Supplementary Information for

Blue light-activated 5,10,15,20-tetrakis(4-bromophenyl)porphyrin for photodynamic eradication of drug-resistant *Staphylococcus aureus*

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Materials and Methods

Reagents

DiBAC4(3) (D141133) was purchased from Aladdin (Shanghai, China). Fluorescein diacetate (FDA, F8040), crystal violet (C8470), ATP Content Assay Kit (BC0305), Superoxide Anion Content Assay Kit (BC1290) and human serum albumin (A8230) were obtained from Solarbio (Beijing, China). SOSG singlet oxygen fluorescent probe (S0067), Hydrogen Peroxide Assay Kit (S0038), MTT (ST316) and DNA Damage Assay Kit (C2038S) were purchased from Beyotime Biotechnology (Shanghai, China). Methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC43300) strain, *Pseudomonas aeruginosa* (ATCC27853) and *Candida albicans* (ATCC10231) were purchased from Shanghai Bioresource Collection Center (Shanghai, China). SYTO9/PI Live/Dead Bacterial Double Stain Kit were purchased from MaokangBio (MX4234, Shanghai, China). TPP (M115647) and TBPP (B300692) were purchased from Aladdin. TCPP (T856586) and TFPP (T928829) were obtained from Macklin (Shanghai, China). TIPP (Y05442) was purchased from Yanshen Technology (Jilin, China).

Bactericidal activities against *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Candida albicans* (*C. albicans*)

P. aeruginosa monocolony was moved to 20 mL liquid LB culture medium and cultivated for 12 h at 37 °C while shaking. In the experiments of antibacterial activity of photoexcited compounds, the *P. aeruginosa* were attenuated with culture medium to 10^6 cfu/mL. Then compounds with varying concentrations were respectively

blended with *P. aeruginosa* solution and incubated for 20 min. Finally, a 445 nm laser with different power densities was used as the blue light source. The bactericidal effects of blue light alone or compound alone were also assessed. After irradiation for the indicated light doses, the *P. aeruginosa* solution was put on LB agar plates to calculate the number of living bacteria.

C. albicans monocolony was moved to 20 mL liquid YM culture medium and cultivated for 12 h at 37 °C while shaking. In the experiments of antibacterial activity of photoexcited compounds, the *C. albicans* were attenuated with culture medium to 10^6 cfu/mL. Then compounds with varying concentrations were respectively blended with *C. albicans* solution and incubated for 20 min. Finally, a 445 nm laser with different power densities was used as the blue light source. The bactericidal effects of blue light alone or compound alone were also assessed. After irradiation for the indicated light doses, the *C. albicans* solution was put on agar plates to calculate the number of living *C. albicans*.

Uptake of TBPP by MRSA

MRSA and TBPP (4, 8, 12 μM) were incubated in the dark for 20 min. The samples were washed with PBS for 3 times and centrifuged (5000 rpm, 5 min) to remove the supernatant. The lysis buffer (50 mM Glucose, 25 mM Tris-HCl, 10 mM EDTA, 0.2 M NaOH, 1% SDS) was added into the samples and fully mixed. Then the samples were centrifuged (12000 rpm, 5 min) to obtain supernatant. The fluorescence of the supernatant was recorded by using a spectrophotometer (FLS1000, Edinburgh Instruments, UK) with the excitation wavelength at 429 nm.[1]

The accumulation of TBPP in MRSA was also assessed by fluorescence microscope. Briefly, MRSA and TBPP (4, 8, 12 μM) were incubated in the dark for 20 min, washed thrice with PBS, and visualized under fluorescence microscopy (Axio Scope5, ZEISS, Germany).

ATP (adenosine triphosphate) content assay

The level of cellular ATP is an important parameter for evaluating the available energy in a microorganism. MRSA was incubated with TBPP (12 μM) for 20 min followed by 445 nm laser irradiation (0, 20, 60 J/cm^2). The ATP levels were detected using the ATP Content Assay Kit (BC0305, Solarbio).

RNA extraction and qPCR

Total RNA was extracted from MRSA using the Total RNA Extraction Kit (R1200, Solarbio, China) following the manufacturer's guideline. 1 μg of the total RNA was reverse transcribed using the All-in-One First-Strand cDNA Synthesis SuperMix (AU341, TransGen Biotech, China). The quantitative real-time PCR (qPCR) amplification mixture contained cDNA, 2×PerfectStart Green qPCR SuperMix (AQ601, TransGen Biotech), and forward and reverse primers (0.2 μM each). Primers for amplification are shown in Table S2.[2] qPCR was carried out with the PIKOREAL 96 Real Time PCR System (Thermo, Finland). Every assay was performed in triplicate and all experiments included analysis of 16S rRNA mRNA levels as internal standard. Relative expression was determined by the Ct method and levels were expressed as percentage relative to the 16S rRNA mRNA levels.

