Electronic Supplementary Information (ESI)

Bi-functional quercetin/copper nanoparticles integrating bactericidal and anti-quorum sensing properties for preventing the formation of biofilms

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S1 Supporting Experiments

S1.1 Materials

Quercetin (Qe) and copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$) were purchased from Aladdin. Concentrated hydrochloric acid (HCl) were purchased from Sinopharm Chemical Reagent Co. Sodium hydroxide (NaOH) and sodium dodecyl sulfate were purchased from Macklin. Triton X-100 was purchased from Sigma. Phosphate buffered saline (PBS, 10×) was purchased from HyClone. The deionized water used in all experiments was purified using a Millipore water purification system to give a resistivity of 18.2 MΩ·cm. All chemicals were used as received without further treatment.

S1.2 In vitro antibacterial and antibiofilm experiments

S1.2.1 Bacterial culture

Pseudomonas aeruginosa (P. aeruginosa, ATCC-15692) and *Staphylococcus aureus* (*S. aureus*, ATCC-6538) were supplied by the China General Microbiological Culture Collection Center (Beijing, China). Prior to the experiments, *P. aeruginosa* and *S. aureus* were incubated in Luria Bertani gravy medium (LB, Sigma-Aldrich) and Nutrient broth (NB, Solarbio), respectively, grown overnight under shaking at 37°C and harvested during the exponential growth phase via centrifugation. The supernatant was discarded, and the cell pellet was re-suspended in PBS. The final concentration of bacteria was adjusted to approximately 1×10^7 cells/mL before use.

S1.2.2 Eradication of planktonic bacteria

380 μL of bacterial suspension (*P. aeruginosa* or *S. aureus*, 1×10^7 cells/mL in PBS) were mixed with 20 μL of QC NPs solution (0.1 mg/mL, 0.2 mg/mL, 0.5 mg/mL) or Qe solution (0.6 mg/mL) per well for incubation at 37°C for 3 h. After that, the bacterial suspensions were appropriately diluted with PBS and placed on gelatinous Luria agar plates (Luria nutrient medium containing 1.5 wt% agar) and incubated at 37°C for 18 h. The number of viable cells was then determined in colony-forming units (CFU). Ideally, each surviving cell should develop into a distinct colony after incubation, thus providing a direct measure of bacterial viability.

S1.2.3 Fluorescence staining assay

The viability of the bacteria in biofilm after various treatments was qualitatively assessed using a standard fluorescence staining assay. Bacterial biofilms were grown in 48-well microplates as described above. After different treatments, 20 μL of SYTO-9 staining solution (3.34 mmol/L, Invitrogen, USA) was dropped on the sample surface and kept in the dark for 15 min. The surfaces were then gently rinsed with sterile water. After being fully air-dried, the stained biofilm was observed using fluorescence microscopy (IX71, Olympus, Japan) and images of 10 randomly chosen fields were captured.

S.1.2.4 Scanning electron microscope (SEM)

The morphology of the formed biofilm was observed using SEM. Bacterial biofilms were grown on the surface of silicon wafers according to the above method. After different treatments, the samples were gently rinsed with sterile water to remove

unattached cells, fixed in a 2.5% glutaraldehyde solution for 2 h, dehydrated in a series of ethanol solutions (30%-100%) for 20 min each, and air-dried. Before characterization, the samples were sputter coated with a 5-nm layer of gold. The attached bacteria and formed biofilms on the surfaces were observed using field emission scanning electron microscopy (FESEM, S4700, Hitachi, Japan) at an accelerating voltage of 15.0 kV.

S1.2.5 Colony counting assay

The viability of bacteria in biofilms was quantitatively evaluated using a standard colony counting assay. After cultivation as described above, the culture medium was removed, and the biofilms were gently washed with PBS. Subsequently, the biofilms were dispersed in PBS by 10 min of sonication, followed by serial dilution and plating on agar plates. The plates were then incubated at 37°C for 18 h, and the CFUs on the different plates were imaged and counted.

