Electronic Supplementary Information

Nanosensor quantitative monitoring of ROS/RNS homeostasis in single phagolysosomes of macrophages during bactericidal process

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1. Experimental Sections

1.1 Materials and Instruments

$H_2PtCl_6 \cdot 6H_2O$, peroxynitrite (sodium), lipopolysaccharide (LPS), calcein acetoxymethyl ester (calcein AM), propidium iodide (PI), $Ru(NH_3)_6Cl_3$ were bought from Sigma-Aldrich (St. Louis, U.S.A.). SiC nanowires (SiC NWs) were purchased from Nanjing/Jiangsu XFNANO Materials Tech Co., Ltd (Nanjing, China). LB agar (powder) and LB Broth (powder) were purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). IFN-γ was bought from Peprotech (U.S.A.). DAF-FM DA, DCFH-DA, and Hoechst 33242 were bought from Beyotime Biotechnology Co., Ltd (Shanghai, China). Formic acid was bought from Chinasun Specialty Products Co., Ltd (Changshu, China). (3-Aminoprooyl) triethoxysilane (APTES), and Fluorescein isothiocyanate (FITC) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). 4% paraformaldehyde (PFA) was purchased from Labgic Technology Co., Ltd (Beijing, China). YF®555-Phalloidin obtained from US EVERBRIGHT (Suzhou, China). PEG-catalase (PEG-CAT) was obtained from Xi'an Ruixi Biotechnology Co., Ltd (Xi'an China). *Escherichia coli* (*E. coli*) CCTCC AB 91112 was bought from the China Center for Type Culture Collection (Wuhan, China). RAW 264.7 macrophages and cell culture medium were purchased from Pricella Life Science&Technology Co., Ltd (Wuhan, China). The other chemical reagents were all obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Transmission Electron Microscope (TEM) images were taken by field emission transmission electronic microscope (JEM-F200, JEOL). Scanning electron microscopy (SEM) images were obtained from field emission scanning electron microscopes (Zeiss SIGMA and Zeiss Merlin Compact, Zeiss). Confocal laser scanning microscope (LSM900, Zeiss) and inverted fluorescent microscope (AxioObserver Z1 and Axiovert 200M, Zeiss) were used to obtain fluorescent images. A microforge (World Precision Instruments, ×40 objective) was used to fabricate NWEs. Amperometric recordings were obtained with a patch clamp amplifier (EPC-10, HEKA Electronics) and a

micromanipulator (TransferMan 4r, Eppendorf).

1.2 Bacterial experiments

1.2.1 Bacterial culture

Escherichia coli (E. coli) was inoculated in LB liquid medium and shaken overnight at 37℃ and 200 rpm in a shaker. The *E. coli* solution was washed 3 times with PBS buffer solution (5000 rpm, 3 min), and then the 4% paraformaldehyde fixative solution was added. It was fixed at room temperature for 20 min and then fixed at 4℃ overnight. After fixation, the *E. coli* solution was washed with PBS buffer solution five times (5000 rpm, 3 min), dispersed in PBS, and stored at 4℃.

1.2.2 Preparation of FITC-labeled *E. coli*

Centrifuged the bacterial solution to discard the supernatant and added 1mg/mL FITC solution, which was uniformly dispersed and incubated at 37℃ for 1 h. FITC-labeled *E. coli* could be obtained after washing with PBS until the supernatant was clear and colorless. The FITC-*E. coli* should be kept away from light.

1.3 Preparation of SiC@Pt NWE

The platinum-black nanowire electrodes (SiC@Pt NWEs) were prepared in the same way as before[1], which simply means that SiC@Pt NWs were prepared by the redox reaction of H_2PtCl_6 and HCOOH. SiC@Pt NWEs were prepared by inserting the SiC@Pt NWs into the pre-prepared borate nanotubes, which were injected with liquid metal under the microscope with micromanipulation.

1.4 Cellular experiments

1.4.1 Cell culture

RAW 264.7 macrophages were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37℃ in a 5% CO_2 atmosphere. For electrochemical and fluorescence experiments, the cells were evenly dispersed in 35 mm cell culture dishes or plates, and the appropriate amount of bacterial solution was added after cell adhesion. The subsequent experiments were performed after co-culture for a certain period of time.

1.4.2 Cell Staining Experiment

1.4.2.1 ROS/RNS staining of RAW 264.7 macrophages

RAW 264.7 macrophages were evenly distributed in 24-well plates and were replaced with medium containing *E. coli* after adhesion, which were co-cultured for 24 h and then stained. The cells were stained with DAF-FM DA and DCFH-DA respectively for 20 min, washed 3 times with PBS, and photographed with an inverted fluorescence microscope.

