Supporting Information

Tuning Molecular Assembly Behavior to Amplify Sonodynamic Activity of Porphyrin for Efficient Antibacterial Therapy

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1. Experimental Procedures

Materials and measurements. All chemicals for synthesis were purchased from Energy Chemical Commercial Company. All probes for ROS detection and other biochemical reagents were obtained from Shanghai Maokang Biotechnology or Solarbio Biotechnology Co., Ltd. The three common microbes were from the China General Microbiological Culture Collection Center. All nutrient medium for growth of microbes were purchased from Qingdao Hope Bio-Technology Co., Ltd. The female rats were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. NMR and MS spectra were obtained on Bruker AVANCE III HD series spectrometer and ultrafleXtreme MALDI-TOF/TOF (Germany). UV-Vis absorption and fluorescence emission spectra were measured on Hitachi UH5300 and F-4600 spectrophotometer (Japan). Hydrated particle size distribution and zeta potentials were recorded by a Winner 802 analyzer (China) and Malvern Nano ZS90 (America), respectively. A JEOL JEM-F200 transmission electron microscope and JSM-7001F scanning electron microscope (Japan) was used to observe the morphology of assemblies and microbes, respectively. Fluorescence images of microbes were obtained on a Zeiss LSM 880 confocal laser scanning microscope (Germany). The Malvern ITC 200 Isothermal titration calorimeter (America) was empolyed to investigate the intermolecular interactions. The CHI660E electrochemical workstation (China) was used to measure sonocurrent density and electrochemical impedance spectroscopy of molecules. The sonodynamic therapy was performed by an ultrasonic therapeutic apparatus (Chattanooga 2776, America)

Detection of total ROS. Probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was used as the indicator of total ROS to perform the experiment. After activated by NaOH solution (30 min, room temperature) in the dark, PBS (10.0 mM) solution was added to obtain DCFH solution (40.0 μM). Then solutions (20 μL) containing sonosensitizers (500 μM) was mixed with 980 μL of DCFH solution were irradiated with US (1.5 W/cm^2, 1 MHz, 50% duty cycle, 20 min). The fluorescence intensity of various solution was recorded every 2 minute at 525 nm using an excited wavelength of 488 nm. The solution without sonosensitizers was set as control group.

For the ROS detection of T1 and T2 in monomer state, the water was replaced by methanol. That is to say, 0.5 mL of methanol solution containing DCFH-DA (1 mM) was mixed with 2 mL of NaOH solution (10.0 mM) to activate for 30 min in the dark. Then 10 mL of methanol solution was added to obtaining DCFH solution. Then the experiment procedure was the same with that of abovementioned assembly state in water.

Determination of $^1O_2$. 1, 3-Diphenylisobenzofuran (DPBF) was employed as the indicator to perform the experiment. 100 μL of ethanol solution containing DPBF (3 mM) and 40 μL of solution containing sonosensitizers (500 μM) was mixed in 1860 μL of ethanol solution. Then mixed solutions containing different sonosensitizers were irradiated with US (1.5 W/cm^2, 1 MHz, 50% duty cycle, 20 min). The absorbance at 410 nm was recorded every 2 min. The solution without sonosensitizers was set as a control group.

Determination of •OH. Aminophenyl fluorescein (APF) was employed as the indicator to perform the experiment. 40 μL of solution containing sonosensitizers (500 μM) was added to 980 μL of solution containing APF (10 μM) followed by a US irradiation (1.5 W/cm^2, 1 MHz, 50% duty cycle, 20 min). The fluorescence intensity of various solution was recorded every 2 minute at 514 nm using an excited wavelength of 490 nm. The solution without sonosensitizers was set as a control group.

Determination of $\cdot O_2^-$. Nitrotetrazolium Blue chloride (NBT) was employed as the indicator to perform the experiment. 40 μL of solution containing sonosensitizers (500 μM) was added to 1960 μL of solution containing NBT (10 μM) followed by a US irradiation (1.5 W/cm^2, 1 MHz, 50% duty cycle, 20 min). The fluorescence intensity of various solution was recorded every 2 minute at 514 nm using an excited wavelength of 490 nm. The solution without sonosensitizers was set as a control group.

Determination of H_2O_2. A hydrogen peroxide fluorescent probe was employed as the indicator to perform the experiment. 20 μL of solution containing sonosensitizers (500 μM) was added to 980 μL of test solution (5 μM) followed by a US irradiation (1.5 W/cm^2, 1 MHz, 50% duty cycle, 20 min). The fluorescence intensity of various solution was recorded every 2 minute at 520 nm using an excited wavelength of 490 nm. The solution without sonosensitizers was set as a control group.

Ultrasonic electrochemistry test. A three-electrode including platinum electrode as the opposite pole, Ag/AgCl electrode as the reference electrode, and the sample group as the working electrode was used to determine the sonocurrent density and electrochemical impedance spectroscopy of different samples under US treatment (1.5 W/cm^2, 1 MHz, 50% duty cycle) or not. The electrolyte was chosen as 0.5 M of $NaSO_4$ solution. The working electrode was prepared dispersing 200 μL of mixed liquid drop containing sample on conductive glass followed by dried in the air overnight.

Cell viability assay. L929 cells are chosen as the representative cells to perform the standard MTT assay. L929 cells were inoculated in 96-well plates (8 ×10^3 cells/well) for overnight. Then fresh medium containing different concentrations of T1 and T2 was added to cells to

replace the old medium for another culture of 24 h. 100.0 μL of fresh medium containing MTT was added to cells to replace the old medium and cells were incubated for 3 h. Then the supernatant of each well was removed and 100.0 μL of DMSO was added. After shaken for 2 min, a microplate reader (Bio-Rad) was used to measure the absorbance value (490 nm) of each well.

