Electronic Supplementary Information

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

Hongqing Wei,[a] Rongjin Zeng,*[,a] Shenglan Wang,[a] Chong-Hua Zhang,[a] Shu Chen,[a] Peisheng Zhang,*[,a,b] and Jian Chen*[,a,c]

[a]Key Laboratory of Theoretical Organic Chemistry and Functional Molecule of Ministry of Education, Hunan Provincial Key Laboratory of Controllable Preparation and Functional Application of Fine Polymers, Hunan Province College Key Laboratory of QSAR/QSPR, Hunan Provincial Key Lab of Advanced Materials for New Energy Storage and Conversion, School of Chemistry and Chemical Engineering, Hunan University of Science and Technology, Xiangtan, Hunan 411201, China. E-mail: zrjxh2@126.com; pshzhang07@gmail.com; cj0066@gmail.com.
[b]State Key Laboratory of Chemo/Biosensing and Chemometrics, Hunan University, Changsha 410082, PR China.
[c]State Key Laboratory of Luminescent Materials and Devices, South China University of Technology, Guangzhou 510640, P. R. China

Experimental Section

Synthesis of TPP-OH

The propionic acid solution (250 mL) of 4-hydroxybenzaldehyde (4.01g, 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 overnight 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. ^{1}H NMR (500 MHz, $CDCl_3$) 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). ^{13}C NMR (125 MHz, $CDCl_3$) 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)carbonothioyl)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 were 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%). ^{1}H NMR (500 MHz, $CDCl_3$) 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), 2.23-1.81 (m, 1H), 1.88-1.74 (m, 1H), 1.15-1.02 (m, 1H), -2.80 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) 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*-PS_{68}

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*-PS_{68} was obtained. (a modena powder; 61 mg; Mn = 4.6 kDa, M_w/M_n=1.27).

Synthesis of TPP-*b*-PS_{68}-*b*-$PEGMA_{300}$

TPP-*b*-PS_{68} (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*-PS_{68}-*b*-$PEGMA_{300}$ was obtained. (oily substance; 30 mg; Mn = 34 kDa, Mw/Mn=1.52).

Synthesis of PEO_{113}-*b*-P(AEMH_6-*co*-EANI_1-*co*-St_{30}).

PEO_{113}-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 PEO_{113}-*b*-(PS_6-*co*-$AEMH_1$-*co*-$EANI_{30}$) was obtained, a faint yellow powder; 270 mg; Mn = 10.1 kDa, M_w/M_n=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*-PS_{68}-*b*-$PEGMA_{300}$ and 2.4 mg·mL^{-1} of PEO_{113}-*b*-(PS_{30}-*co*-$AEMH_6$-*co*-$EANI_1$) 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

^{1}H NMR and ^{13}C 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 (M_n) 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 an appropriate 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.

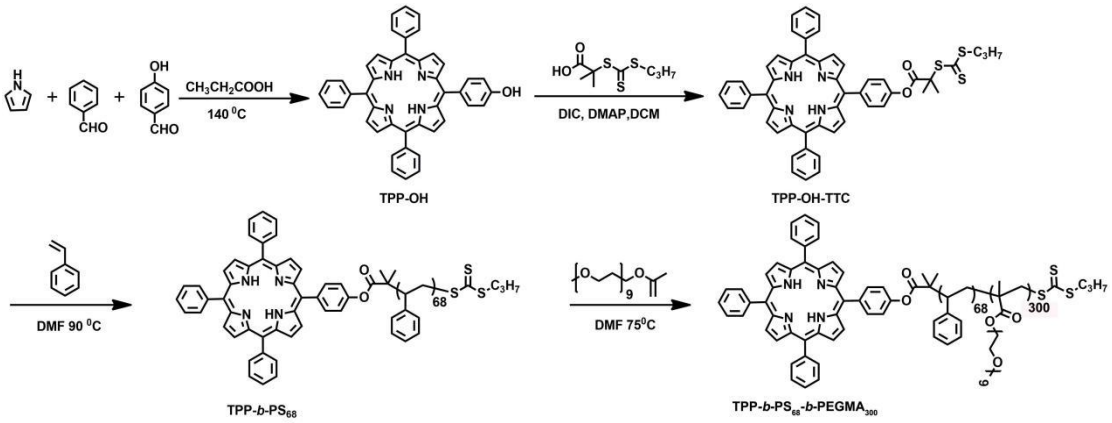
For the co-localization imaging, the cells were stained firstly with the fresh cell growth medium supplemented with ZC-FPN (final concentration: 48 μg•mL^{-1}) for 3 h, then Lyso Tracker green (500 nM) in cell culture medium was added to prewashed cells and incubated at 37°C for 30 min, rinsed with PBS 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

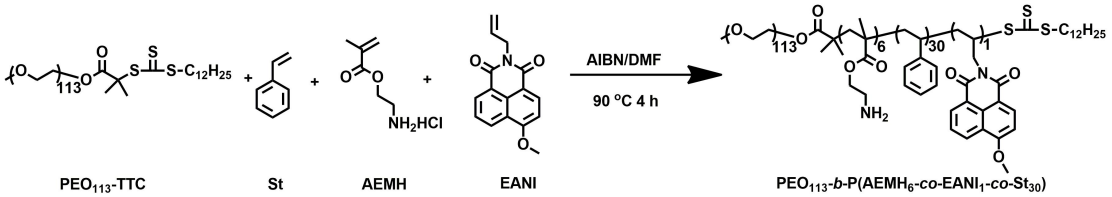
medium supplemented with 10% fetal bovine serum (FBS, Invitrogen). One day before imaging, the cells are passed and plated on polylysine-coated cell culture glass slides inside 30 mm glass culture dishes and allowed to grow to 50-70% confluence. Afterwards, the cells (on glass slides) are washed with 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% CO_2 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).

