) were passed on a 6-well plate and incubated for 24h. Immediately before the staining experiment, cells were washed twice with PBS, incubated with 5$\mu$M Rh-TPP or Rh-Py for 30 min at 37 °C. Then, 100$\mu$M NaClO was added and incubated for another 10 min. After the cells were washed twice with PBS, MitoTracker Green (1 $\mu$M) was added and incubated for 20 min and the confocal fluorescent images were captured.

Fluorescence imaging in living mice. Female Balb/c-nu mice (5–6 weeks old) were purchased from Beijing HFK bioscience CO. Ltd, Beijing, China. Animal experiments were approved by the Institutional Animal Care and Treatment Committee of Sichuan University (Chengdu, China). The mice were acclimated for 1 week before the experiment. Representatively, a nude mouse was given a skin-pop injection of Rh-TPP or Rh-Py (50 $\mu$L, 100 $\mu$M in PBS (pH 7.4, 10 mM, containing 0.1% DMSO)), and a subsequent skin-pop injection of NaClO (50 $\mu$L, 1mM in PBS (pH 7.4, 10 mM)). Images were taken after incubation for different time in Bio-Real in vivo imaging system (Quick View 3000, Bio-Real,AUSTRIA), with an excitation laser of 534 nm and an emission filter of 586±20 nm.
To a solution of rhodamine B (2 g, 4.2 mmol) dissolved in 15 mL of methanol, an excessive hydrazine hydrate (2.5 mL) was added and then the reaction solution was refluxed till the pink color disappeared. After that, the cooled reaction solution was poured into distilled water and extracted with ethyl acetate (3 × 30 mL). The combined extracts were dried with sodium sulfate anhydrous. The solvent was removed under the reduced pressure and the residue was further purified by column chromatography over silica gel eluting with petroleum ether/CH$_2$Cl$_2$ = 1:1 to afford Rh-1 (1.0 g, 2.2 mmol) as a white solid. Yield: 52.4%. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.94 (d, $J$ = 8.0 Hz, 1H), 7.51 – 7.38 (m, 2H), 7.11 (d, $J$ = 7.4 Hz, 1H), 6.46 (d, $J$ = 8.8 Hz, 2H), 6.42 (s, 2H), 6.29 (d, $J$ = 8.8 Hz, 2H), 3.61 (s, 2H), 3.34 (q, $J$ = 7.0 Hz, 8H), 1.17 (t, $J$ = 7.0 Hz, 12H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 165.7, 153.5, 152.3, 148.6, 132.8, 130.1, 128.6, 128.2, 123.9, 122.6, 108.3, 105.9, 97.9, 65.2, 44.1, 12.9. ESI-MS: m/z 457.2 [M + H]$^+$

**Preparation and Characterization of Rh-2**

To a solution of Rh-1 (815 mg, 1.8 mmol) dissolved in 15 mL of anhydrous CHCl$_3$, triethylamine (332 $\mu$L, 2.4 mmol) was added at 0 °C and the reaction solution was stirred for several minutes. Then, a solution of 2-chloroacetyl chloride (180 $\mu$L, 2.4 mmol) in 5 mL of dry CHCl$_3$ was added dropwise. After that, the mixture was warmed to room temperature and stirred for 4 h. Then the reaction solution was poured into distilled water and extracted with CH$_2$Cl$_2$ (3 × 30 mL). The combined organic layer was washed with saturated aqueous NaCl (50 mL) successively and dried over anhydrous Na$_2$SO$_4$. The solvent was removed under the reduced pressure and the residue was further purified by column chromatography over silica gel eluting with petroleum ether/ethyl acetate = 3:1 to afford Rh-2 (860 mg, 1.6 mmol) as a white solid. Yield: 88.9%. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 9.92 (s, 1H), 7.83 (d, $J$ = 7.6 Hz, 1H), 7.62 – 7.48 (m, 2H), 7.02 (d, $J$ = 6.7 Hz, 1H), 6.55 – 6.45 (m, 2H), 6.34 (dd, $J$ = 7.2, 2.3 Hz, 4H), 4.00 (s, 2H), 3.32 (q, $J$ = 7.0 Hz, 8H), 1.08 (t, $J$ = 7.0 Hz, 12H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 165.1, 163.9, 153.5, 152.3, 148.9.
Preparation and Characterization of Rh-TPP