S1.2.6 Crystal violet (CV) staining assay

The biofilm mass was determined using a CV staining assay. After cultivation as described above, the culture medium was removed, and the biofilms were washed gently with PBS. Then, 200 μL of a CV solution (0.1 %, w/v) was added to each well to stain the biofilms for 20 min. The CV solution was then removed, and each well was rinsed with PBS. After drying for 10 min in the air, 200 μL of 90% ethanol was added to each stained biofilm to ensure complete dissolution of the CV dye. 100 μL of staining solution was transferred to a 96-well plate, and the absorbance of each solution at 570 nm was recorded by a microplate reader (100-240 V AC, USA).

S.1.2.7 Bacterial cell membrane integrity

The 1×10^7 CFU/mL bacterial suspension was incubated with various samples for 24 h, and the supernatant was collected. After filtering through a 0.22 μm membrane, the absorbance of obtained suspension was measured using microporous plate apparatus (Varioskan Flash, Thermo Scientific, USA) at 260 nm.

S1.2.8 Evaluation of expression of QS-related genes

The expression of QS-related genes (*lasI*, *lasR*, *rhlI*, and *rhlR* for *P. aeruginosa*; *icaA* and *argA* for *S. aureus*) was evaluated by reverse transcription quantitative polymerase chain reaction (RT-qPCR). The primers used in this study were listed below.

Primer	Sequence(5'-3')
16S	AGGTGGTTCAGCAAGTTGGATGTG
RNA	TCTACGCATTTCACCGCTACACAG
lasI-F	CGCACCTGAAGATCGGCATCG
lasI-R	ATGAAACCGCCAGTCGCTGTTC
lasR-F	AGGACAGCCAGGACTACGAGAAC
lasR-R	TTCCCAGAAAATCGGCAGTACGC
rhlI-F	CCATCCGCAAACCCGCTACATC
rhlI-R	GCTGCACAGGTAGGCGAAGAC
rhlR-F	CGCCACACGATTCCCTTCACC
rhlR-R	TCCAGACCACCATTTCCGAGGAG
gyrB-F	ACATTACAGCAGCGTATTAG
gyrB-R	CTCATAGTGATAGGAGTCTTCT

icaA-F	TTTCGGGTGTCTTCACTCTAT
icaA-R	CGTAGTAATACTTCGTGTCCC
agrA-F	TGATAATCCTTATGAGGTGCTT
agrA-R	CACTGTGACTCGTAACGAAAA

After incubation for 24 hours, the bacteria within biofilms were collected and lysed with Trizol reagent. Total RNA was extracted according to manufacturer's instructions. The purity and concentration of RNA was assessed by The ND1000 spectrophotometer. Messenger RNA processed by the DNase is then reverse-transcribed into complementary DNA (cDNA). The RT-qPCR was performed according to the instructions of the SYBR Green qPCR Mix kit. The RT-qPCR experiment consisted of 30 cycles, each of which lasted for 30 seconds at 95°C, 5 seconds at 60°C, and 30 seconds at 95°C. As internal reference of *P. aeruginosa* and *S. aureus*, *16S* RNA and *gyrB* genes were used as housekeeping genes, respectively. The data were standardized and the changes in gene expression was analyzed using a threshold cycle method.

S1.3 Biocompatibility of QC NPs

S1.3.1 Cell culture

Mouse fibroblasts (L929 cells) were purchased from the cell bank of the Chinese Academy of Sciences. Prior to the experiments, the cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640, Thermo Fisher Scientific, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin (Genview) and 100 μg/mL streptomycin (Solarbio) in a 37°C incubator

(Eppendorf Galaxy 170R) with 5% (v/v) CO_2. Culture medium was changed every two days. Cells were detached from the cell culture flask by trypsinization (0.25% Trypsin-0.53m MEDTA), collected by centrifugation at 1200 r/min for 5 min and re-suspended in fresh cell culture medium before experiments.

