Supporting Information

Combining Fluorogenic ZnSalen and Myeloperoxidase (MPO) to Detect Lysosomal Hydrogen Peroxide in Live Cells

Jing Jing[1] and Jun-Long Zhang[*,1]

[1] Beijing National Laboratory for Molecular Sciences, State Key Laboratory of Rare Earth Materials Chemistry and Applications, College of Chemistry and Molecular Engineering, Peking University, Beijing, P.R. China

E-mail: zhangjunlong@pku.edu.cn

Content

1. Experimental methods

1.1 General information

All solvents and chemicals were purchased from Alfa Aesar or J&K and used as received without further purification. Doubly distilled water was used for all experiments. Oxidases like Myeloperoxidase (MPO, M6908-5UN, from human leukocytes), horse radish peroxidase (HRP), catalase, lacase and lactoperoxidase (LPO) were purchased from SIGMA. Cellular imaging trackers were purchased from Invitrogen (Life Technologies). The absorbance were obtained with an Agilent 8453 UV-*vis* spectrophotometer in 1cm path length quartz cells and the pH values were determined by using a DELTA 320 PH dollar. Single-photon luminescence spectra were recorded using fluorescence lifetime and steady state spectrophotometer (Edinburgh Instrument FLS920). Confocal fluorescent images of living cells were performed using Nikon A1R-si Laser Scanning Confocal Microscope (Japan), equipped with lasers of 405/488/543/638 nm.

1.2 Generation of ROS

Various ROS and RNS including H_2O_2, OCl^-, ^{t}BuOOH, O_2^-, $^{\cdot}OH$, $ROO^{\cdot}$, and ONO_2^- were prepared according to the following methods.

OCl^-:

0.25 μL of 8 M NaOCl (for a final stored concentration of 2 mM) was added to 1000 μL of 50 mM PBS buffer at pH 7.4.

H_2O_2:

A 30% H_2O_2 solution was quantified by measuring the UV absorbance at 240 nm. 20 μL of 1 M H_2O_2 (for a final stored concentration of 20 mM) was added to 980 μL of 50mM PBS buffer at pH 7.4. 200 μL of 1 M H_2O_2 (for a final stored concentration of 200 mM) was added to 800 μL of 50mM PBS suffer at pH 7.4.

^{t}BuOOH:

A 70% *t*BuOOH in H_2O solution was diluted for a final concentration of 200 μM.

O_2^-:

0.03mg KO_2 was dissolved in DMSO for a final concentration of 200 μM.

OH•:

2 mL PBS buffer at pH 7.4 was degassed for about 15 minutes by bubbling N_2. Ammonium iron (II) sulfate (10 mM) was added and the solution was degassed for 30 min by bubbling N_2. 20 μL of 20 mM H_2O_2 was added (for a final concentration of 200 μM).

***t*BuO•:**

2 mL PBS buffer at pH 7.4 was degassed for about 15 minutes by bubbling N_2. Ammonium iron (II) sulfate (10 mM) was added and the solution was degassed for 30 min by bubbling N_2. A 70% ^{t}BuOOH in H_2O solution was diluted and then added for a final concentration of 200 μM.

ONO_2^-:

Solutions of $NaNO_2$ (0.6 M), H_2O_2 (0.6 M) acidified by HCl (0.7 M), and NaOH (0.6 M) were cooled in an ice bath for 15-30 min. The $NaNO_2$ solution (10 ml) was stirred rapidly in an ice bath, and to this solution was added the acidified H_2O_2 solution (10 ml), followed in rapid succession by the NaOH solution (10 ml). A yellow solution rapidly formed and decomposed to a colorless solution. Final

product was generated by addition of the NaOH solution as quick as possible. The concentration of ONO_2^- was determined by measuring the UV absorbance of the solution at 302 nm (ε=1.670*10^3 M cm^{-1}). The ONO_2^- solutions were stored at -20°C before use.

1.3 Kinetic assay

Reaction activities were measured at 25°C in 50 mM sodium phosphate buffer (pH 5.0). Steady-state kinetic constants were obtained by measuring the initial velocity while varying the substrates concentration. A Hanes plot of [s]/v versus [s] was used to estimate the V_{max} and K_m values for the activation. The reaction rate was determined from the increase in the fluorescence at 610 nm. The 2 mL final assay volume contained 1 nM MPO, 1 μM **J-S** and variable amounts of H_2O_2 (0.1 μM to 20 μM). 1μM of **J-S** and Various concentrations of H_2O_2 were dissolved in 2 mL total volume of 50 mM PBS buffer, pH 5.0, containing 1% DMSO as co-solvent. MPO (0.01U) was added to the solution, and the fluorescence intensity was recorded continuously as described above. Initial reaction velocity was calculated plotted against H_2O_2 concentration, plotted against probe concentration, and fitted to a Michaelie Menten curve (Figure S6). The kinetic parameters were calculated by use of the Michaelis-Menten equation shown below:

$$V = V_{max} * [S]/(K_m + [S])$$

1.4 Cell culture

All HeLa cells were incubated in complete medium (Dulbecco's modified Eagle's Medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin) at 37 °C in atmosphere containing 5% CO_2.

