**Supporting information for**

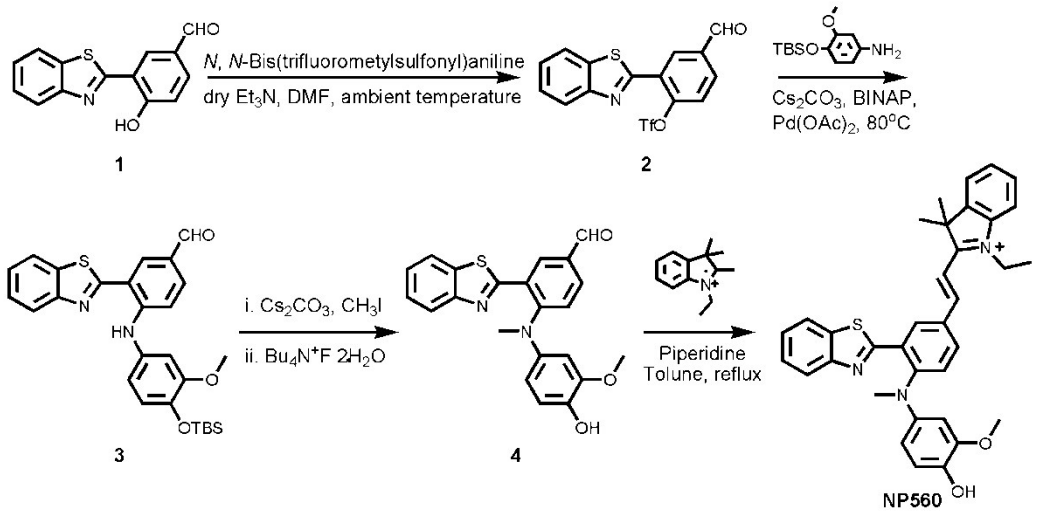
# Bis-Reaction-Trigger as a Strategy to Improve the Selectivity of Fluorescent Probes

## 1. Materials and methods

### Reagents and apparatus

All chemicals were from commercial supplies and used without further purification except otherwise indicated. Dry DMF and dry $Et_3N$ were distilled from $CaH_2$. Dry toluene was distilled from Na. $^1H$ NMR spectra were recorded on a Bruker 500 Fourier transform spectrometer (500 MHz). $^{13}C$ NMR spectra were obtained on a Bruker 500 Fourier transform spectrometer (125 MHz) spectrometer. All NMR spectra were calibrated using the residual solvent ($CDCl_3$) as internal reference ($^1H$ NMR = 7.26, $^{13}C$ NMR = 77.16). All chemical shifts were reported in parts per million (ppm) and coupling constants (*J*) in Hz. The following abbreviations were used to explain the multiplicities: d = doublet, t = triplet, m = multiplet. Mass spectra (MS) were measured on a SHIMADZU LCMS-2020 spectrometer. UV-vis spectra were taken on a HITACHI U-3010 Spectrophotometer. Fluorescence spectra were measured on an Agilent Cary Eclipse Fluorescence Spectrophotometer.

### Probe synthesis and characterization




### Synthesis of intermediate 2

Compound **1** (1.0 g, 3.9 mmol) and N,N-Bis (trifluoromethylsulfonyl)aniline (2.1 g, 5.9 mmom) was dissolved in dry DMF (10 mL) and the mixture was cooled to 0 °C with an ice bath. Dry triethylamine (1.1 mL, 7.9 mmol) was added to the mixture dropwise under a nitrogen atmosphere. The reaction was stirred at ambient temperature overnight. After completion of the reaction as monitored by TLC analysis, $H_2O$ (10 mL) was added to quench the reaction. The mixture was then diluted with ethyl acetate (20 mL) and then transferred to a separatory funnel. The organic phase was

washed subsequently with $H_2O$ (4×10 mL) and brine (1×10 mL), dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure to give the crude product which was purified by flash column chromatography ($SiO_2$, petroleum ether/EtOAc, 25:1) to yield the product as a white solid (1.2 g, 79% yield).

**M.p.:** 79.6-80.7 °C.

**$R_f$**= 0.44 (1:25, EtOAc:PE).

**IR ($cm^{-1}$):** 3074, 2923, 2842, 1710, 1581, 1491, 1427, 1217, 1135, 868

**$^1$H NMR (500 MHz, $CDCl_3$):** δ 10.12 (s, 1H), 8.70 (d, *J* = 2.1 Hz, 1H), 8.17 (d, *J* = 8.2 Hz, 1H), 8.07 (dd, *J* = 8.5, 2.1 Hz, 1H), 7.97 (d, *J* = 8.0 Hz, 1H), 7.67 (d, *J* = 8.5 Hz, 1H), 7.61 - 7.54 (m, 1H), 7.53 -7.44 (m, 1H).

**$^{13}$C NMR (126 MHz, $CDCl_3$)**: δ 189.70, 159.79, 153.24, 150.24, 136.00, 135.79, 133.53, 131.84, 128.28, 127.03, 126.46, 124.19, 123.48, 121.76.