S1.3.2 Live/Dead staining assay

L929 cells were cultured on 48-well plates (2×10^4 cells/well) at 37℃ for 12 h to allow full spreading. The original cell medium was then replaced by RPMI-1640 containing QC NPs with varying concentrations (0, 5, 25, 50 μg/mL), and the cells were continuously cultured at 37°C for 72 h. After that, the RPMI-1640 was replaced by PBS containing a mixture of Calcin-AM and Propidium iodide (PI) dyes (200 μL, 500×), and the cells were stained by the mixture dye for 20 min. Cells were observed by fluorescence microscopy (IX71, Olympus, Japan) and images of 10 randomly chosen fields were captured.

S1.3.3 Cell counting kit-8 (CCK-8) assay

L929 cells were cultured on 48-well plates (2×10^4 cells/well) at 37℃ for 12 h to allow full spreading. The original cell medium was then replaced by RPMI-1640 containing QC NPs with varying concentrations (0, 5, 25, 50 μg/mL), and the cells were continuously cultured at 37°C for 72 h. After washing with PBS for 3 times, 200 μL RPMI-1640 and 20 μL CCK-8 dye were added to each well and cultured at 37℃ for another 2 h. The 100 μL mixture was then transferred to the 96-well plate. The absorbance of each solution at 450 nm was recorded using a microporous plate apparatus (Varioskan Flash, Thermo Scientific, USA). For each sample, six parallel

replicates were performed. The relative cell viability (%) was evaluated by comparing the optical density value of each experimental group with the control group.

S1.3.4 Hemolysis experiment

100 μL of QC NPs solution with different concentrations (0-50 μg/mL in PBS) were added to 100 μL of a 4% (V1/V2) suspension of red blood cells (RBCs) in PBS. Negative and positive controls were prepared using RBCs treated with PBS and Triton X-100, respectively. The resulting RBC suspensions were incubated at 37°C for 3 h and then centrifuged at 2000 r/min for 15 min. A 100 μL aliquot of the supernatant was collected and the optical density (OD) value at 545 nm was recorded. The hemolysis rate was determined as $[(OD_{sample}-OD_{negative\ control}) / (OD_{positive\ control}-OD_{negative\ control})] \times 100\%$.

S1.4 Preparation of BC membranes

Hestrin-Schramm (HS) medium was prepared through dissolving 2.5 g of peptone, 2.5 g of yeast extract, 1.35 g of Na_2HPO_4, 0.75 g of citric acid, and 10 g of glucose in 500 mL deionized water; the pH of the medium was then adjusted to 5.0. The HS medium was sterilized by utilizing an autoclave at 121°C for 30 min. Bacterial cellulose (BC) was bio-synthesized by *Gluconacetobacter xylinus* (ATCC 53582) in HS medium under static culture at 30 °C for 3 days. The as-prepared BC membranes were immersed in 0.1 mol/L NaOH at 60°C for 4 h to remove the microorganism and remaining culture medium, followed by being rinsed with ultrapure water repeatedly until neutral pH was re-established.

S1.5 Statistical analysis

The data were derived from at least three independent experiments, and the results were expressed as mean ± standard deviation. The Student's *t*-test was used for comparisons between the two groups. One-way analysis of variance (ANOVA) with Tukey's post hoc test was performed to analyze the variance between more than two groups. Differences with $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ were considered statistically significant.