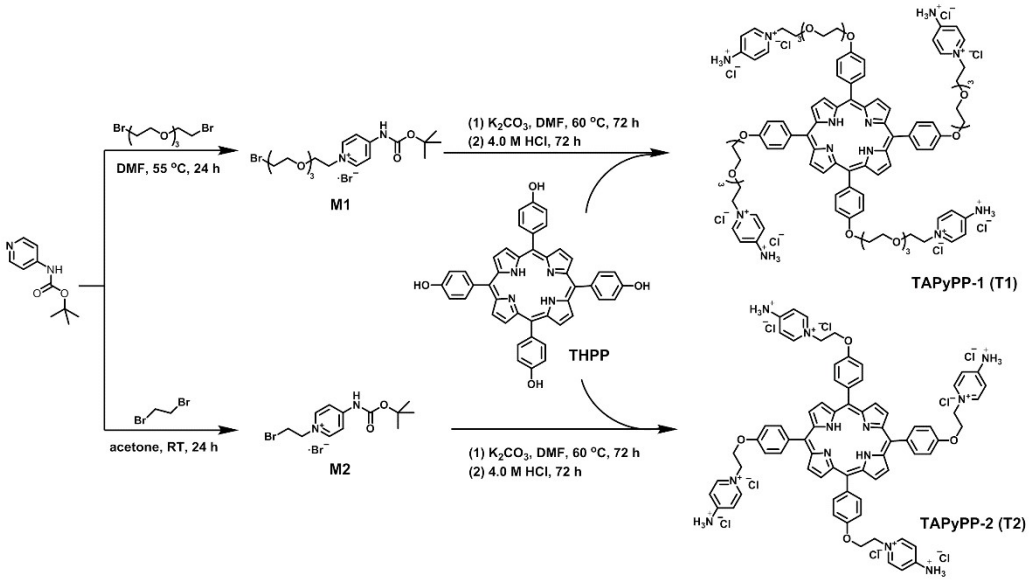
Hemolytic rate assay. The red blood cells were obtained from whole blood of rats by centrifugation (3500 rpm, 15 min) followed by washment using PBS (4-6 times). The obtained red blood cells were dispersed in PBS for subsequent experiment. Then the cell susupension was mixed with different concentrations of T1 and T2 PBS solution, respectively. After cultured at 37 °C for 4 h, the solution was centrifugated to obtain the supernatant. A microplate reader was used to measure the absorbance value (570 nm) of the supernatant. Cell susupension treated by Triton-X and PBS was set as the positive and negative controls, respectively. The hemolytic rates (RHR%) were calculated according to a formula like this: RHR (%) = $(OD_{Sample}–OD_{PBS})/(OD_{Triton\text{-}X}–OD_{PBS})$ ×100%. OD_{Sample} represents the absorbance value (570 nm) of sample groups, OD_{PBS} represents the absorbance value (570 nm) of the negative control group, and $OD_{Triton\text{-}X}$ represents the absorbance value (570 nm) of the positive control group.

Confocal laser scanning microscopy (CLSM) imaging of microbes. (1) Live/dead microbial imaging: After the antimicrobial treatment, the microbes were centrifuged (10000 rpm, 2 min) and washed by PBS. The collected microbes were further stained by a BacLight Live/Dead viability kit for 30 min according to the procedure. Then the stained microbes were centrifuged (10000 rpm, 2 min) and collected to observe by CLSM. A 488 nm laser was used to excite SYTO9 and a 559 nm laser was for PI. After the antibiofilm treatment, the biofilms were stained by the BacLight Live/Dead viability kit for 30 min according to the procedure. Then the biofilms were slightly washed by PBS and subsequently observed by CLSM as abovementioned. (2) Microbial fluorescence imaging: Microbes (10^8 CFU mL^{-1}) were incubated with T2 (0.5 μM) for 20 min at 37 ºC. The microbes without T2 treatment were set as the control. Then the microbes were collected by centrifugation (7100 rpm, 2 min) and washed with PBS twice. Suspended microbes were spread on a glass and observed by CLSM with an excitation wavelength of 438 nm for T2.

Bacterial membrane integrity evaluation. *S. aureus* were incubated with T2 (0.05 μM) for 20 min and those bacteria without T2 treatment was set as the control. Then all bacteria were divided into four groups: control, control+US, T2, T2+US. In the ultrasound groups, bacterial solution was irradiated with US for 20 min (1.5 W/cm^2, 1 MHz, 50% duty cycle). Then

bacterial solution in all groups was filtered through a 0.22 μm filter membrane (water phase). The absorbance value (260 nm) of filtrate was recorded.

2. Supporting Figures



Scheme S1. Synthesis routes of T1 and T2.

