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Electronic Supplementary Information (ESI)

Nitrite-enhanced copper-based Fenton reactions for biofilm removal

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Materials and methods:

Materials

DNA (herring sperm), bovine serum albumin (BSA), sodium nitrite ($NaNO_2$, 99.99%), and Coomassie blue G250 were purchased from Sigma-Aldrich, China. GelRed was supplied by Biotium, USA. Glutaraldehyde (50%), hydrogen peroxide (H_2O_2, 30 wt%), crystal violet (0.1%), fluorescein diacetate (97%), ethanol, dichloromethane, copper sulfate, acetic acid (HOAc, ≥99.5%), nitric acid, 3,3',5,5'-tetramethylbenzidine, L-tyrosine disodium salt hydrate (98%), 3-nitro-L-tyrosine (98%), neocuproine (98%), sodium acetate (NaOAc), terephthalic acid, morpholine ethanesulfonic acid (MES), bicinchoninic acid (BCA), tryptone soy broth (TSB), nutrient agar, peptone from yeast, and yeast extract were purchased from Aladdin (Shanghai, China). N-(3-Oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) was purchased from Sengon Biotech (Shanghai, China). All chemicals and reagents were of analytical or higher grade and used without further purification. Ultrapure water was prepared by a Milli-Q purification system (Millipore, USA).

Instrumentation

A VICTOR X3 microplate reader (PerkinElmer, USA) was used to obtain the optical density of bacterial cells and absorbance values (flat bottom clear 96-well polystyrene plates). Biofilms were visualized with an inverted fluorescence microscope (Nikon Eclipse Ti, Tokyo, Japan), a field emission scanning electron microscope (FE-SEM, Carl Zeiss vltra55, Germany), and a wide-field high-content analysis system (Image Xpress Micro XLS, Molecular Devices, USA) with a FITC filter cube (excitation and emission wavelengths and bandpass widths of 482 and 35 nm and 536 and 40 nm, respectively). The color of the crystal-violet-stained biofilm was recorded with a digital camera. Identification of nitro compounds was performed with an Agilent 1200 LC coupled to a 6210 TOF MS.

Experimental procedures

Antibacterial efficacy study

The antibacterial efficacy of the nitrite-Cu-Fenton reagent was tested on DH5α cells of *Escherichia coli (E.coli).* DH5α cells were cultured in liquid LB medium and incubated at 37 ºC overnight, where the OD_{600} measurement was ~0.88. Next, 5 mL of DH5α cells were transferred to centrifuge tubes for centrifugation at 3000 rpm for 2 min. In each tube, the supernatant was then removed and DH5α cells were resuspended in 0.8 mL of sterile water to obtain concentrated bacterial cells. Thereafter, 5 µL of concentrated bacterial cells were pipetted into HOAc-NaOAc buffer (0.1 M, pH 5.0, final volume 50 µL), containing different combinations of the following reagents: 45 µM of Cu^{2+}, 3 mM of H_2O_2, and 2.5 mM of NO_2^-. After incubation for 1 h at 37 ºC, the mixture was centrifuged, and the supernatant was discarded. The pellet was then resuspended in 1 mL of LB medium before being partially (100 µL) plated onto LB agar and cultured for 12 h at 37 ºC. Colonies were then imaged and counted (Fiji/imageJ). The experiments were performed in triplicate. The killing of *Staphylococcus aureus (S. aureus)* by the nitrite-Cu-Fenton reagent was tested using D48 cells following the aforementioned procedure.

Biofilm removal study

Biofilm formation assay of *Pseudomonas aeruginosa* (PA01) on plastic disks (made of silicone, 12 mm diameter, 2 mm thickness) was performed both in a batch static mode and in a simulated flow cell model. Briefly, PA01 cells were cultured in liquid LB medium and incubated at 37 ºC overnight, where the OD_{600} measurement was ~1.0. PA01 cells were diluted in LB liquid media at a ratio of 1 to 100 and cultured at 37 ºC for 1 h. For quantitative analysis of biofilm growth, PA01 cells were transferred to 24-well polystyrene plates (each well contained 1 plastic disk) and then incubated in batch static mode at 37 ºC to form a biofilm. In another batch, PA01 cells were transferred to 50-mL centrifuge tubes (each containing one plastic disk) and incubated at 37 ºC on a horizontal shaker (50 rpm min^{-1}) to form a biofilm. This system effectively stimulated the shearing force and medium convection that the biofilm would experience in a flow-cell system. The old liquid LB medium was discarded and fresh medium was added every 24 h. After 72 h, plastic disks were carefully collected and dried. Thereafter, the plastic disks with attached biofilms were immersed in 1 mL of HOAc-NaOAc buffer (0.1 M, pH 5.0) containing varying combinations of 0.5 mM of Cu^{2+}, 0.3 M of H_2O_2, and 0.3 M of $NaNO_2$ for 1 h at 37 ºC. For visualization of the biofilms on the surface of the plastic disks, the disks were rinsed before being fixed with 2.5% glutaraldehyde overnight; afterwards, the biofilms were dehydrated through a series of graded ethanol baths, then dried and gold-coated, and finally imaged using an FE-SEM.

To image the biofilm with the digital camera, the plastic disk was rinsed, dried, and then stained with 0.1% crystal violet for 0.5 h. Subsequently, 10% acetic acid was used to solubilize the dye for quantification, where the OD_{600} absorbance values of the acetic acid samples were measured using a microplate reader. A biofilm formation assay of PA01 on 24-well plates without the plastic disks in a batch static mode was performed following the same cell culturing and rinsing steps mentioned above. An inverted microscope (Nikon Eclipse Ti, Tokyo, Japan) and widefield high-content analysis system (Image Xpress Micro XLS, Molecular Devices,

USA) with a FITC filter cube at 20× magnification were employed for visual observations. Fluorescein diacetate (FDA) was used to stain the biofilms for imaging with the Image Xpress Micro XLS system.

