Electronic Supplementary Information

Engineering of a zero cross-talk fluorescent polymer nanoprobe for self-referenced ratiometric imaging of lysosomal hypochlorous acid in living cells

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Experimental Section

Synthesis of TPP-OH

The propionic acid solution (250 mL) of 4-hydroxybenzaldehyde (4.01 g, 32.73 mmol) and benzaldehyde (10 mL, 88.0 mmol) was heated to nearly reflux, and then pyrrole (9 mL, 129.6 mmol) was added under stirring. The reaction mixture was stirred under reflux for about 1 h. After cooling, the solvent was evaporated under reduced pressure. The dark residue was firstly recrystal by ethanol over night and then filtered through a silica column with DCM/PE as eluent. The second band was collected and concentrated to afford 641 mg (3.13%) of a violet solid. 1H NMR (500 MHz, CDCl3) δ 8.85 (d, J = 11.0 Hz, 8H), 8.21 (d, J = 7.3 Hz, 6H), 8.04 (d, J = 8.0 Hz, 2H), 7.80-7.69 (m, 9H), 7.11 (d, J = 8.0 Hz, 2H), 0.00 (s, 1H), -2.77 (s, 2H). 13C NMR (125 MHz, CDCl3) δ=135.69, 134.56, 127.70, 126.68, 163.41-7.45, 77.27, 77.27-76.73, 76.73-76.52. MS(ESI): m/z 631.24 [M+H]+.

Synthesis of TPP-OH-TTC

4-(10,15,20-triphenylporphyrin-5-yl)phenol (TPP-OH) (63 mg, 0.1 mmol), the 2-methyl-2-((propylthio)carbonothiol)thio) propanoic acid (119 mg, 0.5 mmol) and the 4-dimethylaminopyridine (12.217 mg, 0.1 mmol) were dissolved in 30 mL DCM. Then, N, N’-diisopropylcarbodiimide (100.96 mg, 0.8 mmol) was added slowly. The reaction mixture was stirred for 12 h at 25 °C. After that, the solution was washed with 100 mL of distilled water, then the organic phase was evaporated under vacuum. The residue was further purified by column chromatography using DCM/MeOH (V:V, 3:2) as the eluent to afford a yellow solid (912 mg, 88%). 1H NMR (500 MHz, CDCl3) δ = 8.85 (d, J = 4.2 Hz, 1H), 8.45-8.10 (m, 1H), 7.98 - 7.69 (m, 1H), 7.49 (d, J = 8.3 Hz, 1H), 3.38 (t, J = 7.3 Hz, 1H), 2.23-2.81 (m, 1H), 1.88-1.47 (m, 1H), 1.15-1.02 (m, 1H), -2.80 (s, 1H). 13C NMR (125 MHz, CDCl3) δ=172.09, 151.03, 142.20, 139.95, 135.30, 135.53-125.88, 126.74, 126.74, 124.43, 123.54, 120.32, 119.86, 118.97, 56.05, 39.06, 31.54, 30.92-28.82, 25.53, 21.56, 13.57.

Synthesis of TPP-b-PS68

TPP-OH-TTC (17 mg, 0.02 mmol), St (364.49 mg, 3.5 mmol) and AIBN (0.8 mg, 4.9 μmol) were dissolved in 1 mL of dried DMF. After three times of the free-pump-thaw cycle, the mixture was stirred under 90°C for 6 h and then terminated by immersing into ice water. The copolymer was precipitated in methanol (20 mL) to remove any unreacted small molecule compounds. After being filtered and fired at 30°C under a vacuum for 48 h. The polymer TPP-b-PS68 was obtained. (a modena powder; 61 mg; Mn = 4.6 kDa, Mw/Mn=1.27).

