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Supporting Materials

Ultrasmall copper nanoclusters as efficient antibacterial agent for primary peritonitis therapy

Yaru Wang[a], Jingrun Ye[b], Kang Liu*[a], Yinghao Wu[a], Jiayi Linghu[a], Ting Feng[a], Yong Liu[a], Xinyue Dou[a], Xun Yuan*[a], and Haiguang Zhu*[a]

[1]School of Materials Science and Engineering, Qingdao University of Science and Technology, Qingdao 266042, PR China. E-mail: yuanxun@qust.edu.cn; zhuhg@qust.edu.cn

[2]School of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, PR China

Part I. Materials

All materials were used as received without further purification. Ultrapure water (18.2 MΩ·cm) was used in the whole experiment. Ethanol was purchased from Tianjin Fuyu Fine Chemical Co., Ltd (China). 4-mercaptobenzoic acid (MBA) was purchased from TCI (Shanghai) Chemical Industry Development Co., Ltd. Copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), sodium hydrate (NaOH), were purchased from Sinopharm Chemical Reagent Co., Ltd. Sodium borohydride ($NaBH_4$, ≥ 96 %) was purchased from Shanghai Shanpu Chemical Co., Ltd (China). Phosphate buffer solution (PBS) was purchased from Qingdao Hope BioTechnology Co., Ltd. Luria-Bertani (LB) nutrient agar and LB broth were purchased from Qingdao Hope Biotechnology Co., Ltd. Standard strains of *Staphylococcus aureus* ATCC 6538 (*S. aureus*), and

Escherichia coli ATCC 25922 (*E. coli*) were purchased from China General Microbiological Culture Collection Center. L929 mouse fibroblast cells were purchased from American Type Culture Collection.

Part II. Instrumentation.

The UV-vis absorption spectrum of Cu nanoclusters (NCs) solution was analyzed using a Shimadzu UV-1800 spectrophotometer, at 25 °C, in the range of 190-1100 nm, with background correction using 35vol% of ethanol. For the transmission electron microscope (TEM) imaging, samples of Cu NCs in ethanol suspensions were introduced onto copper grids, allowing for drop-drying, and subsequently imaged using a JEOL JEM 2100F microscope with an operating voltage of 200 kV. The content of Cu species in organs and tissues was quantified through inductively coupled plasma-mass spectrometry (ICP-MS). Histological section imaging was conducted on a panoramic section scanner (MIDI/SCAN150).

Part III. Synthesis of Cu_{30} NCs

The synthesis of MBA-protected Cu_{30} NCs followed a previously reported method with some modifications.[1, 2] Specifically, solutions of MBA (50 mM) and $CuSO_4$ (50 mM) were prepared using ethanol and ultrapure water, respectively. In the typical synthesis process, aqueous solution of $CuSO_4$ (0.2 mL) as the copper precursor, ethanol solution of MBA (0.4 mL) as the protecting ligand, and NaOH (1 M, 0.05 mL) were mixed in approximately 35vol% of ethanol (2.9 mL of water and 1.35 mL of ethanol). Following this, 0.1 mL of $NaBH_4$ solution (~112 mM, prepared by dissolving 43 mg of $NaBH_4$ powder in 10 mL of 0.2 M NaOH solution) was added drop by drop to the

solution. The $Cu_{30}(MBA)_{16}$ NCs were then collected after 4 hrs of reaction *via* centrifugation.

