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Halogen anion modulated metal-organic frameworks with enhanced nanozyme activities for bacterial biofilm disruption

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Supplement methods

Materials

Zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$), Cobalt nitrate hexahydrate ($Co(NO_3)_2 \cdot 6H_2O$), Sodium fluoride (NaF), Sodium chloride (NaCl), Sodium bromide (NaBr), Sodium iodide (NaI), and 2-methylimidazole (2-MI) were purchased from Sigma-Aldrich. Methanol (100%) were provided by Sinopharm Chemical Reagent Co., Ltd. 3,3',5,5'-tetramethylbenzidine (TMB), 9,10-anthracenediylbis (methylene) dimalonic acid (ABDA), chloroquine and dimethyl sulfoxide (DMSO) were obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. Tryptone soy broth (TSB) was obtained from Hangzhou Microbial Reagent Co., Ltd. All chemicals were used without further purification. Ultrapure water (18.2 MΩ) was used throughout the experiments.

Synthesis and Characterization of ZIF-L, Ch@ZIF-L and their variants

ZIF-L was synthesised by dissolving 145.52 mg of $Co(NO_3)_2 \cdot 6H_2O$ and 148.71 mg of $Zn(NO_3)_2 \cdot 6H_2O$ in 8 mL of deionized water and transferred to a 25 mL single-neck round-bottom flask. Subsequently, 656.84 mg of 2-MI was added to solution, and the mixture was stirred at room temperature (25^{o}C) for 24 hours. The resulting suspension was centrifuged at 7000 rpm for 10 minutes to collect the synthesized ZIF-L. The product was washed three times with water and vacuum-dried at 60^{o}C for 12 hours.

The preparation of ZIF-L variants involves the additional incorporation of anions. Using ZIF-L-H-Cl as an example, 145.52 mg of $Co(NO_3)_2 \cdot 6H_2O$, 148.71 mg of $Zn(NO_3)_2 \cdot 6H_2O$, and 1462.5 mg of NaCl were dissolved in 8 mL of deionized water and transferred to a 25 mL single-neck round-bottom flask. Then, 656.84 mg of 2-MI was added, and the mixture was stirred at room temperature for 24 hours. The resulting suspension was centrifuged at 7000 rpm for 10 minutes to collect the synthesized ZIF-L-H-Cl. The product was washed three times with water and vacuum-dried at 60^{o}C for 12 hours.

To obtain Ch@ZIF-L variants, 300 mg of activated powder and 1000 mg chloroquine powder were dispersed in 50 mL methanol and stirred at room temperature for 48 hours. The mixture was then centrifuged at 7000 rpm for 10 minutes to collect

the Ch@ZIF-L variants product. The product was washed three times with methanol and vacuum-dried at 60ºC for 12 hours. Transmission electron microscopy (TEM), high-angle annular dark-field scanning TEM (HAADF-STEM), and energy-dispersive X-ray (EDX) mapping were performed using a FEI Talos F200X microscope (operating at 200 kV, FEI, Hillsboro, OR, USA). X-ray powder diffraction (XRD) patterns were recorded with a diffractometer (D8 discover, Bruker) with Cu Kα radiation (wavelength 0.15406 nm). Absorbance peaks were monitored using a microplate reader equipped with ultraviolet-visible (UV-Vis) spectroscopy (Nano Drop 2000c, Thermo Fisher Scientific), with an operating voltage of 40 kV and current of 40 mA, respectively. The Brunauer-Emmett-Teller (BET) surface area was determined from nitrogen adsorption/desorption isotherms at 77 K using ASAP 2050 iinstrument (Micromeritics). The release of Zn^{2+}, Co^{2+}, and chloroquine from Ch@ZIF-L-H-Cl was measured using inductively coupled plasma optical emission spectrometer (ICP-OES, Avio 200, PerkinElmer). Stained biofilms were observed using a three-dimensional confocal laser scanning microscope (LSM 800, Zeiss).