1.5 Cell imaging

For imaging, HeLa cells were grown in poly-D-lysine-coated dishes and incubated in 2mL of complete medium for 24 h. Cells were washed with PBS, and stocked dyes (2 mM in DMSO, for a final concentration of 5 μM) and MPO (0.01U/mL) were added to obtain a final concentration of 5 μM. The treated cells were incubated for 24 hours in dark at 37 °C. Then A few minutes prior to confocal imaging cells were washed twice with PBS. A confocal laser scanning microscope (A1R-si, Nikon, Japan) was used to obtain images. Cells were imaged via the fluorescence mode with a 60× immersion lens with the following parameters: laser power 50%, pinhole 1.0 A.U., excitation wavelength 543 nm, detector slit 552-617 nm, resolution 1024×1024, and a scan speed 0.5 frame per second. For endogenous H_2O_2 generation, HeLa cells were incubated with PMA (1 mg mL^{-1}) for an hour.

1.6 Colocalization assay

A stock solution of **J-S** in chromatographic grade, anhydrous DMSO was prepared as 2 mM. The solution was diluted to a final concentration of 5 μM by complete growth medium. Stock solutions of trackers were prepared as 1mM, and the stock solution was diluted to the working concentrations in complete medium (Lyso Tracker: 75 nM, Mito Tracker: 100 nM, Calcein AM: 1 μM, Hoechst 33528: 1 μM). Transfection with LAMP2-EGFP, EHD1-EGFP, and FYVE-EGFP plasmids: Hela cells were grown to about 80% confluency and then reseeded in 24-well plates; cells were transfected with 0.8 μg plasmid, by using LipfectamineTM 2000 according to manufacturer's instruction. HeLa cells were placed onto 0.1 mM poly-D-lysine coated glasses in complete media and the cells were incubated for 24

h. Then MPO (0.01 U/mL) and **J-S** (5 μM) were incubated for 24 hours. After incubation with 20 μM H_2O_2 for half an hour, cells were washed with PBS buffer twice before confocal experiments. Images were taken under conditions as follows: 60× immersion lens with a resolution of 1024×1024 and a speed of 0.5 frame per second, 543 nm excitation wavelength and 552 to 617 nm detector slit, 80% laser power for **J-S**, and 50% laser power for LysoTracker (ex: 488 nm, em: 505-560 nm), MitoTracker (ex: 488 nm, em: 505-560 nm), Calcein AM (ex: 488 nm, em: 505-560 nm), Hoechst 33528 (ex: 405nm, em: 425-460 nm) and EGFP-LAMP2, EHD1-EGFP, and FYVE-EGFP (ex: 488 nm, em: 505-560 nm). Differential interference contrast (DIC) and fluorescent images were processed and analyzed using ImageJ. The Pearson's correlation coefficient, overlap coefficient, ICA plot and ICQ values were calculated by ImageJ with colocalisation analysis plugins.

1.7 Two photon confocal microscopy imaging

Two photon fluorescence microscopy images were performed on a modified Olympus Fluoview FV1000MPE microscope system equipped with an excitation light laser provided by a modelocked Ti: sapphire laser, (Mai Tai, Spectra-Physics Inc., USA). The microscopy settings were as follows: 60× immersion water objective, a resolution of 512×512, 840 nm excitation wavelength and 570 to 620 nm detector slit. HeLa were treated with MPO-**J**-**S** for 12 h and then H_2O_2 for 30 min, and washed with prewarmed PBS buffer before imaging. The quantified fluorescence intensities were calculated by ImageJ, as well as the reported standard deviations.

2. Docking experiments

Preparation of receptor and ligand:

The coordinates deposited in the Protein Data Bank (PDB code: 1CXP) was used as the target structure to endeavor the docking studies. All the X-ray water molecules in the catalytic site of MPO have been removed, and all hydrogens were added. The whole structure was energy minimized by AutoDockTools version 1.5.4 during docking. Ligand **J-S** and **J-SO** was optimized by Gaussian 09 package, with DFT/B3LYP method. Basis set of atoms C, H, O, N, and S was 6-31G (d). Basis set of atom Zn was LANL2DZ. Then the final protein and ligand structure were used for docking experiment directly.

Molecular docking:

For docking compound **J-S** and **J-SO** into MPO, the program AutoDock version 4.2 was used. Only one monomer was considered in our docking. The grids for MPO had dimensions of 28 Å ×32 Å ×26 Å, with the grid spacing of 0.375 Å. The grid box contained most part of the monomer, involving the catalytic site of MPO. As **J-S** and **J-SO** was a rigid molecular, both ligand and protein were considered rigid part during the docking program. Lamarckian genetic algorithm was used for calculation with 100 runs to give 100 individuals. The number of individuals in population was set as 150, maximum number of energy evaluations was 2500000, and maximum of generations was 27000. All other parameters were used as default values. The results rms tolerance was 2.0 Å.

