Electronic supplementary information

Trojan horse in combinational bacterial killing: the virtue of prickly Zn-CuO as *in situ* 'package' for accelerated antibiosis

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Preparation of the Zn-CuO@GO: Zn-CuO@GO was prepared according to our previous reports with modifications. Typically, the process took place as follows: 0.15 g copper acetate monohydrate was dissolved in 10 mL of double distilled water by stirring, after which 0.055 g of zinc acetate dihydrate was added. A 9/1 ethanol/ water volume ratio solution was obtained after addition of 90 mL of ethanol. GO in different weight was then added into the solution and sonochemical irradiation was started with a high intensity ultrasonic Ti-horn at 750 W (XH-300UL, Beijing Xianghu Science and Technology development Co., Ltd.). After 5 min of sonication, 0.8 mL of an aqueous solution of ammonium hydroxide (28-30%) was injected into the reaction cell to adjust the pH to ~ 8. The sonochemical deposition process continued for 1 h. At the end of the reaction, Zn-CuO@GO was obtained via centrifugation and washing twice with double-distilled water and once with ethanol, and then dried under vacuum. (Notably, under similar protocol without the addition of GO sheet, the obtained prickly Zn-CuO was approximately 20 mg in weight. Thus, 5 mg, 10 mg and 20 mg of GO sheets were in fact added to obtain Zn-CuO@GO (4:1), Zn-CuO@GO (2:1) and Zn-CuO@GO (1:1), respectively.)

Bacterial strain: The bacterial utilized for analysis of the antibacterial activity consisted of the following strains: MDR *E. coli* (ST131 lineage) and a methicillin resistant *S. aureus* (MRSA, USA300 lineage), which were generously provided and isolated clinically by Affiliated People's Hospital of Jiangsu University.

Antimicrobial Activity: The antibacterial activity of Zn-CuO@GO was tested against MDR *E. coli* and MRSA while *E. coli* and *S. aureus* were evaluated as references. Overnight cultures of the bacterial strains were transferred into a nutrient broth (NB) medium ("Difco" Detroit, MI) and allowed to grow at 37 ° C with aeration. When the cell number reached 10^8 CFU, the cells were harvested by centrifugation and washed twice with a 0.85% NaCl solution at pH 6.5 (saline). The bacterial suspensions were incubated with Zn-CuO@GO at 0.1 mg mL^{-1} for up to 3h at 37 °C under mild agitation (200 rpm). An aliquot (100 μL) was taken at different time intervals (0, 10min, 30min, 1h, 2h, and 3h) and plated on nutrient agar plates after 8-fold dilution in saline. The plates were allowed to grow for 24 h at 37 °C and then counted for colony forming units (CFU). Reduction in the viability of these bacterial strains was determined by N/N_0, where N_0 and N are the number of CFUs at the initial (N_0) and following treatment (N).

Morphological observation of bacteria by SEM and TEM: Briefly, microbes after incubation with Zn-CuO@GO were fixed by 4% paraformaldehyde, and dehydrated subsequently. The samples were sputter-coated with platinum (60 s, 50 mA) and viewed under scanning electron microscope (SEM, JEOL 7001F) at an accelerating voltage of 10 kV. For TEM measurement, samples of dehydrated MDR *E. coli* and MRSA cell suspensions were immediately deposited on bare 200-mesh copper grids. The grids were then dried in air and

examined using a transmission electron microscope (JEOL JEM-2100 (HR)).

