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Supporting Information for

Insights into the role of nanostructure in sensing properties of carbon nanodots for improved sensitivity to reactive oxygen species in living cells

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Experimental section

Chemicals and materials

Citric acid, urea, thiourea, ascorbic acid (AA), dimethylsulfoxide (DMSO), NaOH, NaClO, $FeSO_4$, KO_2, HCl and H_2O_2 (30%, *v/v*) were purchased from Beijing Chemical Reagent Company (Beijing, China). Tert-butyl hydrogen peroxide (TBHP), 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH), phorbol 12-myristate 13-acetate (PMA), trypsin, pronase, and proteinase K were obtained from Sigma–Aldrich (St. Louis, USA). *p*-Benzoquinone (BQ) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). $NaNO_2$ and NaN_3 were supplied by Tianjin Fuchen Chemical Reagent Co., Ltd. (Tianjin, China). 3-Morpholinosydnonimine (SIN-1) was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Cell Counting Kit-8 (CCK-8) and Lyso-Tracker Red were purchased from Beyotime (Hangzhou, China). HeLa cells were supplied by the American Type Culture Collection (ATCC, Rockville, MD). All aqueous solutions were prepared with deionized water from Millipore Milli-Q system. $^1O_2$ was produced by the on-line mixing of H_2O_2 with NaClO (H_2O_2: NaClO = 1: 1). $^{\bullet}OH$ was generated from Fenton reaction (Fe^{2+}: H_2O_2 = 10: 1). $O_2^{\bullet-}$ was prepared by dissolving KO_2 into DMSO followed by dilution with deionized water. $TBO^{\bullet}$ was produced from the reaction of Fe^{2+} with TBHP solution (Fe^{2+}: TBHP = 10: 1). $ROO^{\bullet}$ was generated by incubating AAPH in deionizer water (37 ˚C) for 30 min. Peroxynitrite ($ONOO^-$) was prepared through on-line reaction of $NaNO_2$ and H_2O_2-HCl solutions, followed by adding NaOH solution to stabilize the $ONOO^-$. The concentration of the working solution of $ONOO^-$ was calculated from the absorbance at 302 nm (ε=1670 $M^{-1}cm^{-1}$).

Synthesis of carbon nanodots (CDs)

CDs with blue/green PL emission were prepared through a microwave route. CD0.2, CD1, CD2, and CD3 were prepared from citric acid and urea in the mass ratios of 1: 0.2, 1: 1, 1: 2, and 1: 3, respectively. Briefly, citric acid (3 g) and urea (0.6 g / 3 g / 6 g / 9 g) were dissolved in 10 mL deionizer water in a glass beaker. The transparent solution was heated in the microwave oven (750 W) for 4.5 min. The

obtained black-brown solid was then heated at 60 ˚C for 1 h in a vacuum oven. Finally, 20 mL deionizer water was added to disperse the solid, followed by centrifugation at 5000 r/min for 20 min. The supernate was further dialyzed (molecular weight cut-off 1000) to remove the large particles. The solution outside the dialysis bag was then freeze-dried for the next applications.

Cytotoxicity assay

The cytotoxicity of CDs on HeLa cells was evaluated by CCK-8 assay. Working solutions of CD3 were freshly prepared by dissolving the dry powder of CD3 into DMEM medium (with 10% FBS, 100 units/mL of penicillin, and 100 μg/mL of streptomycin). HeLa cells were firstly seeded in a 96-well microtiter plate and cultured in DMEM overnight. The growth medium was removed and the cells were incubated in a DMEM medium containing various concentrations of CD3 for 24 h. Then, the culture media were replaced with the DMEM (200 μL) containing CCK-8 (10 μL). After 1 h of incubation, the absorbance at 450 nm was recorded by a Multimode Plate Readers in quadruplicate.