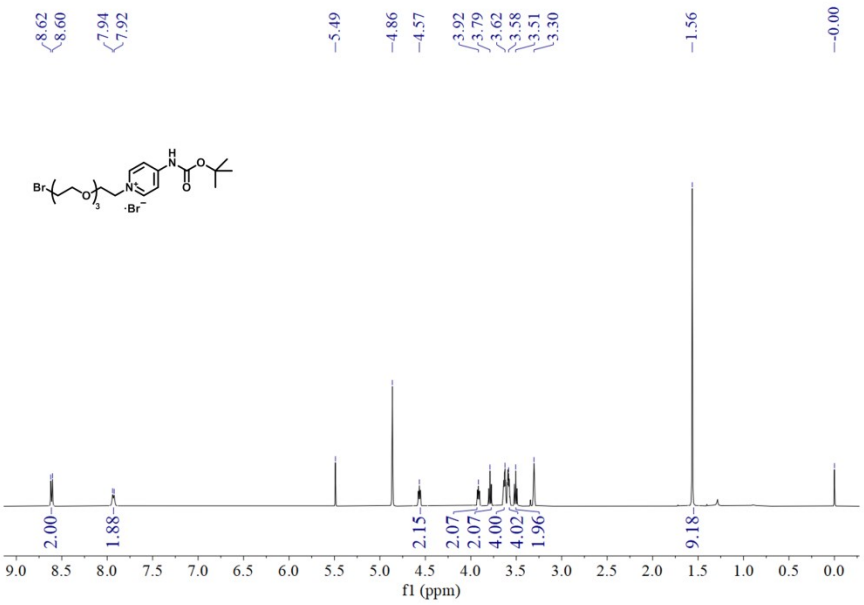


Figure S1. ^{1}H NMR spectrum of M1 in CD_4O.

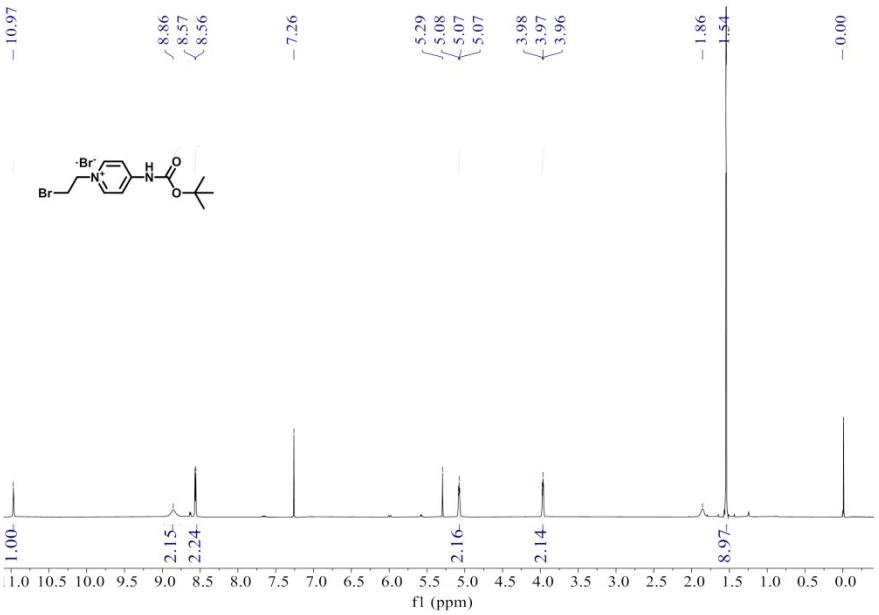


Figure S2. [1] H NMR spectrum of M2 in $CDCl_3$.

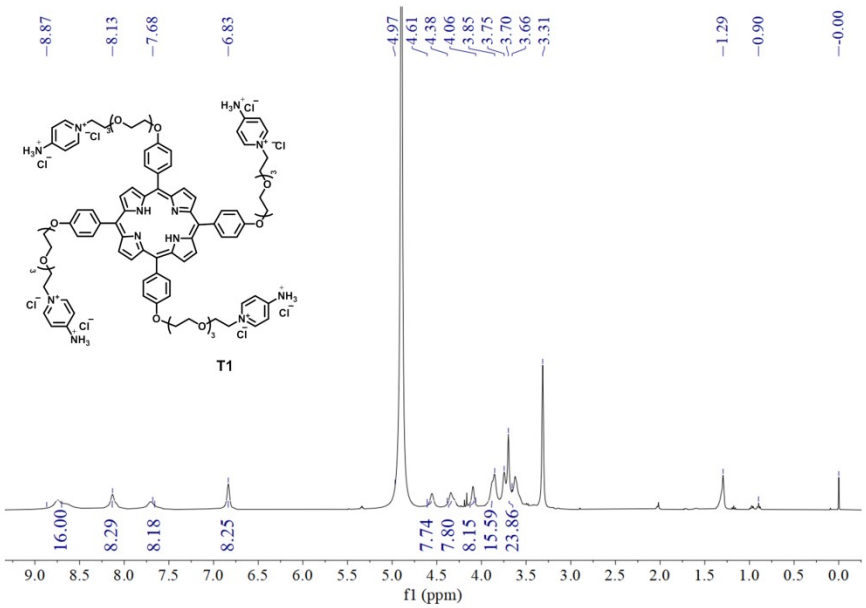


Figure S3. [1] H NMR spectrum of T1 in CD_4O.

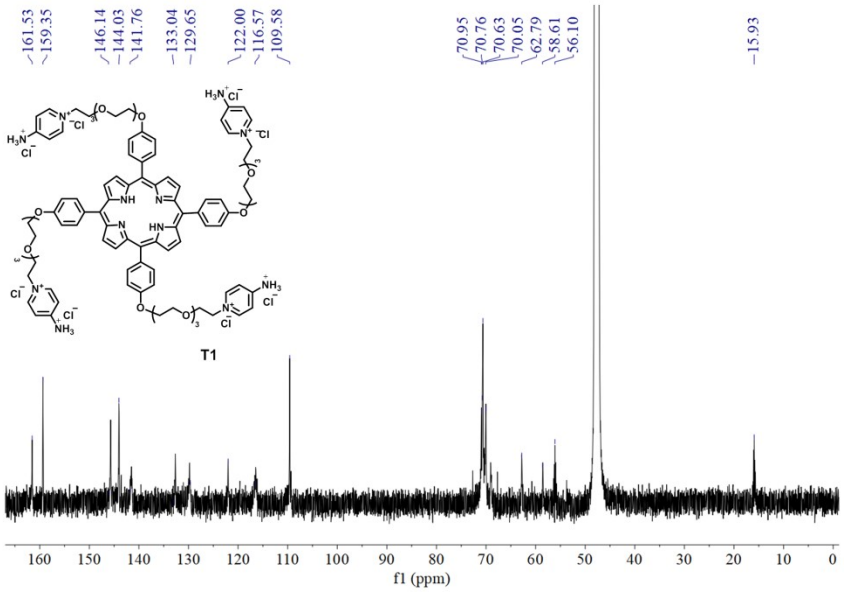


Figure S4. ^{13}C NMR spectrum of T1 in CD_4O.