For the biofilm viability assay, PA01 cells were transferred to 24-well polystyrene plates that did not contain plastic disks in the wells for a 72-h incubation at 37 ºC to form biofilms. Following a similar procedure, the liquid LB medium was discarded and fresh medium was added every 24 h. Finally, the liquid LB medium was carefully removed and the wells were gently rinsed using sterile water. Afterwards, 2 mL of HOAc-NaOAc (0.1 M, pH 5.0) containing 0.5 mM of Cu^{2+}, 0.3 M of H_2O_2, and 0.3 M of $NaNO_2$ was added. After incubation for 1 h at 37 ºC, the wells were rinsed with sterile water before 1 mL of liquid LB medium was added into each well. Dilutions were made and 100 µL aliquots of each dilution were plated onto LB agar plates and cultured for 20 h at 37 ºC. Colonies were counted and imaged, with the number of CFU recorded.

Nucleic acid and protein cleavage study

Unless indicated otherwise, nucleic acid cleavage assays were performed at 37 ºC for 30 min in 50 µL of HOAc-NaOAc buffer (0.1 M, pH 5.0) containing 5 mM of Cu^{2+}, 1 mM of H_2O_2, 0.7 M of $NaNO_2$, and 25 µg of DNA (herring sperm). Nucleic acid cleavage products were identified by agarose gel electrophoresis and GelRed staining. Protein cleavage assays were performed at 37 ºC for 1 h in 50 µL of HOAc-NaOAc (0.1 M, pH 5.0) containing 5 mM of Cu^{2+}, 5 mM of H_2O_2, 0.7 M of $NaNO_2$ and 80 µg of BSA before separation by SDS-PAGE and Coomassie blue G250 staining.

Degradation of the bio-signaling molecule

3-Oxo-C12-HSL was used as the model bio-signal molecule. To prevent potential interference, the reaction was performed in 1 mL of pure water adjusted to pH 5.0 using nitric acid. The concentrations of the reagents were as follows: 3-oxo-C12-HSL (0.408 mM), Cu^{2+} (400 µM), $NaNO_2$ (100 mM), and H_2O_2 (500 mM). The mixtures were incubated at room temperature for 5 h. The products were extracted with 300 µL of acidified ethyl acetate and then dried under a stream of nitrogen. Afterwards, they were reconstituted with a 1 mL mixture of 30% acetonitrile and 70% water. The mixture was homogenized and injected into a liquid chromatography time-of-flight mass spectrometry (LC-TOF MS) system.

Nitration of L-tyrosine

To confirm the generation of nitrogen dioxide ($\cdot NO_2$) by the nitrite-Cu-Fenton system, L-tyrosine was chosen as a model substrate. Tyrosine was mixed with the nitrite-Cu-Fenton reagent and incubated for 24 h at 37 ºC. The concentration of each reagent was as follows: L-tyrosine disodium salt hydrate (10 mg mL^{-1}), Cu^{2+} (500 µM), $NaNO_2$ (25 or 100 mM), and H_2O_2 (100 mM). Afterwards, the mixture was diluted five folds and injected into the liquid chromatography system (Agilent 1200, USA) with the peak of 3-nitrotyrosine (3-NT) identified by TOFMS (Agilent 6210, USA). The presence of 3-NT was evidence of reactive nitrogen species ($\cdot NO_2$) generation.

Hydroxyl radical concentration was monitored using the well-established terephthalic acid (TPA) method where 0.5 mM of TPA was used to monitor ·OH formation under differing $NaNO_2$

concentrations (0–500 mM) in 0.1 M HOAc-NaOAc solution. Fluorescence was recorded for excitation at 310 nm and emission at 420 nm for full-band scanning. The fluorescence intensity of TPA-OH was $NaNO_2$ dose-dependent.

To study the effect of $NaNO_2$ on the initial reaction rates of TMB oxidation by the Cu-Fenton reagent, the absorbance changes were measured at 450 nm within 210 s, which is within the linear phase of the reaction kinetics. The slopes of linear kinetic trend-lines were used to calculate the initial reaction rates, where concentration changes within the first 210 s were calculated using the Beer-Lambert Law with a molar absorption coefficient of 39000 M^{-1} cm^{-1} for ox-TMB. The initial reaction rates for 250 µM of Cu^{2+} were also reported as turnover frequencies (TOF) and are measured in molecules of ox-TMB produced per Cu^{2+} ion per second of reaction time.

Monitoring of Cu^{2+} reduction and Cu^{+} formation and $ONOO^{-}$generation

The monitoring of Cu^{+} formation was based on the 2,9-dimethyl-1,10-phenanthroline (neocuproine) reagent, which produces a stable yellow complex (measured at 455 nm) with Cu^{+}. The monitoring of $ONOO^{-}$ was conducted by UV absorbance measurement at λ = 302 nm

Preparation of nano-CuO coated copper foil Cu(CuO)

Nano-CuO films were deposited on the surface of copper foil using the method developed by Akhavan *et al.*[1] Briefly, copper foils were washed with 3 M HCl and rinsed several times using ethanol and distilled water alternately. The cleaned copper foils were immersed in a 30 mM NaOH solution at 70 ºC for 24 h before washing with distilled water and drying at ambient temperature. A uniform black nano-CuO film was distinguishable on the copper foils, herein named Cu(CuO) foil.