3. Supplementary table

Table S1. Enzymic kinetic parameters.

[NaCl]/ mM	$[H_2O_2]$/ μM	k/min^{-1}	[V]/*10^{-9}Mmin^{-1}
50	0.1	9.28	9.28
	0.2	14.10	14.10
	0.3	18.91	18.91
	0.4	21.02	21.02
	0.5	22.45	22.45
	0.6	24.57	24.57
	0.7	25.16	25.16
	0.8	26.93	26.93
	0.9	30.55	30.55
	1.0	31.73	31.73
V_{max}	$3.6 * 10^{-8}$ Mmin^{-1}		
K_m	0.29 μM		
k_{cat}	36 min^{-1}		
k_{cat}/K_m	2.06 $M^{-1}min^{-1}$		

4. Supplementary figures

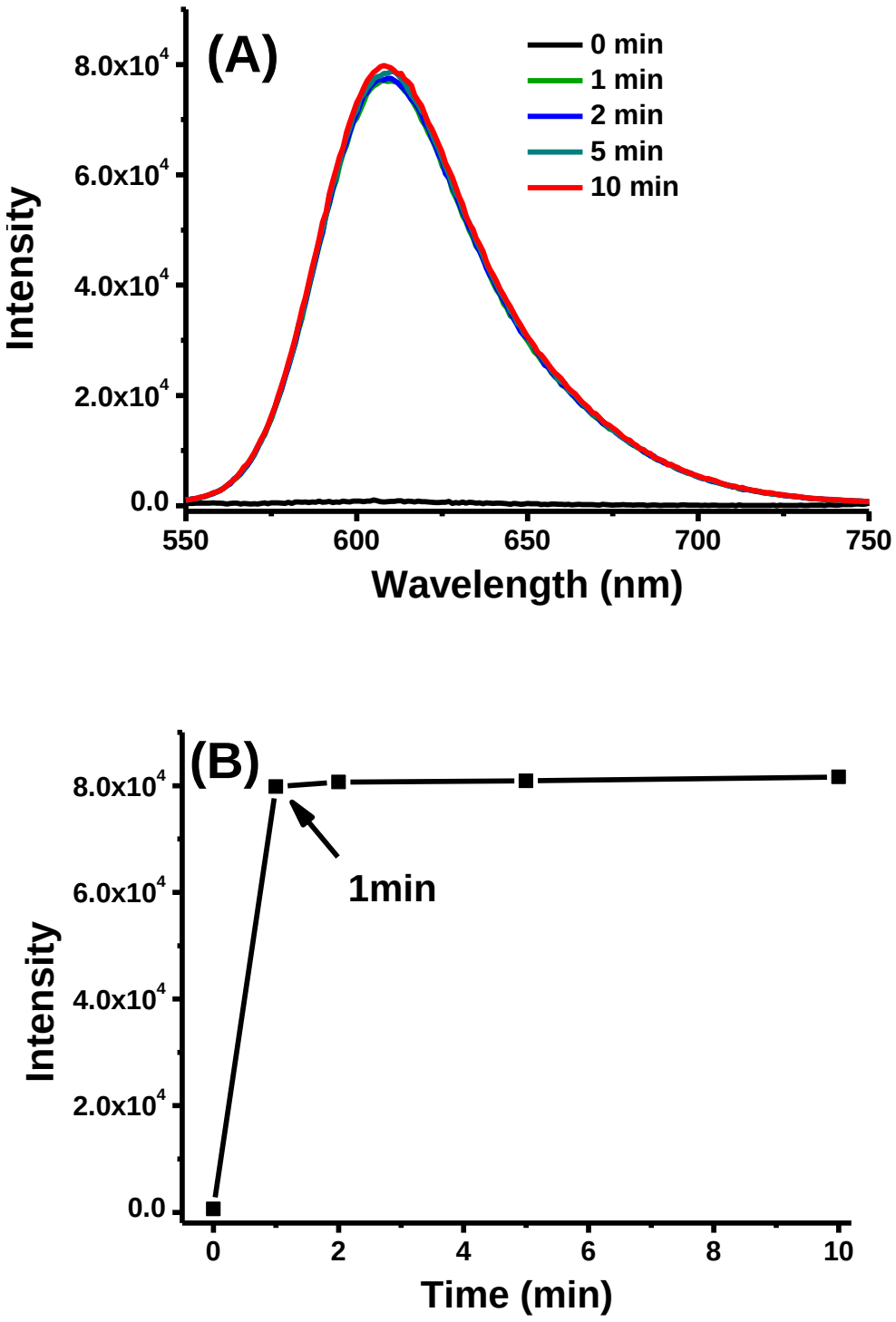

Figure S1. Fluorescence response of **J-S** to NaClO. (A) Fluorescence spectra of **J-S** (final concentration: 10 μM) reacting with NaOCl (20μM). (B) Fluorescence intensity (E_x: 380 nm, E_m:610 nm) plotted against the reaction time.