**ESI-HRMS (*m/z*):** $[M+H]^+$ calc'd. for $C_{15}H_9F_3NO_4S_2$ 387.9925, found 387.9921.

**Synthesis of intermediate 3**

Triflate **2** (3.0 g, 7.7 mmol) was dissolved in dry toluene (30 mL), to which was added sequentially the aniline (2.3 g, 9.2 mmol), $Cs_2CO_3$ (3.5 g, 10.8 mmol), BINAP (23 mg, 0.36 mmol), and $Pd(OAc)_2$ (52 mg, 0.23 mmol). The reaction was then heated to 80 °C under nitrogen atmosphere and was stirred for 16 hours. The reaction was then cooled to ambient temperature and diluted with EtOAc (30 mL). Solid precipitation was removed by filtration and the filtrate was concentrated by rotary evaporation under reduced pressure to yield the crude product which was purified by flash column chromatography on silica gel ($SiO_2$: PE/EtOAc, 25:1) to give the product as a yellow solid (3.5 g, 92% yield).

**M.p.:** 138.9-140.0 °C.

**$R_f$**= 0.54 (1:25, EtOAc:PE).

**IR ($cm^{-1}$):** 3220, 2956, 2929, 2857, 1683, 1614, 1508, 1472, 1280, 913.

**$^1$H NMR (500 MHz, $CDCl_3$)**: δ 9.82 (s, 1H), 8.29 (d, *J* = 1.7 Hz, 1H), 8.00 (d, *J* = 8.1 Hz, 1H), 7.93 (d, *J* = 7.8 Hz, 1H), 7.72 (dd, *J* = 8.8, 1.8 Hz, 1H), 7.49 (td, *J* = 7.8, 1.0 Hz, 1H), 7.43-7.40 (m, 1H), 7.11 (d, *J* = 8.8 Hz, 1H), 6.93 (d, *J* = 8.2 Hz, 1H), 6.86 (d, *J* = 2.3 Hz, 1H), 6.84 (dd, *J* = 8.2, 2.4 Hz, 1H), 3.83 (s, 3H), 1.04 (s, 9H), 0.21 (s, 6H).

**$^{13}$C NMR (126 MHz, $CDCl_3$):** δ 189.63, 168.46, 153.12, 151.88, 150.77, 143.50, 134.13, 133.23, 132.98, 132.70, 126.58, 126.23, 125.71, 122.69, 121.55, 121.53, 118.02, 115.11, 113.69, 110.14, 55.80, 25.87, 18.62, -4.42.

**ESI-HRMS (*m/z*):** $[M+H]^+$ calc'd. for $C_{27}H_{31}N_2O_3SSi$ 491.1825, found. 491.1827.

**Synthesis of intermediate 4**

Compound **3** (1.4 g, 2.9 mmol) was dissolved in dry DMF (10 mL). The solution was cooled to 0°C in an ice bath and $Cs_2CO_3$ (1.4 g, 4.3 mmol) was added in one portion, followed by $CH_3I$ (0.27 mL, 4.3 mmol). The mixture was warmed to ambient temperature slowly (by removing the ice bath). The reaction was monitored by TLC analysis till the disappearance of **3** (ca. 12 hrs). $H_2O$ (10 mL) was added to quench the reaction and the mixture was diluted with EtOAc (20 mL). The biphasic mixture was

then transferred to a separatory funnel and the organic layer was washed sequentially with $H_2O$ (4×10mL) and brine (1×10 mL), dried over anhydrous $Na_2SO_4$, filtered, and concentrated under reduced pressure. The crude product, without further purification, was directly subject to deprotection by being dissolved in THF (10 mL) and then treated with $Bu_4N^+F^-•2H_2O$ (1.7 g, 5.4 mmol). After being stirred at ambient temperature for 20 minutes, the volatile organics were removed by rotary evaporation under reduced pressure and the resulting residue was purified by flash column chromatography ($SiO_2$: PE/EtOAc, 3:1) to give intermediate **4** as a yellow solid (0.8 g, 71% for the two sequential steps).

**M.p.:** 169.1-170.2 °C.

**$R_f$**= 0.54 (1:3, EtOAc:PE).

**IR (cm$^{-1}$):** 3163, 2962, 2834, 2818, 2363, 1702, 1596, 1512, 1371, 1263, 1103, 837,795.

**$^1$H NMR (500 MHz, $CDCl_3$)**: δ 10.09 (s, 1H), 8.85 (d, *J* = 1.9 Hz, 1H), 8.09 (d, *J* = 8.1 Hz, 1H), 7.96 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.83 (d, *J* = 7.9 Hz, 1H), 7.52 - 7.47 (m, 1H), 7.40 - 7.36 (m, 1H), 7.34 (d, *J* = 8.3 Hz, 1H), 6.81 (d, *J* = 8.6 Hz, 1H), 6.44 (dd, *J* = 8.6, 2.6 Hz, 1H), 6.36 (d, *J* = 2.6 Hz, 1H), 3.71 (s, 3H), 3.20 (s, 3H).

**$^{13}$C NMR (126 MHz, $CDCl_3$):** δ 190.98, 163.45, 154.41, 152.41, 147.08, 141.96, 140.86, 136.36, 134.11, 133.39, 131.00, 130.84, 127.42, 126.37, 125.63, 123.25, 121.65, 114.76, 110.77, 102.66, 56.02, 41.84.