Synthesis of TPP-b-PS68-b-PEGMA300

TPP-b-PS68 (21 mg, 0.0046 mmol), PEGMA (250 mg, 0.5 mmol), AIBN (0.41 mg, 0.0025 mmol), were dissolved in 1 mL of dried DMF. After three times of the free-pump-thaw cycle, the mixture was stirred under 75°C for 4 h and then terminated by immersing into ice water. The polymer as precipitated in anhydrous diethyl ether (20 mL) to remove any unreacted small molecule compounds. After being filtered and fired at 30°C under a vacuum for 48 h. The polymer TPP-b-PS68-b-PEGMA300 was obtained. (oily substance; 30 mg; Mn = 34 kDa, Mw/Mn=1.52).

Synthesis of PEO113-b-P(AEMH-co-EAN1-co-St68).

PEO113-TTC (200 mg, 0.04 mmol), St (780 mg,7.5 mmol), EANI (28.1 mg, 0.4 mmol), AEMH (52 mg, 0.4 mmol), AIBN (1.6 mg, 0.0098 mmol) were dissolved in 1.5 mL of dried DMF.
After three times of the free-pump-thaw cycle, the mixture was stirred under 90°C for 4 h and then terminated by immersing into ice water. The copolymer as precipitated in anhydrous diethyl ether (40 mL) to remove any unreacted small molecule compounds. After being filtered and fired at 30°C under a vacuum for 48 h. The polymer PEO113-b-(PS68-co-AEMH1-co-EANI30) was obtained, a faint yellow powder; 270 mg; Mn = 10.1 kDa, Mw/Mn=1.14.

### Preparation of ZC-FPN

Typical procedures employed for the preparation of ZC-FPN are as follows: a mixture tetrahydrofuran (THF) solution (1 mL) of 12 mg·mL⁻¹ of TPP- b-PS68-b-PEGMA100 and 2.4 mg·mL⁻¹ of PEO113-b-(PS68-co-AEMH1-co-EANI1) is prepared first. Subsequently, the above mixture solution is quickly injected into 10 mL of water under vigorous sonication by ultrasound. Then the THF were evaporated under vacuum and filtrated by a 0.22 µm syringe driven filter. Finally, the stable ZC-FPN was obtained. The concentration of ZC-FPN is determined as 1.44 mg·mL⁻¹ by weighing total mass of nanoparticles after freeze-drying.

### Instruments and methods

¹H NMR and ¹³C NMR spectra were recorded on a Bruker Avance 500 MHz NMR spectrometer. UV-Vis spectra were recorded on a Shimadzu UV-2501PC spectrophotometer at room temperature (298 K). MS was conducted with a Finnigan LCQ Advantage MAX mass spectrometer. Fluorescence spectra were measured on RF-5301PC fluorescence spectrophotometer at room temperature. The number-average molecular weight (Mn) and PDI were determined by using a Waters 2695 gel permeation chromatograph (GPC) at 30°C and THF was used as the eluent (1.0 mL·min⁻¹). The calibration curve was obtained by using polystyrene (PS) as the standard. The IR spectrum was recorded on Nicolet 6700. The diameter of nanoparticles was determined by a Malvern Nano-ZS90 instrument, and their morphology was recorded on a JEM-2100F transmission electron microscope (TEM, JEOL USA, Inc).

### General Procedure for HClO detection

Unless otherwise noted, all the absorbance and fluorescence spectra fluorescence measurements were performed in 10 mM PBS buffered solution (pH 5.0) according to the following procedure. In a 5 mL cuvette, 2.80 mL of PBS and 0.1 mL of ZC-FPN (final concentration: 48 µg·mL⁻¹) are mixed, followed by addition of a predetermined volume of NaClO solution (HClO donor, 0 µM, 10 µM, 30 µM, 40 µM, 50 µM, 60 µM, 70 µM, 80 µM, 100 µM, 120 µM, 160 µM) respectively. The final volume of liquid in the cuvette was adjusted to 3.0 mL with PBS. After incubation at room temperature for 7 min, the solution was transferred to a quartz cell of 1 cm optical length to measure the absorbance and fluorescence spectra.

### Cell viability assay and cell imaging

HeLa cells and and RAW264.7 cells were used to evaluate the cytotoxicity of ZC-FPN by MTT assay according to ISO 10993-5.