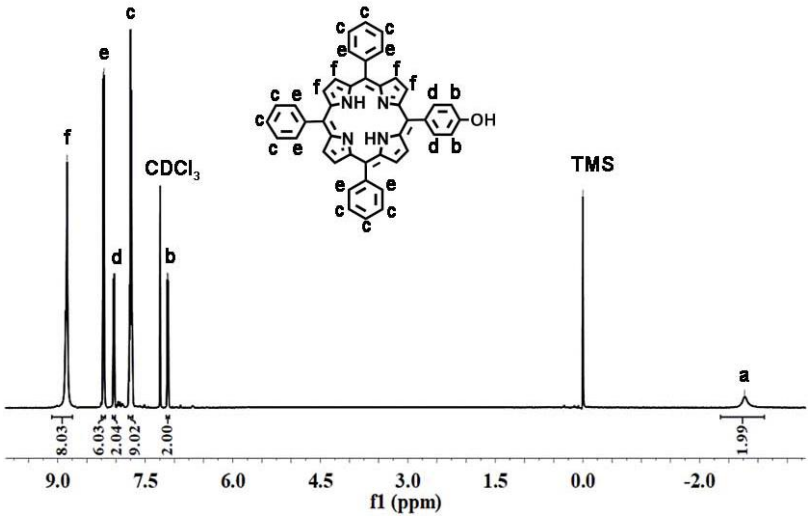
Synthesis routes:



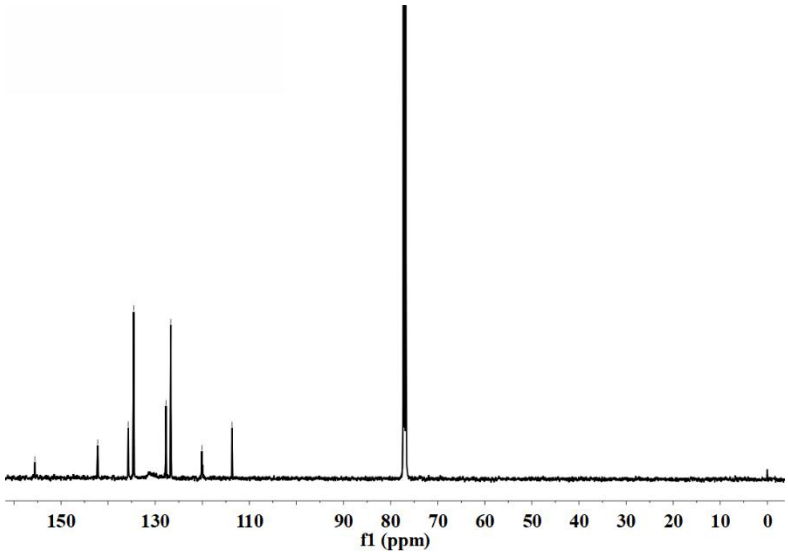
Scheme S1. Synthesis route of **TPP-*b*-PS$_{68}$-*b*-PEGMA$_{300}$**.



Scheme S2. Synthesis route of **PEO$_{113}$-*b*-P(AEMH$_6$-*co*-EANI$_1$-*co*-St$_{30}$)**.



(A)



(B)

Figure S1. ^{1}H NMR (A) and ^{13}C NMR (B) spectrum (in $CDCl_3$) of **TPP-OH**.

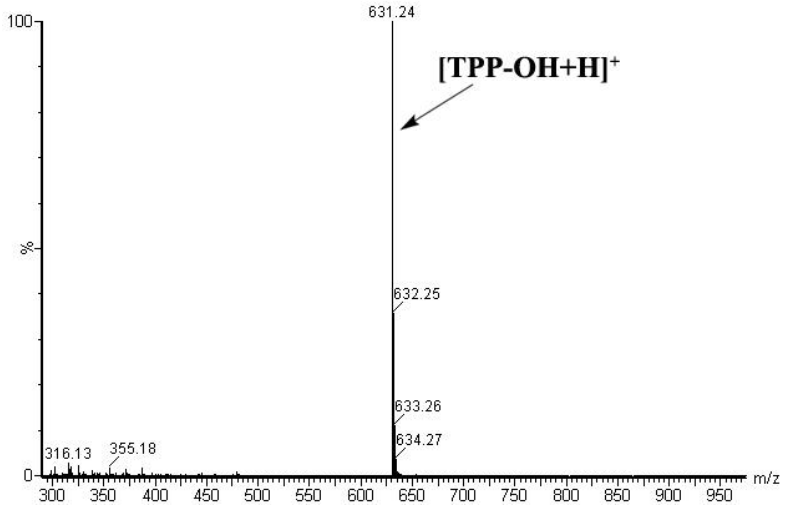
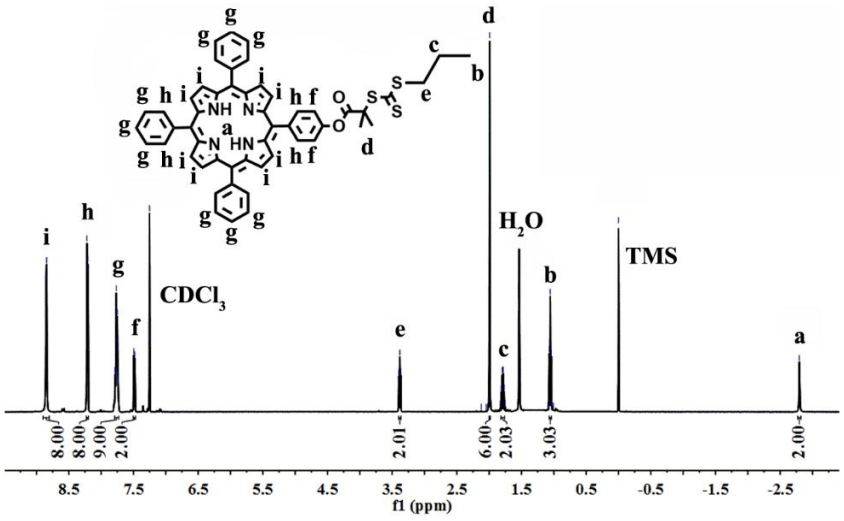
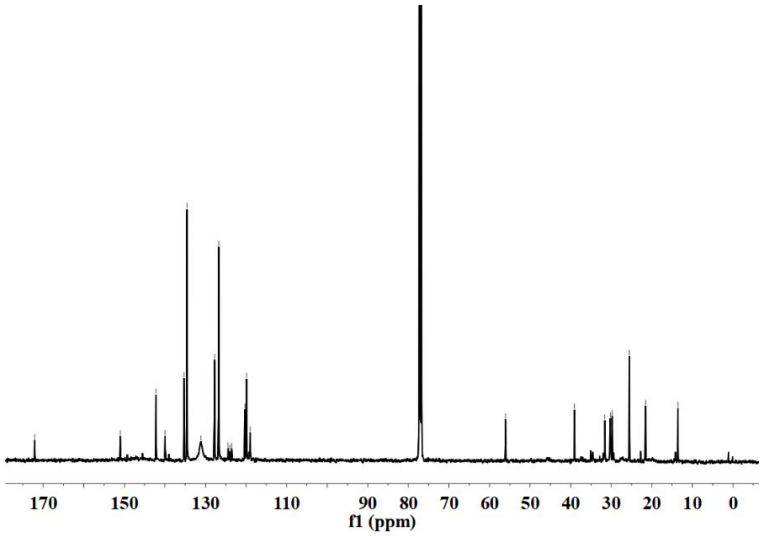