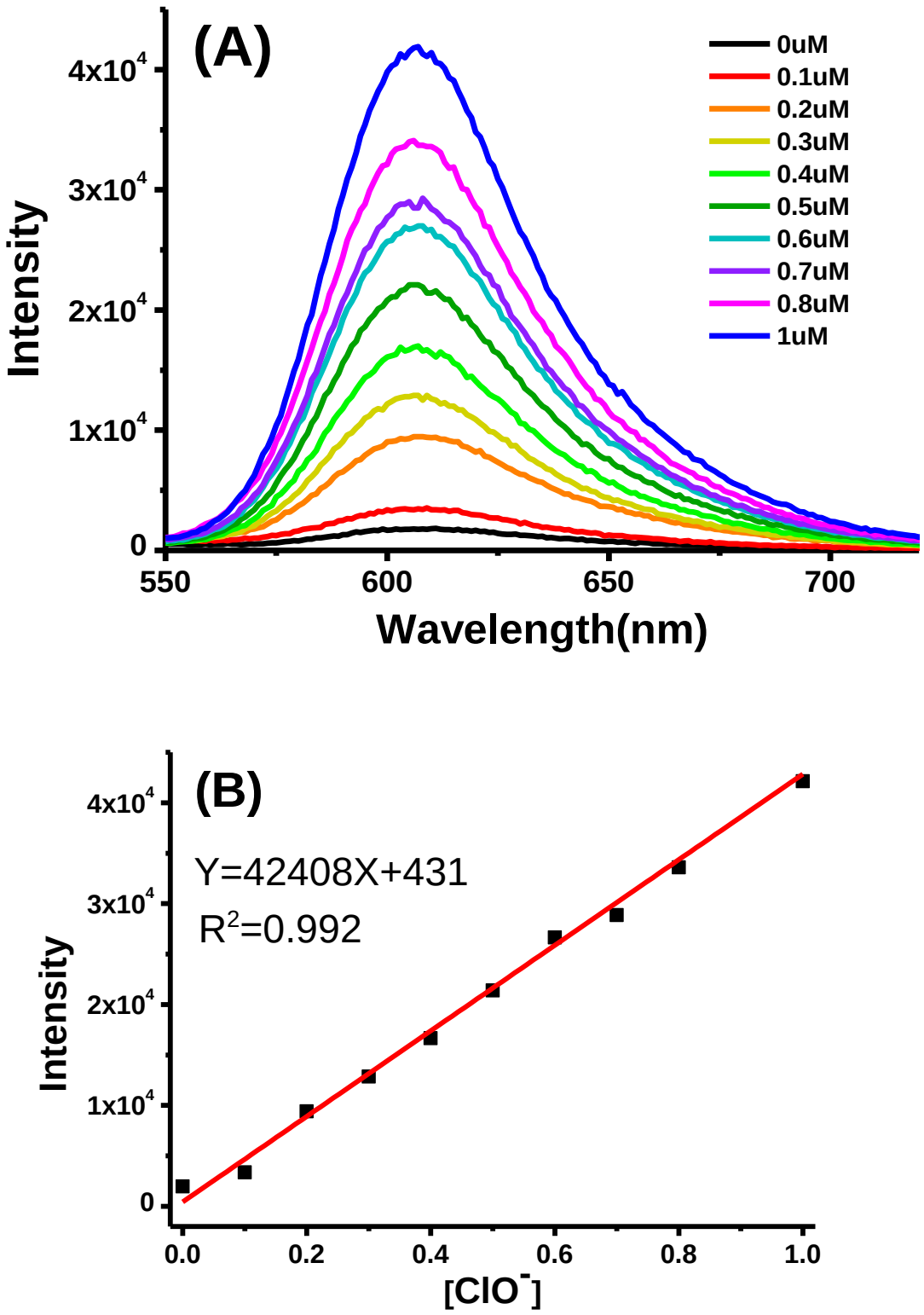

Figure S2. The limit of detection for NaClO. (A) Fluorescence spectra of **J-S** (final concentration: 1μM) recorded 1 min after the addition of NaOCl (ranging from 0 to 1 μM). The fluorescence intensity was determined with excitation at 380 nm. (B) Fluorescence intensity (λ_{ex}: 380 nm, λ_{em}: 610 nm) plotted against the concentration of NaOCl. The calculated detection limit is 49 nM.