Evaluation of Chloroquine Loading Rate in ZIF-L-H-Cl

UV-Vis spectroscopy was employed to confirm the loading of chloroquine in ZIF-L-H-Cl, and to evaluate the chloroquine loading rate. The chloroquine solutions with gradient concentrations of 5, 10, 20, 30, 40, and 50 μg/mL were prepared, followed by UV-Vis spectra measurements for the calibration curve using the absorbance at 330 nm. Then Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) was employed to quantify the metal contents of ZIF-L-H-Cl for determination of the mass. A differential analysis of the UV-Vis absorbance at 330 nm (before and after chloroquine loading) was performed to quantify the mass of chloroquine loaded in ZIF-L-H-Cl. The loading efficiency of chloroquine in ZIF-L-H-Cl was calculated according to the following formula,

$$\text{Loading Rate (\%)} = \frac{\text{Mass}_{\text{chloroquine}}}{\text{Mass}_{\text{chloroquine}}+\text{Mass}_{\text{ZIF-L-H-Cl}}} \times 100\%$$

where, $\text{Mass}_{\text{chloroquine}}$ and $\text{Mass}_{\text{ZIF-L-H-Cl}}$ represent the mass of the substance as specified, in the tests using 1 mL sample suspensions.

In Vitro Release of Chloroquine, Co^{2+}, and Zn^{2+} from Ch@ZIF-L-H-Cl

Briefly, 1 mL of Ch@ZIF-L-H-Cl (100 μg/mL) was dispersed in dialysis bags (8 kDa), immersed in 10 mL of phosphate buffer (pH 6.0, biofilm microenvironment pH) and shaken at 37°C. At predetermined time intervals (0, 0.5, 1, 2, 4, and 8 hours), 1 mL of buffer was removed, and 1 mL of buffer was added at the same time. Finally, the concentrations of Co^{2+}, Zn^{2+}, and Cl- ions at different time points were determined by ICP-OES.

Oxidase-like Activity of Ch@ZIF-L and its variants

The oxidase-like activities of Ch@ZIF-L and its variants were evaluated based on the oxidation of TMB. In this process, 10 μL of 50 mM TMB was mixed with 1 mL of acetate buffer (0.1 M acetic acid, 0.1 M sodium acetate, pH 4.0, 25°C), followed by the addition of 10 μL of 20 μg/mL chloroquine or 100 μg/mL Ch@ZIF-L variants. After thorough mixing, 200 μL of the solution was transferred into a clean 96-well plate, and the absorbance peak at 652 nm was monitored using a microplate reader equipped with UV-Vis spectroscopy (Synergy H1, Bio-Tek).

Kinetic of Enzymatic Catalytic Reaction

Kinetic measurements were performed by monitoring the change in absorbance at 652 nm using a microplate reader (Synergy H1, Bio-Tek). Absorption spectra were recorded at 60 seconds intervals over a total duration of 300 seconds. Samples contained 10 μg of Ch@ZIF-L variants or Ch@ZIF-L, along with varying concentrations of TMB in 0.1 m acetate buffer (pH = 4.0). Kinetic parameters were calculated using the Michaelis–Menten equation: $V = Vmax \times [S] / (Km + [S])$, where $Vmax$ represents the maximum reaction velocity, $[S]$ is the substrate concentration, and Km is the Michaelis constant. The values of Km and $Vmax$ were determined from the double reciprocal plot. The molar absorption coefficient of TMB at 652 nm is 39000 $m^{-1} \cdot cm^{-1}$.

Bacterial Culture

S. aureus (ATCC 25923) was cultured on tryptone soy agar at 37°C in ambient air. After 24 hours, a single colony was picked from the agar plate and inoculated in 8 mL of TSB, then incubated at 37°C for 24 hours in ambient air. This preculture was diluted 1:20 in 8 mL of TSB and grown for an additional 16 hours at 37°C. *Staphylococci* were

collected by centrifugation (4000 rpm for 5 minutes) and washed twice with PBS. Finally, the bacteria were resuspended in 8 mL of PBS and their concentration was estimated by measuring the optical density at 600 nm (OD 600 of 0.1 corresponds to approximately 10^8 CFU mL^{-1}).