S2 Supporting Results

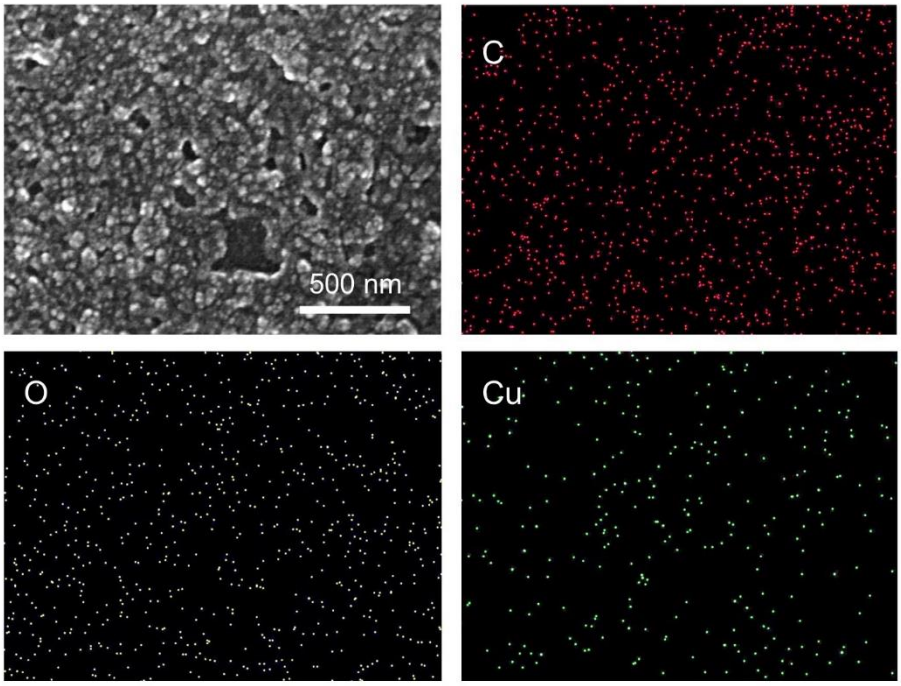

Figure S1. EDS elemental mapping images of QC NPs.