Fluorescein diacetate (FDA) staining assay

MRSA biofilms grew in 24 well plates were washed three times with PBS. 500 μL 20 μg/mL FDA solution was added and incubated in the dark for 30 min. After washed with PBS for two times, the biofilms were observed using confocal microscopy (Nikon C2, Japan).

Quantification of MRSA in biofilms

The biofilms grew in 24 well plates were washed with PBS. After washing, 200 μL PBS was added to wells and biofilms were detached by scraping wells with a 200 μL pipette tip 15 times across the wells. The scraped biofilms were then homogenized in solution by repeatedly pipetting the solution five times. Serial dilutions were then created by adding 100 μL to 900 μL PBS. Finally, 100 μL of homogenized biofilm suspension was spread out onto agar plates at different dilutions. Plates were incubated for 24 h under static conditions at 37℃. Colonies on plates were enumerated and expressed as cfu (colony forming units)/mL.[3]

Animal models

All the mice (male ICR mice weighted about 20.0 g) used in this study were purchased from Shanxi Medical University (Shanxi, China). All in vivo experiments were approved by the Animal Ethics Committee of Shanxi Medical University. All animal procedures were in accord with the guidelines of the Institutional Animal Care and Use Committee of Shanxi Medical University (Shanxi, China). The mice were housed in a temperature-controlled, ventilated and standardized disinfected animal room, and allowed to acclimatize for 1 week before the start of experiments. To assess the effects of TBPP-PDT on wound healing, a wound (about 1 cm) was created on the

back of the mice, and 10^7 methicillin-resistant *Staphylococcus aureus* (MRSA) were injected for 2 d to construct biofilm infection model.[4] The mice were divided into four groups (n = 6). Then the mice were subcutaneously administrated by PBS, blue light alone, TBPP alone (100 μL, 0.5 mg/kg), TBPP (100 μL, 0.5 mg/kg) + blue light (120 J/cm^2) every two days during an 8 days administration period. The wounds of the mice were recorded by camera every two days. At day 8, the wound tissues were removed from mice, and the numbers of bacteria in the wounds were determined by plate count methods.

Skin irritation potentials of TBPP-PDT were evaluated in male ICR mice weighted about 20.0 g. The backs of mice were clipped free of hair, and were subcutaneously administrated by PBS, blue light alone, TBPP alone (100 μL, 0.5 mg/kg), TBPP (100 μL, 0.5 mg/kg) + blue light (120 J/cm^2) every two days for 8 days. The skins of the mice were recorded by camera every two days.

Cell viability assay

NIH/3T3 cells (mouse embryonic fibroblasts) were inoculated into 96-well plates with 10^4 cells per well, and cultured in 5% CO_2 incubator at 37℃ for 24 h.[5] The old medium was discarded, and 200 μL of fresh medium containing TBPP (12 μM) was added. After incubation for 20 min, the cells were treated with different doses (40, 80, 120 J/cm^2) of blue light, and cultured for 24 h. Then 10 μL MTT (5 mg/mL) was added into each well. After incubation for 4 h, 100 μL DMSO was added into each well. The light absorption was measured at 570 nm.

DNA damage assay

NIH/3T3 cells were incubated with TBPP (12 μM) for 20 min followed by irradiation with blue light (120 J/cm^2). For positive controls, NIH/3T3 cells were treated with 50 μM camptothecin (SC0141, Beyotime Biotechnology) for 3 hours. After fixation with 4% paraformaldehyde for 30 min at room temperature, the cells were permeabilized with 0.5% Triton X-100 for 5 min, blocked with 5% bovine serum albumin at room temperature for 30 min and probed overnight with primary antibodies against γ-H2AX (C2038S, Beyotime Biotechnology) at 4 ºC.[6] After rinsing three times with PBS for 5 min each, cells were incubated with a fluorochrome-conjugated secondary antibody, diluted in antibody dilution buffer, for 30 min at room temperature in the dark. Labeled cells were then rinsed with PBS and incubated with DAPI for 15 min. Finally, the cells were rinsed with PBS and analyzed under a confocal microscope (Nikon C2, Japan).

Protein denaturation detection

Human serum albumin (HSA, 2 μM) was incubated with TBPP (12 μM) for 20 min followed by irradiation with blue light (120 J/cm^2). The fluorescence spectra of HAS were performed on a fluorescence spectrophotometer (FLS1000, Edinburgh Instruments, UK) equipped with a 1.0 cm quartz cell. The excitation and emission slits with a band pass of 5.0 nm were used for all the measurements. The excitation wavelength was set at 295 nm to selectively excite the Trp residue, and the emission spectra were recorded in the wavelength range from 290 to 450 nm.[7]

Statistical analysis

All experiments were repeated at least three times. SPSS 17.0 software was used to

calculate the statistical significance of the experimental results. The significance level was set as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$. Error bars denote the standard deviation (SD).