1.4.2.2 ROS staining of RAW 264.7 macrophages after pre-incubation with CAT

RAW 264.7 macrophages and bacteria were co-cultured as described above. CAT was pre-incubated for 30 min before staining, and DCFH-DA solution was added to stain for 20 min. The cells were washed 3 times with PBS, and then imaging was performed under an inverted fluorescence microscope, while the inhibitor-free group was set up as a control group.

1.4.2.3 Characterization of bacterial phagocytosis by RAW 264.7 macrophages

RAW 264.7 macrophages were evenly dispersed in confocal dishes and were replaced with the culture medium containing FITC-*E. coli* for a certain period after adhesion. After the cells were fixed with 4% paraformaldehyde, 0.5% Triton X-100 was added for permeation, then YF®555-Phalloidin and Hoechst 33342 mixed solution was added to stain for 20 min at room temperature. After staining, the cells were washed 3 times with PBS and then rapidly placed under a confocal fluorescence microscope for imaging.

1.4.2.4 Characterization of RAW 264.7 macrophages killing bacteria

The procedure for co-culture of RAW 264.7 macrophages and bacteria was the same as above. After 24 h of co-culture, the medium was discarded and the free bacteria was washed with PBS, then the bacteria-free medium was added to continue incubation for 12 h. The co-staining of YF®555-Phalloidin with Hoechst 33342 was performed as above. At the same time, a 24 h co-culture group was set up as the control group, and imaging was performed under a confocal fluorescence microscope immediately after staining.

1.5 Amperometric data acquisition and analysis

All cellular electrochemical experiments were performed under an inverted microscope fitted with a well-grounded Faraday cage. SiC@Pt NWE was connected to a micromanipulation instrument, and the NWE was brought close to and inserted into the cell under test by controlling the micromanipulator. The amperometric traces at a series of potentials (+800 mV, +600 mV, and +500 mV vs. Ag/AgCl) were detected by a patch-clamp amplifier in a two-electrode system. The sampling frequency is 50 kHz and the Bessel filter is 2.9 kHz.

The raw amperometric data were processed using Igor Pro software, and the initial (Q_0) and produced (Q_{prod}) ROS/RNS quantities of each spike signal were obtained by deconvoluting mathematically the current time variation of shoulder spikes in Matlab software using a self-made program as previously described[2, 3].

To determine the source of the measured signals, a previously developed carbon-coated SiC nanowire electrode (SiC@C NWE) was used[4], which had good detection performance for high concentrations of electroactive substances (ascorbic acid, uric acid, and dopamine) commonly found in cells, while not detecting ROS/RNS. No spike signal was detected when the SiC@C NWE was inserted into macrophages after phagocytosis of *E. coli* and a voltage of +800 mV was applied. It is suggested that the detected signal originated from ROS/RNS in the phagolysosomes rather than from other electroactive substances (uric acid, ascorbic acid, dopamine, etc.).

1.6 Transformation equations for the four primary ROS/RNS

$$2O^{\cdot\bullet}_2{}^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

$$O^{\cdot\bullet}_2{}^- + NO^\bullet \rightarrow ONOO^- \quad (2)$$

$$2ONOO^- \rightarrow 2NO_2^- + O_2 \quad (3)$$

1.7 Calculation of four primary ROS/RNS

The amount of ROS/RNS material in phagolysosomes at different potentials was calculated by Faraday's law: $Q = nzF$, where the charge Q was obtained by integration over the i-t curve, $F = 96500\ C\ mol^{-1}$ is the Faraday constant and z is the electron

transfer number ($z_{H_2O_2} = z_{NO_2^-} = 2,\ z_{NO} = z_{ONOO^-} = 1$).

The amount of individual ROS/RNS in a single phagolysosome of a RAW 264.7 macrophage can be obtained by solving the following system of equations:

$$Q_{800\,mV} = Q_{ONOO^-} + Q_{H_2O_2} + Q_{NO} + Q_{NO_2^-} \quad (4)$$

$$Q_{600\,mV} = Q_{ONOO^-} + Q_{H_2O_2} + Q_{NO} \quad (5)$$

$$Q_{500\,mV} = Q_{ONOO^-} + 0.95Q_{H_2O_2} \quad (6)$$

$$Q_{500\,mV+CAT} = Q_{ONOO^-} \quad (7)$$

where $Q_{potential}$ is the total charge measured under the selected voltage, and $Q_{species}$ is the charge of the named species.