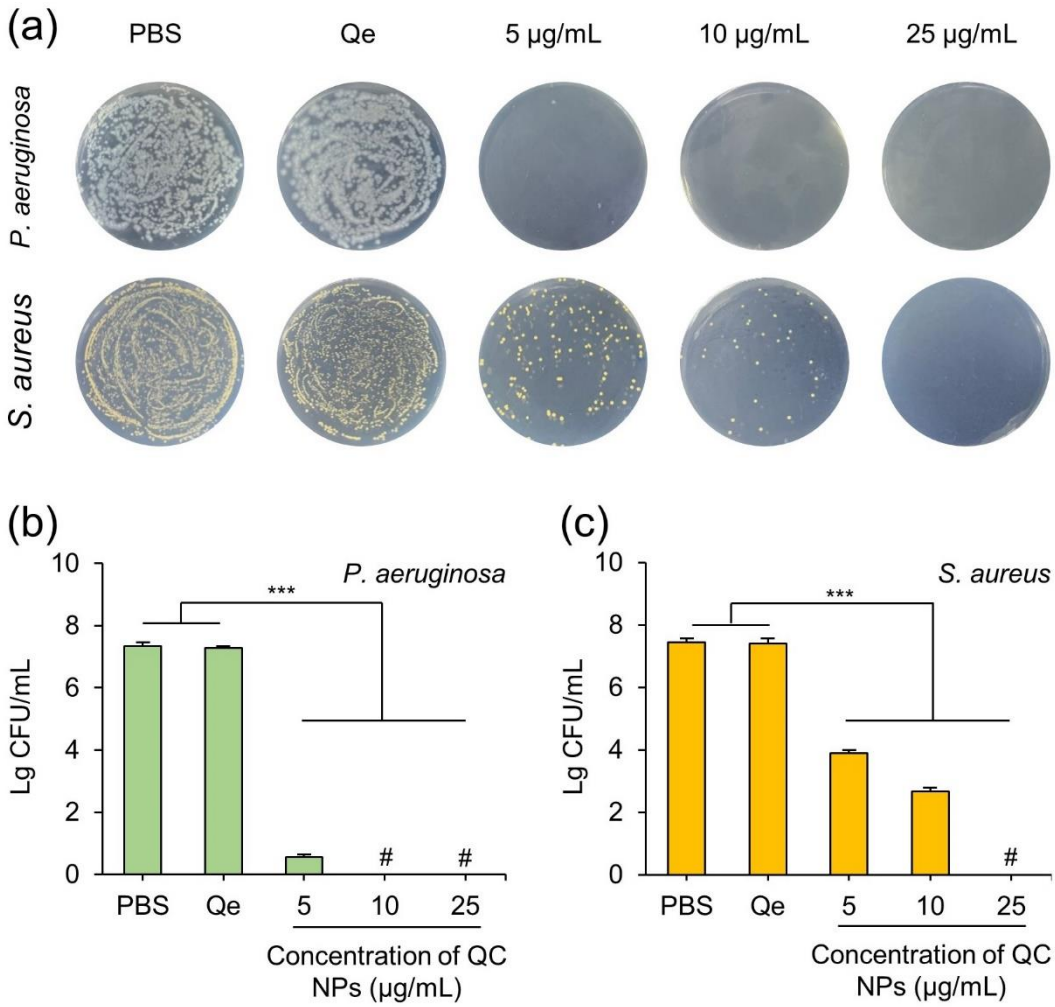

Figure S2. (a) Representative photographs of bacterial colony formation on agar plates after being treated with different samples. (b) Corresponding results of CFU counting. Data are mean ± SD (n = 3, *** $p < 0.001$). # indicates negligible colony formation.

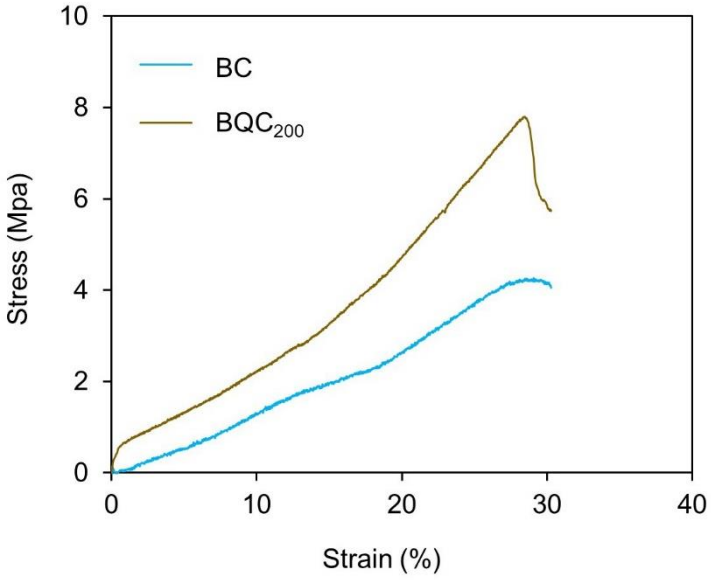

Figure S3. Stress-strain curves of BC and BQC_{200} membranes in the wet state.

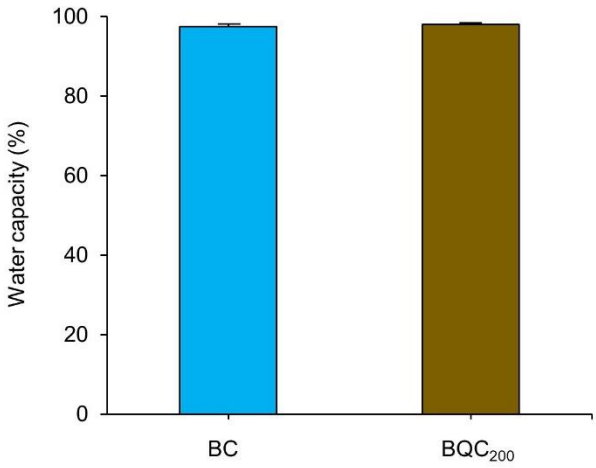

Figure S4. Water capacity of BC and BQC_{200} membranes. Data are mean ± SD (n = 6).

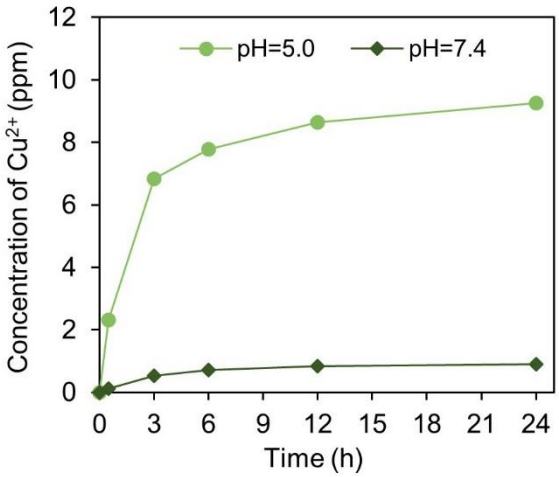

Figure S5. Cumulative release of Cu^{2+} ions from the BQC_{200} immersed in PBS under different pH conditions.