For the co-localization imaging, the cells were stained firstly with the fresh cell growth medium supplemented with ZC-FPN (final concentration: 48 µg·mL⁻¹) for 3 h, then Lyso Tracker green (500 nM) in cell culture medium was added to prewashed cells and incubated at 37°C for 30 min, rinsed with PBS three times and were further incubated with NaOCl (HClO donor, 120 µM) for 30 min at 37°C, and then the fluorescence images were acquired through a Nikon A1MP confocal microscope equipped with a cooled CCD camera. Channel 1: excitation: 405 nm, blue emission collected: 440-500 nm; Channel 2 (Lyso Tracker or Mito Tracker red): excitation: 559 nm, emission collected: 575-675 nm.

For the ratiometric imaging of exogenous HClO, HeLa cells are incubated in the RPMI1640
medium supplemented with 10% fetal bovine serum (FBS, Invitrogen). One day before imaging, the cells are passed and plated on polylysine-coated cell culture glass slides inside 30 mm glass culture dishes and allowed to grow to 50-70% confluence. Afterwards, the cells (on glass slides) are washed with RPMI1640 and re-incubated in the RPMI1640 medium containing ZC-FPN (final concentration: 48 μg•mL⁻¹) for 3 h at 37°C and then washed with PBS three times. After incubating with NaOCl (HClO donor, 50 μM, 100 μM) for another 30 min at 37 °C, the culture dishes are washed with PBS and then imaged on a Nikon A1MP confocal microscope equipped with a cooled CCD camera. Excitation: 405 nm, blue emission collected: 440-525 nm (Channel A); red emission collected: 630-675 nm (Channel B).

For the ratiometric imaging of endogenous HClO, RAW264.7 murine macrophages were cultured in Dulbeccos Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO₂ and 95% air at 37°C. The cells were plated in 35 mm glass-bottom culture dishes and allowed to adhere for 24 h. For the detection of endogenously produced HClO, the living RAW264.7 macrophages were treated with LPS (1 μg•mL⁻¹) for 12 h, and then further co-incubated with PMA (1 μg•mL⁻¹) and ZC-FPN (final concentration: 48 μg•mL⁻¹) for 3 h. Prior to imaging, the cells were washed three times with PBS (1 mL). In a control assay, the living RAW264.7 cells were co-treated with LPS (1 μg•mL⁻¹) and NAC (2 mM) for 12 h and then co-incubated with PMA (1 μg•mL⁻¹) and ZC-FPN (final concentration: 48 μg•mL⁻¹) for 3 h, followed by washing three times with PBS (1 mL) and the fluorescence images were imaged on a Nikon A1MP confocal microscope equipped with a cooled CCD camera. Excitation: 405 nm, blue emission collected: 440-525 nm (Channel A); red emission collected: 630-675 nm (Channel B).
Synthesis routes:

Scheme S1. Synthesis route of \( \text{TPP-}b\text{-PS}_{68}\text{-b-PEGMA}_{300} \).

Scheme S2. Synthesis route of \( \text{PEO}_{113}\text{-b-P(AEMH}_{6}\text{-co-EANI}_{1}\text{-co-St}_{30}) \).
Figure S1. $^1$H NMR (A) and $^{13}$C NMR (B) spectrum (in CDCl$_3$) of TPP-OH.

Figure S2. Mass spectrum of TPP-OH (m/z 631.24 [M+H]$^+$).
Figure S3. $^1$H NMR (A) and $^{13}$C NMR (B) spectrum (in CDCl$_3$) of TPP-OH-TTC.
Figure S4. $^1$H NMR (in CDCl$_3$) of TTCA (1), TPP-OH (2), TPP-OH-TTC (3), TPP-\textit{b}-PS$_{68}$ (4), TPP-\textit{b}-PS$_{68}$-\textit{b}-PEGMA$_{300}$ (5).
Figure S5. GPC trace of TPP-OH-TTC, TPP-b-PS$_{68}$, TPP-b-PS$_{68}$-b-PEGMA$_{300}$. 