Live and dead staining: Live and dead staining were conducted according to our previous reports. Briefly, an aliquot (900 μL) of bacterial suspension was transferred into a 1.5-mL Eppendorf tube, and then 100 μL of known concentrations of Zn-CuO@GO suspensions were added. Control samples contained 900 μL of bacterial suspension and 100 μL of 0.1% Tween-80 solution.[1, 2] After 30 min incubation, the cells were stained with propidium iodide (PI; excitation/emission at 535-nm/615-nm; Sigma-Aldrich) for 10 min, fixed with 4% paraformaldehyde for 1.0 h and then counter-stained with 4',6-diamidino-2-phenylindole (DAPI, excitation/emission at 358-nm/461-nm; Sigma-Aldrich) for 10 min in the dark. The stained samples were then washed extensively with PBS. Subsequently, 20 μL of the cell suspension was dipped onto a glass slide and air dried. 20 μL of resin in dimethylbenzene was then deposited onto the glass slide and put a cover slip over it. The fluorescence images were taken under a laser confocal scanning microscopy (LCSM; Leica TCS SP5 II). (Notaly, the Zn-CuO@GO were prestained with fluorescein isothiocyanate isomer I (FITC; excitation/emission at 490-nm/520-nm; Sigma-Aldrich) via incubation with FITC solution. Briefly, 10 mg of bare Zn-CuO@GO were suspended in 10 mL of dimethylformamide (DMF). Subsequently, 5 μL aliquot of aminopropyltriethoxysilane (APTS) dissolved in 100 μL DMF was added to the Zn-CuO suspensions and stirred for 24 h. To introduce the Zn-CuO@GO composites with FITC for imaging, they were pre-suspended in a solution of 4 mg of FITC in 2 mL of DMF for 4 h. The obtained nanoparticles were then washed thoroughly with DMF at least five times to remove unbounded FITC molecules. Subsequently, 20 mL of DI water were added to wash the FITC labeled Zn-CuO@GO composites. The process was continued until no fluorescence was detected in the supernatant.)

ICP-MS measurement: The metal component of Zn-CuO@GO with the deposition ratio of 4:1, 2:1 and 1:1 was determined by ICP (VISTA-MPX). Basically, the nanoparticles (5 mg) were immersed in strong acid (H_2SO_4) (1 m, 5 mL) until completely dissolved; the ion concentrations in the solution were subsequently determined by ICP for further calculation of their weight ratios.

For time-lapse metal ions concentration evaluation upon immersed in saline, 5 mg of the Zn-CuO@GO (4:1) were dispersed in 50 mL saline (in dialysis bag) and incubated at 37 °C under mild shaking (100 rpm). At predetermined time intervals, 0.1 mL of solution was collected and diluted into 10 mL H_2O, and the metal components was determined by inductively coupled plasma (ICP) (VISTA- MPX).

ESR Measurements: •OH production was detected using the ESR spin trapping technique coupled with a spin trap DMPO (Sigma, St. Louis, MO). Excessive DMPO was added to Zn-CuO@GO (4:1) aqueous suspensions with different concentrations before and after antibacterial measurement, and was drawn into a gas-permeable capillary. Each capillary was folded and inserted into a narrow quartz tube that was open at both ends, and then placed into the ESR cavity. The ESR spectra were recorded on a Bruker ER 100d X-band spectrometer. The EPR measurements conditions were set as follows: the microwave of the ESR was set at frequency of 9.852 GHz and the power at 1.156 mW. Measurement conditions were as follows: sweep width, 100 G; resolution, 1024; receiver gain, 5.64e+003; conversion time, 36 ms; time constant, 20.480 ms.

GSH assay: Following a previous study, the concentration of thiols in GSH was quantified by the Ellman's assay. GO, or Zn-CuO@GO dispersions(225 μL at 0.1 mg/mL) in 50 mM bicarbonate buffer (pH 8.6) was added into 225 μL of GSH (0.8 mM in the bicarbonate buffer) to initiate oxidation. [3] All samples were prepared in triplicate. The mixtures were transferred into a 24-well plate, covered with alumina foil, and incubated at room temperature for 2 h under mild shaking (150 rpm). After incubation, 785 μL of 0.05 M Tris-HCl and 15 μL of DNTB (Ellman's reagent, 5,50-dithio-bis-(2-nitrobenzoic acid), Sigma-Aldrich) were added into the mixtures to yield a yellow product. GO, or Zn-CuO@GO was removed from the mixtures by filtration through a 0.22 μm polyethersulfone filter. The filtered solutions from each sample (250 μL) was then placed in a 96well plate and measured at 412 nm on a Biorad. GSH solution without graphene-based materials was used as a negative control.

GSH (0.4 mM) oxidization by H_2O_2 (1 mM) was used as a positive control. The loss of GSH was calculated by the following formula: loss of GSH % = (absorbance of negative control - absorbance of sample)/absorbance of negative control × 100.