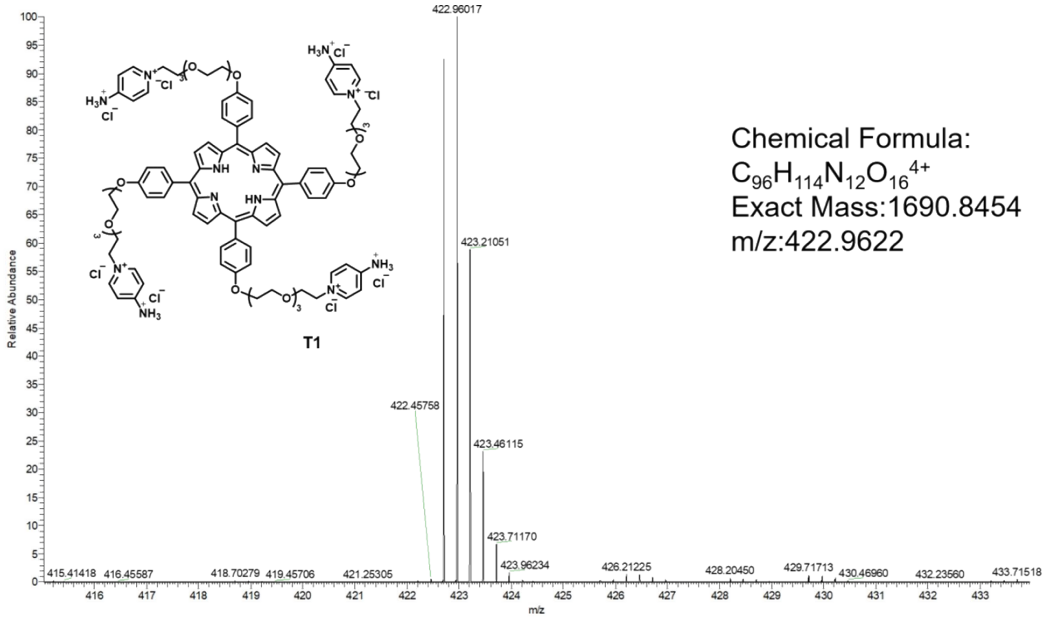


Figure S5. HRMS-ESI spectrum of T1.

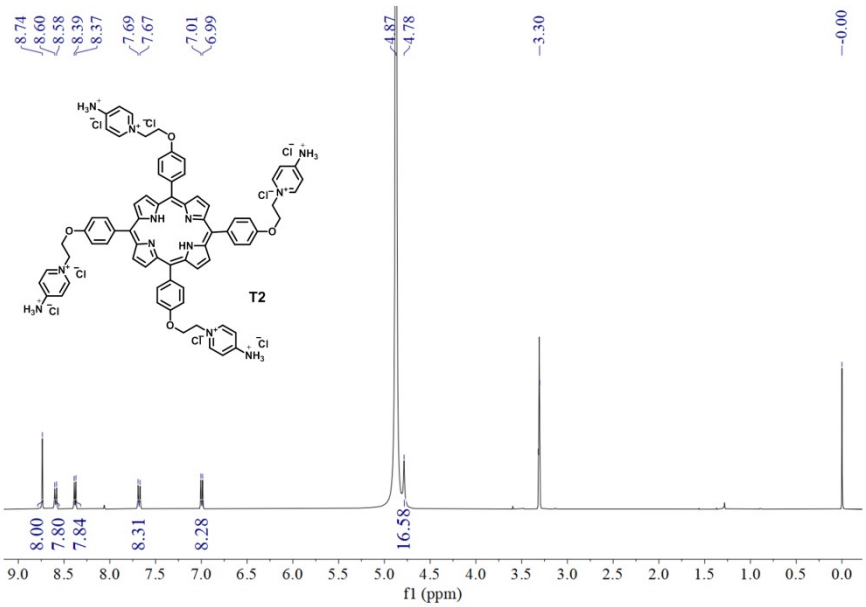


Figure S6. [1] H NMR spectrum of T2 in CD_4O.