Figure S2. Mass spectrum of **TPP-OH** (m/z 631.24 $[M+H]^+$).



(A)



(B)

Figure S3. ^{1}H NMR (A) and ^{13}C NMR (B) spectrum (in $CDCl_3$) of **TPP-OH-TTC**.

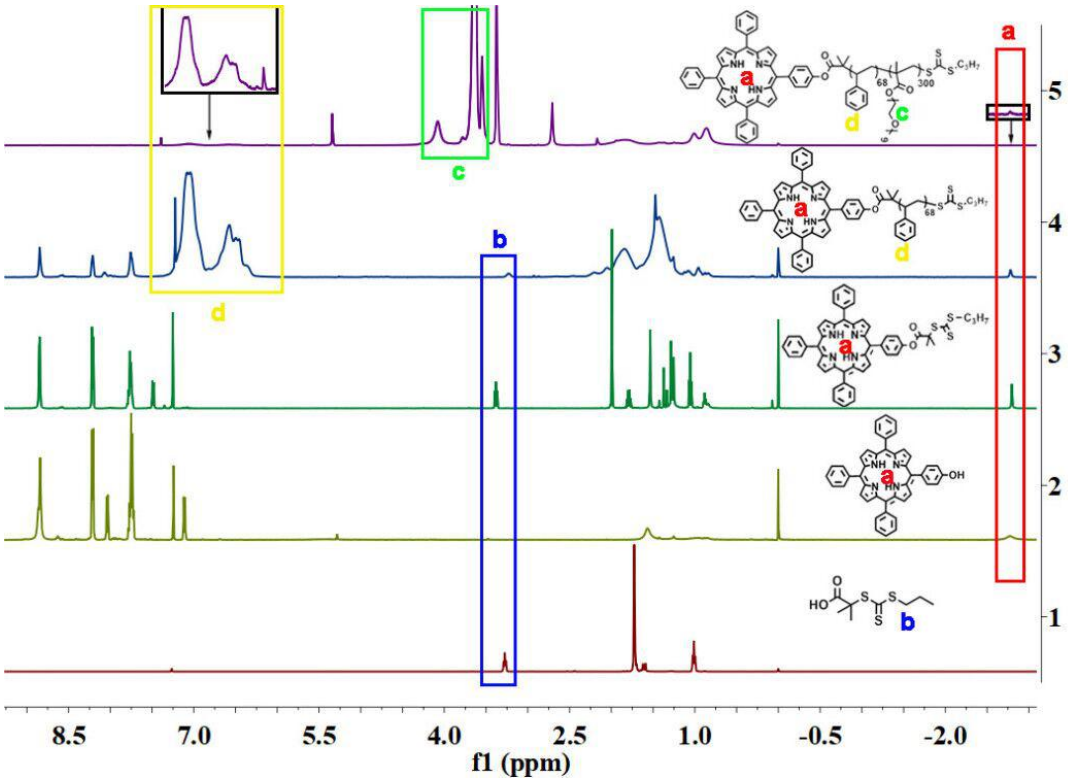


Figure S4. ^{1}H NMR (in $CDCl_3$) of **TTCA (1), TPP-OH (2), TPP-OH-TTC (3), TPP-*b*-PS$_{68}$ (4), TPP-*b*-PS$_{68}$-*b*-PEGMA$_{300}$ (5)**.

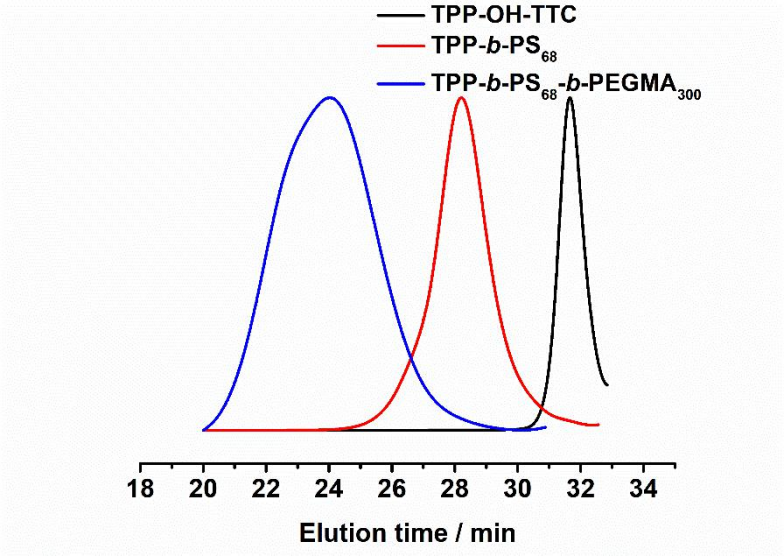


Figure S5. GPC trace of **TPP-OH-TTC**、**TPP-*b*-PS$_{68}$**、**TPP-*b*-PS$_{68}$-*b*-PEGMA$_{300}$**.