Part IV. Antibacterial experiment

The gram-negative *Escherichia coli* ATCC 25922 (*E. coli*) and gram-positive *Staphylococcus aureus* ATCC 6538 (*S. aureus*) were selected as bacteria models to assess the bactericidal efficacy of the Cu_{30} NCs. Prior to the antibacterial test, all glassware, media solutions, and reagents were sterilized in an autoclave at 121°C and 103 kPa for 30 minutes. The bacteria from solid LB agar plates were then transferred to 10 mL of liquid Luria-Bertani (LB) culture medium and incubated at 37°C for 12 hrs. Subsequently, the bacterial culture underwent centrifugation at 8000 rpm for 5 minutes to eliminate metabolites, and was then diluted with PBS solution to produce a bacterial suspension with an OD_{600} of 0.1 (equivalent to a bacteria concentration of approximately 10^6 CFU·mL^{-1}; where CFU denotes colony forming units). For the *in vitro* antimicrobial assay, 1 mL of Cu_{30} NCs at varying concentrations (0.2 mM, 0.1 mM, 0.05 mM and 0 mM) were added to 4 mL of bacterial suspension and incubated in an orbital shaker for 8 hrs. A control group without any antibacterial agents was similarly processed. The bacterial suspension was then diluted by a factor of 1000. Subsequently, 100 μL of the diluted bacterial suspension was pipetted and spread on fresh agar plates, followed by incubation at 37°C for 12 hrs. The number of viable bacteria was determined by counting the colonies growing on the plates after incubation, allowing for the calculation of the antibacterial activity.

Part V. Animal experiments

1. Model establishment

The successful induction of peritonitis in mice was achieved through the intraperitoneal injection of *E. coli* (OD_{600}=1.667, 0.3 mL injection), resulting in evident symptoms such as depression, curling up into a ball, and the discharge of mucus stool. The observations were conducted over a period of 5 consecutive days following the injection of various concentrations of *E. coli*, and the lethality was recorded to determine the minimum volume required for effective induction. Subsequently, once the modeling process was completed, mice were injected with varying concentrations of Cu_{30} NCs and monitored over 5 consecutive days, with the survival rate being recorded in order to establish the minimum effective dosage of Cu_{30} NCs.

2. *In vivo* antibacterial assay

Each mouse in the healthy (or normal) control group was injected intraperitoneally with 0.1 mL of saline.

The *in situ* intraperitoneal injection of Cu_{30} NCs was administered to each mouse in the diseased group, with a dose of 0.1 mL (0.6 mM), and the monitoring was started once after the intraperitoneal injection of Cu_{30} NCs (recorded as 0). Intraperitoneal fluids were then extracted at various time points, namely 0, 8, 16, 24, 32, 40, and 48 hrs. Subsequently, these extractions were diluted in PBS at intervals of 10^0, 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 times, followed by the use of coated plates to calculate the bacterial counts. Furthermore, the spleen and peritoneum of the mice were collected, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, stained with

hematoxylin and eosin (H&E), and imaged using a fluorescence microscope to monitor any inflammatory changes. In contrast, peritoneal fluids were drawn from both the healthy and diseased control groups at 48 hrs, diluted in PBS, and counted using coated plates at dilution intervals of 10^0, 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6, in order to serve as the control.

3. Biocompatibility

To evaluate the *in vivo* biocompatibility of Cu_{30} NCs, organs (heart, liver, spleen, lungs, kidneys, and peritoneum) were taken from the three groups of mice mentioned above at 48 hrs, and photographs of the intactness of each organ were taken. 4% paraformaldehyde fixation was followed by paraffin embedding, slicing, H&E staining, and fluorescence microscopy imaging.

4. Biodistribution

Organs (heart, liver, spleen, lungs, kidneys and peritoneum) were taken from mice in the treatment group at each time period, dried at 60°C, ablated by excess aqua regia immersion, and diluted for copper ion detection by ICP-MS.