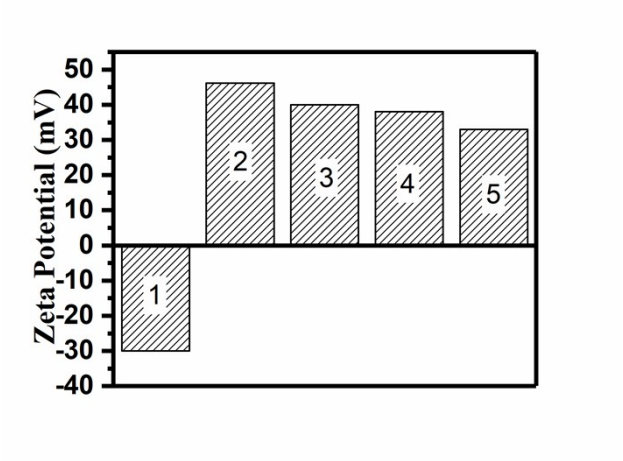

Fig. S1 Zeta potential of GO, Zn-CuO and Zn-CuO@GO in saline, respectively.

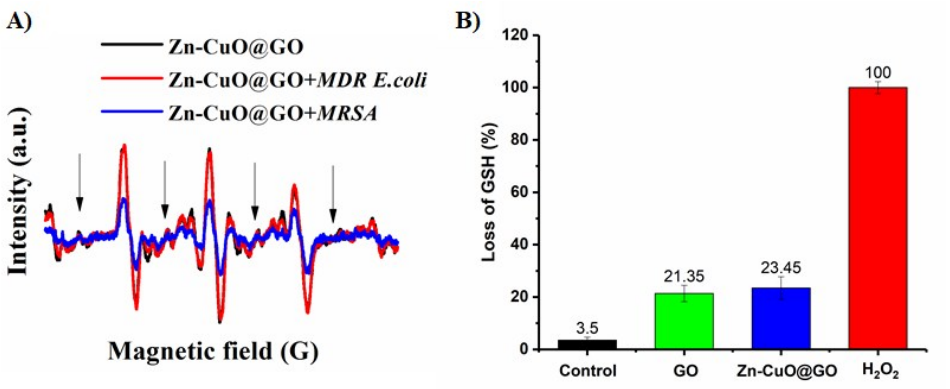

Fig. S2 (A) ESR spectra of hydroxyl radical (•OH) detection in a suspension of Zn–CuO@GO (0.1 mg mL^{-1}) under experimental doses upon antibacterial treatment with MDR *E. coli* and *MRSA*. (B) GSH assays evaluating GO and Zn-CuO@GO (0.1 mg mL^{-1}). H_2O_2 was used a reference.

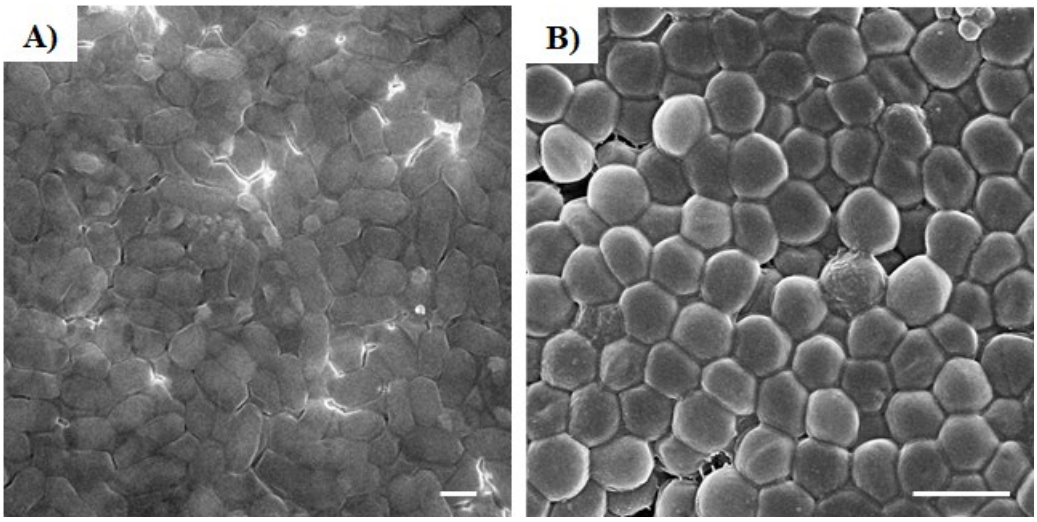

Fig. S3 SEM images of MDR *E. coli* and *MRSA* without any treatment. Scale bar: 1 µm.