Tables and Figures

Table S1. MBC values for PDT (120 J/cm^2) mediated by compounds against MRSA

PDT	MBC (μM)
TPP-PDT	40
TBPP-PDT	12
TCPP-PDT	32
TFPP-PDT	28
TIPP-PDT	16

Table S2. Primer sequences for qPCR experiments

Gene	**Sequence (5'-3')**
TSST-1	Sense Strand: ACCCCTGTTCCCTTATCATC
	Antisense Strand: AAAAGCGTCAGACCCACTAC
agrA	Sense Strand: TGATAATCCTTATGAGGTGCTT
	Antisense Strand: CACTGTGACTCGTAACGAAAA
16S rRNA	Sense Strand: GCTGCCCTTTGTATTGTC
	Antisense Strand: AGATGTTGGGTTAAGTCCC

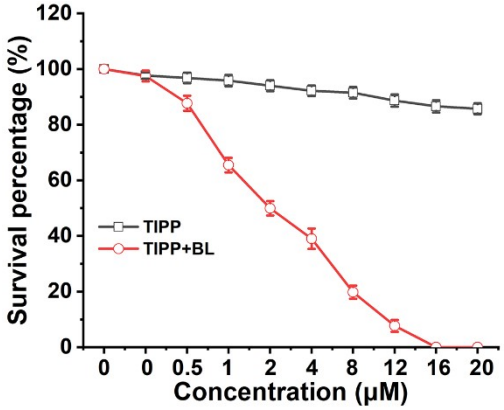

Fig. S1. The viability of MRSA exposed to different concentrations (0, 0.5, 1, 2, 4, 8, 12, 16, 20 μM) of TIPP for 20 min followed by irradiation at 120 J/cm^2 with blue light (420 nm) (mean ± SD, n = 3). BL, blue light. MRSA, methicillin-resistant *Staphylococcus aureus*.

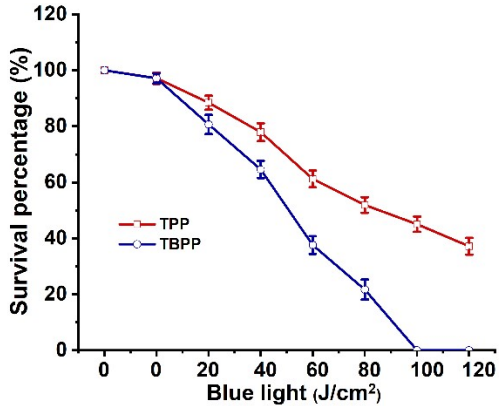

Fig. S2. The viability of MRSA exposed to TPP and TBPP (12 μM) for 20 min followed by irradiation with different doses (0, 20, 40, 60, 80, 100, 120 J/cm^2) of blue light (mean ± SD, n = 3). BL, blue light. MRSA, methicillin-resistant *Staphylococcus aureus*.

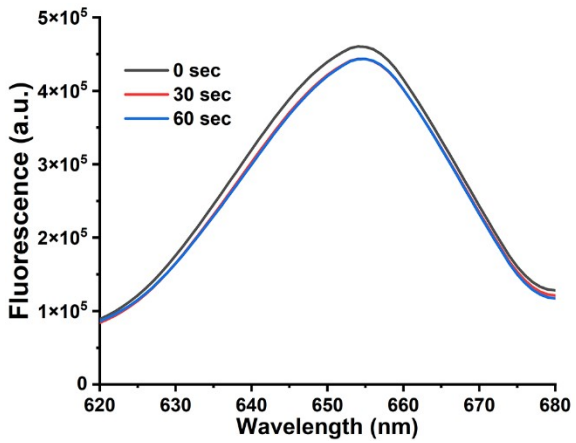

Fig. S3. The fluorescence spectra of TBPP (12 μM) exposed to blue light (0.2 W/cm^2) for 0, 30 and 60 sec. After blue light irradiation for 30 sec, there was a small decrease in the maximum absorbance of TBPP and no shift of the peak. After another 30 sec of irradiation, the fluorescence intensity of TBPP did not continue to decline, indicating that TBPP exhibited relatively good stability.