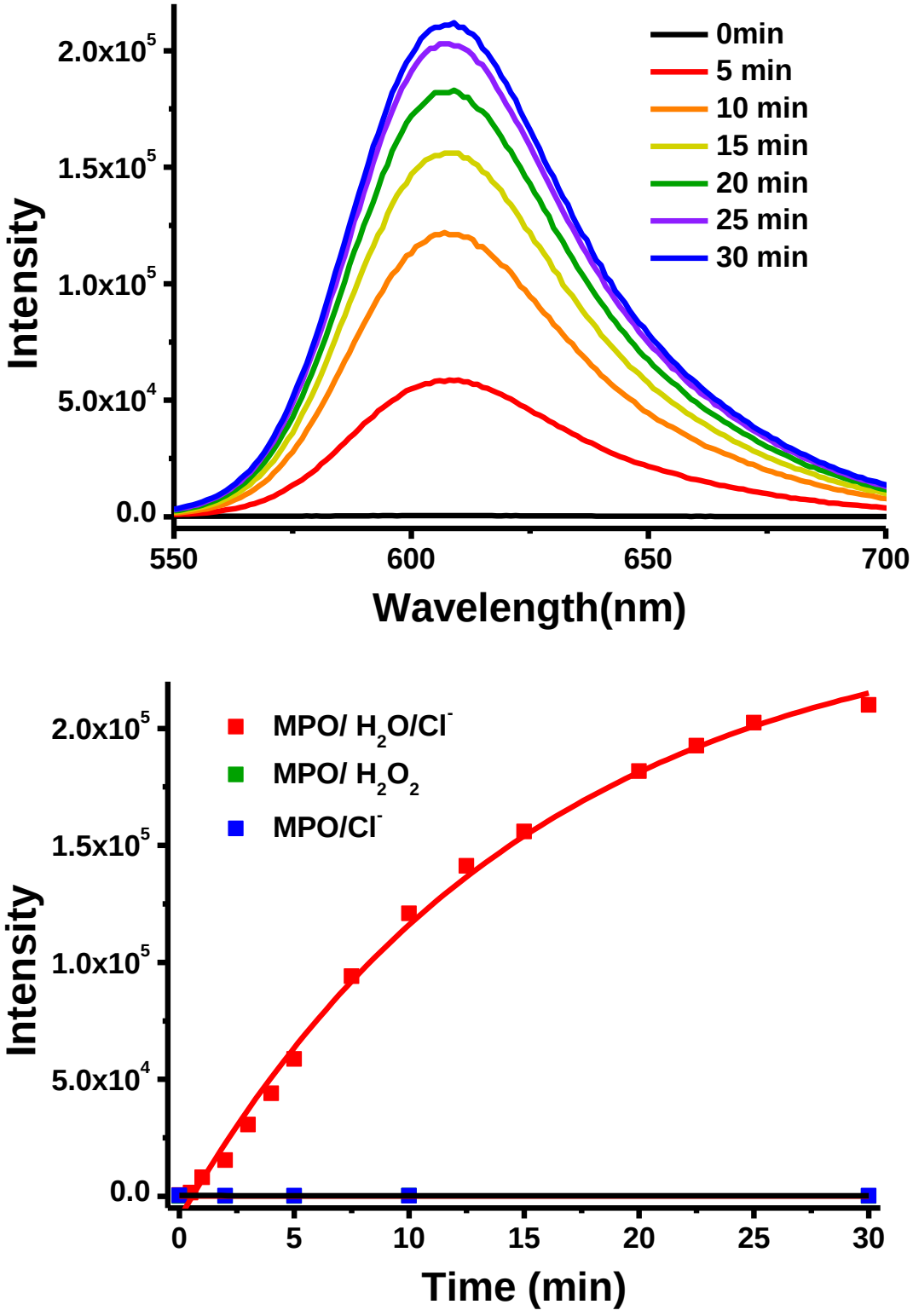

Figure S3. Application of "MPO-**J**-**S**" to detect H_2O_2. Data shown are for 10 μM for **J**-**S**, 0.01 U/mL for MPO, 150 mM for Cl^-, and 20 μM for H_2O_2 in PBS buffer pH 5.0 at 37 ^oC. (A) Fluorescence changes of "MPO-**J**-**S**" with the addition of H_2O_2 within 30 minutes. (B) Time course of fluorescence intensity at 610 nm in the presence or absence of H_2O_2 or Cl^- (λ_{ex}: 380 nm, λ_{ex}: 610 nm).