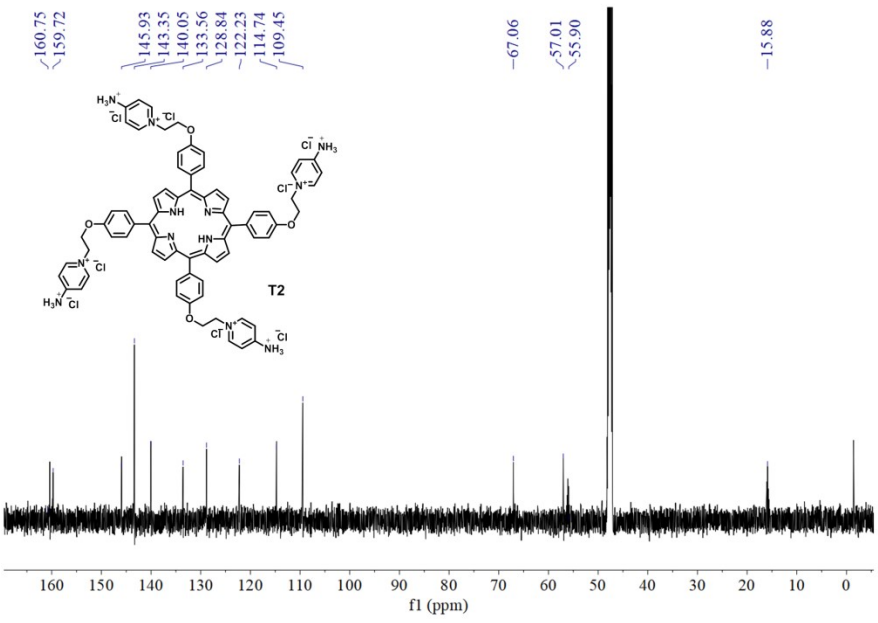


Figure S7. ^{13}C NMR spectrum of T2 in CD_4O.

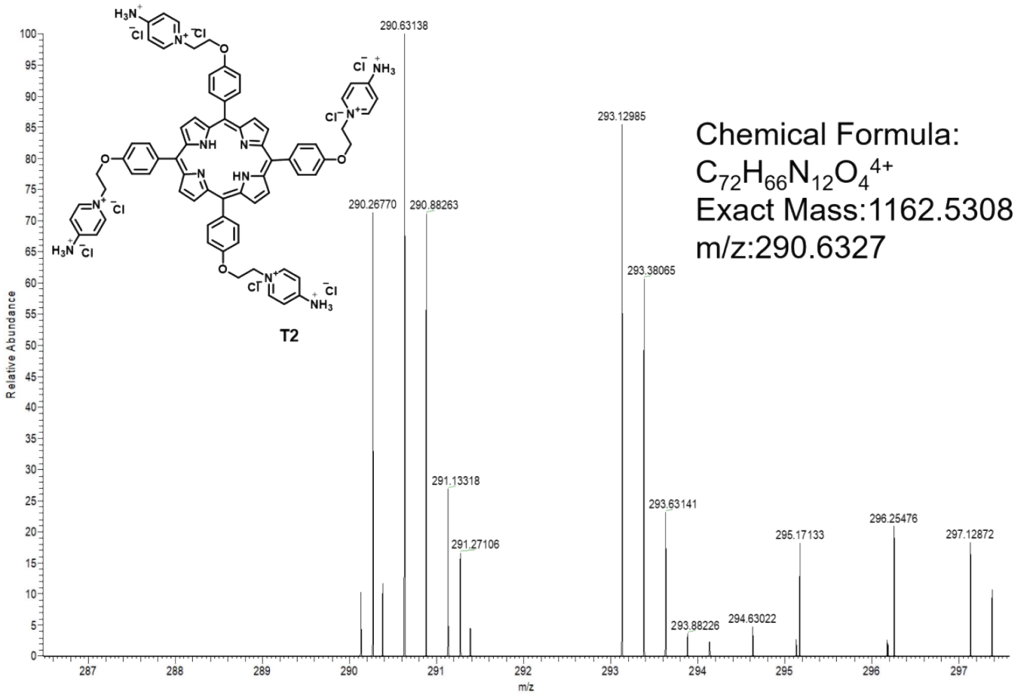


Figure S8. HRMS-ESI spectrum of T2.

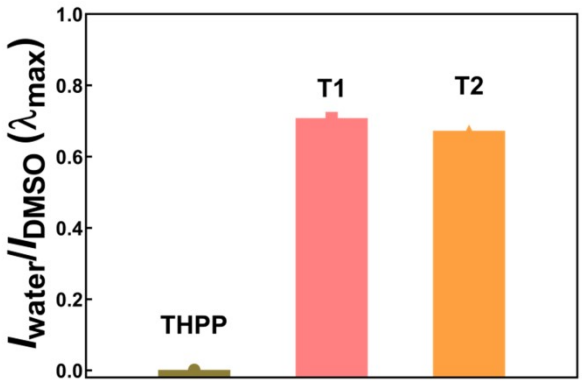


Figure S9. The quantitative data of fluorescence quenching of three molecules in water compared to in DMSO.

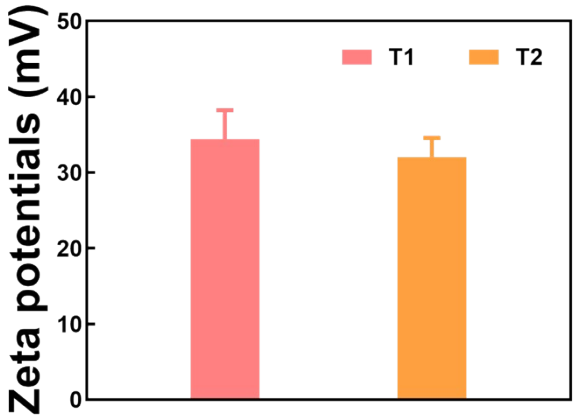


Figure S10. The Zeta potentials of T1 and T2.