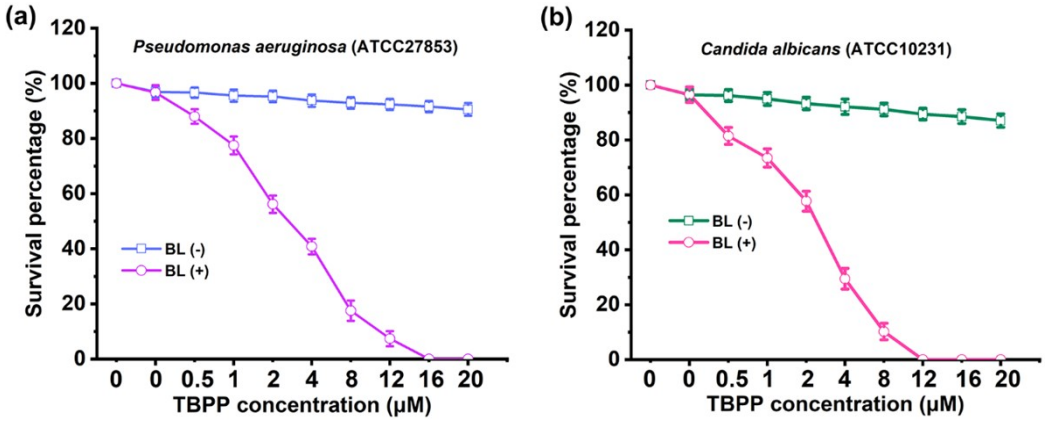

Fig. S4. Survival curve of *P. aeruginosa* and *C. albicans* treated with TBPP + BL. (a) The viability of *P. aeruginosa* exposed to different concentrations of TBPP for 20 min followed by 445 nm laser irradiation (100 J/cm^2) (mean ± SD, n = 3). (b) The viability of *C. albicans* exposed to different concentrations of TBPP for 20 min followed by 445 nm laser irradiation (100 J/cm^2) (mean ± SD, n = 3). BL, blue light.

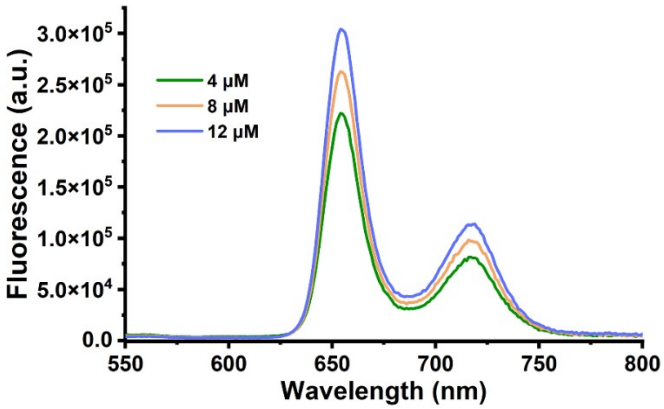

Fig. S5. The fluorescence intensity of TBPP (4, 8, 12 μM) in MRSA at 20 min. MRSA, methicillin-resistant *Staphylococcus aureus*.

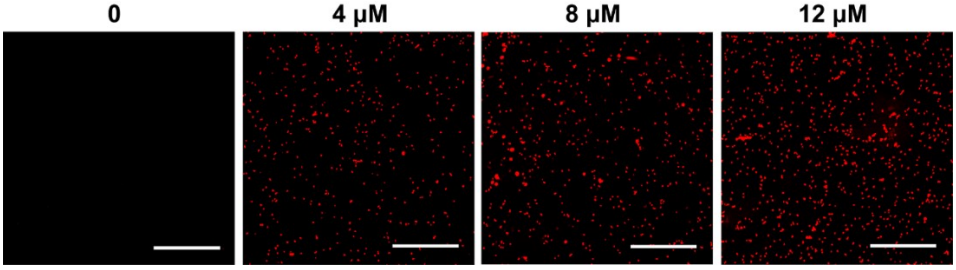

Fig. S6. Uptake of TBPP (4, 8, 12 μM) in MRSA at 20 min by fluorescence microscope. Scal bar = 250 μm. MRSA, methicillin-resistant *Staphylococcus aureus*.

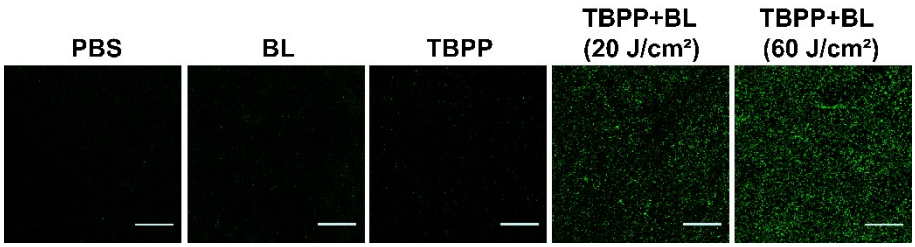

Fig. S7. ROS production in MRSA exposed to TBPP (12 μM) for 20 min followed by irradiation with different doses (0, 20, 60 J/cm^2) of blue light was tested using 2,7-dichlorofluorescein diacetate (DCFH-DA, 10 μM) as a probe by fluorescence microscope. BL, blue light. MRSA, methicillin-resistant *Staphylococcus aureus*.