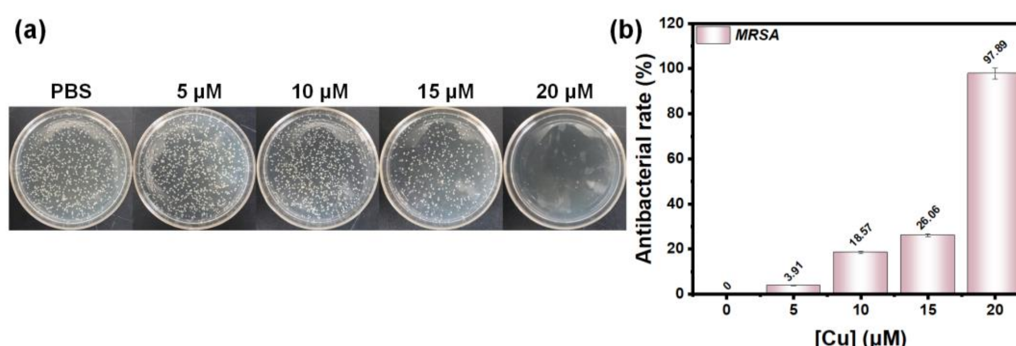

Fig. S1. (a) *In vitro* antibacterial results of Cu_{30} NCs with the concentration range of 5-20 μM against *MRSA* with the usage of PBS as control. (b) Antibacterial rate of Cu_{30} NCs against *MRSA*. Error bars, mean ± SD (n = 3).

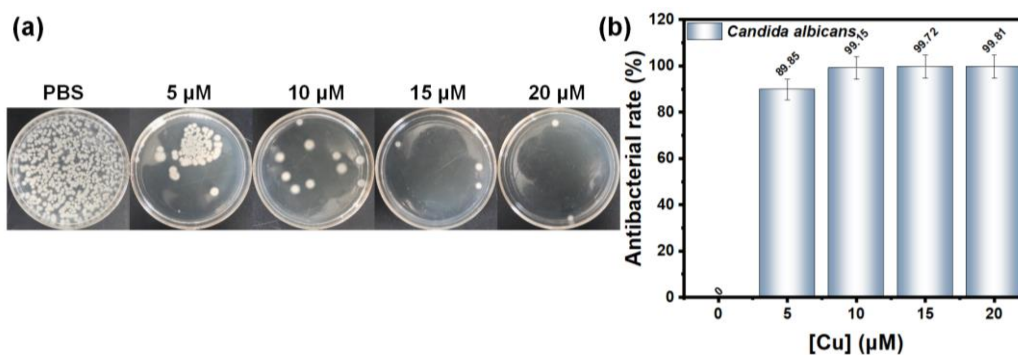

Fig. S2. (a) *In vitro* antifungal results of Cu_{30} NCs with the concentration range of 5-20 μM against fungus *Candida albicans* with the usage of PBS as control. (b) Antifungal rate of Cu_{30} NCs against fungus *Candida albicans*. Error bars, mean ± SD (n = 3).

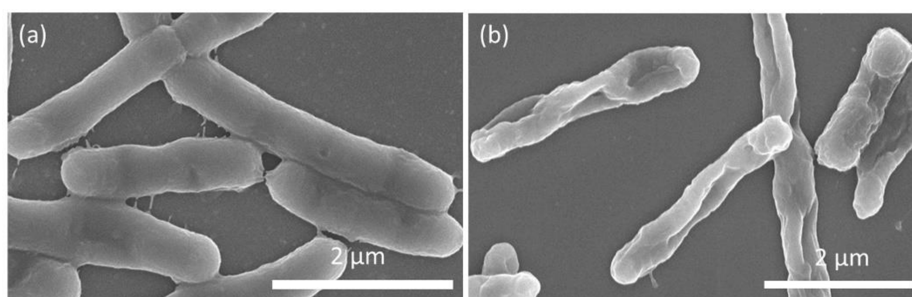

Fig. S3. SEM images of the *E. coli* before (a) and after (b) the treatment with Cu_{30} NCs.

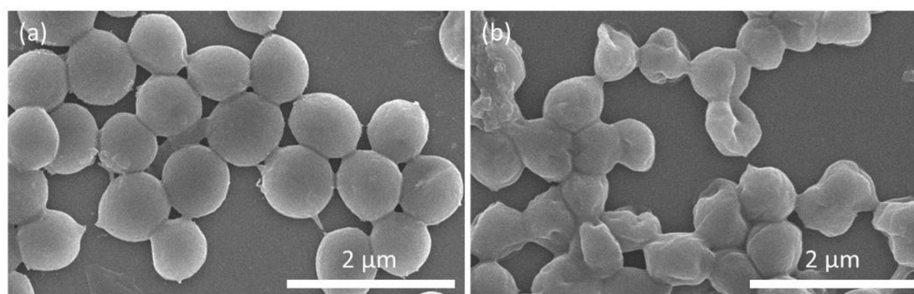

Fig. S4. SEM images of the *S. aureus* before (a) and after (b) the treatment with Cu_{30} NCs.