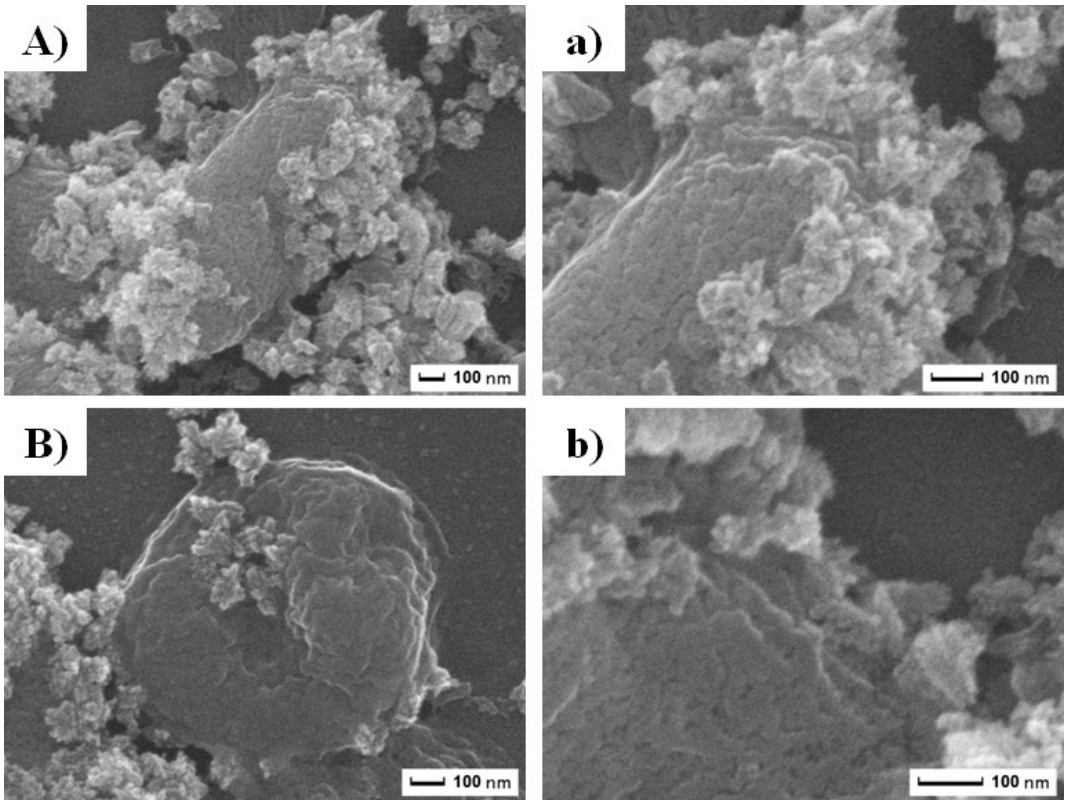

Fig. S4 (A) and (B) were SEM images of MDR *E. coli* and *MRSA* after treatment with Zn-CuO, respectively. (a) and (b) were magnified SEM images revealing detailed cell-material interactions.

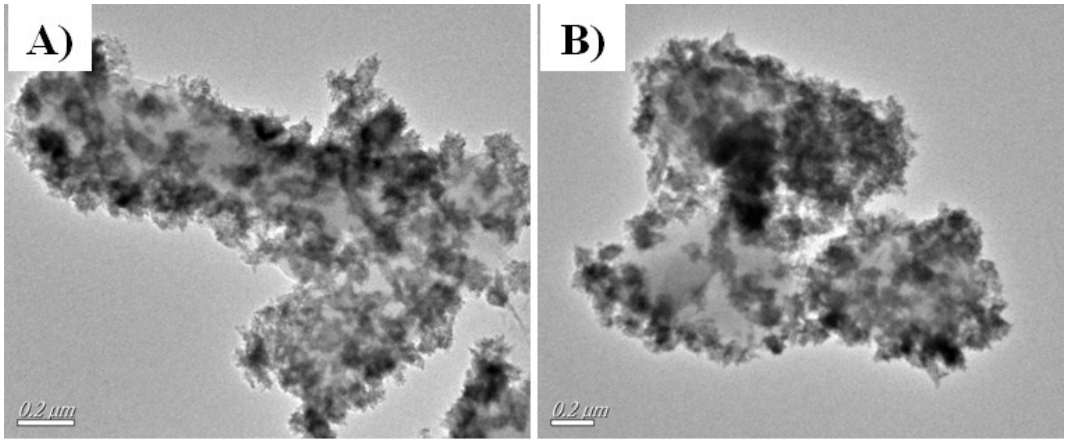

Fig. S5 TEM images of MDR *E. coli* and *MRSA* with Zn-CuO treatment.