Biofilm Growth

Staphylococcus aureus biofilms were cultured using bacterial slides (18 mm) placed in 12-well plates. First, 1mL of *Staphylococcus aureus* suspension (3×10^8 CFU/mL) was added to each well and allowed to culture at 37ºC for 2 hours, enabling the bacteria to settle and adhere. The bacterial slides were then gently washed with sterile PBS to remove any non-adhered planktonic bacteria, and subsequently transfered to another well containing 2 mL TSB. Mature biofilms were allowed to form after 48 hours of culture at 37ºC.

Generation of Singlet Oxygen

The ability of Ch@ZIF-L and its variants to produce singlet oxygen was evaluated by monitoring the decrease in absorbance of a 9,10-anthracenediylbis (methylene) dimalonic acid (ABDA) solution. Singlet oxygen bleached the ABDA, converting it into the corresponding peroxide lactone and causing the reduction in absorbance at 400 nm. To measure this, 100 μg Ch@ZIF-L variants or Ch@ZIF-L was mixed with 10 μL ABDA solution (20 mM) and added to 1 mL of acetate buffer (pH 4.0). The UV-visible absorbance at 400 nm was then measured at regular intervals using a UV-visible spectrophotometer (Nano Drop 2000c, Thermo Fisher Scientific) to quantify the production of singlet oxygen.

***In vitro* Cytotoxicity test**

In vitro biosafety was assessed by culturing Human umbilical vein endothelial cells (HUVEC, ATCC CRL-1730) fibroblasts in petri dishes, filled with dulbecco's modified eagle medium-high glucose (DMEM-HG) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37℃ in a humidified 5% CO_2 incubator. When the cells reached at 70% confluency, they were harvested by adding an ethylenediaminetetraacetic acid-trypsin (EDTA−trypsin) solution, centrifuged at 500 g for 10 minutes, and resuspended in DMEM-HG. The cells were then seeded into 96-

well plates (8000 cells, 100 μL per well) and incubated for 24 hours. After removing the growth medium, fresh medium with different concentrations Ch@ZIF-L-H-Cl was added. After 24 and 48 hours, the medium was replaced, cell viability was assessed adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-containing (MTT-containing) medium for 30 minutes. The absorbance at 490 nm was measured using an enzyme-labeled instrument to determine the number of cells in each well.

***In vitro* Hemolysis test**

Hemolytic effects were evaluated *in vitro* using red blood cells from 8 weeks old, male mice, provided by the Model Animal Research Center of Soochow University. All animal experiments were performed in accordance with the guidelines and with the approval of the Institutional Animal Care and User Committee at Soochow University. Anticoagulant citrate dextrose-supplemented whole blood (800 μL) was taken from the orbital venous plexus and centrifugated at 500 g for 5 minutes at 4ºC to collect red blood cells. Red blood cells were washed three times with phosphate-buffered saline (PBS) and resuspended in 800 μL of PBS. Then, 0.1 mL of red blood cells was mixed with 0.9 mL of Ch@ZIF-L-H-Cl at different concentrations. After incubation for 3 hours at 37ºC, the mixed suspension was centrifuged at 500 g for 5 minutes, and the absorbance of hemoglobin in the supernatant was measured at 540 nm using a microplate reader. Red blood cells suspended in PBS served as a negative control, while cells suspended in ultrapure water were used as a positive control. Percentage hemolysis was calculated according to,

$$\text{Hemolysis (\%)} = \frac{\text{Abs}_{\text{ch@ZIF-L-H-Cl}} - \text{Abs}_{\text{PBS}}}{\text{ABs}_{\text{water}} - \text{Abs}_{\text{PBS}}} \times 100\%$$

where, $\text{Abs}_{\text{Ch@ZIF-L-H-Cl}}$, Abs_{PBS}, and $\text{Abs}_{\text{water}}$ represent the absorbance values of the respective hemoglobin suspensions measured at 540 nm.