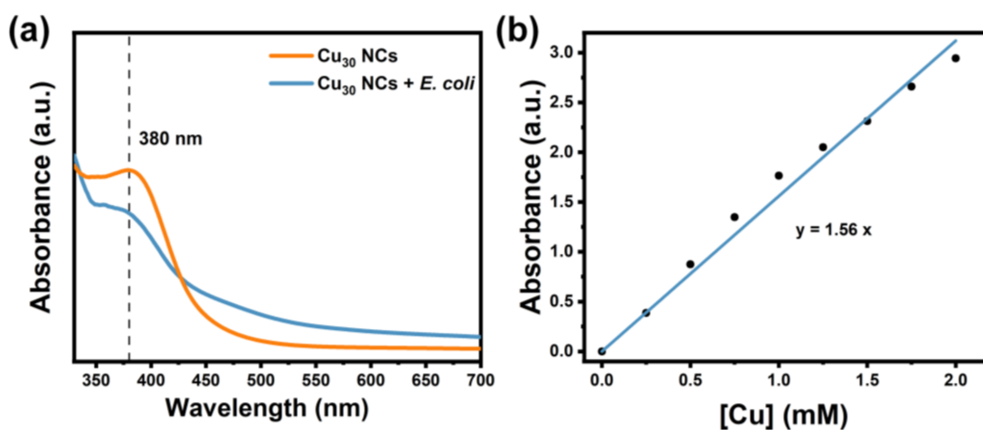

Fig. S5. (a) UV-vis absorption spectra of Cu_{30} NCs before and after incubation with *E. coli*. (b) Linear relationship between the concentration and the absorbance (380 nm) of Cu_{30} NCs.

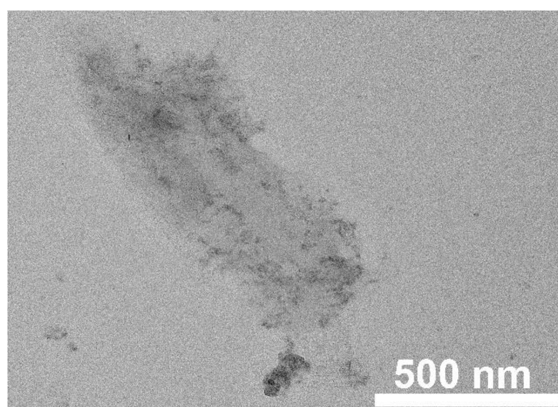

Fig. S6. A representative TEM image of the fixed *E. coli* cell incubated with Cu_{30} NCs.

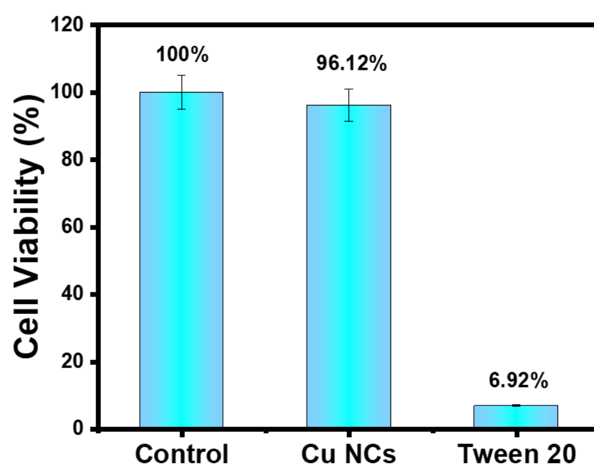

Fig. S7. Cell viability evaluated by MTT assay after 24 hrs treatment with Cu_{30} NCs (12.5 μM), ultrapure water as positive control and Tween 20 as negative control. Error bars, mean ± SD (n = 3).