Test the inhibition of nascent S. aureus biofilm on Cu(CuO) foil upon H_2O_2-NO_2^{-} treatment

A biofilm suppression assay of *S. aureus* (D48) on the Cu(CuO) foil in the absence and presence of H_2O_2 plus nitrite was performed. Glass slides of the same size as the studied Cu(CuO) foil were used as a control. The inoculum was prepared by diluting the bacterial cells (10^7 CFU mL^{-1}) in TSB media at a ratio of 1:75. For quantitative analysis of biofilm growth, 0.1 mL of inoculum was transferred to 24-well polystyrene plates and incubated with Cu(CuO) foils (or the glass slide control) in the presence of varying concentrations of H_2O_2 and nitrite (0, 5, 10, and 15 mM) at 37 ºC to form biofilms in the ten-fold diluted TSB media. After 12 h, the Cu(CuO) foils (or the glass slides) were carefully removed and rinsed using phosphate buffer. The Cu(CuO) foils (or the glass slides) were stained with SYTO 9/PI Live/Dead dye for 15 min and their images were observed and recorded using a fluorescence microscope (Nexcope NE910, China), where living and dead cells can be distinguished by green (live) and red (dead) fluorescence. Mortality, defined as the percentage of dead relative to the total number of cells on the Cu(CuO) foil or on the glass slide was calculated. Surface coverage analysis was performed on images of the bacteria on the glass slides and Cu(CuO) foils using image analysis software (Fiji/ImageJ). The amount of biofilm biomass was sonicated down and evaluated by protein analysis via the bicinchoninic acid (BCA) method using an optical density reader at 562 nm.

Removal of established *E. Coli* Biofilm on the surfaces

Before inoculation, concentrated *E. coli* (DH5α) cells (10^7 CFU mL^{-1}) were diluted in TSB media at a ratio of 1:15. 0.1 mL of the inoculum of DH5α cells were transferred into 24-well polystyrene plates (each well contained either Cu(CuO) foil or the glass slide) and then incubated for 48 h at 37 ºC to form biofilms in the ten-fold diluted TSB media. The TSB medium was replaced every 24 h. After 48 h, the Cu(CuO) foils or glass slides were removed and carefully cleaned using phosphate buffer. To evaluate the biofilm dispersal ability of the Cu(CuO)-H_2O_2-nitrite system, Cu(CuO) foil or the glass slide was immersed in 1 mL of MES buffer (0.1 M, pH 6.0) containing varying concentrations of H_2O_2 and nitrite to disperse the biofilms. After 1 h, the Cu(CuO) foil (or the glass slide) was carefully removed and rinsed using phosphate buffer. Then the biofilm images were recorded, and the biofilm biomass was evaluated using the BCA assay. To evaluate the biological viability of the EPS-protected resident cells within the treated biofilms, after treatment, the Cu(CuO) foil (or the glass slide) was transferred into a 5 mL centrifuge tube and re-incubated with 1 mL of fresh LB medium at 37 ºC for 12 h. 100 µL of re-cultured cell solution was plated on LB agar and the colonies were counted and imaged.

The effect of H_2O_2-NO_2^- treatment time on biofilm growth on Cu(CuO) foil

To test the inhibition of formation of biofilm of either *E.coli* or *S. aureus* on Cu(CuO) surfaces by H_2O_2-NO_2^- treatment over 1, 4, 8, and 12 h, a bacterial suspension was obtained by culturing bacterial cells in LB medium at 37 ºC in microplates until the optical density (OD600) reached 0.7–0.8 (by microplate reader, Infinite 200 Pro, Tecan, Austria). Afterwards, 1.5 mL of bacterial cell suspension was transferred to microcentrifuge tubes before centrifugation at 3000 rpm for 2 min. The supernatant was removed and the D48 cells were re-suspended in 200 µL of sterile water to obtain a concentrated bacterial cell solution. Thereafter, 100 µL concentrated bacterial cells (10^7 CFU mL^{-1}) were pipetted into MES buffer (0.1 M, pH 5.0 final volume 1 mL) containing combinations of the Cu(CuO) foil (or glass slides as the Control) with various concentration of H_2O_2 and nitrite. After incubation at 37 ºC for 1, 4, 8, or 12 h, the mixtures were vortexed and 100 µL aliquots were plated onto LB agar and cultured for 12 h at 37 ºC. After 12 h, the biofilm growth on the Cu(CuO) foils was recorded as described in the section "Test the inhibition of nascent *S. aureus* biofilm on Cu(CuO) foil upon H_2O_2-NO_2^- treatment".

The antibacterial efficacy contributed by nano-CuO surfaces Vs leached Cu ions

To determine the contributions for the cytotoxicity toward planktonic bacteria, the cell-killing ability of the Cu(CuO) foil and its leachate against *S. aureus* was investigated. The leachate was prepared by immersing Cu(CuO) foil in MES buffer solution for 1, 4, 8 or 12 h in the absence and presence of H_2O_2 plus NO_2^-. 100 µL concentrated *S. aureus* (D48) bacterial cells (10^7 CFU mL^{-1}) was incubated with Cu(CuO) foils or the leachate in the presence and absence of H_2O_2 plus NO_2^- at 37 ºC in MES buffer (0.1 M, pH 6.0, final volume 1 mL). After incubation for 1, 4, 8, or 12 h, the mixtures were vortexed and 100 µL aliquots were plated onto LB agar and cultured for 12 h at 37 ºC. The colonies were counted.

Supporting Table:

Table S1. Synergistic antibacterial (*Pseudomonas aeruginosa*) activity of the nitrite–Cu-Fenton system.[2]

Treatment			% Survival*		
Cu^{2+} (mM)	H_2O_2 (M)	$NaNO_2$ (M)	Experimental (E)	Theoretical (T)	T/E
0.5			71.1		
	0.3		55.8		
		0.3	45.2		
0.5	0.3	0.3	5.6	17.9	3.2
0.5	0.3		15.2		
		0.3	45.2		
0.5	0.3	0.3	5.6	6.9	1.2
0.5		0.3	18.5		
	0.3		55.8		
0.5	0.3	0.3	5.6	10.3	1.8
	0.3	0.3	20.1		
0.5			71.1		
0.5	0.3	0.3	5.6	14.3	2.6

*CFU on the plate of treatment group ratio to the CFU on the control plate.