**ESI-HRMS (*m/z*):** $[M+H]^+$ calc'd. for $C_{22}H_{19}N_2O_3S$ 391.1116, found.391.1113.

**Synthesis of probe NP560**

Intermediate **4** (50 mg, 0.13 mmol) and *N*-ethyl-2,3,3-trimethyl-3*H* indol bromide (24 mg, 0.13 mmol) was dissolved in toluene (5.0 mL) and the mixture was cooled to 0 °C with an ice bath. Piperidine (12 μL, 0.13 mmol) was added to the mixture and the reaction was heated to reflux. The reaction was monitored by TLC analysis till the disappearance of **4**. After cooling to ambient temperature, the mixture was diluted with EtOAc (20 mL) and was washed with with $H_2O$ (1×10mL) and brine (1×10 mL), dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography ($SiO_2$: DCM/MeOH, 20:1) to give **NP560** as a purple black solid (62 mg, 86%).

**M.p.:** 240.1-241.5°C

**$R_f$**= 0.34 (1:20, MeOH:DCM)

**IR (cm$^{-1}$):** 3142, 2986, 2930, 2360, 1606, 1594, 1518, 1471, 1272, 791

**$^1$H NMR (500 MHz, $CDCl_3$)**: δ 9.01 (d, *J* = 8.5 Hz, 1H), 8.37 (d, *J* = 1.6 Hz, 1H), 8.22 (d, *J* = 16.0 Hz, 1H), 8.04 (d, *J* = 8.1 Hz, 1H), 7.89 (d, *J* = 16.0 Hz, 1H), 7.83 (d, *J* = 7.9 Hz, 1H), 7.68 (d, *J* = 7.7 Hz, 1H), 7.61 – 7.55 (m, 3H), 7.51 – 7.44 (m, 2H), 7.38 (t, *J* = 7.5 Hz, 1H), 6.75 (d, *J* = 8.5 Hz, 1H), 6.47 (dd, *J* = 8.5, 2.3 Hz, 1H), 6.42 (d, *J* = 2.2 Hz, 1H), 5.09 (q, *J* = 7.1 Hz, 2H), 3.72 (s, 3H), 3.27 (s, 3H), 1.85 (s, 6H), 1.63 (t, *J* = 7.5 Hz, 3H)

**$^{13}$C NMR (126 MHz, $CDCl_3$):** δ 180.79, 164.34, 154.56, 153.81, 152.39, 146.99, 143.42, 141.76, 141.39, 140.59, 136.19, 133.67, 129.94, 129.83, 129.40, 129.03, 126.57, 126.38, 125.62, 122.99, 122.78, 121.73, 114.95, 114.76, 112.92, 111.74, 104.26, 56.16, 52.36, 44.59 (d, *J* = 8.1 Hz), 42.54, 27.28, 21.93, 14.62.

**ESI-HRMS (*m/z*):** $[M]^+$ calc'd. for $C_{35}H_{34}N_3O_2S^+$ 560.2372, found 560.2389.

**General experimental for photophysical property characterization**

All the photophysical characterization experiments were carried out in Tris-buffer solution (10 mM, pH 7.4) at ambient temperature. Probe **NP560** was dissolved in DMSO to make a 5 mM stock solution. $ONOO^-$ and other bio-relevant species were prepared as described previously (*J. Am. Chem. Soc.* 2015, 137, 12296−12303).

To test the photophysical response of **NP560** towards $ONOO^-$ or other reactive species, aliquots of probe stock solutions were diluted with Tris-buffer solution and treated with analytes to make sure both probes and analytes were kept at desired final concentrations. After quick and vigorous shaking, the mixture was allowed standing in the dark for 15 min and then the photophysical properties were recorded. All experiments were performed in triplicate, and data shown were the average.

**Confocal fluorescence staining and analysis**

EA.hy926 cells were used in the study. Briefly, the cells were grown in DMEM with 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. After culture on glass cover slips overnight, cells were treated with $ONOO^-$ or SIN-1 for indicated time, then stained with **NP560** probe (5 μM) at 37 °C for 30 min before being fixed in 4 % PFA and imaged by confocal microscope (Nikon A1R).

For time-lapse confocal imaging in live cells, endothelial cells were cultured on glass-bottom plates overnight, then were incubated with **NP560** probe (5 μM) at 37 °C for 30 min. The dynamic change of **NP560** fluorescence in live cells with SIN-1 (2 mM) stimulation was captured by time-lapse confocal microscope for 60 min. The fluorescence density was analyzed using Image J software (NIH, Bethesda, MD, USA).

**Cytotoxicity assay**

The Cell Counting Kit-8 (CCK-8; Beyotime, Shanghai, China) was used to analyze the effects of **NP560** on EA.hy926 endothelial cells viability according to the manufacturer's protocols. Briefly, equal numbers of cultured EA.hy926 endothelial cells were incubated with or without **NP560** (2.5, 5, 10, 15 μM) in a 96-well plate for 24 h. Then cells were incubated with CCK-8 solution (10 μl per well) at 37 $^oC$ with 5% $CO_2$ for 3 h. Absorbance at 450 nm was measured with a microplate reader (ELx800, Bio-Tek, Winooski, VT, USA). The well with medium and CCK-8 solution but without cells was used as a blank control. Cell viability of vehicle-treated control groups not exposed to **NP560** was defined as 100%. Data are expressed as mean ± S.E.M. ns $P>0.05$ versus control.