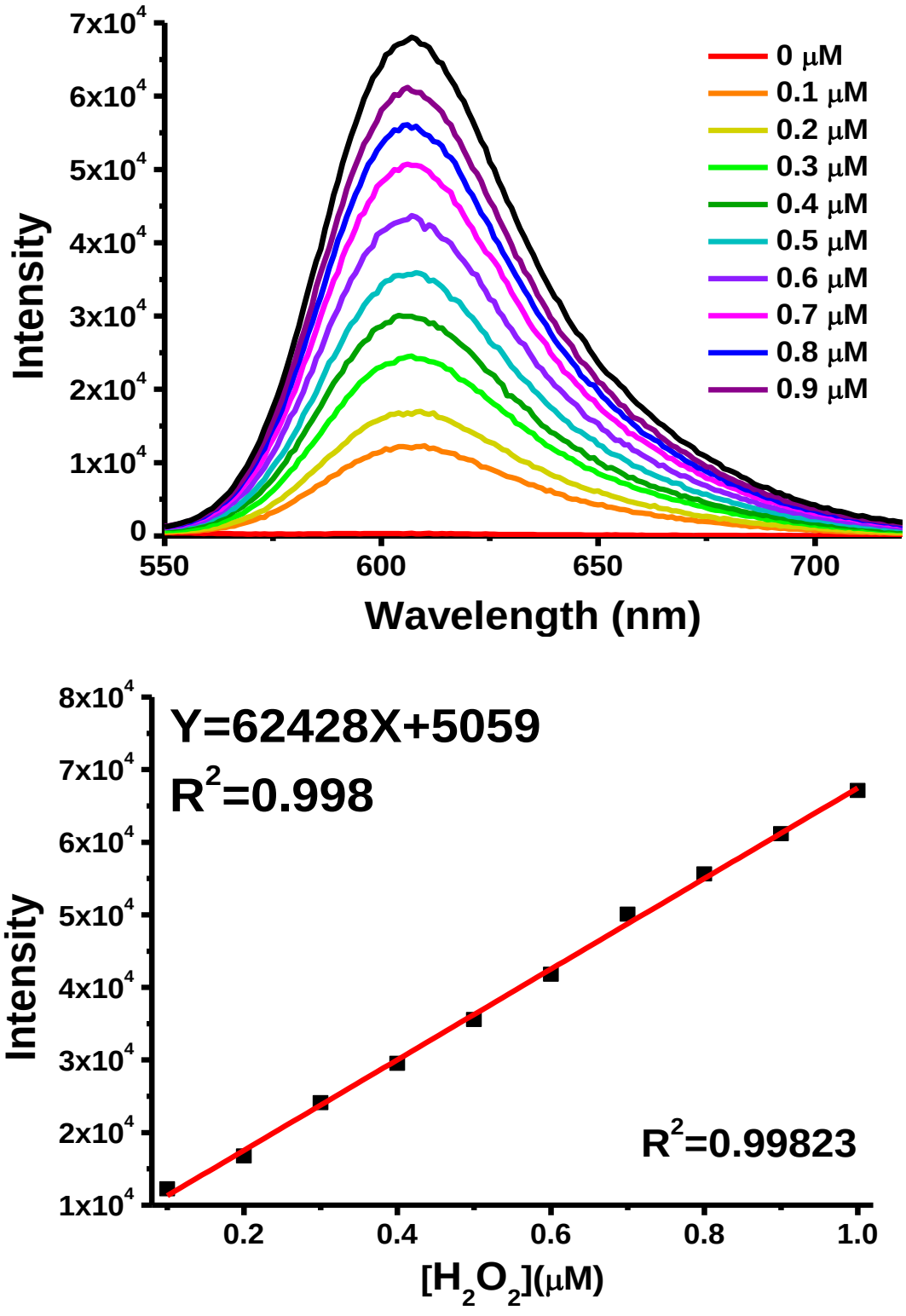

Figure S4. The limit of detection of "MPO-**J**-**S**" for H_2O_2. (A) Fluorescence spectra of **J**-**S** (final concentration: 10 μM) in recorded 30 min after the addition of H_2O_2 (ranging from 0 to 1μM). The fluorescence spectra were recorded with excitation at 380 nm. (B) Fluorescence intensity (λ_{ex}: 380nm, λ_{ex}: 610 nm) plotted against the concentration of H_2O_2.

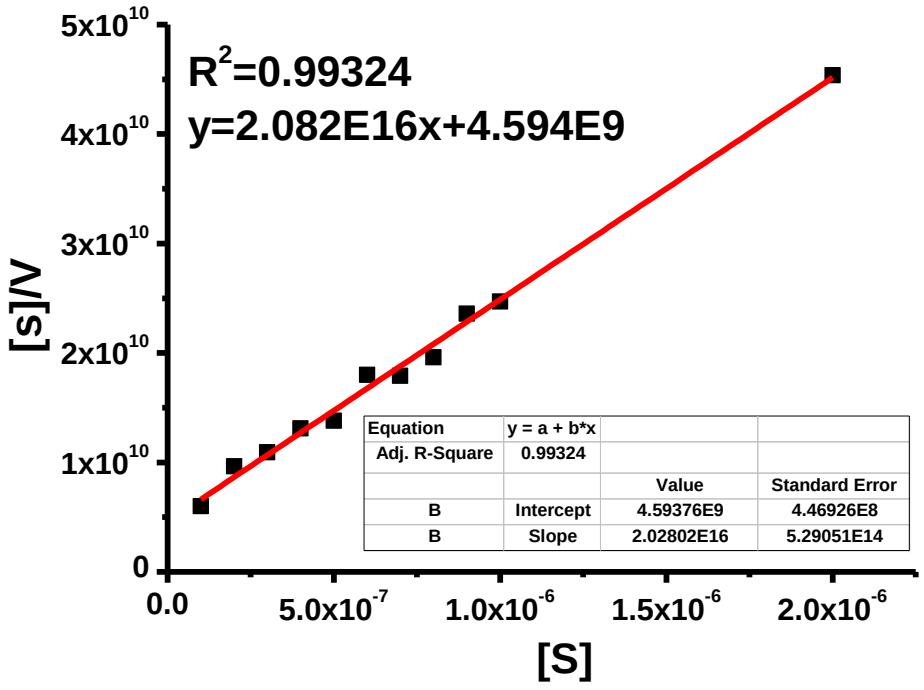

Figure S5. Plotted Michaelis Menten curve.