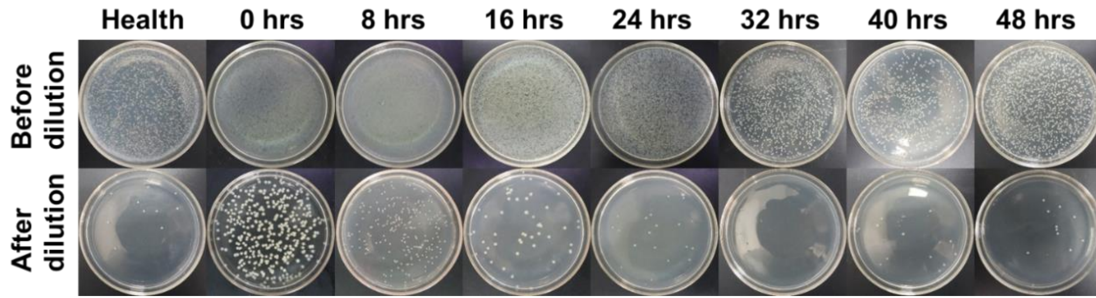

Fig. S8. *In vivo* antibacterial results of Cu_{30} NCs against *E. coli* at different time (0~48 hrs) from the intraperitoneal injection of Cu_{30} NCs. The upper panel shows agar plates without bacteria suspension dilution, while the lower panel shows corresponding agar plates with the bacteria suspension of diluted 10^6 times for 0 hr, 8 hrs and 16 hrs, 10^3 times for 24 hrs and 32 hrs, and 10^2 times for healthy group, 40 hrs and 48 hrs.

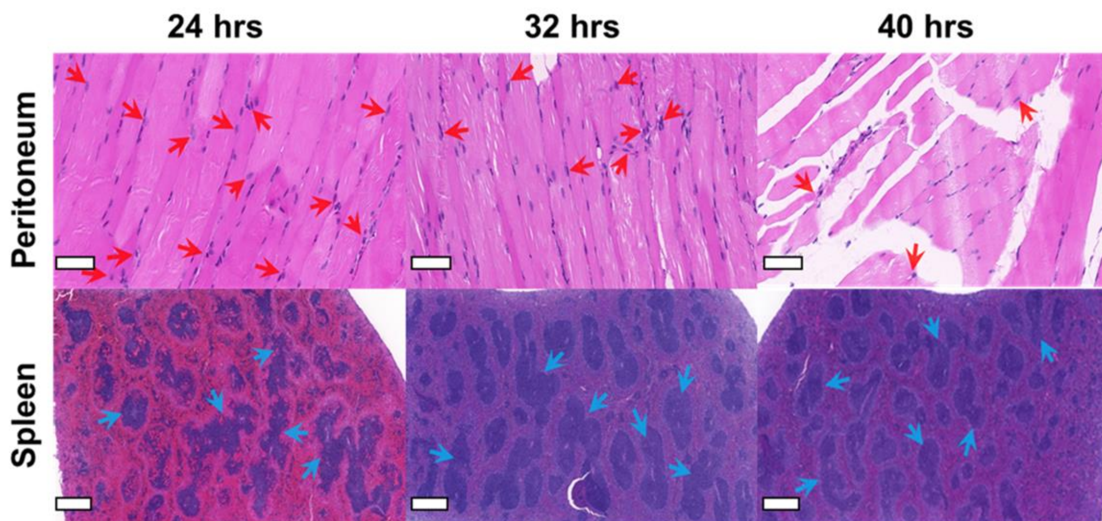

Fig. S9. H&E staining of histological sections including spleen and peritoneum of mice with an acute peritoneal infection after treatment with Cu_{30} NCs for 24 hrs, 32 hrs, and 40 hrs. The increasing inflammatory cells on peritoneum and the enlarged lymphoid nodules on spleen are marked with red and blue arrows, respectively. Scale bar, 50 µm (peritoneum group), 500 µm (spleen group).