**2. Supplementary figures**

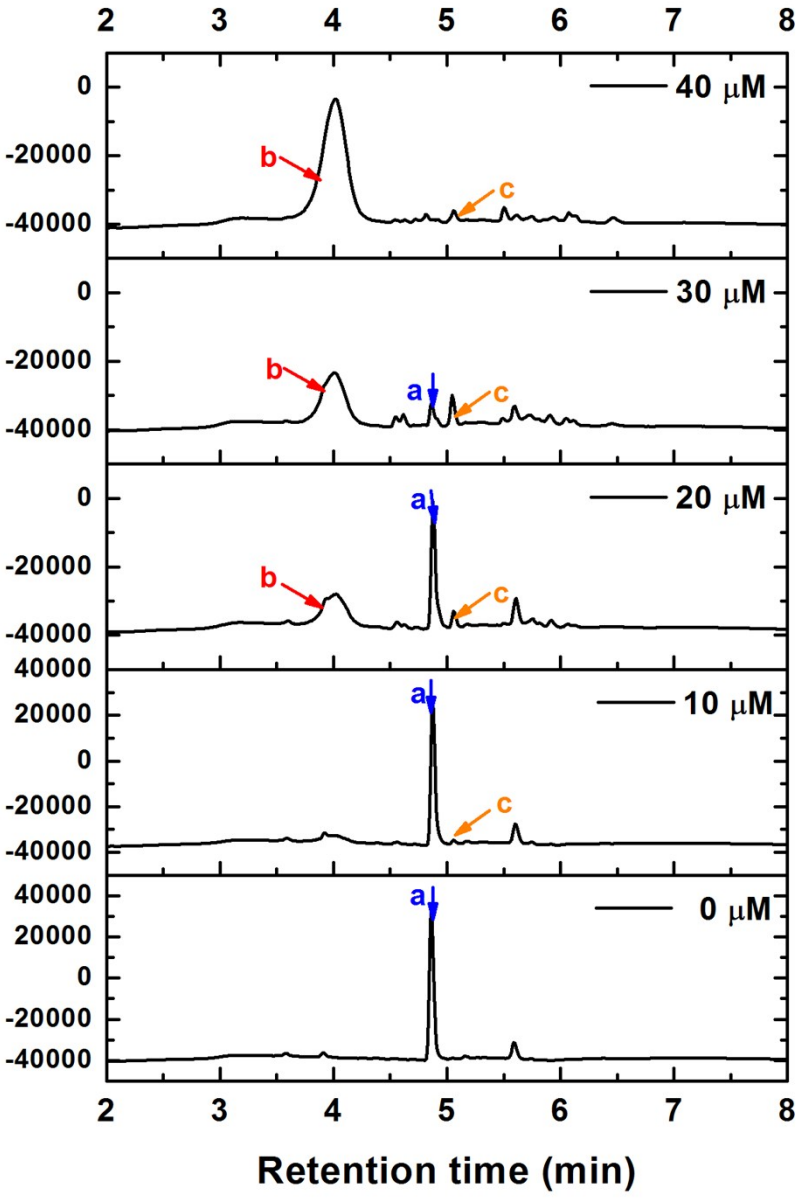



Fig. S1 Liquid chromatography spectra of probe **NP560** (20 μM, peak a) after the treatment of various amounts of $ONOO^-$. The intensity of peak a decreased in a $ONOO^-$ -dose dependent way, accompanied by the emergence of peak b and peak c.

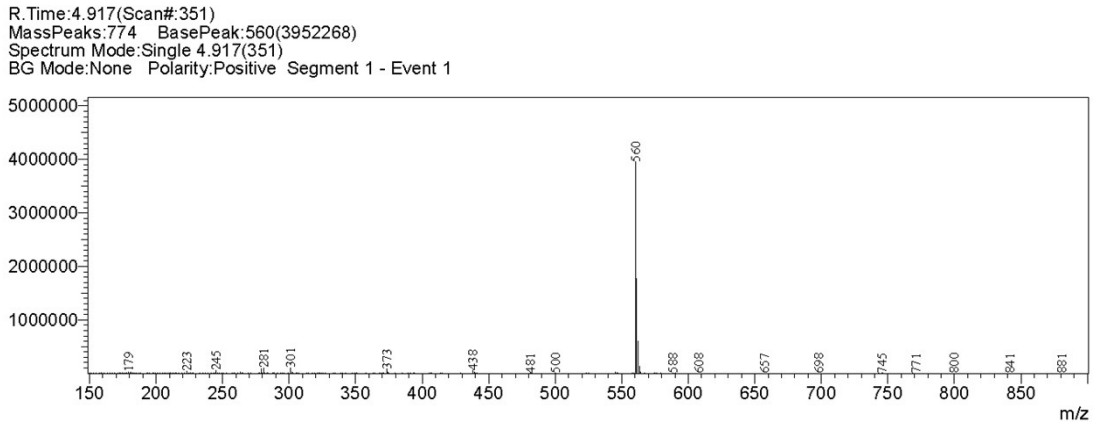



Fig. S2 Mass spectra of peak a in Fig S1. The *m/z* 560 signal was corresponding to the structure of **NP560**.