Cell culture and cellular uptake of CD probes

HeLa cells were seeded in a 6-well microtiter plate at a density of 10^5 cells/well and cultured overnight in DMEM medium. As for Fluorescence imaging, HeLa cells were firstly seeded in confocal dishes (Coverglass Bottom Dish) and cultured overnight, followed by incubation in a DMEM medium containing 500 μg/mL of CD3 for 4 h. Then the cells were washed twice with PBS (pH 7.4), and cultured in 2 mL of PBS (pH 7.4) during the imaging. The fluorescence images were acquired by using a 40× objective, excitation at 405 nm.

Subcellular localization of the CD probes

The location of the CD probes was confirmed by costaining with Lyso-Tracker Red. HeLa cells were seeded in confocal dishes and cultured overnight, and then incubated with 500 μg/mL of CD3 for 4 h. Subsequently, the culture media were replaced with the DMEM containing Lyso-Tracker Red and the

cells were incubated for another 1 h. Then the cells were washed twice with PBS (pH 7.4), and cultured in 2 mL of PBS (pH 7.4) for the fluorescence imaging. The excitation wavelengths for CD3 and lysosomes were 405 nm and 542 nm, respectively.

Chemiluminescence (CL) measurements and determination of $ONOO^-$ in living cells

SIN-1 was applied to generate exogenous $ONOO^-$ in living cells. Working solutions of SIN-1 were prepared by dissolving SIN-1 into NaOH solution (0.01 M) and incubated at 37 ˚C for 45 min. Firstly, HeLa cells were incubated with 500 μg/mL of CD3 for 4 h. The CD-labeled cells were then suspended with pancreatin treatment and resuspended in PBS (pH 7.4) at a density of 5×10^5 cells/mL. CL signals were detected by a static injection setup (Figure S14). 100 μL of CD-labeled cells were added into the quartz vial, followed by injection of 100 μL of $ONOO^-$ or SIN-1 working solution. The CL signals were detected by a PMT adjacent to the quartz vial with a work voltage of −1000 V and data integration time of 0.1 s.

For the real-time monitoring dynamic process of $ONOO^-$ production in living cells, PMA was used to stimulate the endogenous $ONOO^-$ generation. The CL signals were recorded while 1.0 mL of CD-labeled cells was added into the quartz vial. After 0.5 h, PMA (5.0 μg/mL) was injected into the cells, and the CL signals were recorded at 6-minute intervals throughout the stimulation period.

Instruments and methods

The photoluminescence (PL) spectra of the samples were detected by using Hitachi F−7000 fluorescence spectrophotometer (Tokyo, Japan). The UV−vis absorption spectra were recorded by a Shimadzu UV−3600 spectrophotometer (Tokyo, Japan). The morphology of the CDs was examined by a high-resolution transmission electron microscope (HRTEM, JEM-3010, JEOL). The surface functional groups of CDs were confirmed by Fourier-transform infrared (FT−IR) spectra collected with a Nicolet 6700 FT−IR spectrometer (Thermo, America). The surface states of CDs were detected by X-ray photoelectron spectroscopy (XPS) using a Thermo-VG Scientific ESCALAB 250 spectrometer with a monochromatic

Al Kα X-ray source (1486.6 eV). The energy level of CDs was measured by cyclic voltammetry (CV) using a CHI660E electrochemical workstation (CH Instruments, USA). CL measurements were performed on an ultra-weak biophysics chemiluminescence (BPCL) analyzer (Institute of Biophysics, Chinese Academy of Science, Beijing, China). CL spectra were obtained using a Hitachi F−7000 fluorescence spectrophotometer without an excitation light. The confocal fluorescence images were acquired using Leica TCS SP8 Laser Scanning Confocal Microscope (Germany Leica Co., Ltd). In CCK-8 assay, the absorbance was recorded by a Multimode Plate Readers (PerkinElmer Enspire, USA).