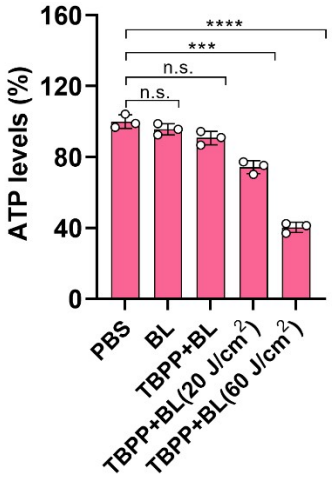

Fig. S8. ATP (adenosine triphosphate) levels of MRSA incubated with TBPP (12 μM) for 20 min followed by irradiation with different doses (0, 20, 60 J/cm^2) of light (mean ± SD, n = 3). ***P < 0.001, ****P < 0.0001 (two-way ANOVA, Dunnett's *posthoc* test). BL, blue light. MRSA, methicillin-resistant *Staphylococcus aureus*.

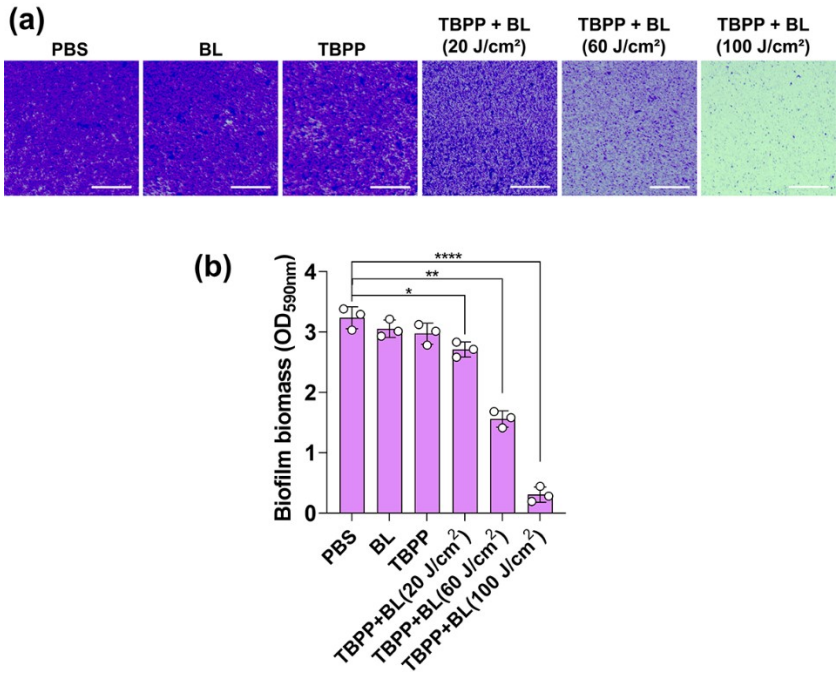

Fig. S9. (a) Biofilm formation tested by crystal violet staining. MRSA were exposed to TBPP (12 μM) for 20 min followed by irradiation with different doses (0, 20, 60, 100 J/cm^2) of blue light. Then the bacteria were allowed to culture for 36 h at 37°C for biofilm formation. The biofilms were stained with crystal violet and visualized under microscopy. Scale bar = 100 μm. (b) Quantitative analysis of the crystal violet-stained biofilms with pretreatments compared with PBS-treated control group (mean ± SD, n = 3). *P < 0.05, **P < 0.01, ****P < 0.0001 (twoway ANOVA, Dunnett's *posthoc* test). BL, blue light. MRSA, methicillin-resistant *Staphylococcus aureus*.

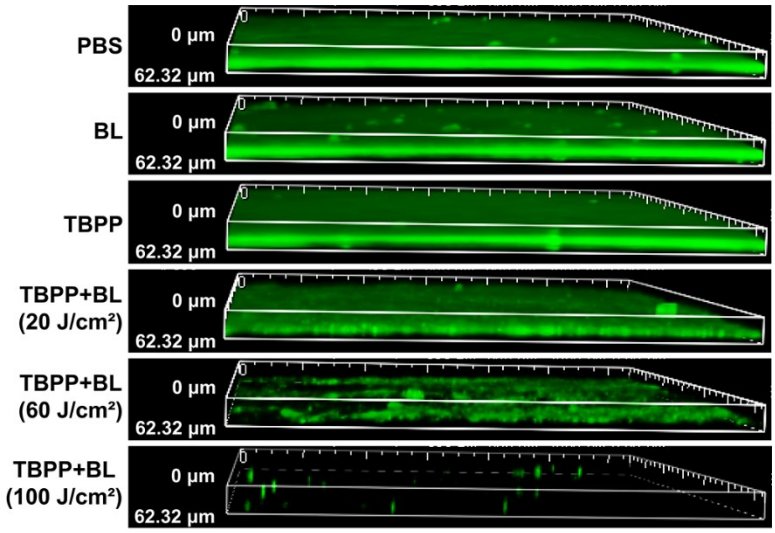

Fig. S10. 3D confocal images of the biofilms. MRSA were exposed to TBPP (12 μM) for 20 min followed by irradiation with different doses (0, 20, 60, 100 J/cm^2) of blue light. Then the bacteria were allowed to culture for 36 h at 37°C for biofilm formation. The biofilms were stained with fluorescein diacetate (FDA). BL, blue light. MRSA, methicillin-resistant *Staphylococcus aureus*.