***In vivo* Biosafety**

After shaving the back of the mice, 100 μL of Ch@ZIF-L-H-Cl solution (1000 μg/mL) and 100 μL of PBS were injected subcutaneously into two groups of mice. The injections were administered once every 24 hours and repeated three times. Ten days after the first injection, the mice were sacrificed and blood was collected. The blood

samples were left undisturbed for 30 minutes and then centrifuged at 2000 rpm for 10 minutes to obtain plasma for biochemical analysis. Simultaneously, the mice were dissected, and the main internal organs (heart, liver, spleen, lung and kidney) were collected for histological analysis and stained with hematoxylin-eosin (H&E).

Different Treatments of Biofilm Infection Wound

The bacteria infected wound models were established on the back of female mice (Balb/c, 6-8 weeks). The mice were anesthetized by intraperitoneal injection of 100 μL of 5% chloral hydrate. Circular wounds (d = 8 mm) were created using surgical scissors after removing the dorsal hair of the mice. Each wound was inoculated with 100 μL of *Staphylococcus aureus* (3×10^7 CFUs) and left for 30 minutes while the mice remained anesthetized. After 24 hours, the wounds were infected with *Staphylococcus aureus*. The mice with infected wounds were randomly divided into four groups, with three mice in each group. On day 0 (24 hours post-infection), the mice were anesthetized for 30 minutes, and their wounds were treated by dropping 100 μL of PBS, 100 μL chloroquine (20 μg/mL), 100 μL ZIF-L-H-Cl (100 μg/mL) or 100 μL ZIF-L-H-Cl (100 μg/mL) to the wound surface. The treatment was repeated on both day 0 and day 1.

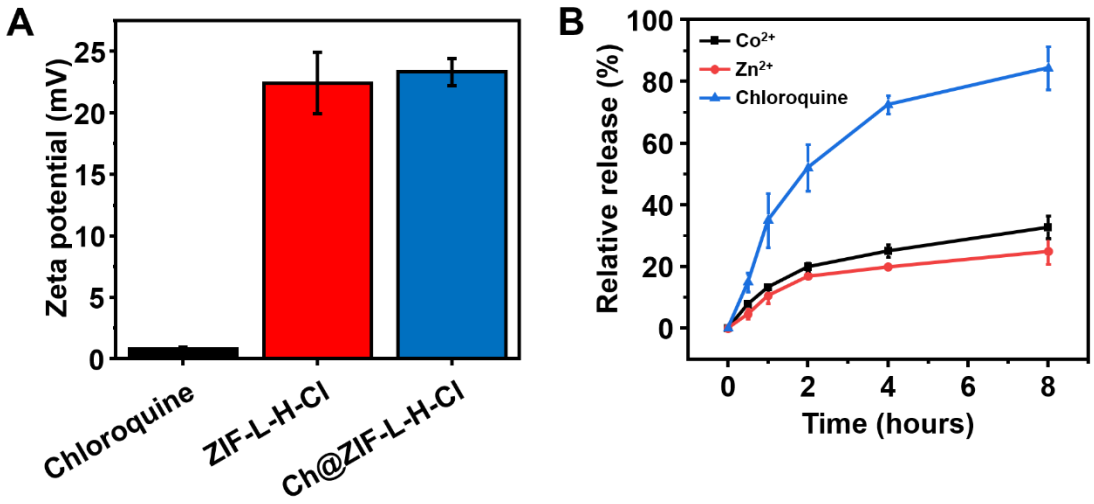

Figure S1. Zeta potentials and relative release of Ch@ZIF-L-H-Cl. (A) Measurements of zeta potentials of chloroquine, ZIF-L-H-Cl and Ch@ZIF-L-H-Cl. Error bar: standard deviation (n = 3). (B) Release rates of Co^{2+}, Zn^{2+} and chloroquine from Ch@ZIF-L-H-Cl at pH 6.0 (simulating the biofilm microenvironment). The amounts of chloroquine, Zn^{2+}, and Co^{2+} in Ch@ZIF-L-H-Cl at 0 h were normalized to 100%. The release efficiency of chloroquine was 84.3% at 8 h. Error bars: standard deviations (n = 3).