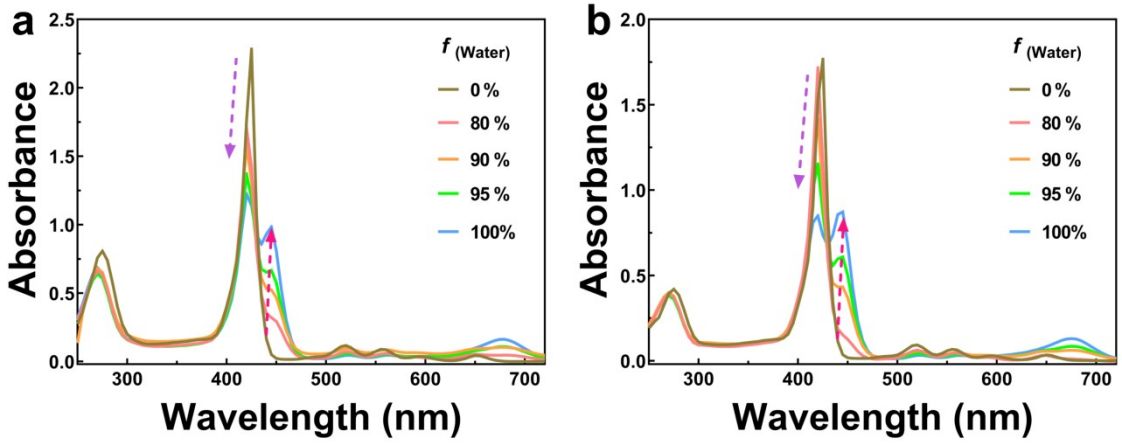


Figure S11. UV-vis absorption spectra changes of (a) T1 and (b) T2 in mixture of water/DMSO with different f_{water}.

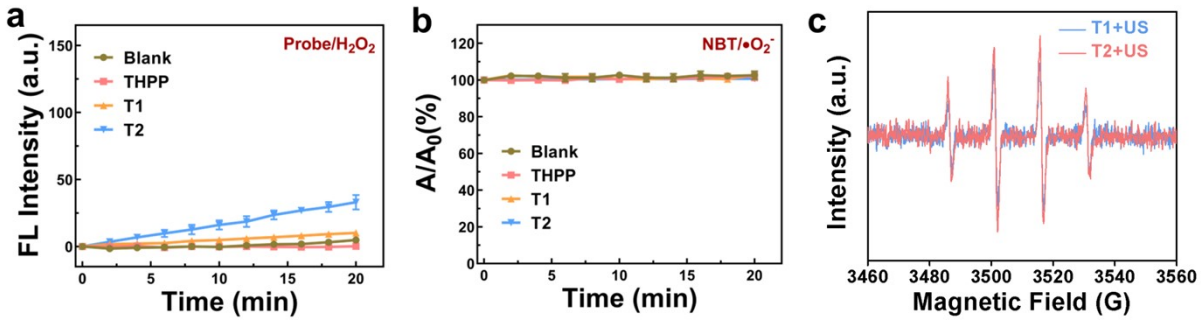


Figure S12. (a) H_2O_2 and (b) $\cdot O_2^-$ generated by different sonosensitizer under ultrasound irradiation (1.0 MHz, 1.5 W cm^{-2}, 50% duty cycle). (c) ESR spectra of ·OH spin adducts with DMPO generated by T1and T2 under ultrasound irradiation.

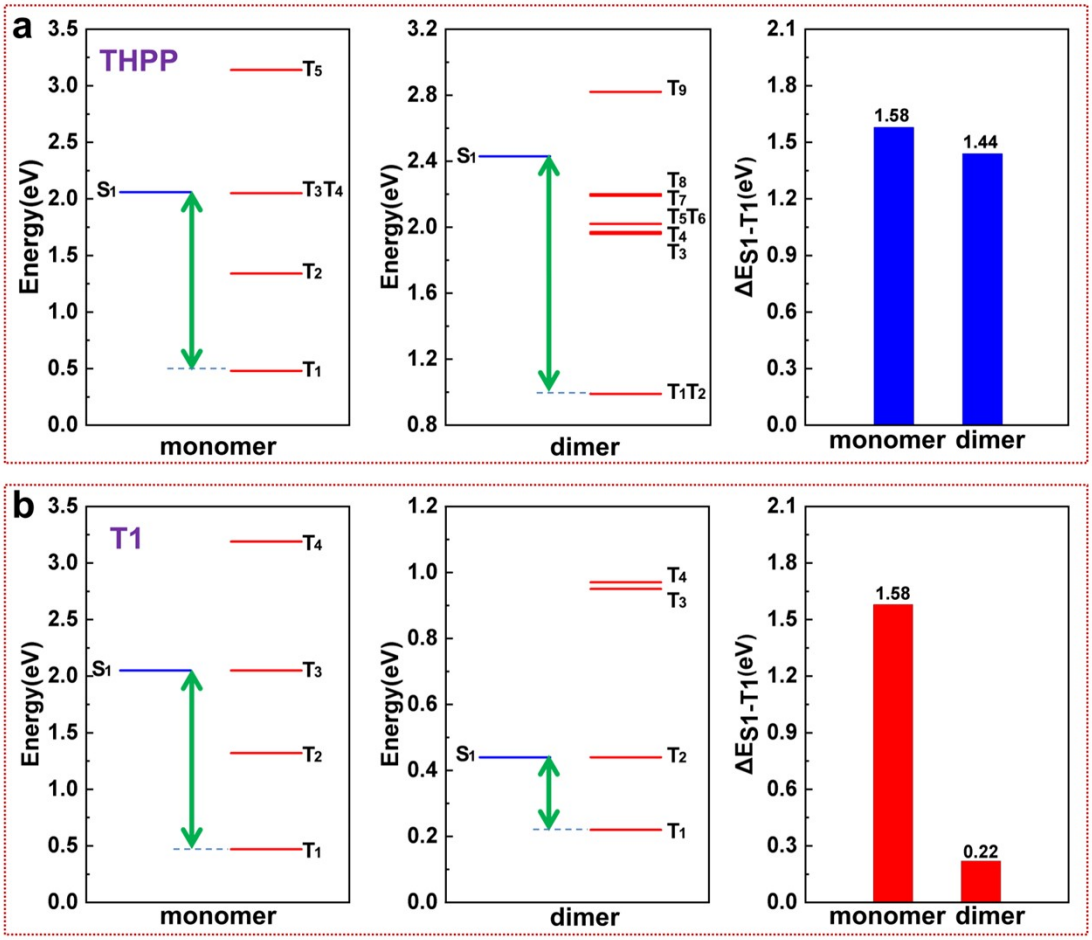


Figure S13. TD-DFT calculated energy-level diagrams of the lowest excited singlet (blue) and nearby triplet (red) states of monomer and dimer structures of (a) THPP and (b) T1 (left) and corresponding S_1-T_1 energy gap (right).