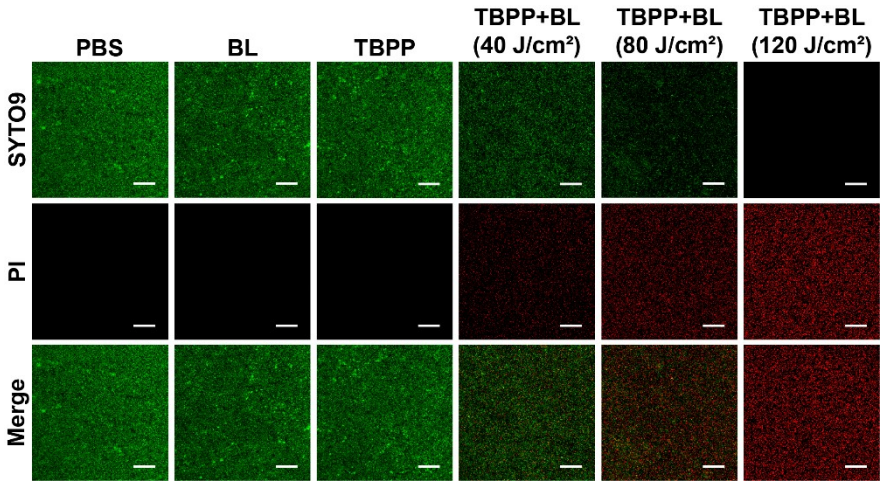

Fig. S11. Live/dead (SYTO9/PI) stain images of MRSA embedded in biofilms exposed to TBPP (12 μM) for 20 min followed by irradiation with different doses (0, 40, 80, 120 J/cm^2) of blue light using confocal microscopy. Red, dead bacteria. Green signal, living bacteria. PI, propidium iodide. BL, blue light. MRSA, methicillin-resistant *Staphylococcus aureus*. Scale bar = 20 μm.

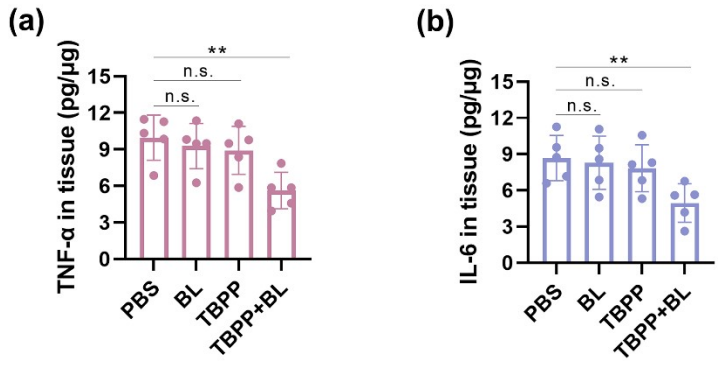

Fig. S12. Secretion levels of TNF-α (a) and IL-6 (b) in the infected skin tissues after treatments (mean ± SD, n = 5). $^{**}P < 0.01$ (two-way ANOVA, Dunnett's *posthoc* test). TNF-α, tumor necrosis factor α. IL-6, interleukin 6. BL, blue light.