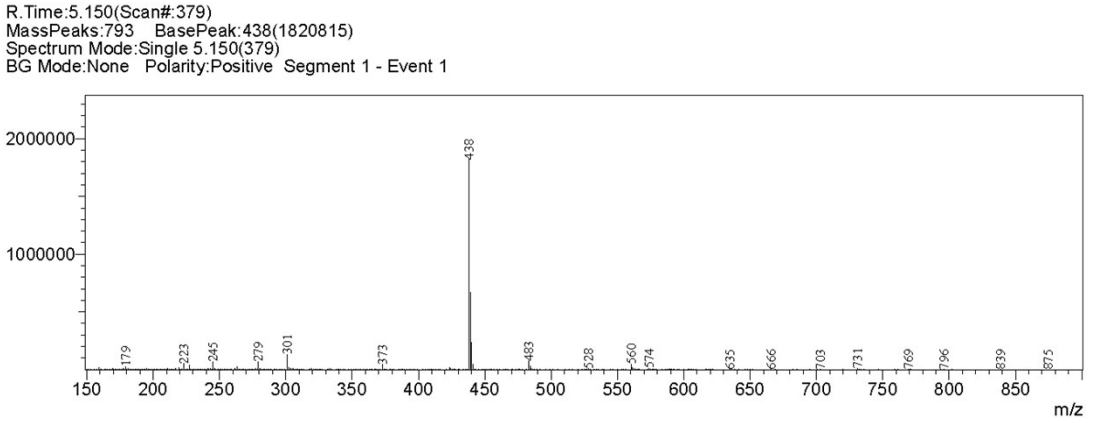



Fig. S3 Mass spectra of peak c in Fig S1. The *m/z* 438 signal was corresponding to the structure of **NP560-I**.

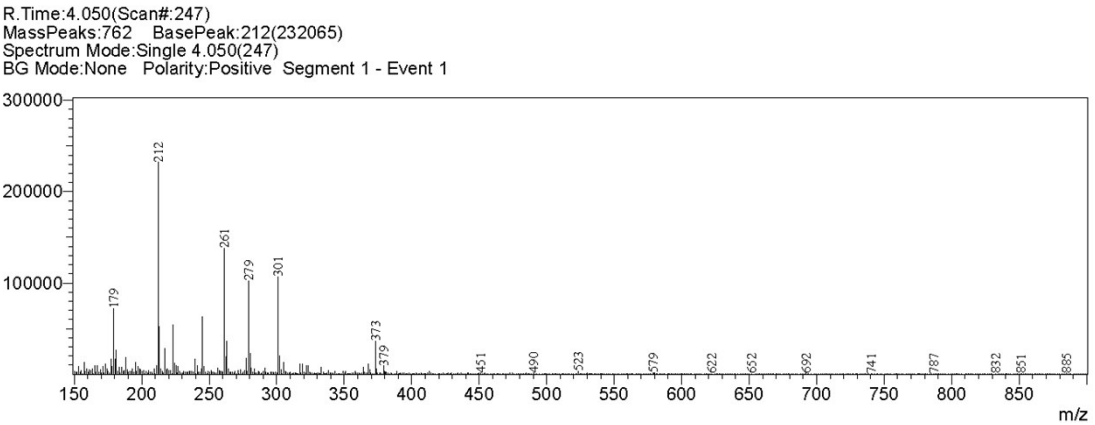



Fig. S4 Mass spectra of peak b in Fig S1. The *m/z* 212 signal was corresponding to the structure of 1-ethyl-3,3-trimethyloxindole ($[M+Na]^+$).

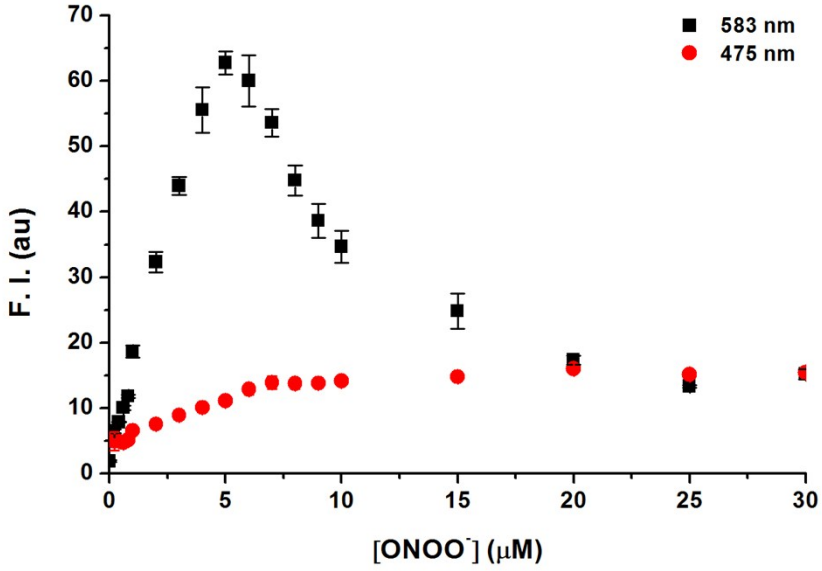



Fig. S5 Fluorescence response of **NP560** towards various amounts of $ONOO^-$. Data shown were the emission intensity at 583 nm or 475 nm of **NP560** (5 μM) after the treatment of $ONOO^-$ of indicated concentrations, λex 375 nm.