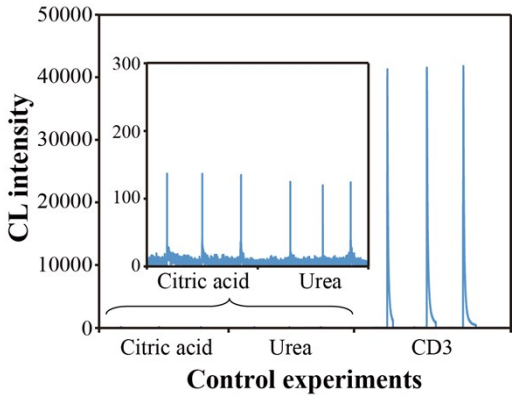

Fig. S1 CL signals of $ONOO^-$ system in the presence of different samples including citric acid, urea, and CD3.

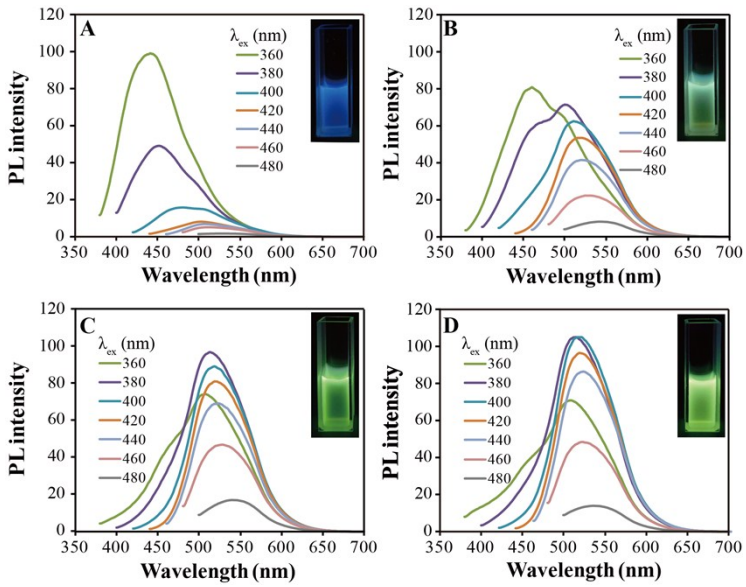

Fig. S2 (A−D) PL spectra of CD0.2, CD1, CD2, and CD3, respectively. Inset: photographs of the corresponding CDs under 365 nm UV light.

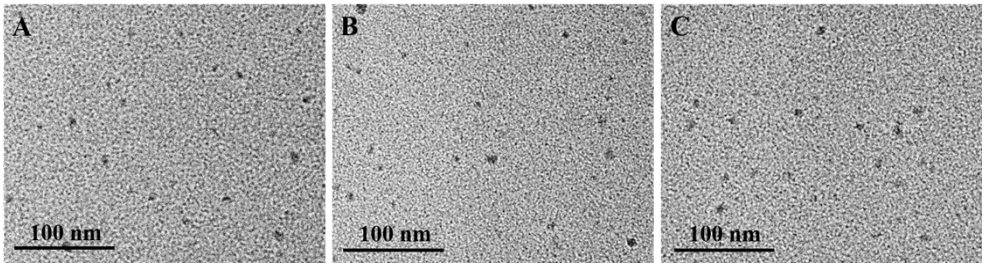

Fig. S3 (A−C) TEM images of CD1, CD2, and CD3, respectively.