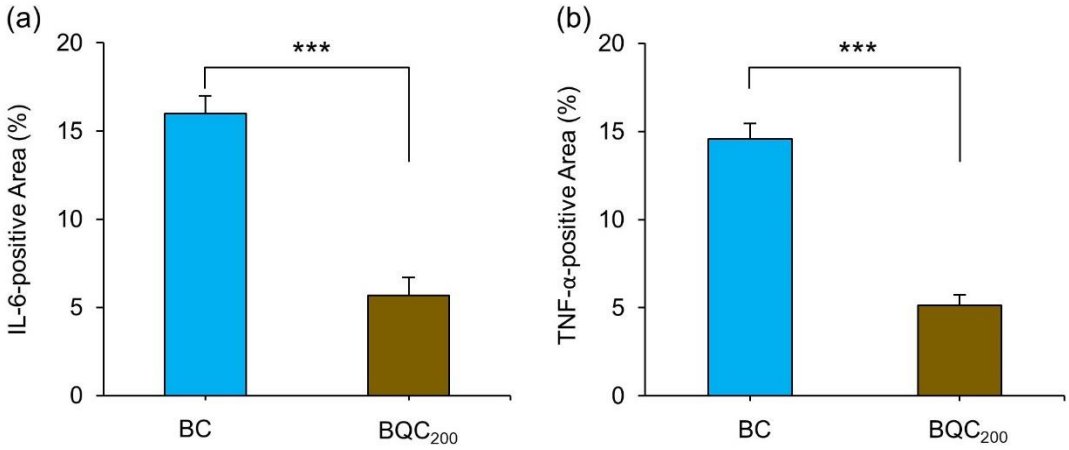

Figure S6. The quantified IL-6 and TNF-α positive cells positive area of the infected skin wound tissues 9 days after different treatments. Data are mean ± SD (n = 6, *** p < 0.001)

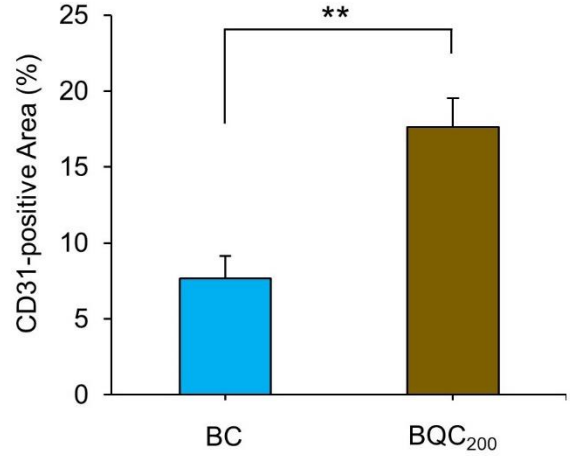

Figure S7. The quantified CD31 positive area (green area) of the infected skin wound tissues 9 days after different treatments. Data are mean ± SD (n = 6, ** p < 0.01)

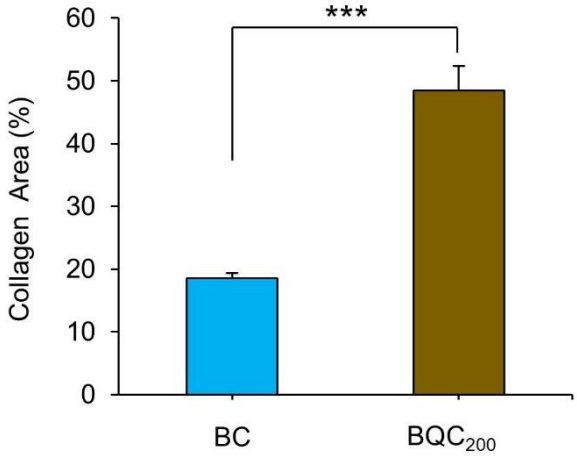

Figure S8. The collagen deposition area of the infected skin wound tissues 9 days after different treatments. Data are mean ± SD (n = 6, *** p < 0.001)