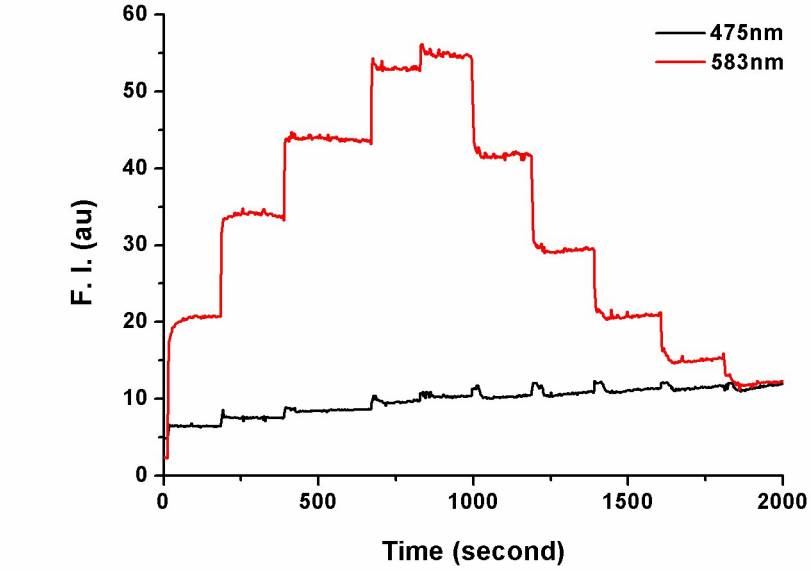
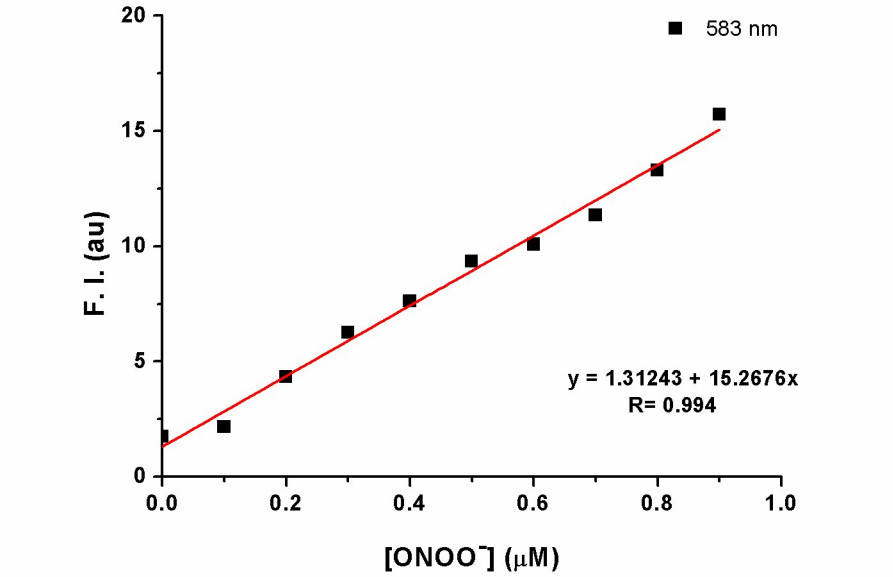



Fig. S6 Fluorescence response of **NP560** towards the sequent treatment of small portions of $ONOO^-$. Data shown were the time-lapsed emission at 583 nm or 475 nm of **NP560** (5 μM) after the sequent treatment of $ONOO^-$ (1 μM each time), λex 375 nm.

Step 1: Blank measurements (n=20). Mean: FB = 1.51433 ± 0.196429;

Step 2: Linear regression analysis on fluorescence intensity (583 nm) and the corresponding $ONOO^-$ concentrations (0-1 μM):




Step 3: Detection limit calculation: $C_{LOD} = 3\sigma/s$

Where σ is the standard deviation of the blank measurements;

s is the slope of the calibration curve line obtained from the linear regression analysis.

Fig. S7 Procedures for limit of detection determination.

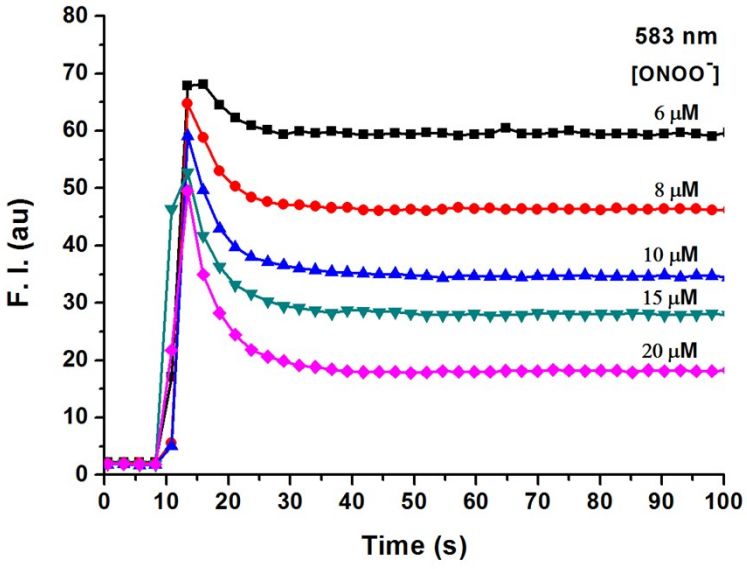



Fig. S8 Time-lapsed response of **NP560** towards $ONOO^-$. Data shown were the time-lapsed emission intensity change of **NP560** at 583 nm after the treatment of $ONOO^-$.