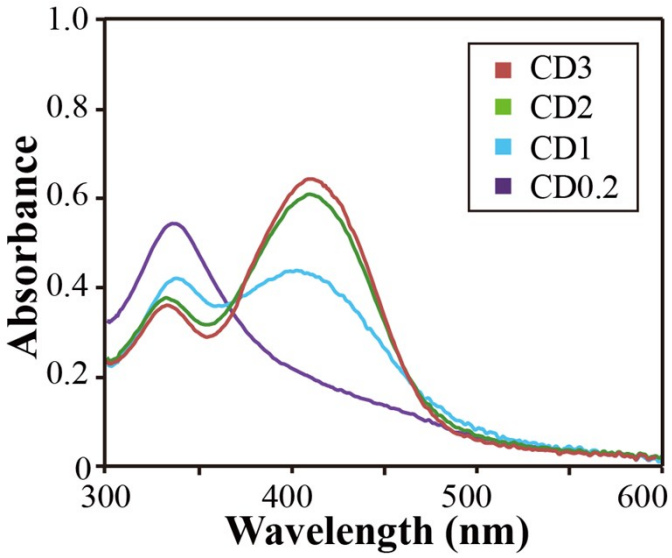

Fig. S4 UV-vis absorption spectra of the four CD samples.

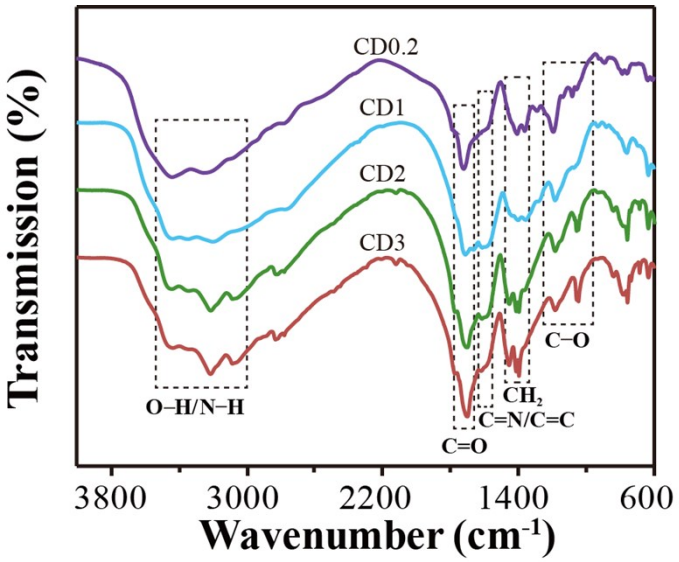

Fig. S5 FT-IR spectra of the four CD samples.

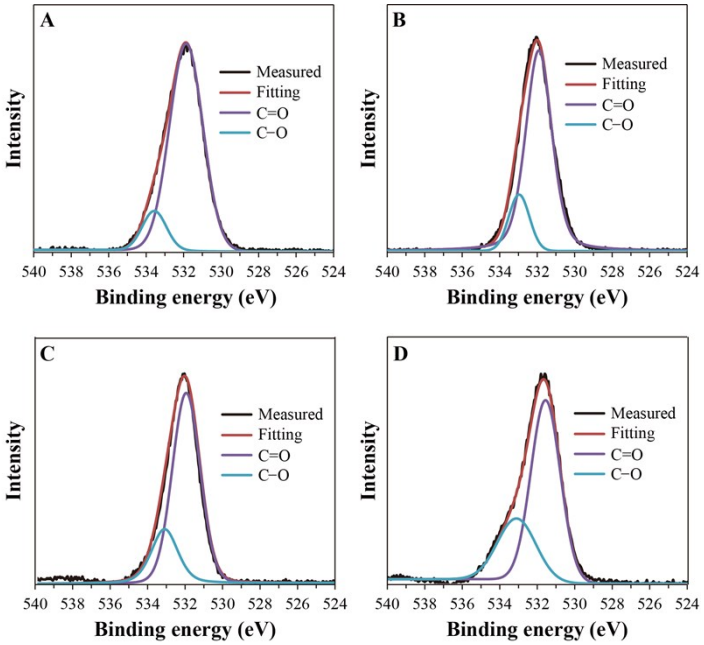

Fig. S6 (A−D) High-resolution XPS spectra of the O 1s peaks of CD0.2, CD1, CD2, and CD3, respectively.