Table S1. Molecular weight distribution data of starting linear polymers.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$M_n$, GPC $^a$</th>
<th>$M_w$, GPC $^a$</th>
<th>PDI</th>
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</thead>
<tbody>
<tr>
<td>TPP-OH-TTC</td>
<td>570</td>
<td>600</td>
<td>1.06</td>
</tr>
<tr>
<td>TPP-b-PS$_{68}$</td>
<td>4600</td>
<td>5800</td>
<td>1.27</td>
</tr>
<tr>
<td>TPP-b-PS$<em>{68}$-PEGMA$</em>{300}$</td>
<td>34000</td>
<td>51600</td>
<td>1.52</td>
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</table>

$^a$The data were acquired using SEC based on a polystyrene calibration curve and obtained from GPC analysis was using THF as eluent at a flow rate of 1.0 mL/min.
Figure S6. IR spectrum of TPP-\textit{b}-PS_{68}-\textit{b}-PEGMA_{300}.

\textbf{Note:} The IR spectrum of TPP-\textit{b}-PS_{68}-\textit{b}-PEGMA_{300} is shown in Fig. S6, the characteristic bands of PS blocks C=\textit{C} stretching at 1450–1600 cm\textsuperscript{-1}, C=\textit{H} stretching at 3020–3080 cm\textsuperscript{-1}, C=\textit{H} bending at 690–754 cm\textsuperscript{-1}) indicate the successful polymerization of St monomer; the characteristic PEGMA stretching vibration of C-H, C=O and C-O are observed at 2888 cm\textsuperscript{-1} and 1728 cm\textsuperscript{-1} 1137 cm\textsuperscript{-1} indicate the successful polymerization of PEGMA monomer.
Figure S7. $^1$H NMR of the PEO-$b$-P(AEMH$_6$-co-EANI$_1$-co-St$_{30}$).

Figure S8. GPC trace of PEO$_{113}$-$b$-P(AEMH$_6$-co-EANI$_1$-co-St$_{30}$) and PEO$_{113}$-TTC.
Note: The IR spectrum of PEO<sub>113</sub>-b-P(AEMH<sub>6</sub>-co-EANI<sub>1</sub>-co-St<sub>30</sub>) is shown in Fig. S9, the characteristic PEO stretching vibration of C-H, C=O and C-O are observed at 2888 cm<sup>-1</sup>, 1730 cm<sup>-1</sup> and 1100 cm<sup>-1</sup>; the characteristic bands of PS blocks C=C stretching at 1450–1600 cm<sup>-1</sup>, C=H stretching at 3020–3080 cm<sup>-1</sup>, C=H bending at 690–754 cm<sup>-1</sup> indicate the successful polymerization of St monomer; the characteristic bands of AEMH blocks C=O stretching at 1730 cm<sup>-1</sup>, C-O stretching at 1100 cm<sup>-1</sup>, C=N bending at 1380 cm<sup>-1</sup> indicate the successful polymerization of AEMH monomer.

Table S2. Molecular weight distribution data of starting linear polymers.

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<tr>
<th>Sample</th>
<th>( M_n, \text{GPC}^a )</th>
<th>( M_w, \text{GPC}^a )</th>
<th>PDI</th>
</tr>
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<tbody>
<tr>
<td>PEO&lt;sub&gt;113&lt;/sub&gt;-TTC</td>
<td>7800</td>
<td>8200</td>
<td>1.04</td>
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<tr>
<td>PEO&lt;sub&gt;113&lt;/sub&gt;-b-P(AEMH&lt;sub&gt;6&lt;/sub&gt;-co-EANI&lt;sub&gt;1&lt;/sub&gt;-co-St&lt;sub&gt;30&lt;/sub&gt;)</td>
<td>10100</td>
<td>10800</td>
<td>1.07</td>
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</table>