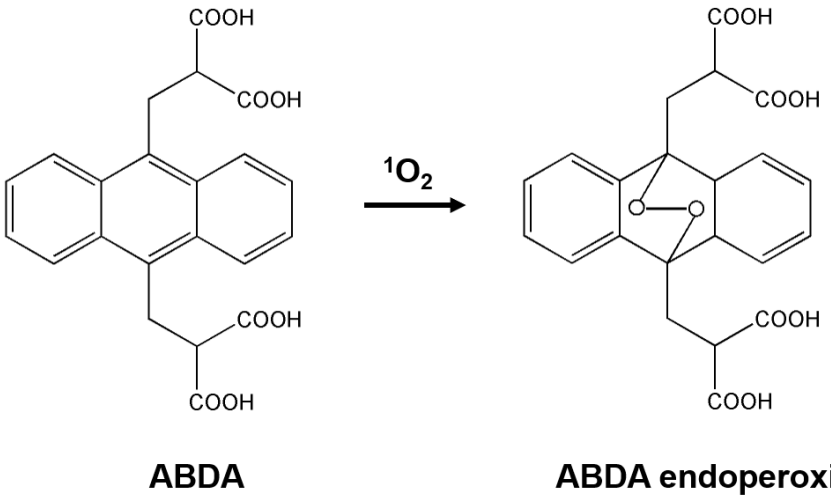

Figure S2. Oxidation of 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) by singlet oxygen into ABDA endoperoxide.

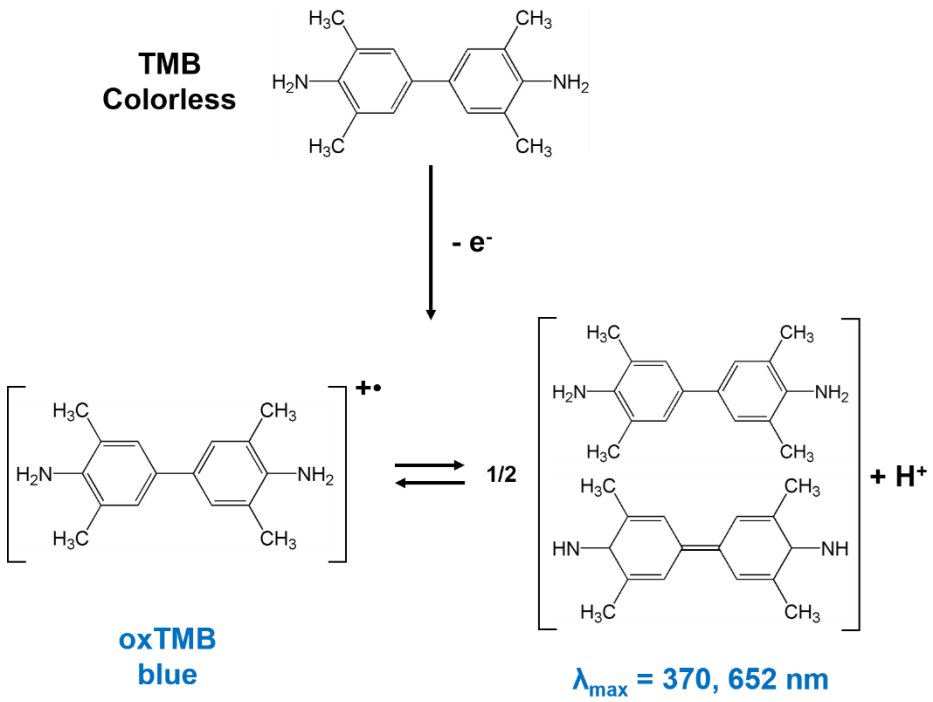

Figure S3. Oxidation of 3,3',5,5'-tetramethylbenzidine (TMB, colorless) into oxTMB (blue).