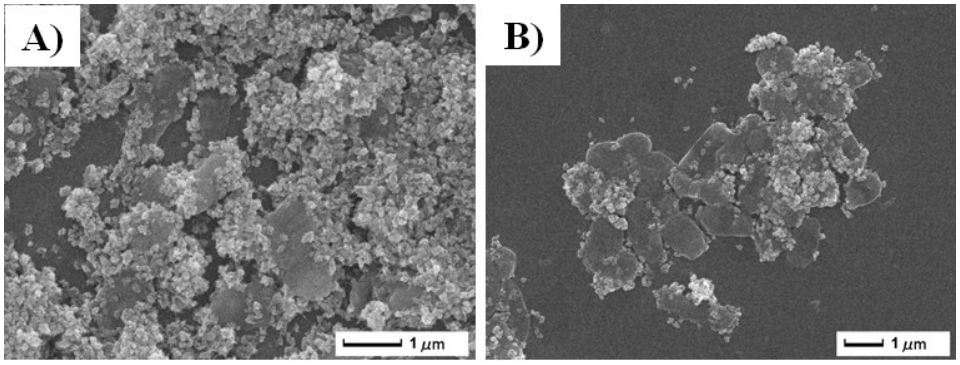

Fig. S6 SEM images of MDR *E. coli* after incubation with Zn-CuO@GO, which indicated that the bacteria were accumulated and attached into Zn-CuO@GO.

Additional Notes:

The leakage of DNA/RNA was not detectable due to their adsorption into the composite, as the GO layer has large specific surface area preferable for small molecule adsorption.

Table S1 Weight ratio of Zn-CuO deposited into GO as determined by inductively coupled plasma (ICP).

Sample	Amount of Zn-CuO measured from ICP per 5 mg of Zn-CuO	Measured deposition weight ratio (Zn-CuO:GO)	Denoted deposition weight ratio (Zn-CuO:GO)
Zn-CuO@GO (4:1)	3.970 mg	3.854 : 1	4 : 1
Zn-CuO@GO (2:1)	3.343 mg	2.018 : 1	2 : 1
Zn-CuO@GO (1:1)	2.345 mg	0.883 : 1	1 : 1

Table S2 Antibacterial test results from literatures, taking ZnO, CuO and GO into consideration, based on their nanostructure, particle size, bacterial stain, dosages and time of completion to achieve ~100% inhibition.

References	Material	Nanostructure	Particle size (nm)	Bacterial strain	Dosage (mg/mL)	Time of completion
Applerot et. al[4]	ZnO	N/A	260±40	*E. coli* 1313	0.1	3 h
Applerot et. al[4]	ZnO	N/A	260±40	*S. aureus* 195	0.1	3 h
Raghupathi et al[5]	ZnO	N/A	30	Methicillin sensitive *S. aureus* strain	0.488	6 h
Raghupathi et al[5]	ZnO	N/A	88/142/212	Methicillin sensitive *S. aureus* strain	0.488	N/A (> 8h)
Applerot et. al[4]	CuO	Sphere	20	*E. coli*	0.1	3 h
	CuO	Sphere	20	*S. aureus*	0.1	3 h
Liu et. al[6]	GO	Nanosheets	N/A	*E. coli*	20	2 h
He et. al[7]	GO	Nanosheets	300	*S. mutan*	0.8	N/A (> 2 h)
Zhang et. al (our result)	Zn-CuO@GO	Nanosheets	N/A	*E. coli*	0.1	10 min
Zhang et. al (our result)	Zn-CuO@GO	Nanosheets	N/A	*S. aureus*	0.1	10 min

Table S3 Time lapse record of metal ions concentrations such as Cu^{2+} and Zn^{2+} ion released from Zn-CuO@GO after immersion in saline (0.1 mg mL^{-1}) determined by inductively coupled plasma (ICP).

Sample	Cu^{2+} mmol L^{-1} in saline						Zn^{2+} mmol L^{-1} in saline					
	0h	0.1h	0.3h	1h	2h	3h	0h	0.1h	0.3h	1h	2h	3h
Zn-CuO@GO	0	0.15	0.43	0.83	0.99	1.2	0	0	0.001	0.002	0.003	0.004

References:

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