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

Sample	Mn, $_{GPC}$ [a]	Mw, $_{GPC}$ [a]	PDI
TPP-OH-TTC	570	600	1.06
TPP-*b*-PS$_{68}$	4600	5800	1.27
TPP-*b*-PS$_{68}$-PEGMA$_{300}$	34000	51600	1.52

[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.

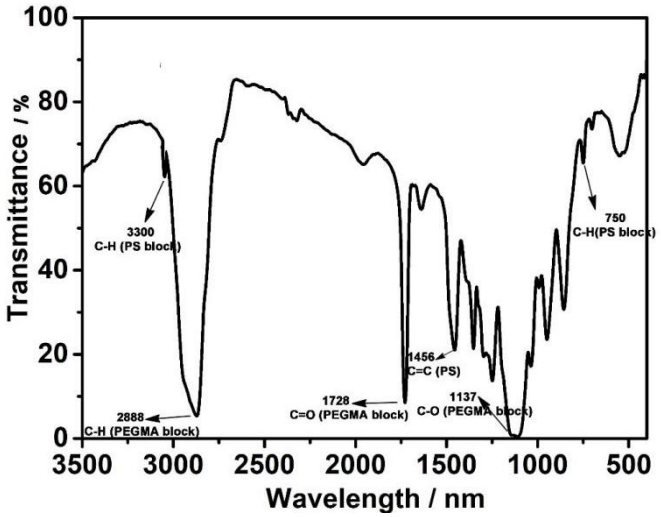


Figure S6. IR spectrum of **TPP-*b*-PS$_{68}$-*b*-PEGMA$_{300}$**.

Note: The IR spectrum of TPP-*b*-PS$_{68}$-*b*-PEGMA$_{300}$ is shown in Fig. S6, the characteristic bands of PS blocks C=C stretching at 1450~1600 cm^{-1}, C=H stretching at 3020~3080 cm^{-1}, C=H bending at 690~754 cm^{-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^{-1} and 1728 cm^{-1} 1137 cm^{-1} indicate the successful polymerization of PEGMA monomer.

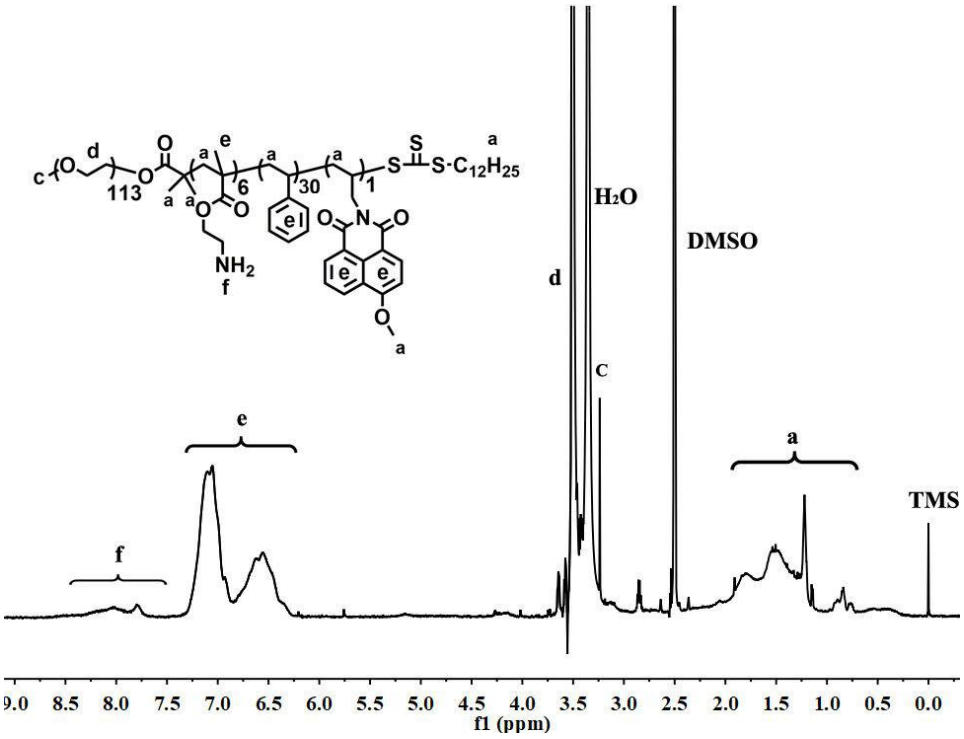


Figure S7. ^{1}H NMR of the **PEO-*b*-P(AEMH$_6$-*co*-EANI$_1$-*co*-St$_{30}$)**.

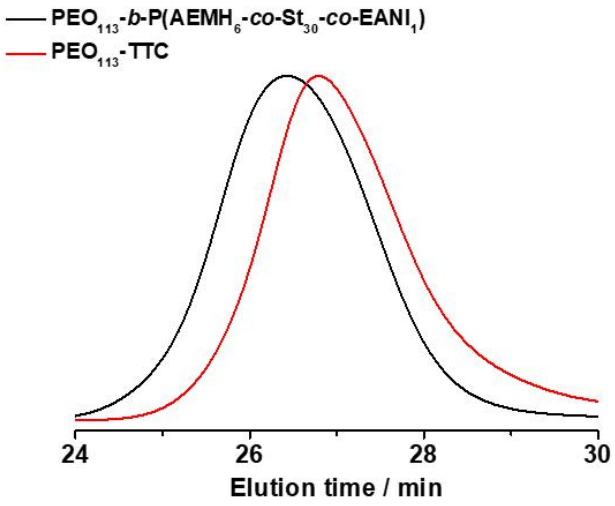


Figure S8. GPC trace of **PEO$_{113}$-*b*-P(AEMH$_6$-*co*-EANI$_1$-co-St$_{30}$)** and **PEO$_{113}$-TTC**.