Supporting Figures:

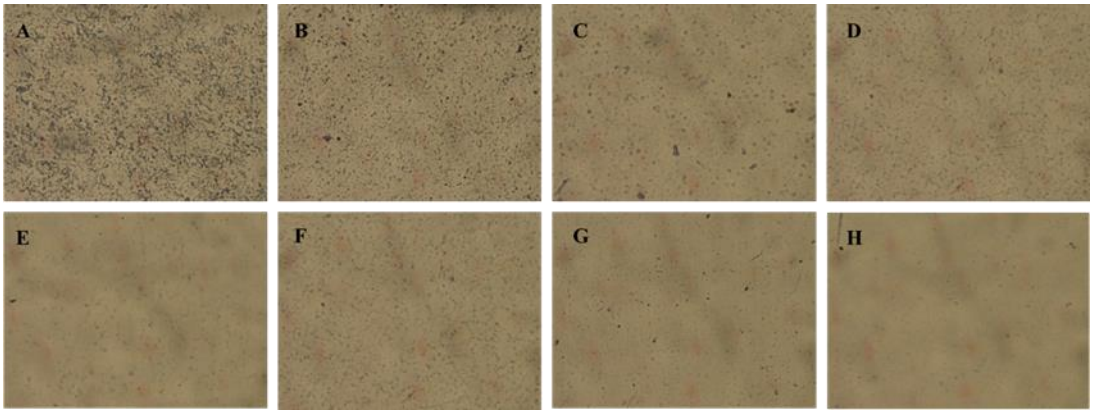

Fig. S1. Microscope images of *Pseudomonas aeruginosa* biofilms following nitrite–Cu-Fenton reagent treatment. 0.1 M HOAc-NaOAc (pH 5.0), [Cu^{2+}] = 0.50 mM, [H_2O_2] = 0.30 M, and [$NaNO_2$] = 0.30 M. A, control; B, Cu^{2+}; C, NO_2^-; D, H_2O_2; E, Cu^{2+}-H_2O_2; F, H_2O_2-NO_2^-, G, Cu^{2+}-NO_2^-; and H, Cu^{2+}-H_2O_2-NO_2^-.

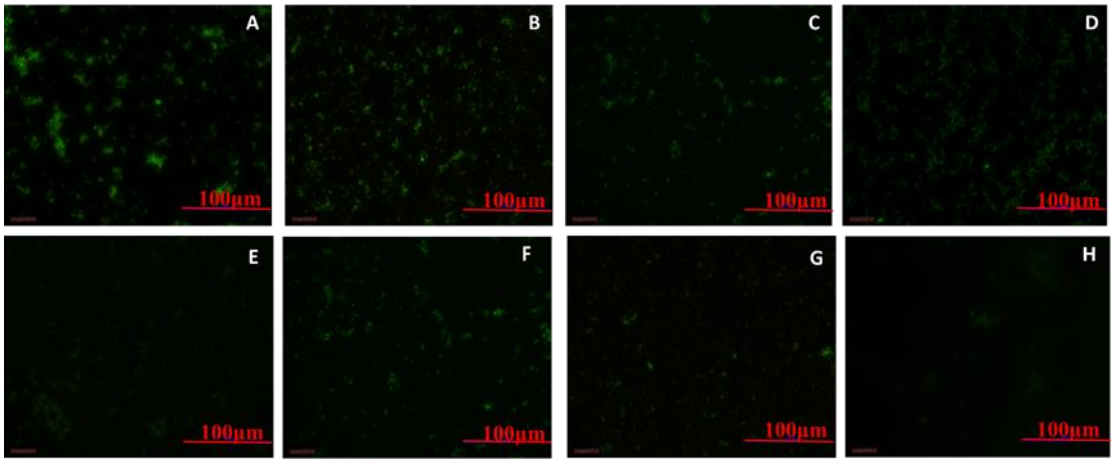

Fig. S2. Wide-field high-content analysis images of *Pseudomonas aeruginosa* biofilms after treatments by A) control; B) Cu^{2+}; C) NO_2^-; D) H_2O_2; E) Cu^{2+}-H_2O_2; F) H_2O_2- NO_2^-; G) Cu^{2+}-NO_2^-; and H) Cu^{2+}-H_2O_2- NO_2^-. 0.1 M HOAc-NaOAc (pH 5.0), [Cu^{2+}] = 0.50 mM, [H_2O_2] = 0.30 M, and [$NaNO_2$] = 0.30 M.