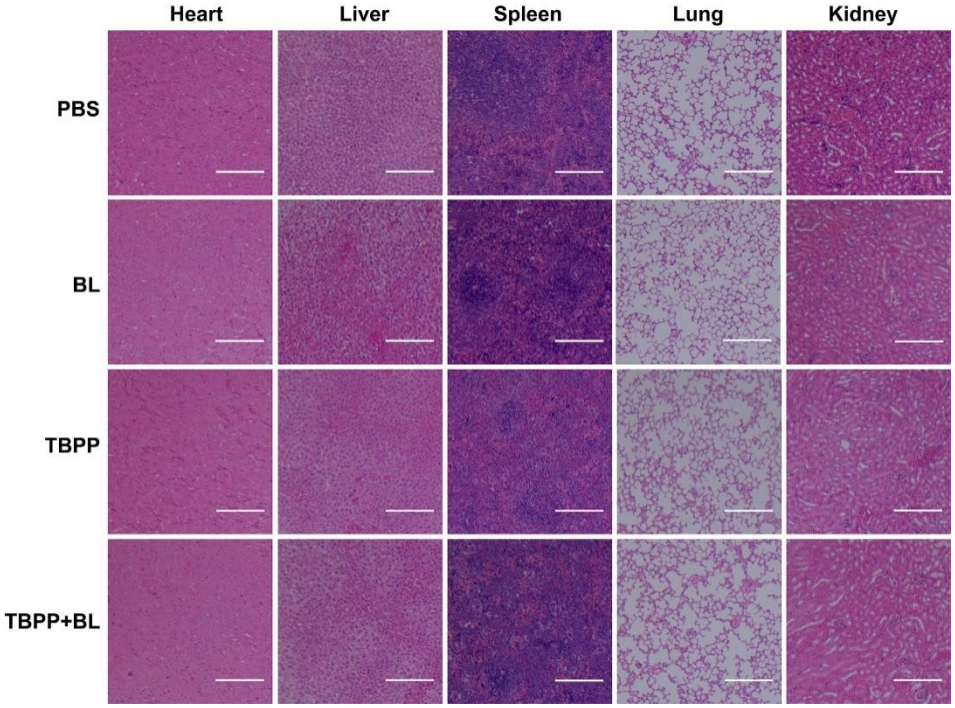

Fig. S13. Representative micrographs of H&E (haematoxylin and eosin) staining of five important organs (lung, liver, spleen, kidney and heart) with different treatments. BL, blue light. Scale bar = 200 μm.

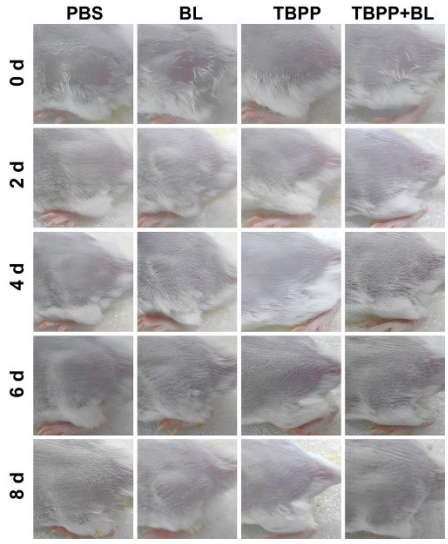

Fig. S14. Digital images of mouse skins. The mice were administered with PBS, blue light alone, TBPP alone (100 μL, 0.5 mg/kg), TBPP (100 μL, 0.5 mg/kg) + blue light (120 J/cm^2) every two days during an 8 days administration period. The skins were surveyed every two days. BL, blue light.

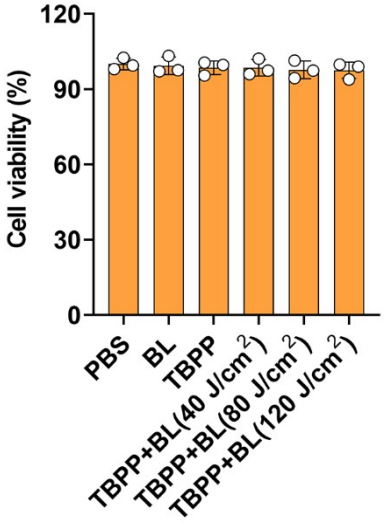

Fig. S15. The cell viability of NIH/3T3 cells incubated with TBPP (12 μM) for 20 min followed by irradiation with different doses (40, 80, 120 J/cm^2) of blue light (mean ± SD, n = 3). BL, blue light.

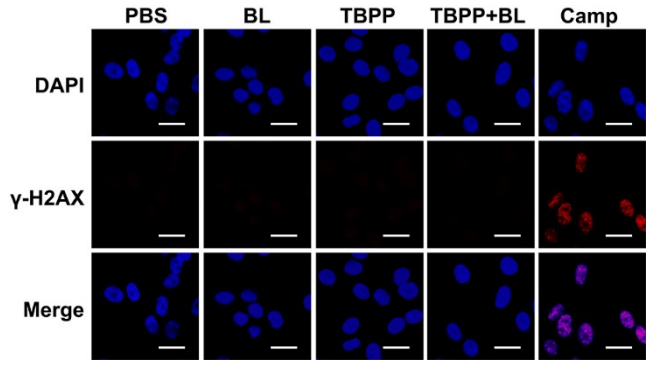

Fig. S16. Representative images of γ-H2AX (DNA damage marker) in NIH/3T3 cells incubated with TBPP (12 μM) for 20 min followed by irradiation (120 J/cm^2) of blue light. The levels of γ-H2AX were not upregulated obviously, suggesting that TBPP-PDT did not induce significant DNA damage in NIH/3T3 cells. BL, blue light. DAPI, 4',6-diamidino-2-phenylindole, DNA dye. Camp, camptothecin, positive control. Scale bar = 5 μm.