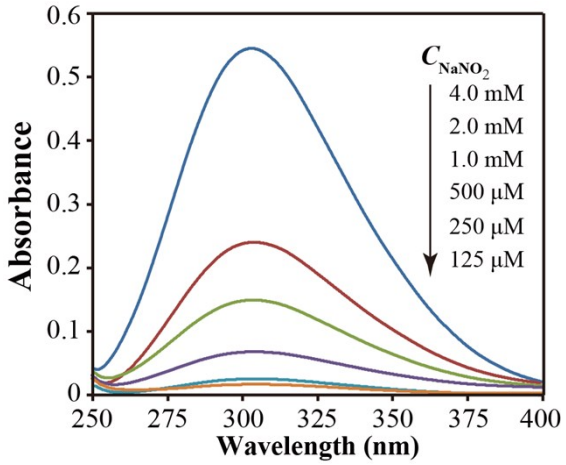

Fig. S7 UV−vis absorption spectra of $ONOO^-$ generated from the reaction of $NaNO_2$ with acidified H_2O_2.

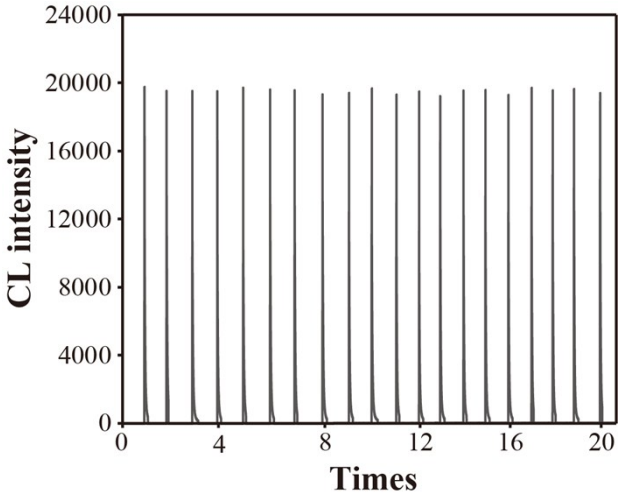

Fig. S8 CL signals of CD3 for 20 repeated measurements of 2.0 μM of $ONOO^-$. The relative standard deviation was 1.0%.

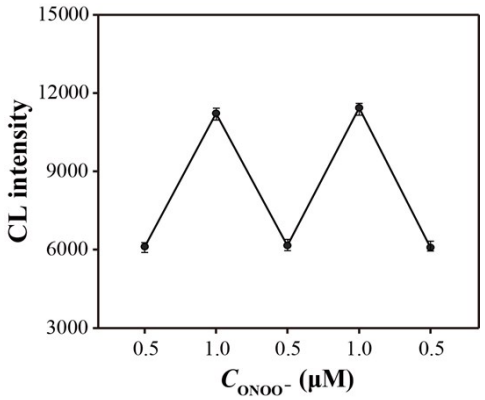

Fig. S9 Reversible response of CD3 for the determination of $ONOO^-$.

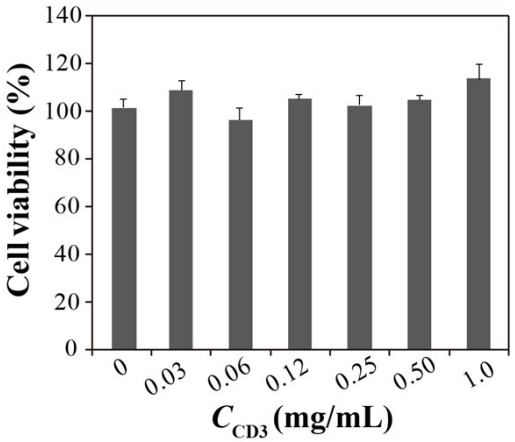

Fig. S10 Cells viability of HeLa cells incubated with different concentrations of CD3 for 24 h.