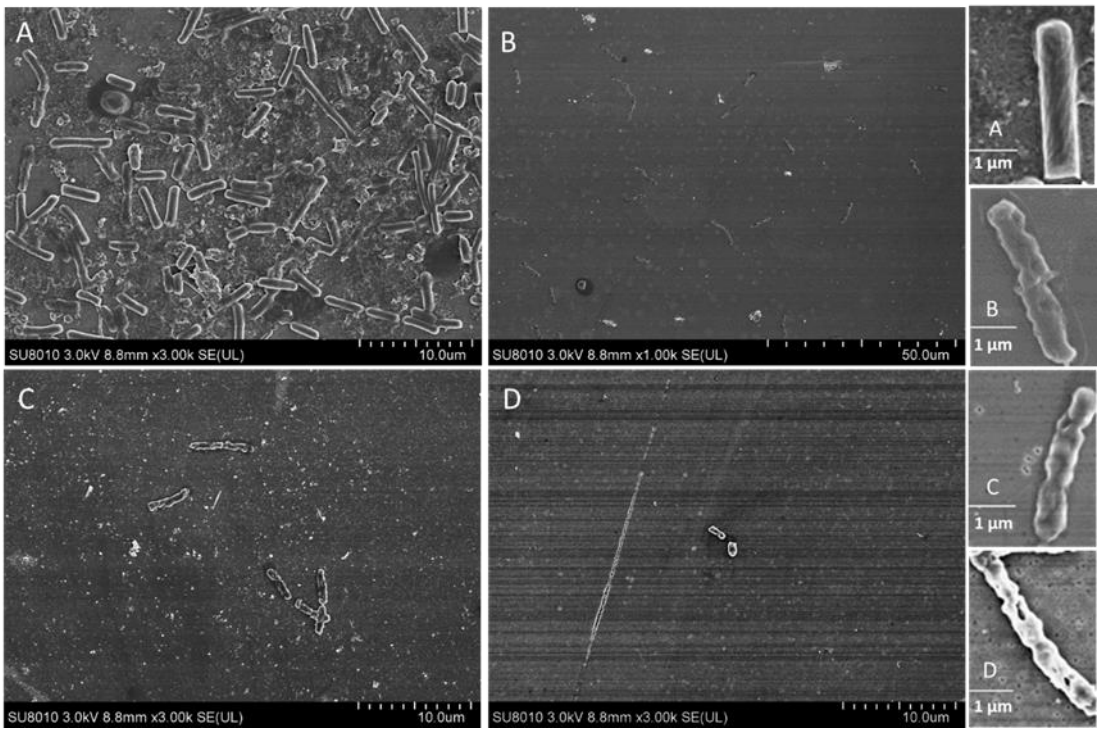

Fig. S3. Field emission scanning electron microscope (FE-SEM) images of *Pseudomonas aeruginosa* biofilms after treatment by A) control; B) H_2O_2; C) Cu^{2+}-H_2O_2; and D) Cu^{2+}-H_2O_2-NO_2^-. 0.1 M HOAc-NaOAc (pH 5.0), [Cu^{2+}] = 0.50 mM, [H_2O_2] = 0.30 M, and [$NaNO_2$] = 0.30 M.

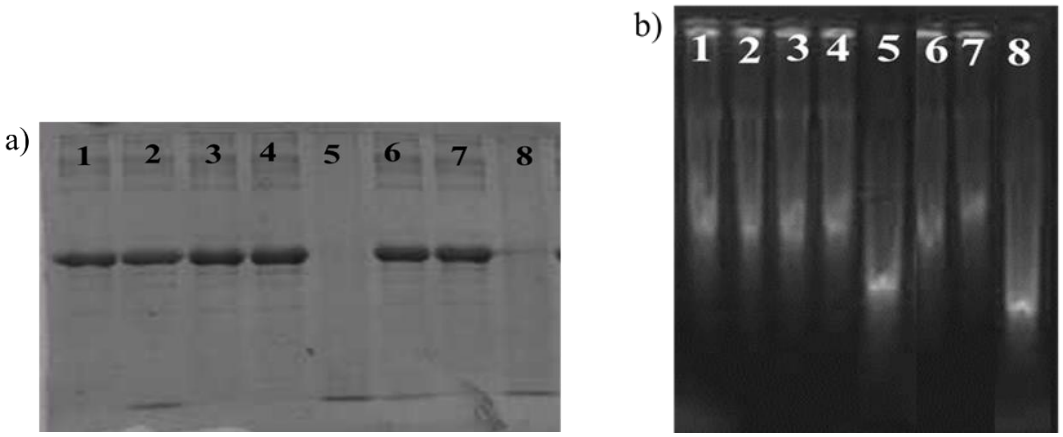

Fig. S4. Electrophoresis images of a) bovine serum albumin and b) genomic DNA after treatment by the nitrite-Cu-Fenton reagent. The diagram shows the sequence of bands from 1 to 8 treated by 1, control; 2, Cu^{2+}; 3, NO_2^-; 4, H_2O_2; 5, Cu^{2+}–H_2O_2; 6, H_2O_2–NO_2^-; 7, Cu^{2+}–NO_2^-; and 8, Cu^{2+}–H_2O_2–NO_2^-. 0.1 M HOAc-NaOAc (pH 5.0), [Cu^{2+}] = 5 mM, [H_2O_2] = 1 mM, and [$NaNO_2$] = 0.7 M.

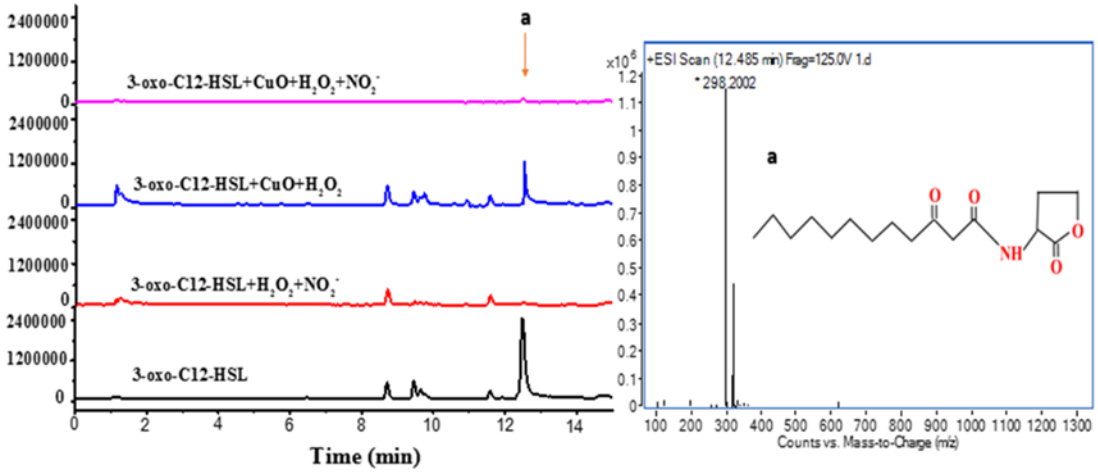

Fig. S5. The degradation of 3-oxo-C12-HSL (a) by nitrite-Cu-Fenton reagent as detected by liquid chromatography mass spectrometry. 0.1 M HOAc-NaOAc (pH 5.0), [Cu^{2+}] = 400 μM, [H_2O_2] = 0.50 M, [$NaNO_2$] = 0.10 M, and [3-oxo-C12-HSL] = 0.40 mM.