2. Supporting Figures

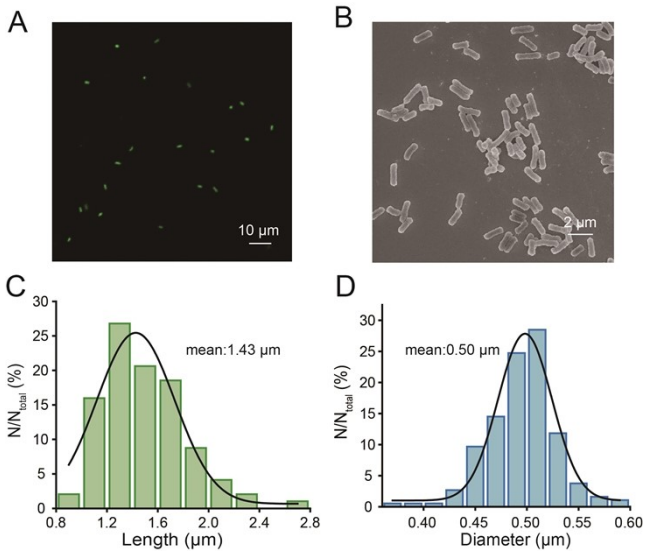

Fig. S1 (A) The fluorescence image of FITC-labeled *E. coli.* The SEM image (B) and length-diameter (C, D) distribution of *E. coli*.

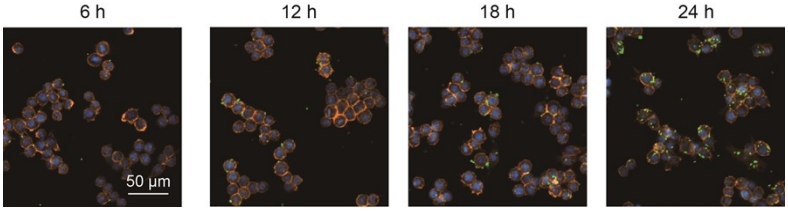

Fig. S2 Fluorescence images of RAW 264.7 macrophages after phagocytosis of *E. coli* at different times (6 h, 12 h, 18 h, and 24 h). Orange, actin filaments; green, *E. coli*; blue, nucleus.

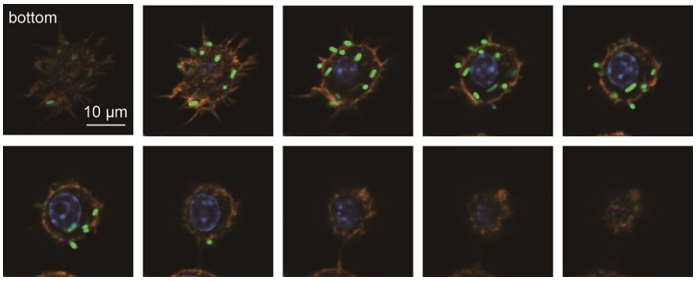

Fig. S3 Z-stacked confocal images of RAW 264.7 macrophages after 24 h phagocytosis of *E. coli*. *E. coli* (green), actin filaments (orange), nucleus (blue).

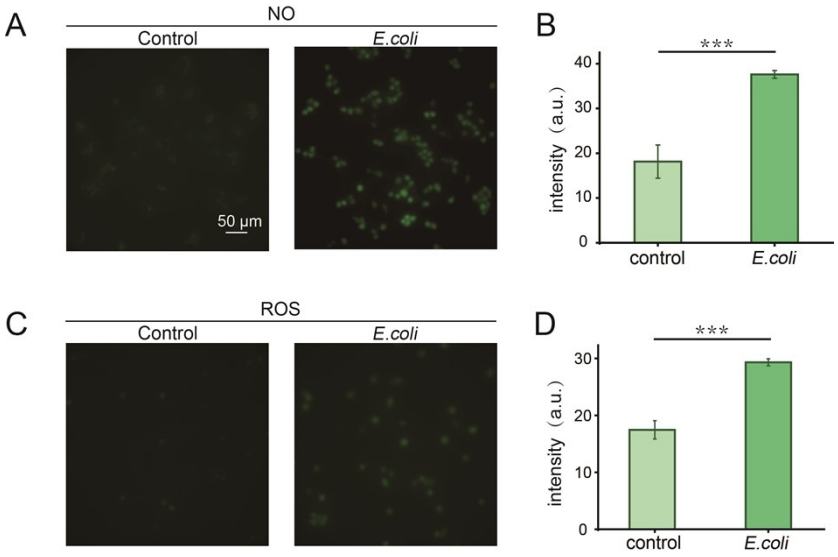

Fig. S4 Fluorescence images and statistical analysis of RAW 264.7 macrophages after 24 h phagocytosis of *E. coli* stained with DAF-FM DA (A, B) and DCFH-DA (C, D) (means and SEM, n=5; ***$P < 0.001$).

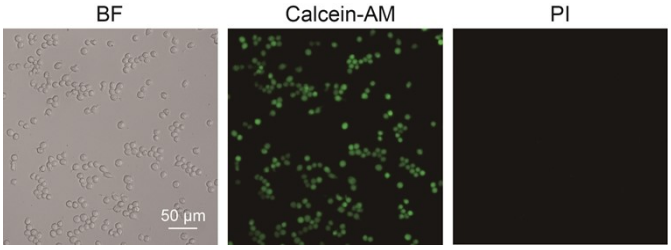

Fig. S5 Bright-field and fluorescence images of RAW 264.7 macrophages after 24 h phagocytosis of *E. coli* stained with calcein-AM (green) and propidium iodide (PI, red).