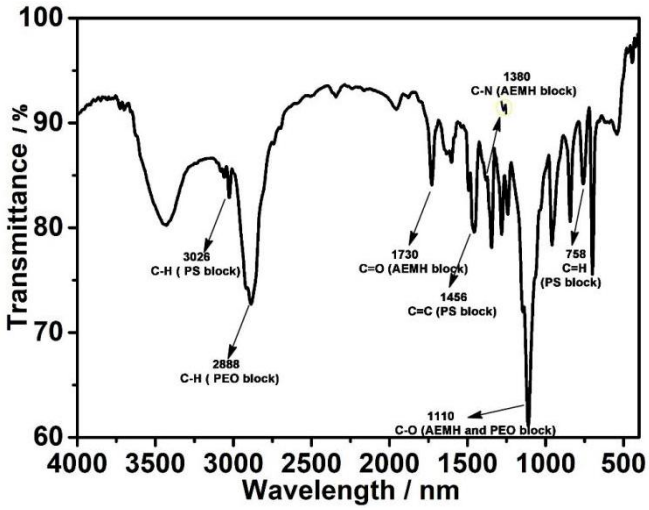


Figure S9. IR spectrum of **PEO_{113}-*b*-P($AEMH_6$-*co*-$EANI_1$-co-St_{30})**.

Note: The IR spectrum of PEO_{113}-*b*-P($AEMH_6$-*co*-$EANI_1$-*co*-St_{30}) is shown in Fig. S9, the characteristic PEO stretching vibration of C-H,C=O and C-O are observed at 2888 cm-1， 1730 cm-1 and 1100 cm-1; the characteristic bands of PS blocks C=C stretching at 1450~1600 cm^{-1}, C=H stretching at 3020~3080 cm^{-1}, C=H bending at 690~754 cm^{-1} indicate the successful polymerization of St monomer; the characteristic bands of AEMH blocks C=O stretching at 1730 cm-1, C-O stretching at 1100 cm^{-1}, C=N bending at 1380 cm^{-1} indicate the successful polymerization of AEMH monomer.

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

Sample	$M_{n,\ GPC}$ [a]	$M_{w,\ GPC}$ [a]	PDI
PEO_{113}-TTC	7800	8200	1.04
PEO_{113}-*b*-P($AEMH_6$-*co*-$EANI_1$-*co*-St_{30})	10100	10800	1.07

[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.

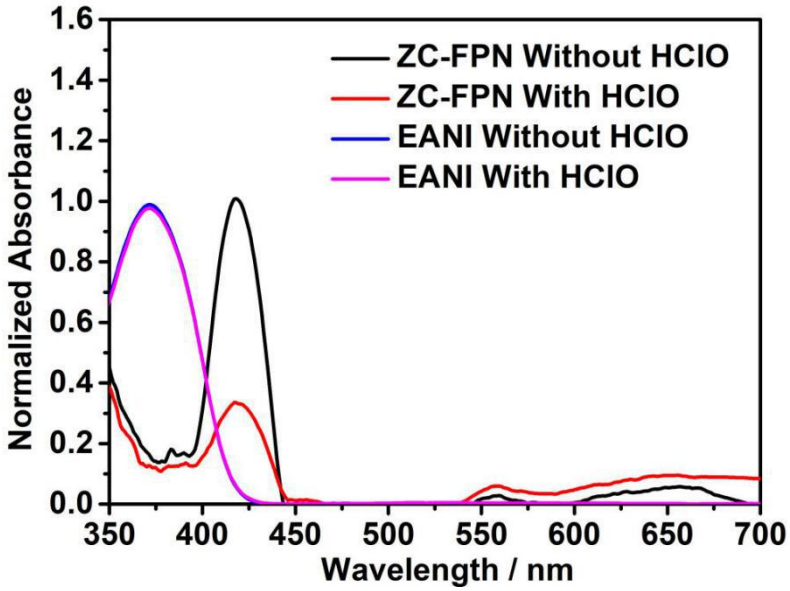


Figure S10. The normalized absorbance spectra of ZC-FPN (48 μg•mL^{-1}) and EANI without and with HClO (160 μM).

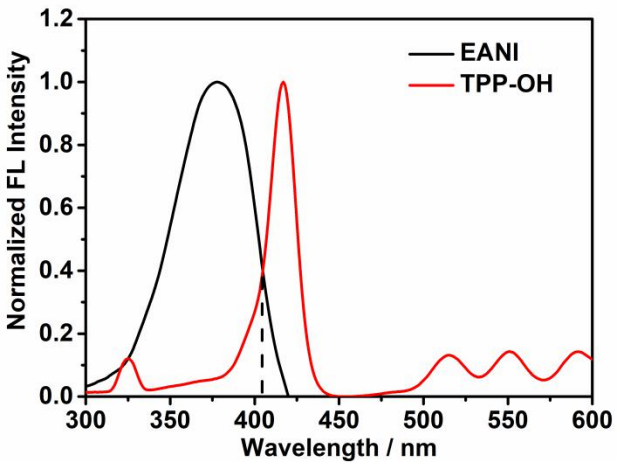


Figure S11. The normalized excitation spectra of EANI (black curve) and TPP-OH (red curve).