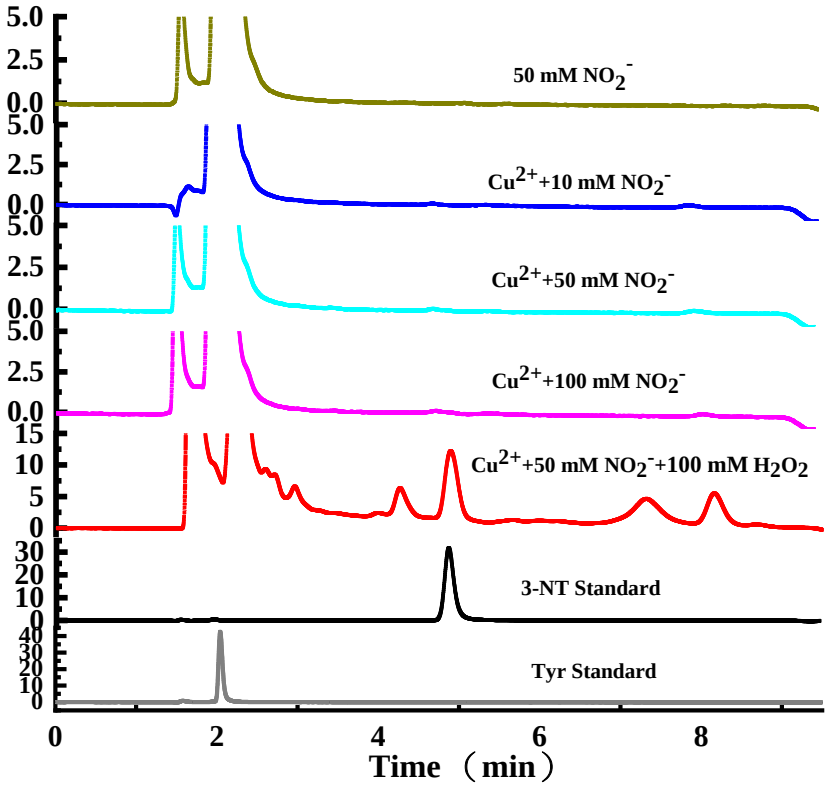

Fig. S6. Liquid chromatography of tyrosine treated by the nitrite-Cu-Fenton reagent, where [Cu^{2+}] = 400 μM.

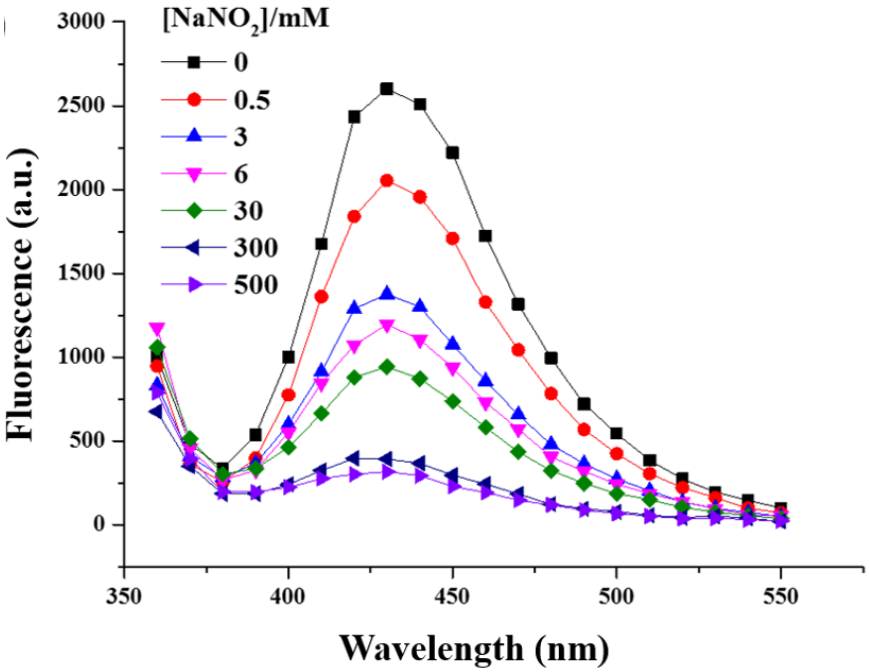

Fig. S7. The hydroxyl radical yield as detected by the conversion of terephthalic acid (TPA) to fluorescent hydroxyterephthalic acid as a function of $NaNO_2$ concentration (0–500 mM). 0.1 M HOAc-NaOAc (pH 5.0), [Cu^{2+}] = 3 mM, [H_2O_2] = 0.75 M, and [TPA] = 0.5 mM.

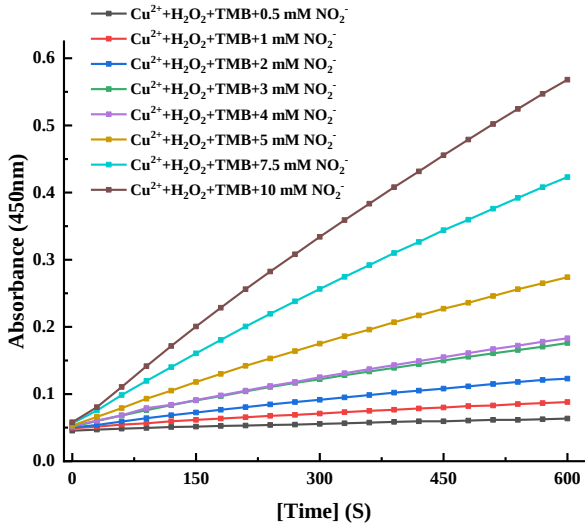

Fig. S8. TMB oxidation by nitrite-Cu-Fenton systems ([H_2O_2] = 0.5 mM, [TMB] = 0.5 mM, [Cu^{2+}] = 0.25 mM, and 0.5 – 10 mM of nitrite) in 0.1 M acetate buffer (pH 5.0), reaction time 10 min.