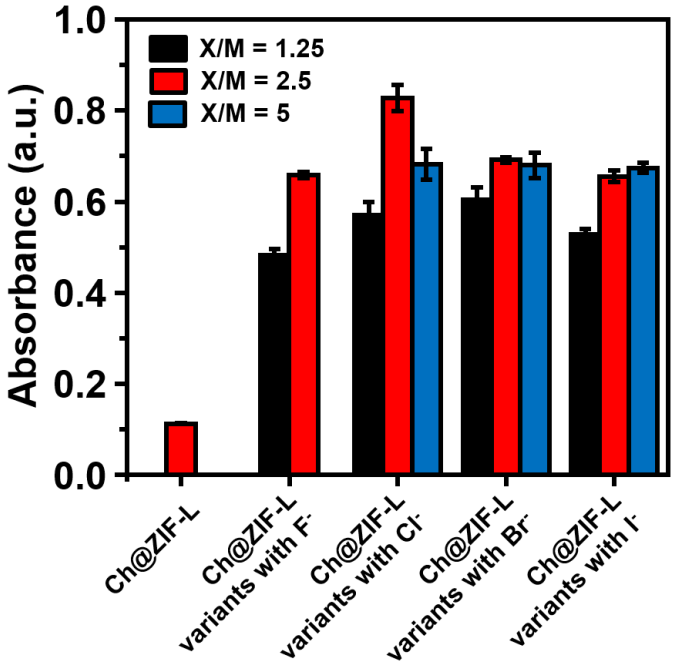

Figure S4. Evaluation of oxidase-like activities assayed with the probe of 3,3',5,5'-tetramethylbenzidine (TMB). Error bars: standard deviations (n = 3).

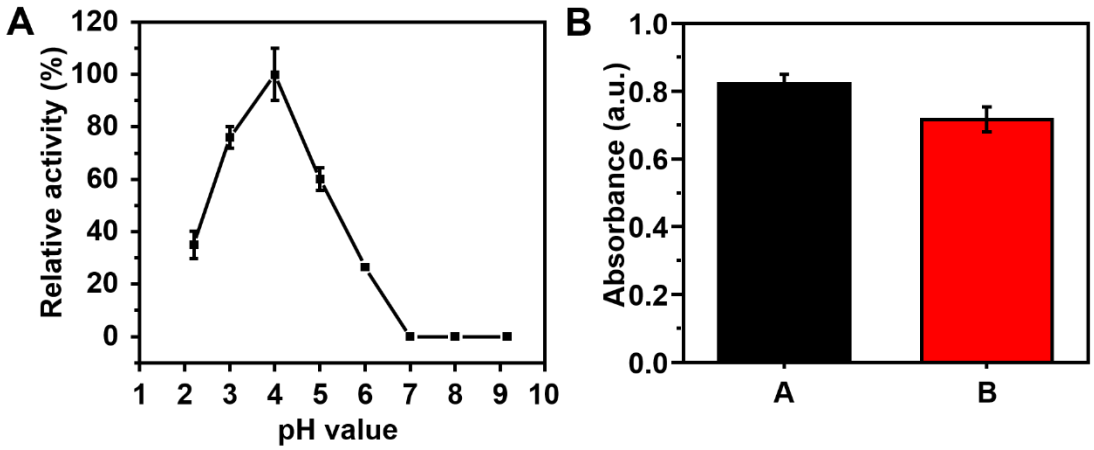

Figure S5. Catalytic activities of Ch@ZIF-L-H-Cl based on oxidation of TMB. (A) Catalytic activities of Ch@ZIF-L-H-Cl dependent on pH values. Error bars: standard deviations (n = 3). (B) Evaluation of the effect of ion releasing on the oxidase activities of Ch@ZIF-L-H-Cl. Column A: the catalytic activity of Ch@ZIF-L-H-Cl before the acidic treatment; Column B: the catalytic activity of Ch@ZIF-L-H-Cl in the parallel identical aliquots after the acidic treatment (pH 6.0). Error bars: standard deviations (n = 3).