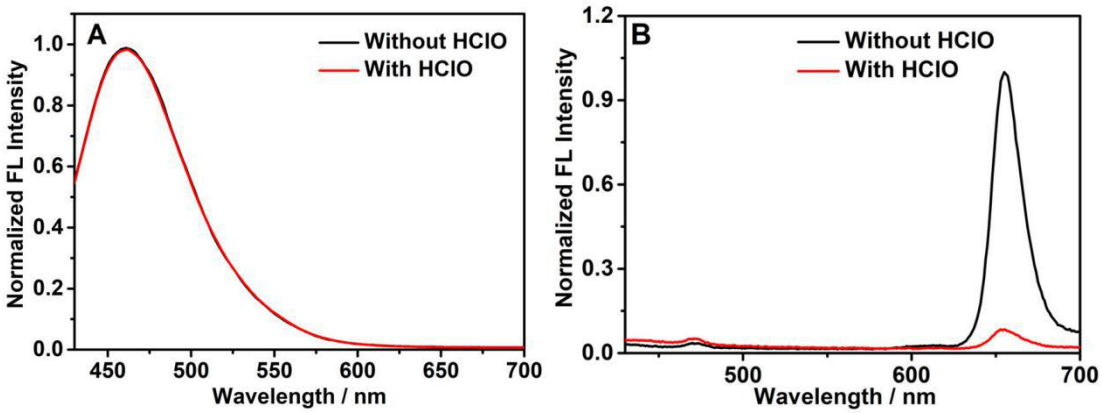
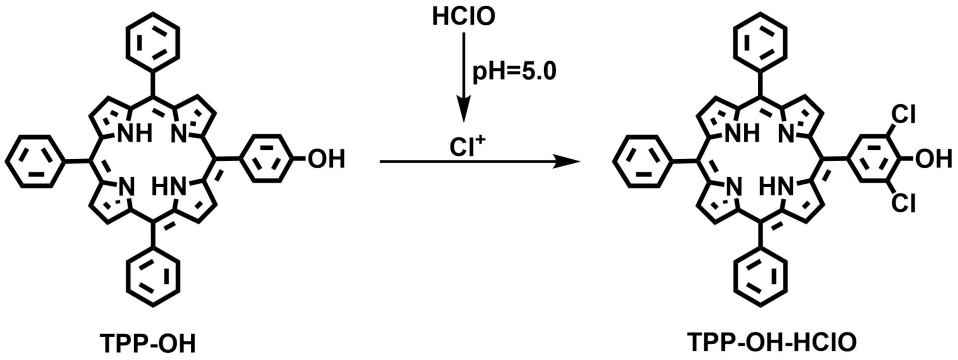


Figure S12. Normalized fluorescence intensity of EANI (A) and TPP-OH (B) without (black curve) and with (red curve) HClO (160 μM).



Scheme S3 Possible reaction mechanism of TPP-OH responding to HClO.

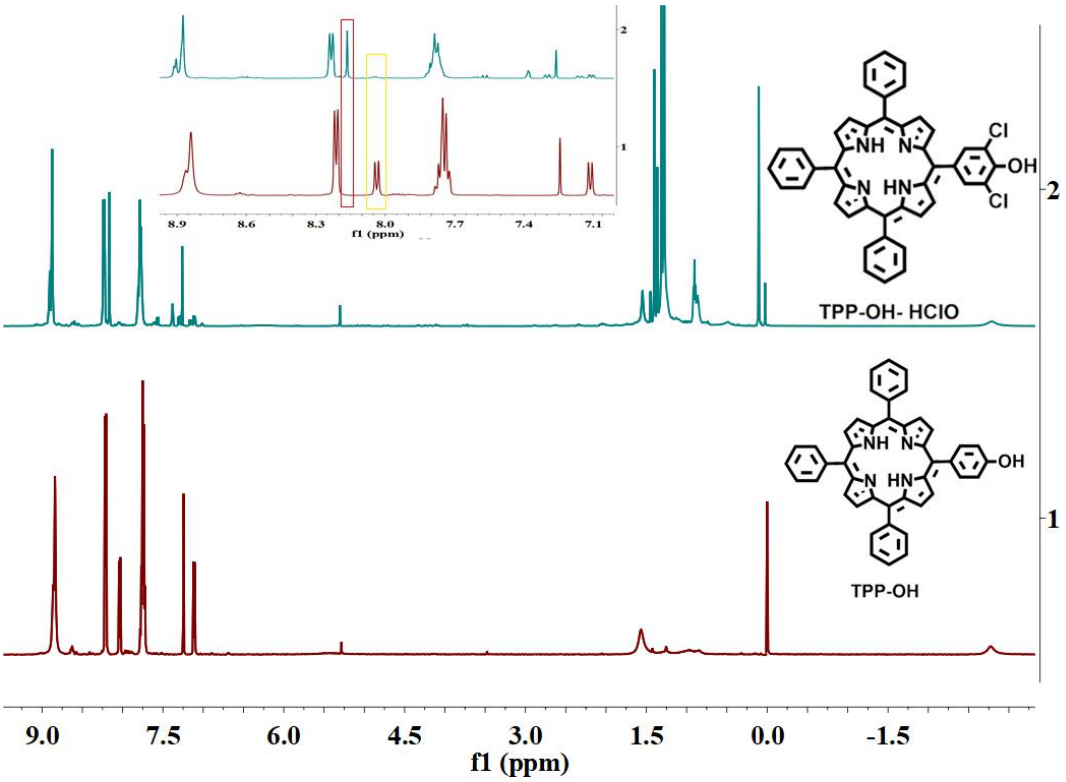


Figure S13. ^{1}H NMR (in $CDCl_3$) of TPP-OH before (up) and after (down) addition of HClO.

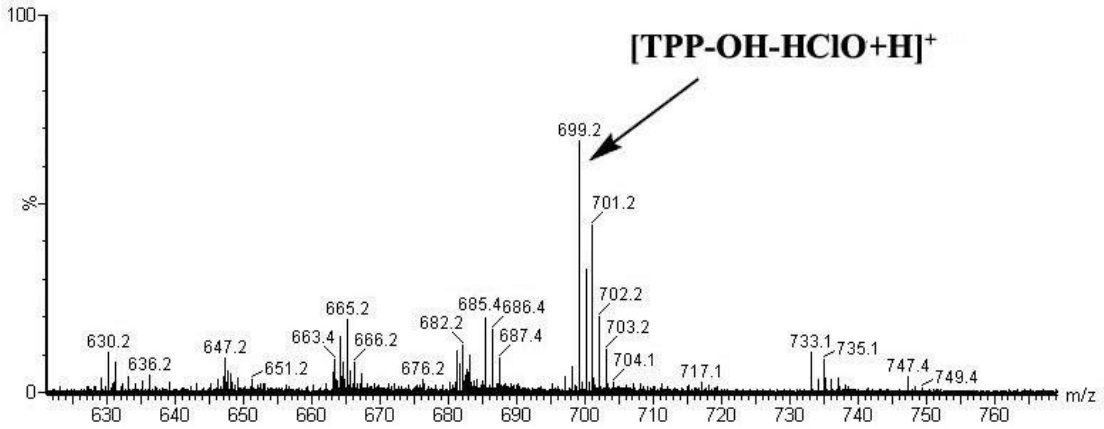


Figure S14. The mass spectrum of **TPP-OH-HClO** (m/z 699.2 $[M+H]^+$).