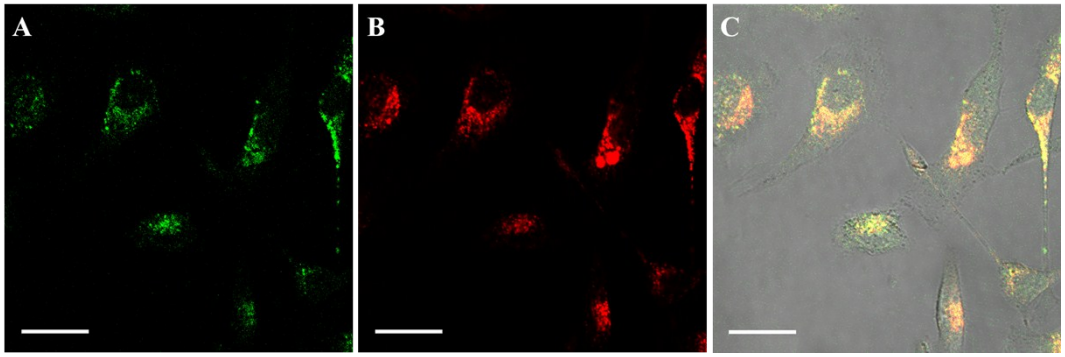

Fig. S11 Intracellular localization of the CD3 in stained HeLa cells. (A) Green channel: CD3; (B) red channel: lysosomes; (C) overlay of the red and green fluorescence channels with the bright field images. Scale bars, 30 μm.

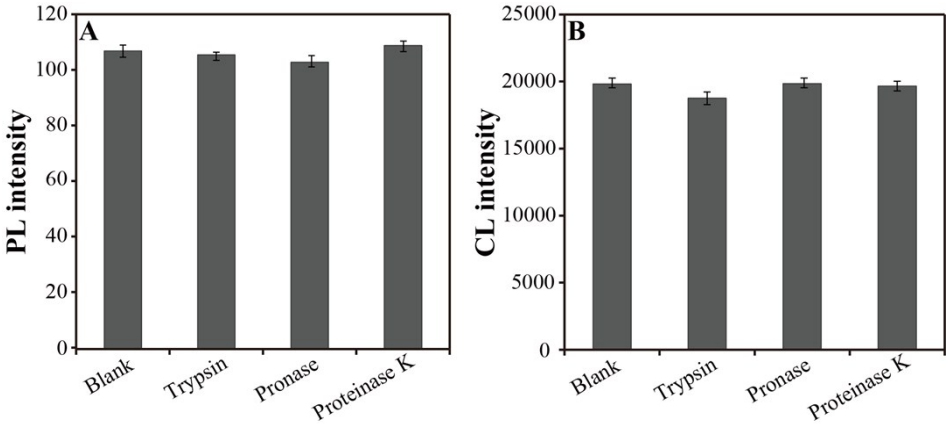

Fig. S12 Influences of various proteases (0.05% trypsin, 0.2 units/mL pronase, 2 units/mL proteinase K) on A) the PL intensity of CD3 and B) the CL intensity of the CD−$ONOO^-$ system. The CD3 were incubated with proteases for 6 h at 37 ˚C. The concentration of $ONOO^-$ was 2.0 μM.

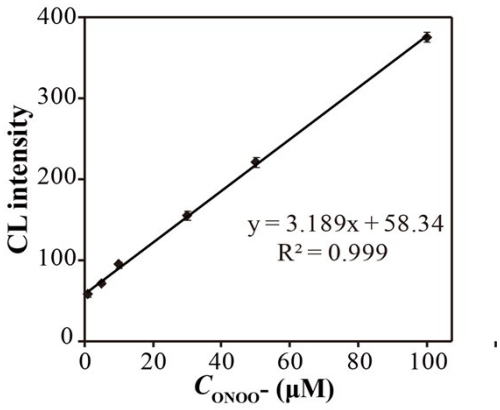

Fig. S13 CL intensity as a function of the $ONOO^-$ concentration in HeLa cells.