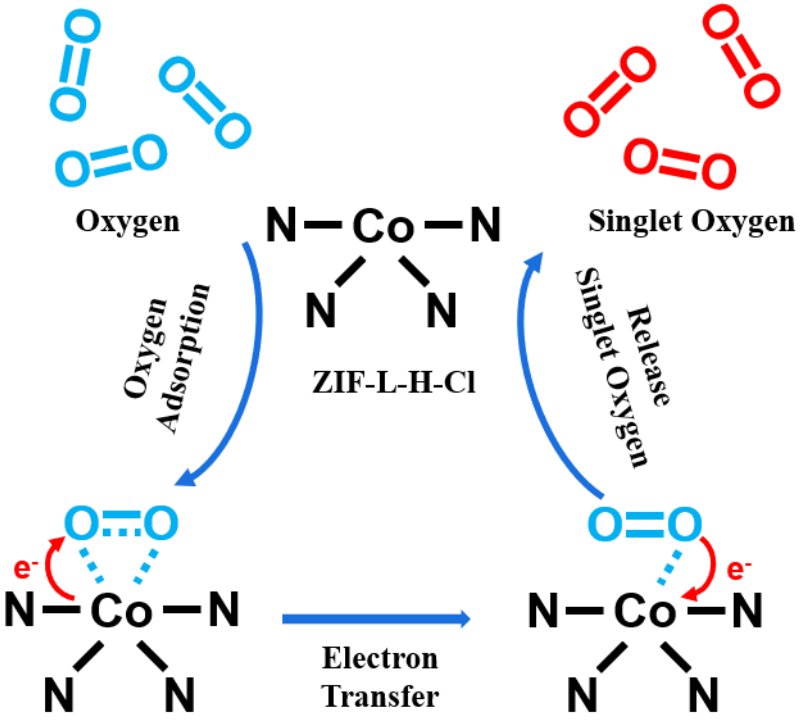

Figure S6. The generation mechanism of singlet oxygen from molecular oxygen.

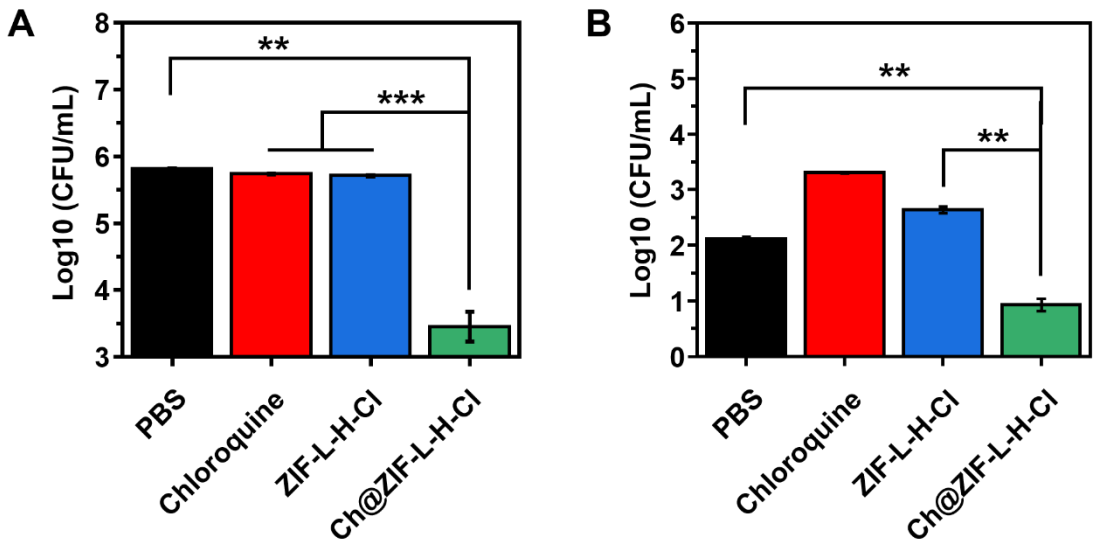

Figure S7. Bacterial counts in mice wound and blood after treatment with PBS, Chloroquine, ZIF-L-H-Cl and Ch@ZIF-L-H-Cl. **A)** Bacterial counts in mice wounds after different treatments. Error bars: standard deviations (n = 3). **B)** Bacterial counts in mice blood after different treatments. Error bars: standard deviations (n = 3). Two-tailed student's *t*-test for statistically significant differences (**$p < 0.01$, ***$p < 0.001$).