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 σbi represents the standard deviation for the logarithm of fluorescence intensity ratio $\log[I_{464}/I_{655}]$ of ZC-FPN in the absence of HClO.

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

LOD = $3\times0.0053/0.008=1.988$ μM

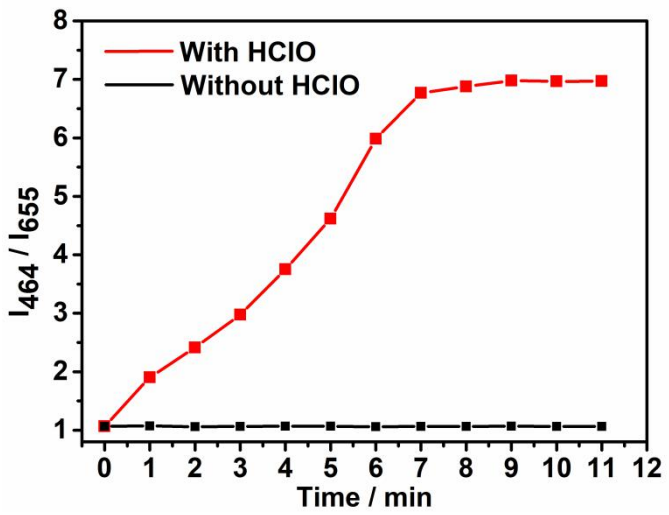


Figure S15. Time course of the ratiometric fluorescence intensity (I_{464}/I_{655}) of ZC-FPN (48 $\mu g \cdot mL^{-1}$) without and with HClO (160 μM)

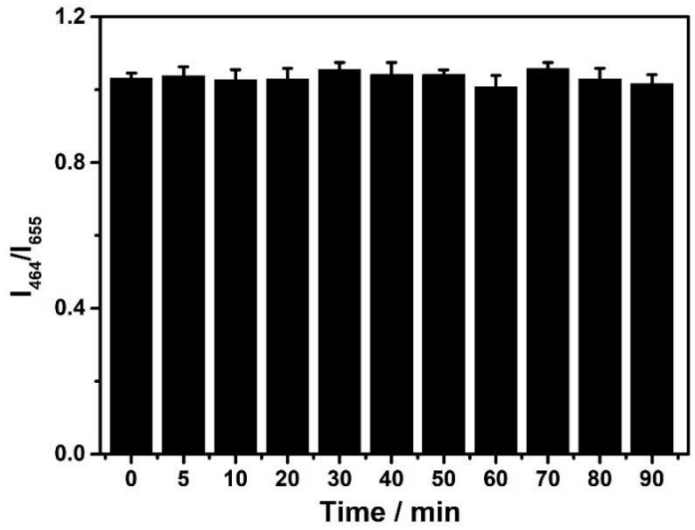


Figure S16. Fluorescence intensity changes (I_{464}/I_{655}) of ZC-FPN (48 μg·mL^{-1}) under a continuous 365 nm UV lamp irradiation (2.8 mW·cm^{-2}).

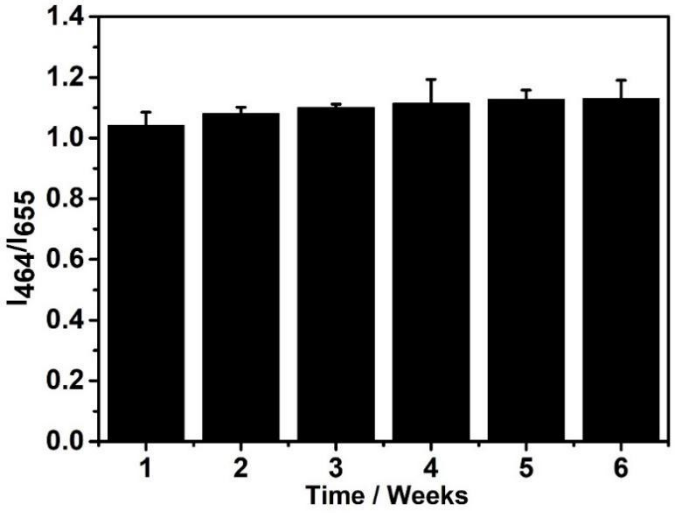


Figure S17. Fluorescence long-term photostability of ZC-FPN (48 μg·mL^{-1}) under ambient temperature and kept in the dark.

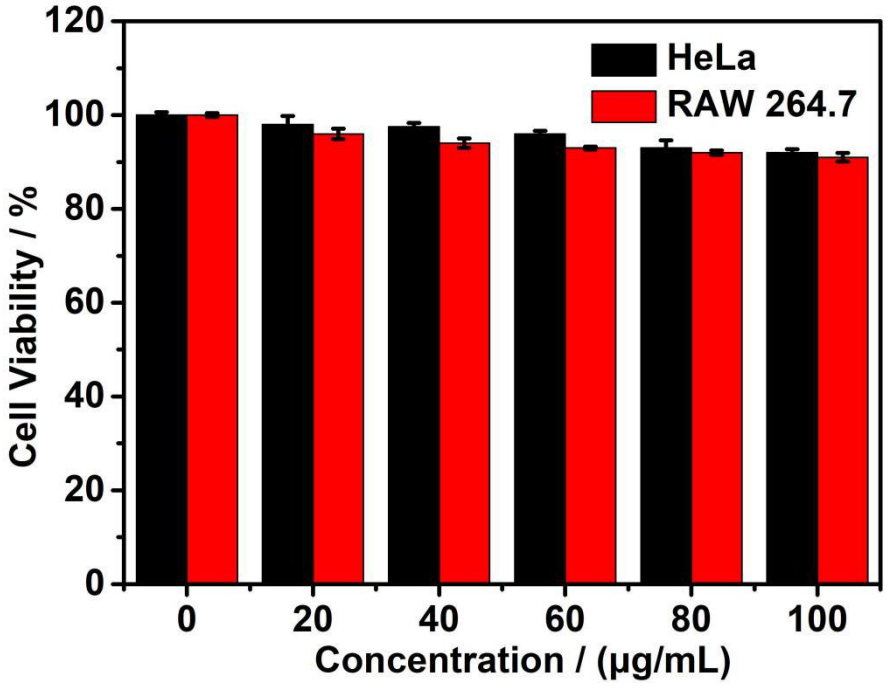


Figure S18. Viability for HeLa cells and RAW264.7 cells treated with the varied concentrations of ZC-FPN for 24 h. The results are the mean standard deviation of eight separate measurements.