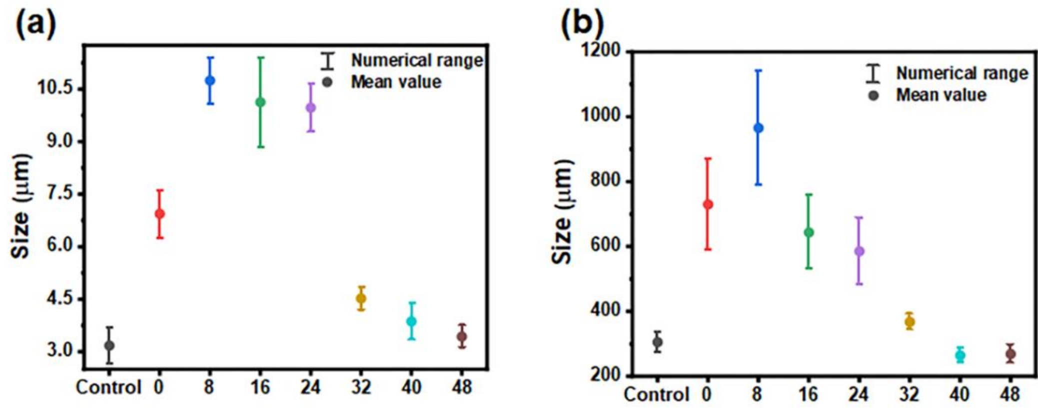

Fig. S10. Size of (a) inflammatory cell nuclei on peritoneum, and (b) splenic lymph node in acute peritonitis mice at different time from the intraperitoneal injection of Cu_{30} NCs. The normal mice without any infection were used as a control group. Error bars, mean ± SD (n = 3).

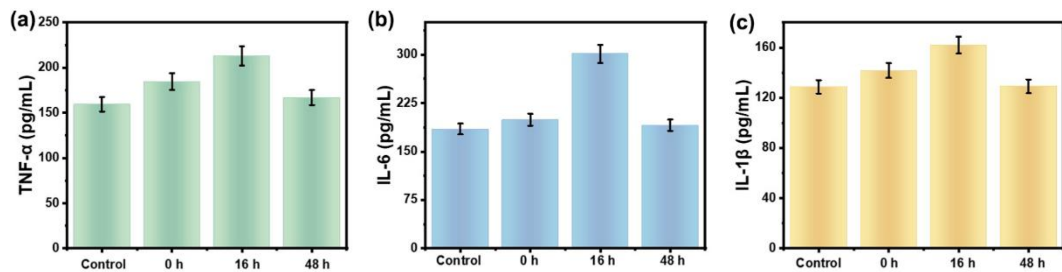

Fig. S11. Time-course inhibition of inflammatory cytokines, including (a) tumor necrosis factor-alpha (TNF-α), (b) interleukin-6 (IL-6), and (c) interleukin-1β (IL-1β) in acute peritonitis mice at different time from the intraperitoneal injection of Cu_{30} NCs. The normal mice without any infection were used as a control group. Error bars, mean ± SD (n = 3).