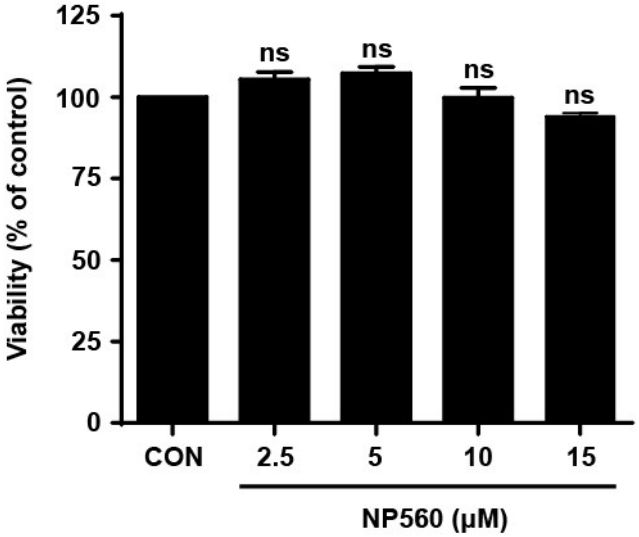



Fig. S9 Effects of **NP560** on cell viability determined with the CCK-8 assay. The Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China) was used to analyze the effects of **NP560** on EA.hy926 endothelial cells viability according to the manufacturer's protocols. Briefly, equal numbers of cultured EA.hy926 endothelial cells were incubated with or without **NP560** (2.5, 5, 10, 15 μM) in a 96-well plate for 24 h. Then cells were incubated with CCK-8 solution (10 μl per well) at 37 °C with 5% $CO_2$ for 3 h. Absorbance at 450 nm was measured with a microplate reader (ELx800, Bio-Tek, Winooski, VT, USA). The well with medium and CCK-8 solution but without cells was used as a blank control. Cell viability of vehicle-treated control groups not exposed to **NP560** was defined as 100%. Data are expressed as mean ± S.E.M. ns P>0.05 versus control.

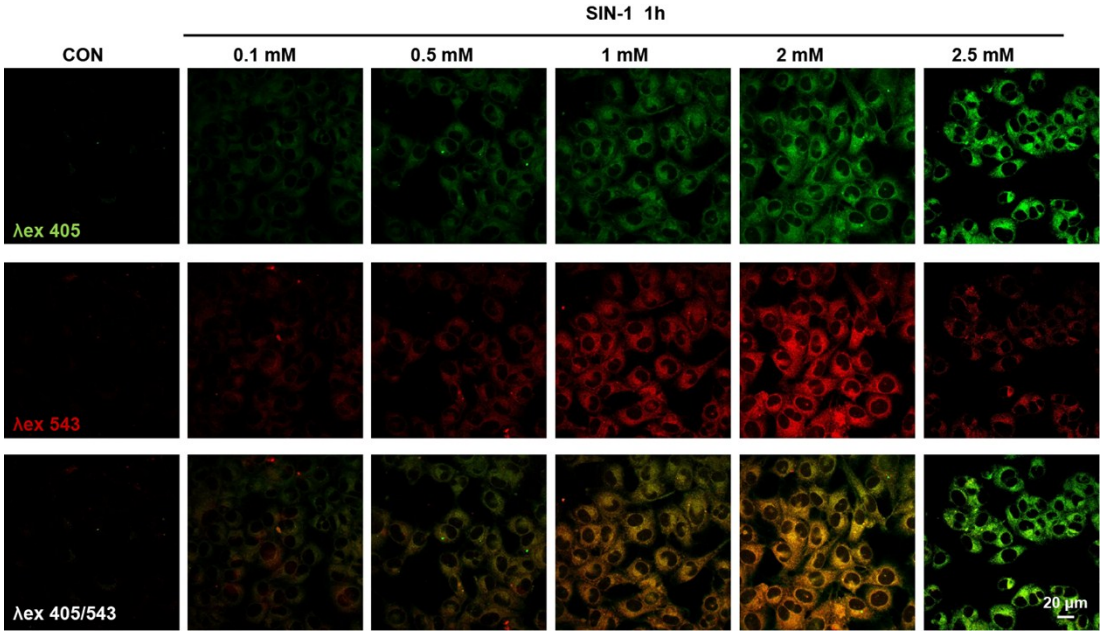



Fig. S10 Intracellular fluorescence of **NP560** in response to SIN-1 treatment. EA.hy926 endothelial cells were cultured on glass cover slips overnight and then incubated with SIN-1 at various concentrations for 1 h, followed by **NP560** ( 5 μM ) staining for 0.5 h. Cells were then fixed in 4 % PFA for 10 min and imaged by confocal microscope. The intracellular **NP560** fluorescence was recorded with excitation at 405 nm and emission at 420-500 nm for the green channel colour, and excitation at 543 nm and emission at 560-630 nm for the red channel colour.