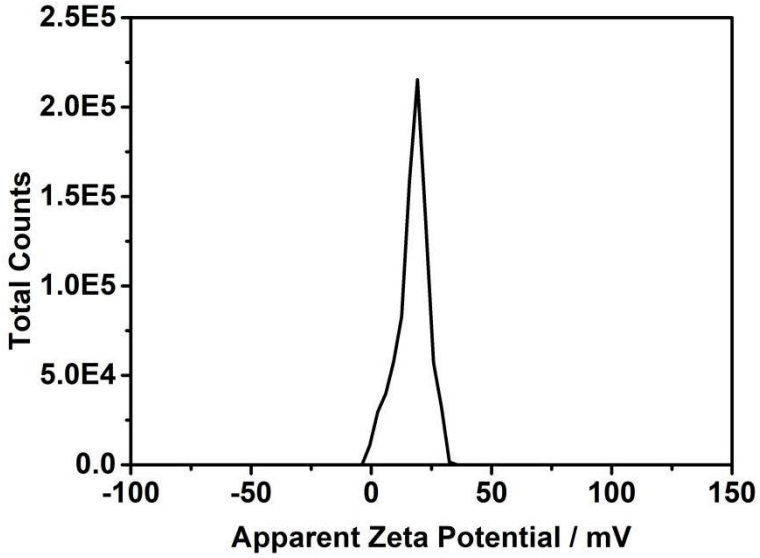


Figure S19. The zeta potential of ZC-FPN.

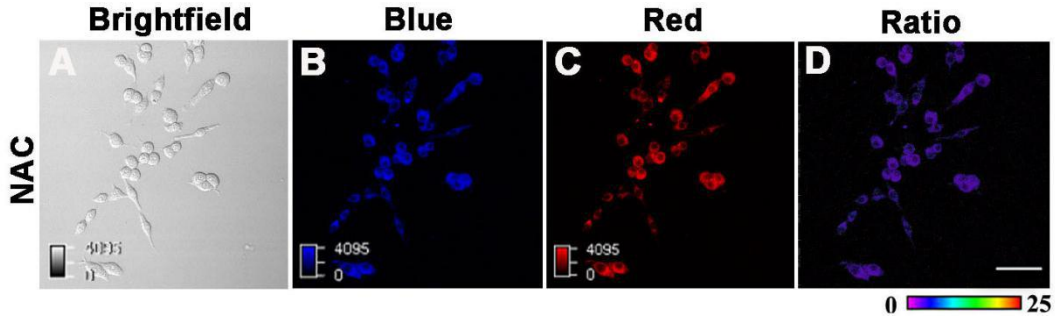


Fig. S20. Confocal fluorescence microscopy images of RAW264.7 macrophage cells incubated with NAC (2 mM) and ZC-FPN (48 μg·mL^{-1}). λ_{ex} = 405 nm, λ_{em1} = 440-500 nm (blue channel), and λ_{em2} = 640-670 nm (red channel). Scale bar: 20 μm.

Table S2. Comparison of the some recently reported ratiometric fluorescent probes for HClO

Probe	Solution	Emission shift (nm)	Targeting	References
XWJ	PBS (pH 7.4, 50% DMF)	120 nm	none	Sens. Actuators B, 2018, 263, 252-257
Lyso-HA	PBS (pH 5.0, 30% DMSO)	136 nm	lysosomal	J. Mater. Chem. B, 2016, 4, 4739-4745
Probe 4	PBS (pH 7.4, 10% CH_3CN)	90 nm	none	Anal. Chem., 2018, 90, 12937-12943.
FPD	PBS (pH 5.0)	94 nm	lysosomal	Polym. Chem., 2017, 8, 5795-5802.
Probe	PBS (pH 7.4, 10% CH_3CN)	90 nm	none	Anal. Chem., 2018, 90, 12937-12943.
QClO	PBS (pH 7.4, 5% DMF)	70 nm	none	Chem. Sci., 2018, 9, 6035-6040
Probe 1	PBS (pH 7.4, 50% EtOH).	57 nm	lysosomal	Spectroc. Acta Pt. A-Molec.Biomolec.Spectr., 2019, 223, 117334.
ZPAC	PBS (pH 7.4, [CTAB] = 5×10^{-4} M)	128 nm	none	Talanta, 2019, 201, 330-334.
Zcp-Me	PBS (pH 7.4, 5% EtOH)	123 nm	mitochondria	Sens. Actuators B, 2018, 276, 8-12.
RNL	B-R (pH 4.5 and 7.2, EtOH 10%)	51 nm	lysosome	J. Mater. Chem. B, 2015, 3, 3260-3266.
BRClO	PBS (pH 7.4, EtOH 10%)	90 nm	none	Ind. Eng. Chem. Res., 2015, 54, 8842-8846.
PM	PBS (pH 5.5)	107 nm	liver	Biosens. Bioelectron, 2018, 99, 318-324.
NR	PBS (pH 5.0 70% EtOH)	50 nm	lysosome	Sens. Actuators B, 2017, 244, 907-913.
BODIPY-P	PBS (pH 7.4)	62 nm	mitochondria	Sens. Actuators B,2019, 299, 126937
ZOC	PBS (pH 7.0 40% EtOH)	132 nm	lysosome and lipid	Anal. Chim. Acta., 2019, 1064, 87-93.
Probe	PB (pH 7.0 20% EtOH)	60 nm	none	Talanta 2017, 170, 496-501.
TPA-ClO	PBS (pH 7.4 50% DMF)	60 nm	liver	Chem. Commun., 2019, 55, 12912-12915.
This work	**PBS (pH 5.0)**	**191 nm**	**lysosome**	