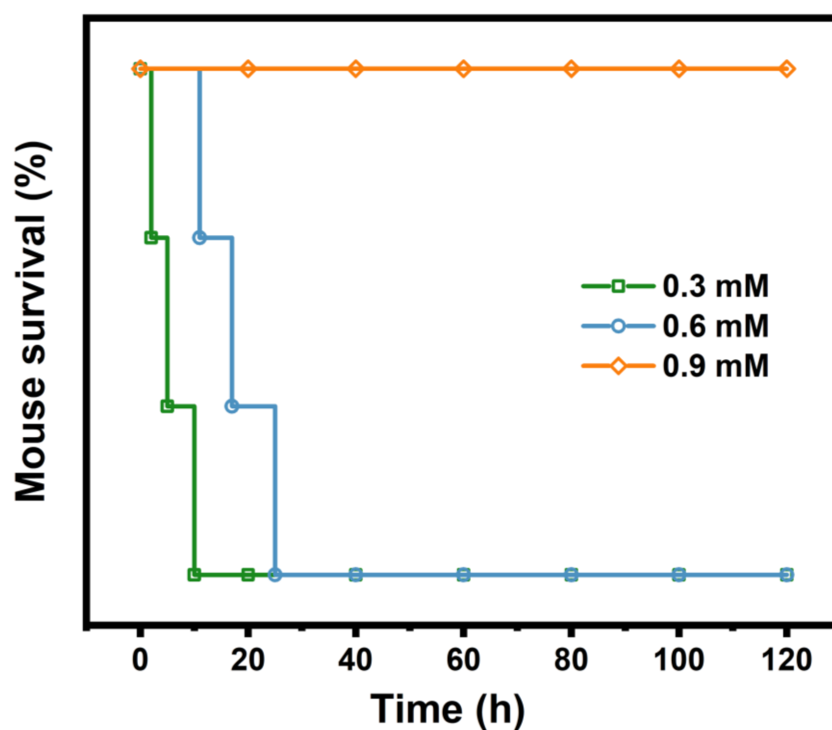

Fig. S12. Survival rate of peritonitis mice after injection with different concentrations of ampicillin.

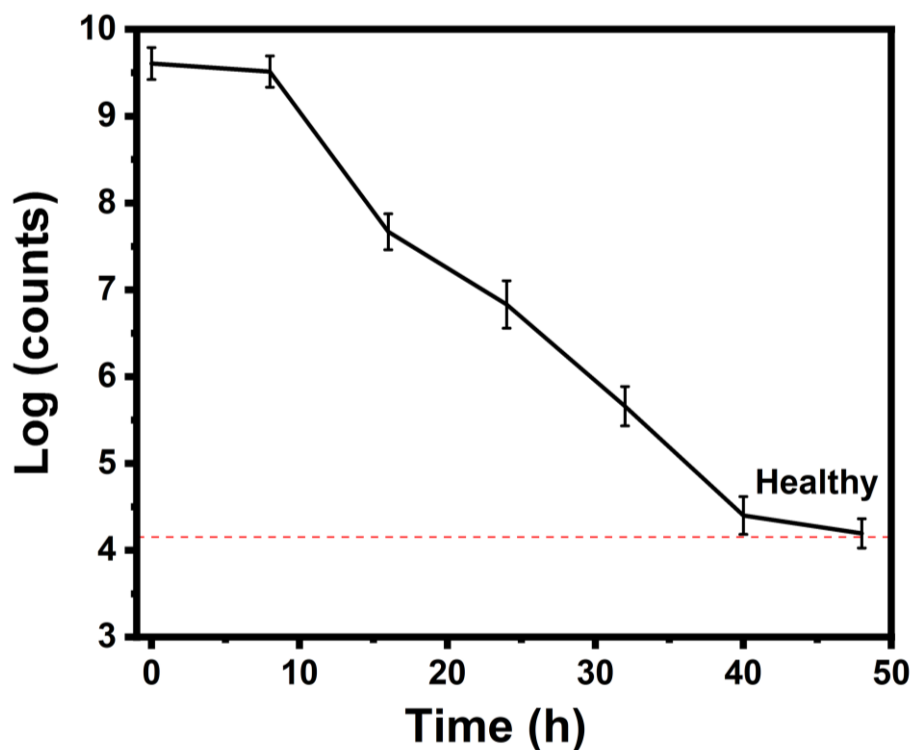

Fig. S13. *In vivo* antibacterial results of mice after injection with ampicillin (0.9 mM).

Reference

1. X. Yuan, B. Zhang, Z. Luo, Q. Yao, D. T. Leong, N. Yan and J. Xie, *Angewandte Chemie International Edition*, 2014, **53**, 4623–4627.

2. Y. Ge, X. Yali, W. Yaru, T. Ying, C. Leng Leng, J. Fuyi, D. Fanglin, Z. Xianfeng, Y. Jackie Y and Y. Xun, *Nano Research*, 2023, **16**, 1748–1754.