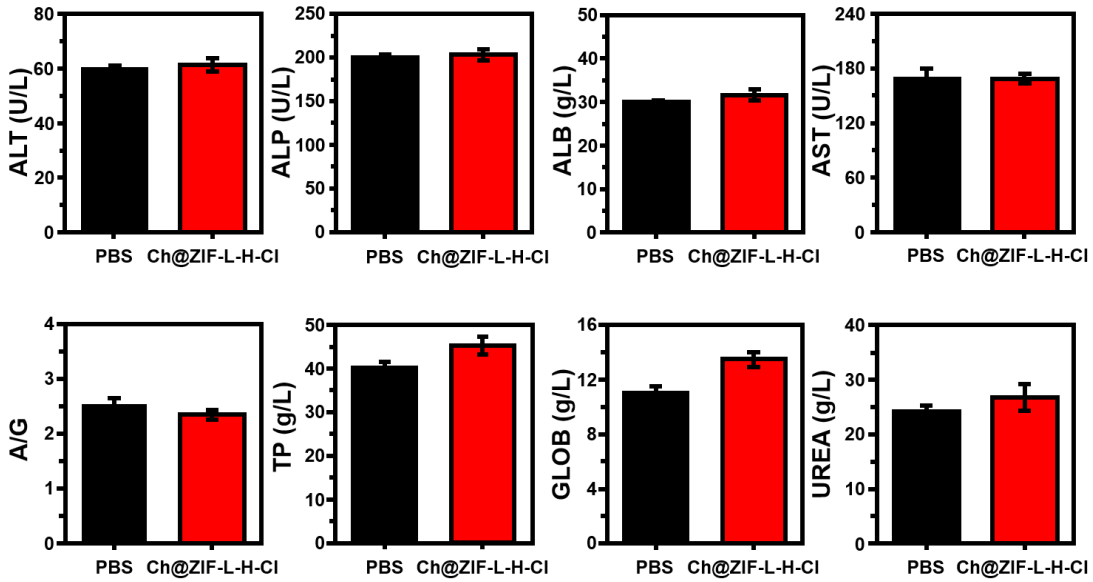

Figure S8. Hematological examinations on mice subcutaneously injected with Ch@ZIF-L-H-Cl or PBS. These results are the means and standard deviations (n = 3) of alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin (ALB), aspartate aminotransferase (AST), albumin to globulin ratio (A/G), total protein (TP), globulin (GLOB), and urea nitrogen (UREA).

Table S1. Atomic fraction and atomic error of energy-dispersive X-ray mapping of N, Cl, Co and Zn in ZIF-L-H-Cl and Ch@ZIF-L-H-Cl.

Species	Element	Atomic Fraction (%)	Atomic Error (%)
ZIF-L-H-Cl	N	20.87	4.50
	Cl	1.35	0.27
	Co	4.2	0.66
	Zn	4.87	0.76
Ch@ZIF-L-H-Cl	N	21.02	4.53
	Cl	1.28	0.25
	Co	4.14	0.64
	Zn	4.95	0.77

Table S2. Comparison of the kinetic parameters of various catalysts toward the oxidation of TMB (αxTMB).

Catalyst	***[S]*µg/mL**	***Substrate***	***Vmax* (M s^{-1})**	***Km* (M)**
Ch@ZIF-L-O	60	TMB	5.2×10^{-10}	5.6×10^{-3}
Ch@ZIF-L-C-Cl	60	TMB	5.5×10^{-10}	1.2×10^{-3}
Ch@ZIF-L-H-Cl	60	TMB	9.7×10^{-10}	8.3×10^{-4}
Ch@ZIF-L-F-Cl	60	TMB	8.6×10^{-10}	1.9×10^{-3}