To a solution of Rh-2 (160 mg, 0.3 mmol) dissolved in 15 mL of anhydrous CH\textsubscript{3}CN, KI (100 mg, 0.6 mmol) and triphenylphosphine (314 mg, 1.2 mmol) was added in one portion and the reaction solution was heated to reflux for 24 h. Then, the solvent was removed under the reduced pressure and the residue was further purified by column chromatography over silica gel eluting with CH\textsubscript{2}Cl\textsubscript{2}/CH\textsubscript{3}OH = 50:1 to afford Rh-TPP (120 mg, 0.14 mmol) as a light purple solid. Yield: 46.7%. 1H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) 9.84 (s, 1H), 7.70 (m, 11H), 7.56 (m, 6H), 7.35 (m, 1H), 7.01 (d, \(J = 7.4\) Hz, 1H), 6.72 (d, \(J = 8.8\) Hz, 2H), 6.34 (d, \(J = 2.3\) Hz, 2H), 6.27 (d, \(J = 8.7\) Hz, 2H), 5.10 (d, \(J = 13.7\) Hz, 2H), 3.33 (q, \(J = 7.0\) Hz, 8H), 1.16 (t, \(J = 7.0\) Hz, 12H). 13C NMR (100 MHz, CDCl\textsubscript{3}) \(\delta\) 164.7, 153.2, 152.8, 148.9, 134.9, 133.9, 133.5, 133.1, 130.1, 129.6, 127.8, 127.4, 123.8, 123.2, 118.4, 117.5, 108.2, 103.9, 97.9, 66.1, 53.5, 44.4, 12.8. HRMS calcd for C\textsubscript{48}H\textsubscript{48}N\textsubscript{4}O\textsubscript{3}P\textsuperscript{+} [M]+: 759.3459, found: 759.3088.

Preparation and Characterization of Rh-Py

To a solution of Rh-2 (160 mg, 0.3 mmol) dissolved in 15 mL of anhydrous CH\textsubscript{3}CN, KI (100 mg, 0.6 mmol) and pyridine (287 \(\mu\)L, 3.6 mmol) was added in one portion and the reaction solution was heated to reflux for 24 h. Then, the solvent was removed under the reduced pressure and the residue was further purified by column chromatography over silica gel eluting with CH\textsubscript{2}Cl\textsubscript{2}/CH\textsubscript{3}OH = 30:1 to afford Rh-Py (100 mg, 0.14 mmol) as a light purple solid. Yield: 46.7%. 1H NMR (400 MHz, DMSO) \(\delta\) 10.47 (s, 1H), 8.67 (dd, \(J = 15.5, 6.8\) Hz, 3H), 8.18 – 8.06 (m, 2H), 7.84 (d, \(J = 7.0\) Hz, 1H), 7.67 – 7.50 (m, 2H), 7.07 (d, \(J = 7.3\) Hz, 1H), 6.47 (d, \(J = 8.9\) Hz, 2H), 6.36 (s, 2H), 6.32 (d, \(J = 8.9\) Hz, 2H), 5.44 (s, 2H), 3.34 (d, \(J = 8.0\) Hz, 8H), 1.09 (t, \(J = 8\) Hz, 12H). 13C NMR (100 MHz, DMSO) \(\delta\) 163.9, 163.7, 153.2, 151.9, 148.9, 146.9, 134.1, 129.6, 129.1, 128.2, 124.4, 123.2, 108.2, 104.3, 97.5, 65.7, 60.6, 44.1, 12.9. HRMS calcd for C\textsubscript{35}H\textsubscript{38}N\textsubscript{5}O\textsubscript{3}\textsuperscript{+} [M]+: 576.2969, found: 576.2685.
Figure S1. Absorption spectra of (left) Rh-TPP and (right) Rh-Py before and after reaction with various ROS in PBS (pH 7.4, 10 mM, containing 0.1% DMSO). [Rh-TPP] = [Rh-Py] = 5 μM, ClO⁻: NaClO (final 50 μM) was added and the mixture was stirred at 20 °C. ·OH: ferrous perchlorate (500 μM) and H₂O₂ (1 mM) were added at room temperature. O₂⁻: KO₂ was dissolved in the anhydrous DMSO and then the appropriate aliquot was added (final 100 μM). H₂O₂: H₂O₂ (final 100 μM) was added and the mixture was stirred at 20 °C. ONOO⁻: ONOO⁻ (final 50 μM) was added and the mixture was stirred at 20 °C. tBuOOH: tBuOOH (final 100 μM) was added and the mixture was stirred at 20 °C. Each spectrum was recorded at 30 min after reaction. Inset: image of (a) Rh-TPP and (b) Rh-Py in the absence or presence of NaClO.