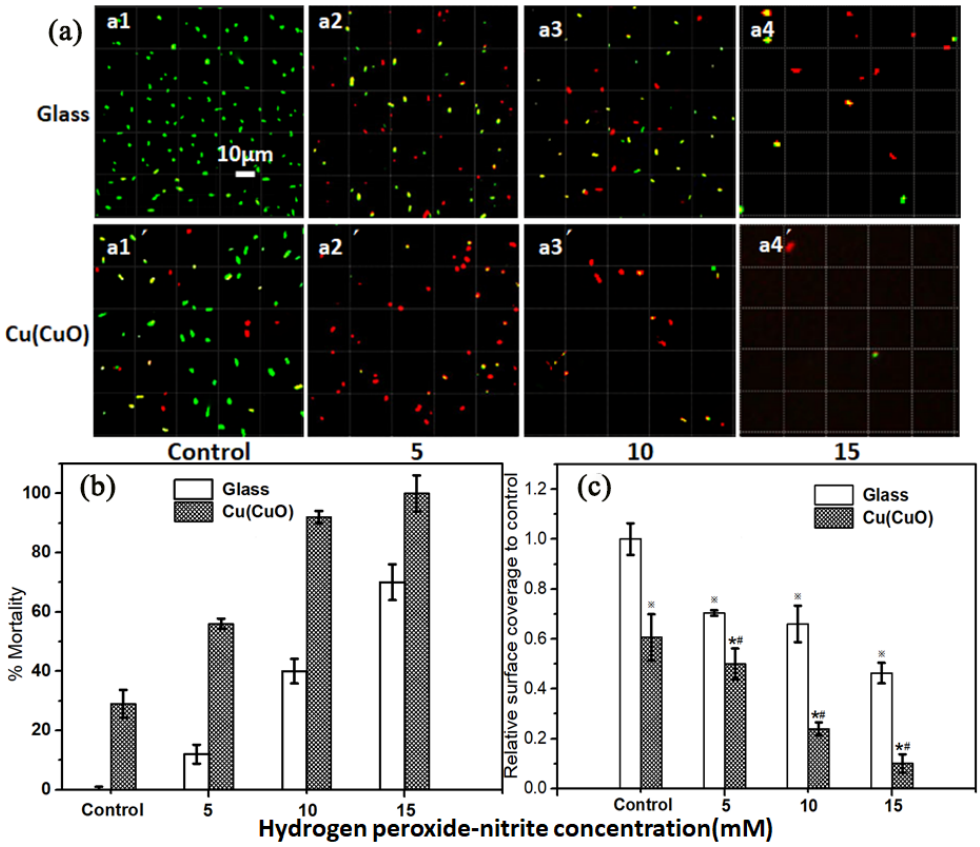

Fig. S9. Inhibition of Cu(CuO)-H_2O_2-NO_2^- system on the development nascent *S. aureus* biofilm. (a) Fluorescence microscope images of biofilm with different treatment: a1–a4 indicate biofilm growth on glass surfaces, while a1'–a4' indicates biofilm on Cu(CuO) film. a1 and a1', a2 and a2', a3 and a3', and a4 and a4' were treated with the different concentrations (0, 5, 10, and 15 mM) of hydrogen peroxide plus nitrite. (b) The mortality of *S. aureus* after contact with H_2O_2 and nitrite mixtures on a glass slide (control) or Cu(CuO) foil after 12 h of incubation. (c). Relative surface coverage analysis of *S. aureus* biofilms normalized to the surface coverage of that on glass slides (control), n=3.