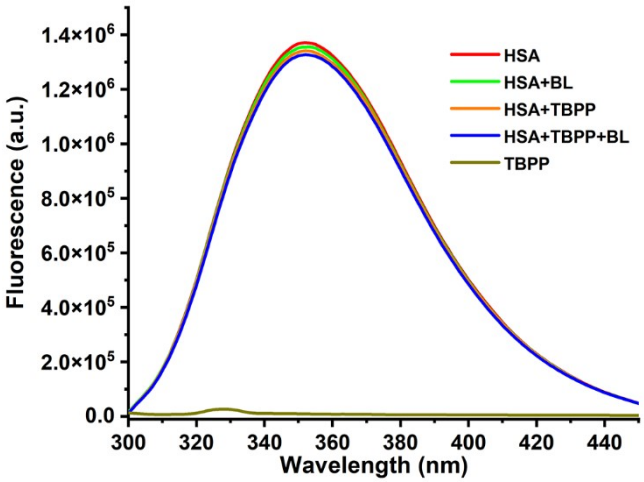

Fig. S17. Fluorescence spectra of human serum albumin (HSA, 2 μM) with different treatments of PBS, BL (120 J/cm^2), TBPP (12 μM) and TBPP (12 μM) + BL (120 J/cm^2). There was no obvious red-shift or blue-shift of the maximum absorption peak and there were slight decreases of fluorescence intensity in the fluorescence spectra of HSA, indicating that TBPP-PDT did not induce significant HSA denaturation. BL,

blue light.

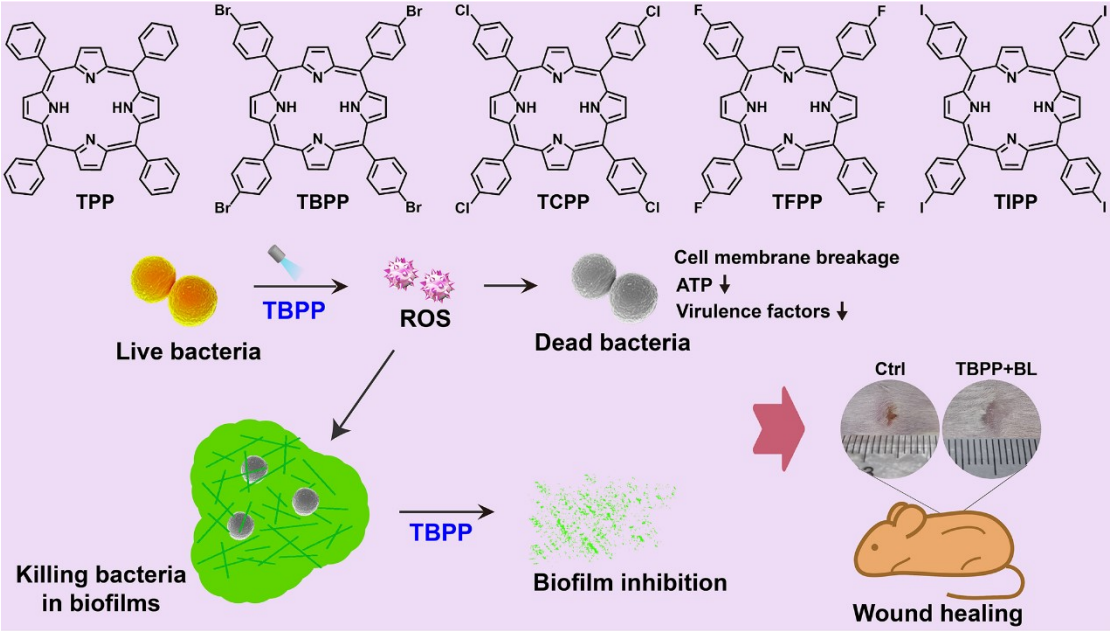

Fig. S18. TBPP-PDT caused cell membrane injury, ATP content reduction as well as virulence factor down-regulation via ROS generation, resulting in bacterial death. Moreover, TBPP-PDT could eradicate the bacteria wrapped in biofilms and disintegrate the mature biofilms. More importantly, TBPP-PDT was able to accelerate wound healing in mouse infection model. PDT, photodynamic therapy. TPP, 5,10,15,20-tetraphenyl porphyrin. TBPP, 5,10,15,20-tetrakis(4-bromophenyl) porphyrin. TCPP, 5,10,15,20-tetrakis(4-chlorophenyl)porphyrin. TFPP, 5,10,15,20-tetrakis(4-fluorophenyl)porphyrin. TIPP, 5,10,15,20-tetrakis(4-iodophenyl)porphyrin. ROS, reactive oxygen species. ATP, adenosine triphosphate.

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