Figure S2. Fluorescence spectra of (left) Rh-TPP and (right) Rh-Py before and after addition of various cations and NaClO in PBS (pH 7.4, 10 mM, containing 0.1% DMSO). [Rh-TPP] = [Rh-Py] = 5 μM, [NaClO] = [M]⁺⁺ = 50 μM. Cations: Na⁺, K⁺, Mg²⁺, Fe³⁺, Cd²⁺, Ni²⁺, Co²⁺, Cr³⁺, Pb²⁺, Hg²⁺, Al³⁺, Mn²⁺, Ag⁺, Cu²⁺, Zn²⁺.
Figure S3. The titration curve plotted with the fluorescence intensity of (a) Rh-TPP at 577 nm and (b) Rh-Py at 575 nm as a function of NaClO concentration in range of 0-10 μM. [Rh-TPP] = [Rh-Py] = 5 μM.

Figure S4. The effect of pH on the fluorescence intensity of (a) Rh-TPP and (b) Rh-Py in the absence or presence of NaClO (50 μM). [Rh-TPP] = [Rh-Py] = 5 μM, λex = 540 nm, slit: 3 nm/3 nm.
Figure S5. Temporal profile of fluorescence intensity of Rh-TPP (blue line) and Rh-Py (red line). NaClO (50 μM) was added at the 60th second.

Figure S6. Time-dependent change of fluorescence intensity of (a) Rh-TPP and (b) Rh-Py in the absence (blue line) or presence (red line) of NaClO (50 μM). [Rh-TPP] = [Rh-Py] = 5 μM, λ<sub>ex</sub> = 540 nm, slit: 3 nm/3 nm.
Figure S7. ESI spectra of Rh-TPP upon addition of 10 equiv NaClO.

Figure S8. ESI spectra of Rh-Py upon addition of 10 equiv NaClO.
Scheme S2. Reaction mechanism of Rh-TPP/Py with NaClO.

Figure S9. Effects of Rh-TPP and Rh-Py at varied concentrations on the viability of Hela cells. The results are the mean standard deviation of three separate measurements.

Figure S10. Confocal imaging of ClO− in HeLa cells with Rh-TPP or Rh-Py. (a) and (e): bright field images of HeLa cells loaded with 5 μM Rh-TPP and Rh-Py for 30 min; (b) and (f): the fluorescence images of (a) and (e), respectively; (c) and (g): fluorescence images of (a) and (e) after incubation with NaClO (100 μM) for 10 min, respectively; (d) and (h): merged images. Bars: 25 μM.
$^{1}$H-NMR Spectrum of $\text{Rh-I}$ in CDCl$_3$ (400 MHz):

\begin{center}
\includegraphics[width=\textwidth]{h_nmr_spectrum.png}
\end{center}

$^{13}$C-NMR Spectrum of $\text{Rh-I}$ in DMSO-$d_6$ (100 MHz):

\begin{center}
\includegraphics[width=\textwidth]{c_nmr_spectrum.png}
\end{center}
$^{1}$H-NMR Spectrum of Rh-2 in DMSO-$_{d_6}$ (400 MHz):

$^{13}$C-NMR Spectrum of Rh-2 in DMSO-$_{d_6}$ (100 MHz):
$^1$H-NMR Spectrum of Rh-TPP in CDCl$_3$ (400 MHz):

$^{13}$C-NMR Spectrum of Rh-TPP in CDCl$_3$ (100 MHz):
$^1$H-NMR Spectrum of **Rh-Py** in DMSO-$d_6$(400 MHz):

- 10.47
- 8.86
- 6.66
- 4.44
- 3.34
- 3.25
- 1.41
- 1.08

$^{13}$C-NMR Spectrum of **Rh-Py** in DMSO-$d_6$(100 MHz):

- 134.07
- 128.54
- 128.43
- 128.22
- 108.16
- 104.25
- 97.40
- -65.70
- -60.85
- -44.12
- 52.92

S13
HRMS spectra of **Rh-TPP**: 

![HRMS spectra of Rh-TPP](image1)

\[ [M]^+ = 759.3459 \]

HRMS spectra of **Rh-Py**: 

![HRMS spectra of Rh-Py](image2)

\[ [M]^+ = 576.2969 \]