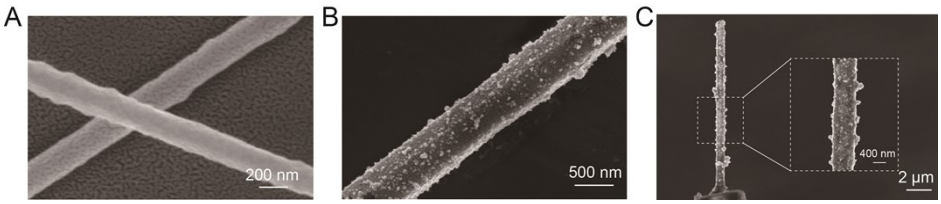

Fig. S6 SEM images of SiC NWs (A), SiC@Pt NW (B), and SiC@Pt NWE (C).

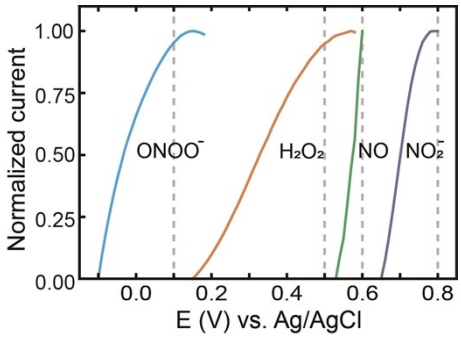

Fig. S7 Optimal detection potentials of four primary ROS/RNS ($ONOO^-$, H_2O_2, NO, and NO_2^-).

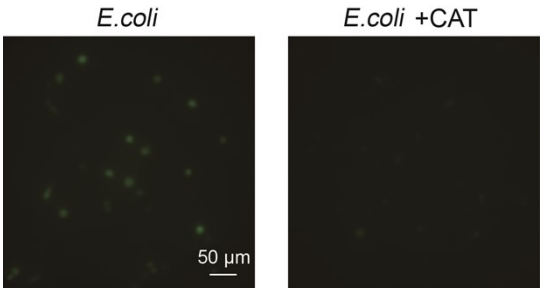

Fig. S8 Fluorescence images of RAW 264.7 macrophages with (right) and without (left) incubation with CAT for 30 min before stained with DCFH-DA.

Fig. S9 Representative amperometric trace recorded at +800 mV inside *E. coli* solution.

Fig. S10 Representative amperometric traces recorded after the insertion of a SiC@C NWE into macrophages after 24 h phagocytosis at +800 mV.

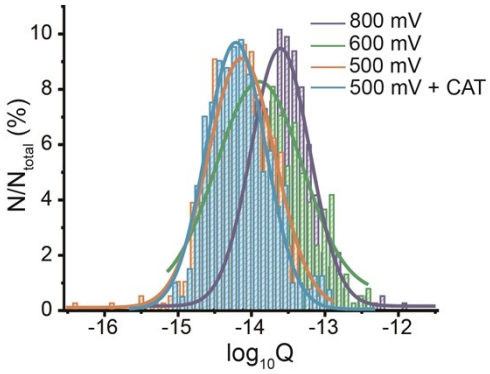

Fig. S11 Log-normal distribution of charge value ($\log_{10}Q$) at different potentials (purple, +800 mV, n = 384 events from 20 cells; green, +600 mV, n = 383 events from 26 cells; orange, +500 mV, n = 385 events from 22 cells; blue, +500 mV + CAT, n = 398 events from 21 cells).

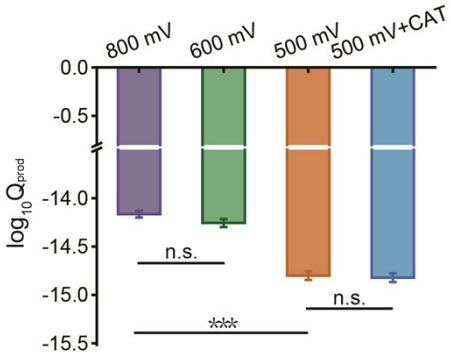

Fig. S12 Statistical analysis of $\log_{10}Q_{prod}$ at different potentials (***$P < 0.001$, n.s.: no significance).

3. References

1 Y.-T. Qi, H. Jiang, W.-T. Wu, F.-L. Zhang, S.-Y. Tian, W.-T. Fan, Y.-L. Liu, C. Amatore and W.-H. Huang, *J. Am. Chem. Soc.*, 2022, **144**, 9723-9733.

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