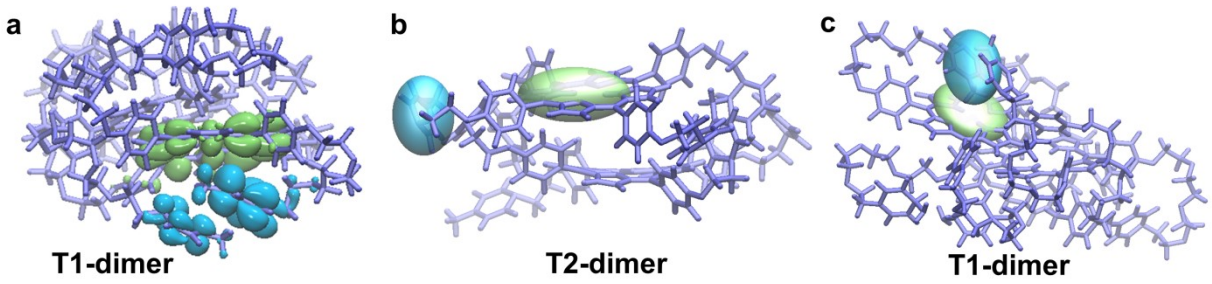


Figure S14. (a) Hole-electron analysis of dimer structure of T1 in front view. The C_{hole}-C_{ele} function diagrams of (b) T2-dimer and (c) T1-dimer.

Table S1. The significant index associated with the excited state properties of T1-dimer and T2-dimer (D represents the distance between the electron centroid and the hole centroid; Sr denotes the overlap degree between holes and electrons; t index refers to the degree of separation between holes and electrons; HDI is the hole delocalization index; EDI is the electron delocalization index).

$S_1 \rightarrow S_0$	D (Å)	Sr	t (Å)	HDI	EDI
T1	4.07	0.229	2.631	8.63	5.47
T2	10.17	0.019	8.345	10.31	5.65

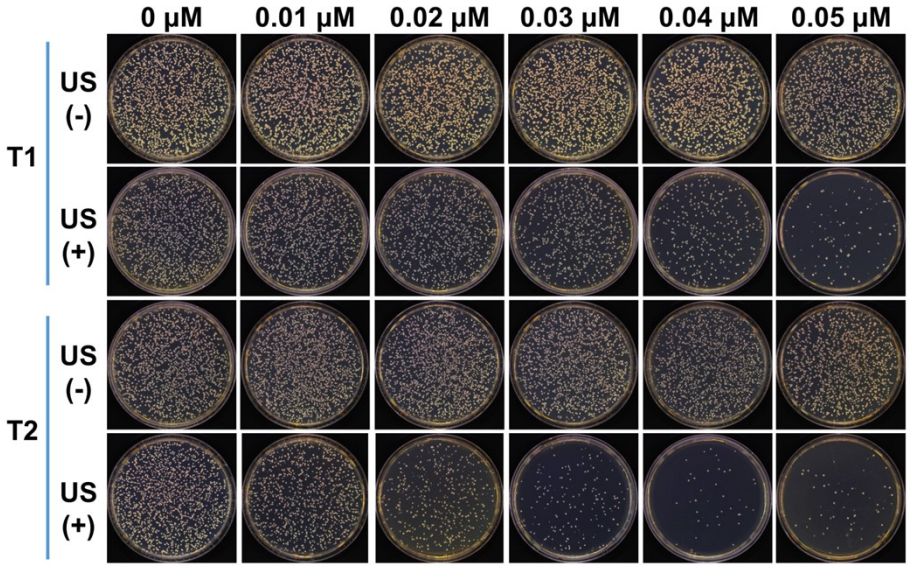


Figure S15. Photographs of *S. aureus* colony formed on agar plate after treatment of different concentrations of sonosensitizers with or without ultrasound.