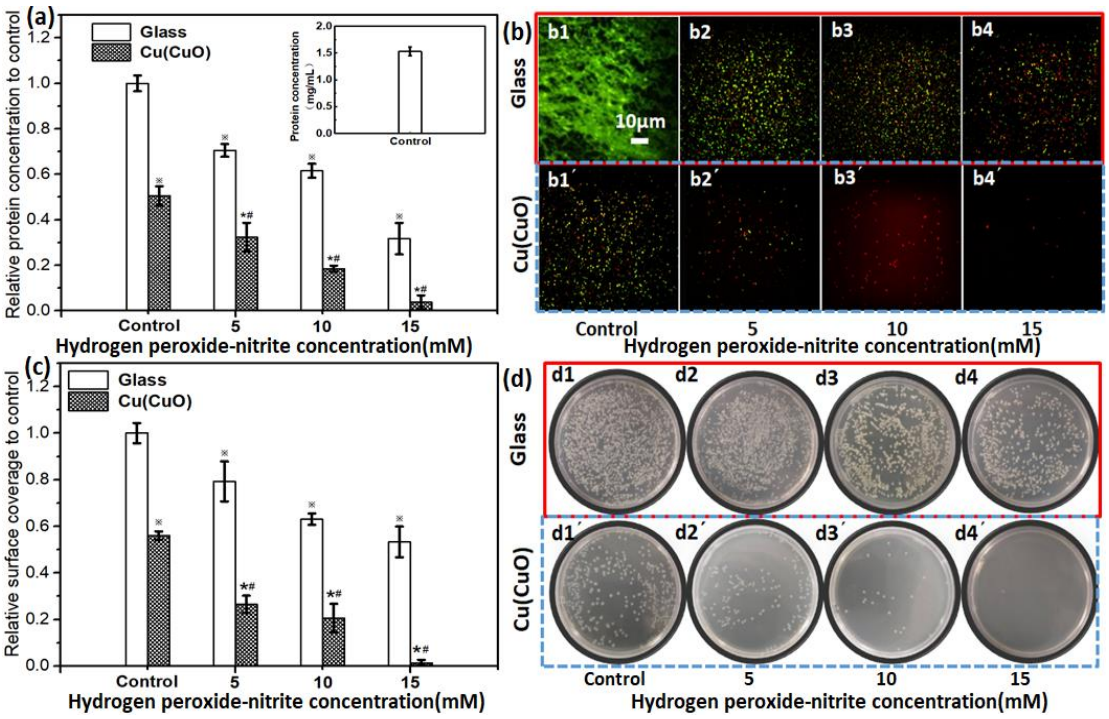

Fig. S10. Disruption of established *E. coli* biofilm by Cu(CuO)-H_2O_2-NO_2^-system. (a) After treatment with different concentrations (0, 5, 10, and 15 mM) of hydrogen peroxide plus nitrite, the relative amount of protein of the biofilm on the glass surface (control) or Cu(CuO) foil was plotted relative to the protein concentration of the control (inset) for n=3. (b) The fluorescence microscopic images of the biofilm; b1–b4 indicates biofilm formed on the glass slides, b1'– b4' indicates that on Cu(CuO) foil. b1 and b1', b2 and b2', b3 and b3', and b4 and b4' were treated with different concentrations (0, 5, 10, and 15 mM) of hydrogen peroxide plus nitrite. (c) Relative surface coverage analysis of *E. coli* biofilms normalized to the surface coverage of those on glass slides (control), n=3. (d) The resulting colony counts for glass slide (control, d1–d4, top) or Cu(CuO) foil (d1'–d4', bottom) used in (b) that were centrifuged in culture medium and re-cultured, indicating the survival of the biofilm-protected *E. coli* cells after treatment.

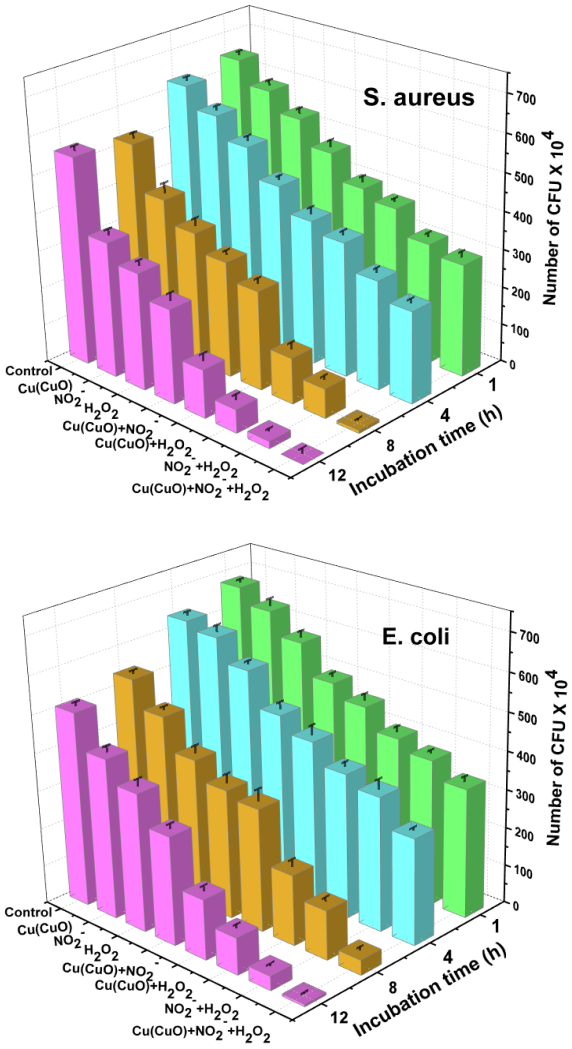

Fig. S11. Inhibition of formation of biofilm of either *E. coli* or *S. aureus* on Cu(CuO) surfaces by H_2O_2-NO_2^- treatment over 1, 4, 8, and 12 h. After 12 h of treatment, the biofilms were stained and imaged as illustrated in Fig.S9.

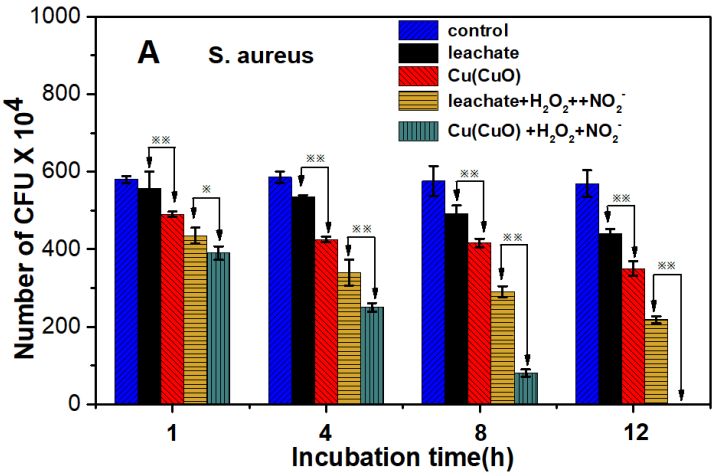

Fig. S12. Planktonic *S. aureus* killing by Cu(CuO) or leachate systems in the absence and presence of H_2O_2 and NO_2^- after 1, 4, 8 and 12 h of incubation. The control contains only bacteria and MES buffer. * ($p \leq 0.05$), **($p \leq 0.01$)

Supporting References:

1. Akhavan, O., Azimirad, R., Safa, S., & Hasani, E. (2011). CuO/Cu (OH) 2 hierarchical nanostructures as bactericidal photocatalysts. *Journal of Materials Chemistry*, *21*(26), 9634-9640.
2. Ragab-Depre, N. J. (1982). Water disinfection with the hydrogen peroxide-ascorbic acid-copper (II) system. *Applied and environmental microbiology*, *44*(3), 555-560.