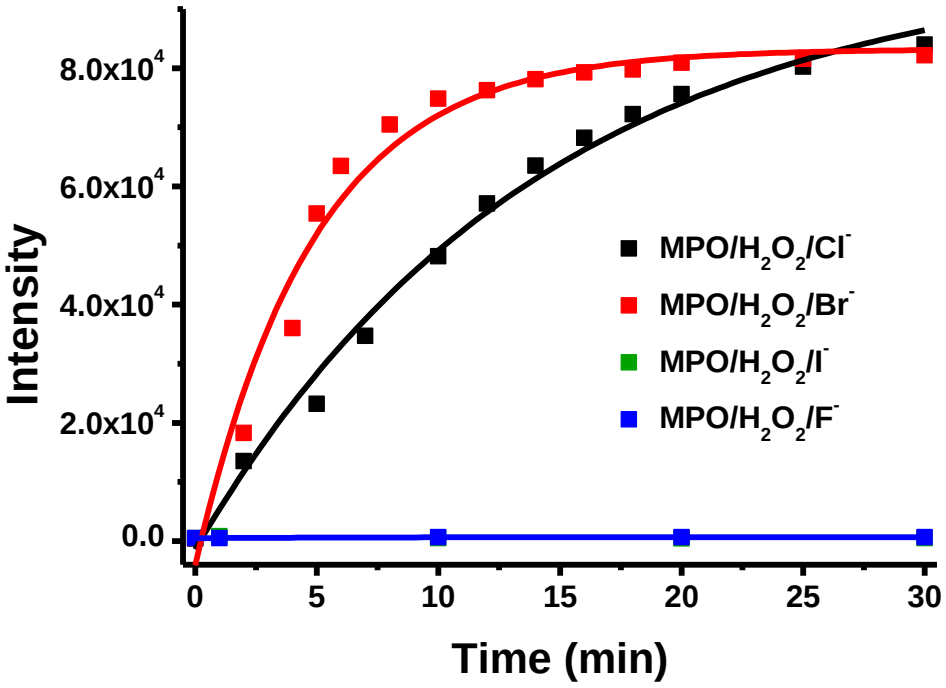

Figure S6. H_2O_2 detection in the presence of different halides. H_2O_2 was added to a solution of **J-S** (10 μM)/ MPO (0.01 U/mL) in the presence of different halide ions (150 mM), including Cl^-, Br^-, I^-, F^-. (λ_{ex}: 380 nm, λ_{em}: 610 nm) at pH 5.0.

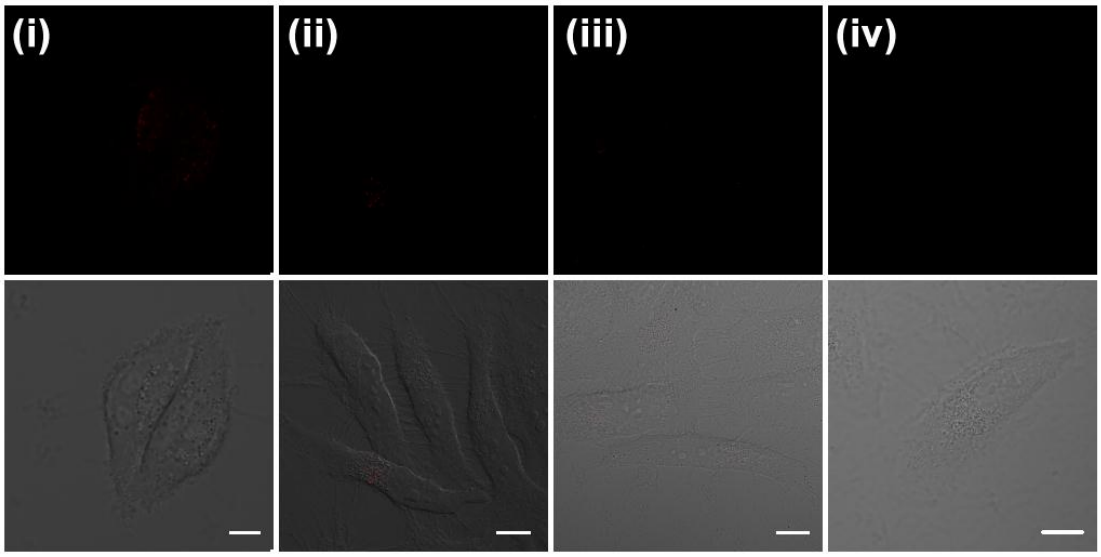

Figure S7. Confocal images of living HeLa cells loaded with **J-S** under different stimulants. HeLa cells were treated with **J-S** (5 μM) and MPO (0.01 U/mL) for 12 hours at 37 ^{o}C, and then incubated with 20 μM H_2O_2 for 30 minutes (i) or 20 μM NaClO for 30 minutes (ii). Cells treated with **J-S** (5 μM) for 12 hours at 37 ^{o}C, and then incubated with 20 μM H_2O_2 for 30 minutes (iii) or 20 μM NaClO for 30 minutes (iv). Scale bar: 10 μm.