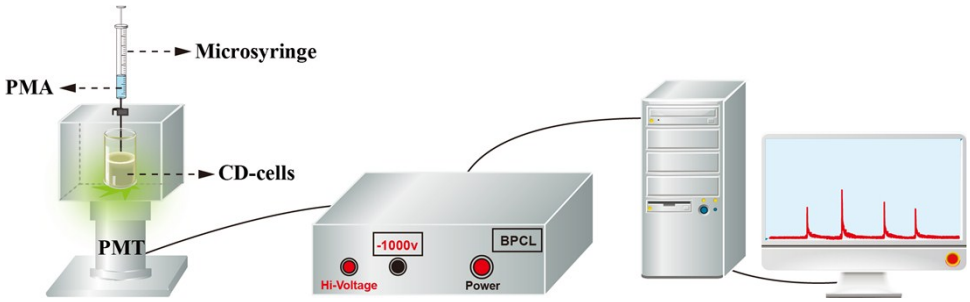

Fig. S14 Schematic diagram of a static injection CL setup for the real-time monitoring $ONOO^-$ in living cells.

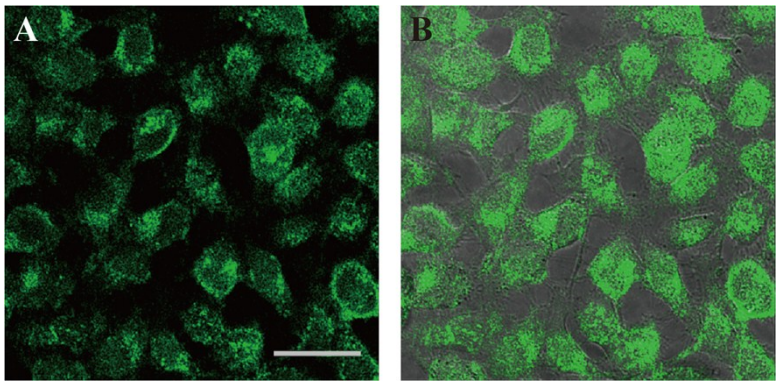

Fig. S15 Confocal fluorescence microscopy images of HeLa cells incubated with 500 μg/mL of CD3 for 4 h, and then treated with 5.0 μg/mL PMA for 2 h. (A) Green fluorescence channel; (B) overlay of the green fluorescence channel with the bright field images. Scale bar, 40 μm.

Table S1. Tolerance limits of various interfering agents on the determination of 2 μM $OONO^-$.

Coexistent substances	Content in living cell	Tolerance (mM)
K^+	140−152 mM	100
Mg^{2+}	0.1−1 mM	20
Al^{3+}	0.5 μM	5
Zn^{2+}	< 0.05 mM	5
Ca^{2+}	0.1 μM	2.5
Ba^{2+}	< 2 mM	1
Fe^{3+}	< 5 μM	1
Cr^{3+}	< 0.4 μM	0.5
Pb^{2+}	< 0.1 μM	0.25
Ni^{2+}	<6 μM	0.1
Mn^{2+}	<5 μM	0.05
Cu^{2+}	10^{-18} M	0.002
NO_3^-	1−10 μM	200
S^{2-}	0.01−0.1 μM	0.1
I^-	< 0.25 μM	0.05
Glucose	2−5 mM	5
Glycine	< 0.1 mM	5
Lysine	< 0.1 mM	5
Cysteine	0.8±4 μM	0.25
Uric acid	2−5 μM	0.05
Glutathione	1−10 mM	0.25
Ascorbic acid	<0.02 mM	0.01

An error of ±5.0% in the relative CL intensity is considered to be tolerable.