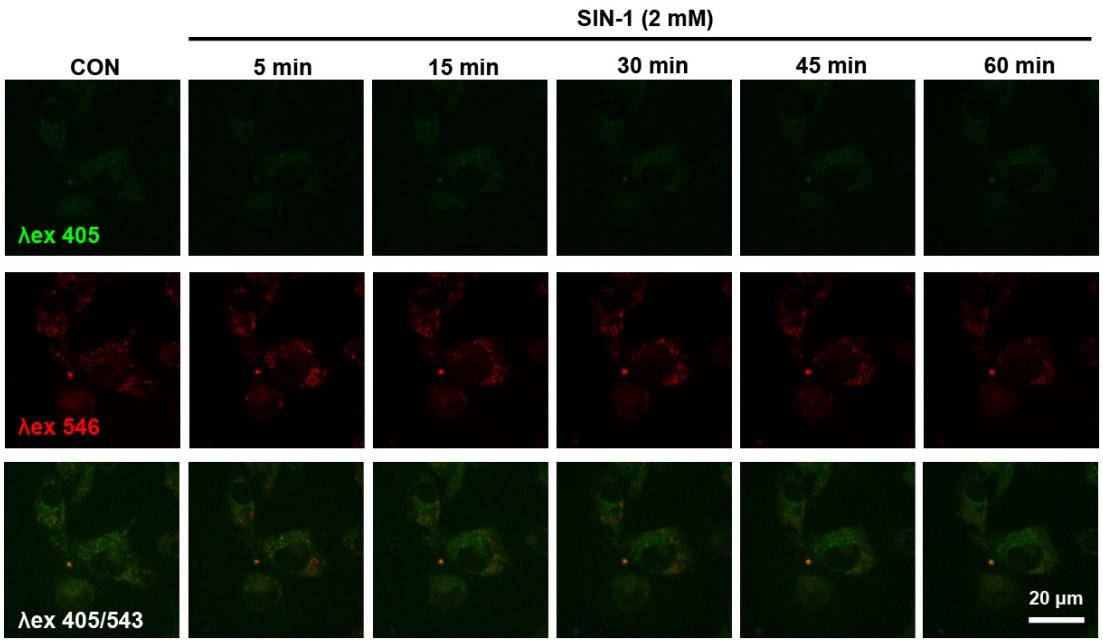



Fig. S11 Real-time tracking of endogenous $ONOO^-$ formation by **NP560**. EA.hy926 endothelial cells were loaded with **NP560** and then treated with SIN-1 (2 mM). Time-lapsed series of single confocal plane images were recorded with excitation at 405 nm and emission at 420-500 nm for the green channel colour, and excitation at 543 nm and emission at 560-630 nm for the red channel colour.

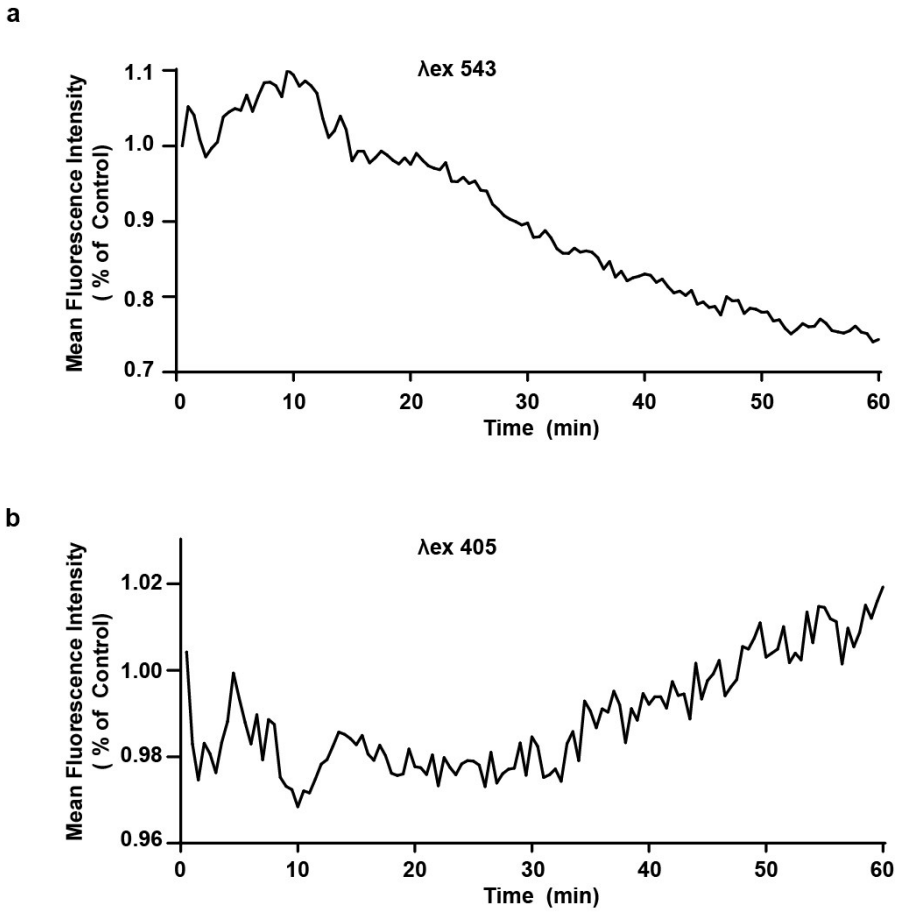



Fig. S12. Quantification of dynamic change of **NP560** mean fluorescence intensity in Fig. S11. Data are presented as ratio to control.

Supplementary Movie 1. Time-lapse series of Fig. S11 with **NP560** fluorescence being recorded with excitation at 405 nm and emission at 420-500 nm for the green channel colour.

Supplementary Movie 2. Time-lapse series of Fig. S11 with **NP560**5 fluorescence being recorded with excitation at 543 nm and emission at 560-630 nm for the red channel colour.