\(^a\)The data were acquired using SEC based on a polystyrene calibration curve and obtained from GPC analysis was using THF as eluent at a flow rate of 1.0 mL/min.
Figure S10. The normalized absorbance spectra of ZC-FPN (48 μg•mL⁻¹) and EANI without and with HClO (160 μM).
Figure S11. The normalized excitation spectra of EANI (black curve) and TPP-OH (red curve).
Figure S12. Normalized fluorescence intensity of EANI (A) and TPP-OH (B) without (black curve) and with (red curve) HClO (160 μM).
Scheme S3 Possible reaction mechanism of TPP-OH responding to HClO.
Figure S13. $^1$H NMR (in CDCl$_3$) of TPP-OH before (up) and after (down) addition of HClO.
Figure S14. The mass spectrum of TPP-OH-HClO (m/z 699.2 [M+H]+).
**Determination of the detection limit:**

**Fluorescence:**

First the calibration curve was obtained from the plot of fluorescence intensity ratio \( \log[I_{464}/I_{655}] \) versus HClO concentration. The regression curve equation was then obtained for the lower concentration part.

The detection limit = \( 3 \times \sigma_{bi} / m \)

where \( m \) is the slope of the curve equation, and \( \sigma_{bi} \) represents the standard deviation for the logarithm of fluorescence intensity ratio \( \log[I_{464}/I_{655}] \) of ZC-FPN in the absence of HClO.

\[
\log[I_{464}/I_{655}] = 0.186 + 0.008 \times [\text{HClO}]
\]

\( \text{LOD} = 3 \times 0.0053 / 0.008 = 1.988 \, \mu\text{M} \)
Figure S15. Time course of the ratiometric fluorescence intensity ($I_{464}/I_{655}$) of ZC-FPN (48 μg·mL$^{-1}$) without and with HClO (160 μM)
Figure S16. Fluorescence intensity changes ($I_{464}/I_{655}$) of ZC-FPN (48 μg·mL$^{-1}$) under a continuous 365 nm UV lamp irradiation (2.8 mW·cm$^{-2}$).
Figure S17. Fluorescence long-term photostability of ZC-FPN (48 μg·mL⁻¹) under ambient temperature and kept in the dark.
Figure S18. Viability for HeLa cells and RAW264.7 cells treated with the varied concentrations of ZC-FPN for 24 h. The results are the mean standard deviation of eight separate measurements.
Figure S19. The zeta potential of ZC-FPN.
Fig. S20. Confocal fluorescence microscopy images of RAW264.7 macrophage cells incubated with NAC (2 mM) and ZC-FPN (48 μg mL⁻¹). λ_ex = 405 nm, λ_em1 = 440-500 nm (blue channel), and λ_em2 = 640-670 nm (red channel). Scale bar: 20 μm.
Table S2. Comparison of the some recently reported ratiometric fluorescent probes for HClO

<table>
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<tr>
<th>Probe</th>
<th>Solution</th>
<th>Emission shift (nm)</th>
<th>Targeting</th>
<th>References</th>
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<td>XWJ</td>
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<td>120 nm</td>
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<td>Sens. Actuators B, 2018, 263, 252-257</td>
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<td>Lyso-HA</td>
<td>PBS (pH 5.0, 30% DMSO)</td>
<td>136 nm</td>
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<td>J. Mater. Chem. B, 2016, 4, 4739-4745</td>
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<tr>
<td>Probe 4</td>
<td>PBS (pH 7.4, 10% CH₃CN)</td>
<td>90 nm</td>
<td>none</td>
<td>Anal. Chem., 2018, 90, 12937-12943.</td>
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<tr>
<td>Probe</td>
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<tr>
<td>Probe 1</td>
<td>PBS (pH 7.4, 50% EtOH)</td>
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<td>ZPAC</td>
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<td>lysosome</td>
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<td>BODIPY-P</td>
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<td>mitochondria</td>
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<td>TPA-CIO</td>
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<td>Chem. Commun., 2019, 55, 12912-12915.</td>
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<td><strong>This work</strong></td>
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<td><strong>lysosome</strong></td>
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