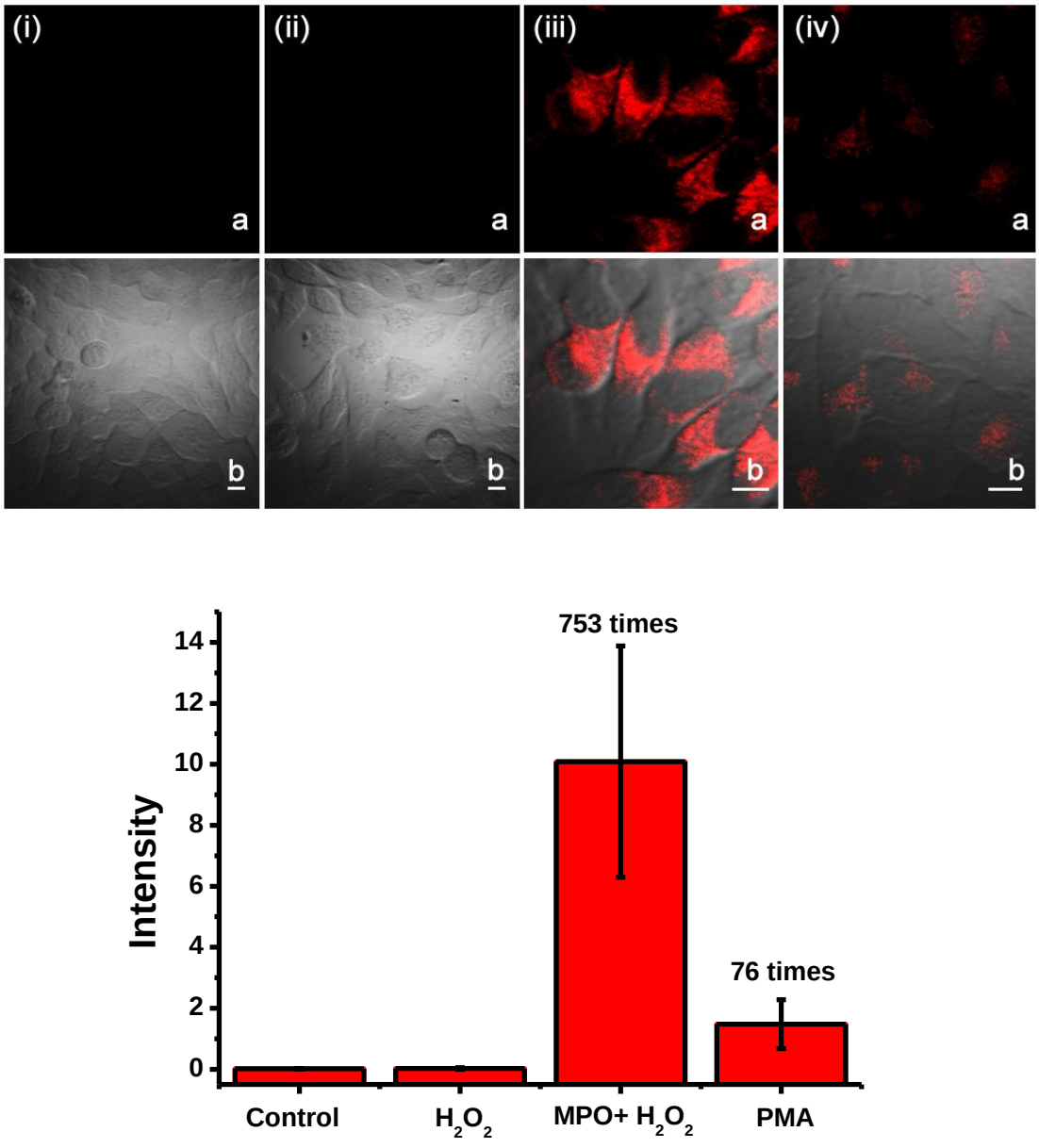

Figure S8. Two photon fluorescence confocal images of living HeLa cells loaded with **J-S** under different stimulants. HeLa cells were loaded with (i) **J-S** (5 μM) for 24 hours and then 20 μM H_2O_2 for 30 minutes; (ii) **J-S** (5 μM) and MPO (0.01 U/mL) for 24 hours; (iii) **J-S** (5 μM) and MPO (0.01 U/mL) for 24 hours were treated with 20 μM H_2O_2 for 30 minutes.(iv) **J-S** (5 μM) and MPO (0.01 U/mL) for 24 hours were treated with 1 μM PMA for an hour; (a) Fluorescence images; (b) merged images of (a) and DIC. Detection channel: λ_{ex}= 840nm, λ_{em}=570-620nm. Laser power: 10 mW. Scale bar: 10 μm.