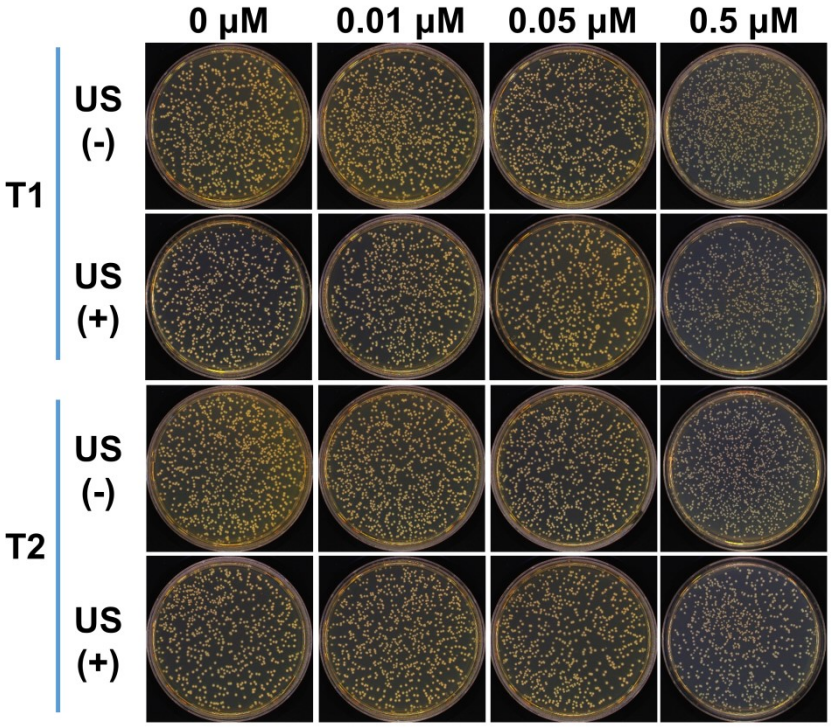


Figure S16. Photographs of *E. coli* colony formed on agar plate after treatment of different concentrations of sonosensitizers with or without ultrasound.

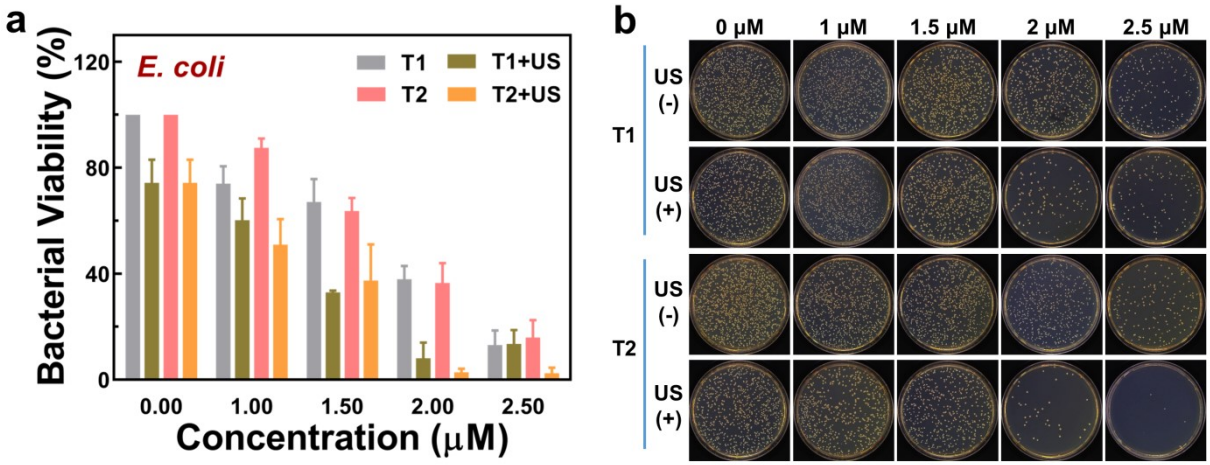


Figure S17 (a) The *E. coli* viability after treatment of higher concentrations of sonosensitizers with or without ultrasound and (b) corresponding photographs of colony formed on agar plate.

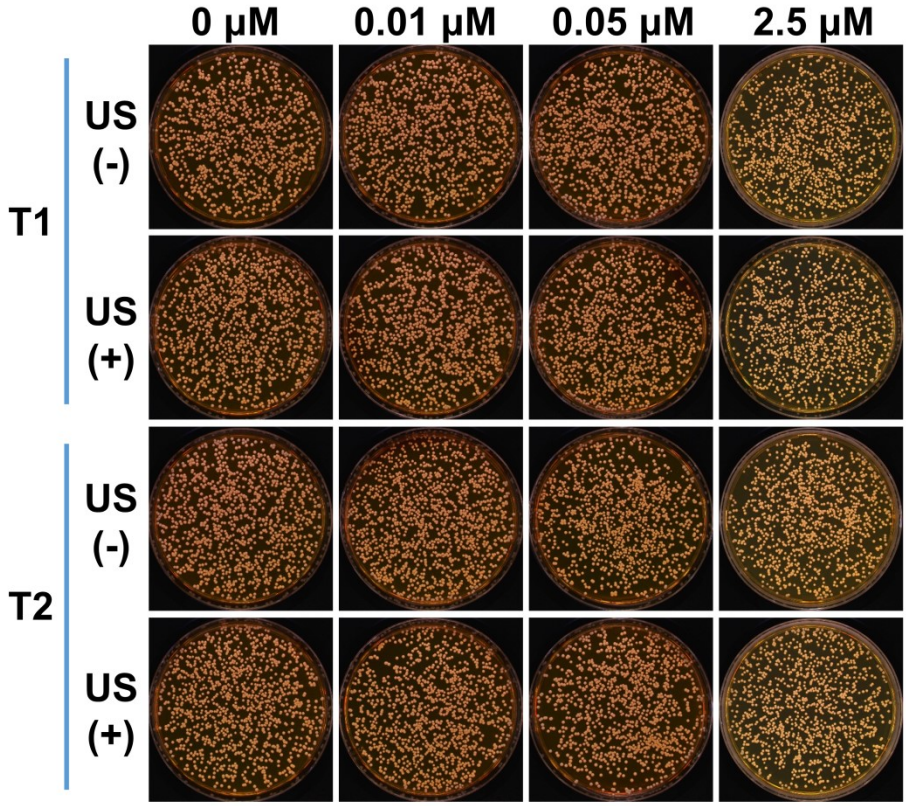


Figure S18. Photographs of *C. albicans* colony formed on agar plate after treatment of different concentrations of sonosensitizers with or without ultrasound.

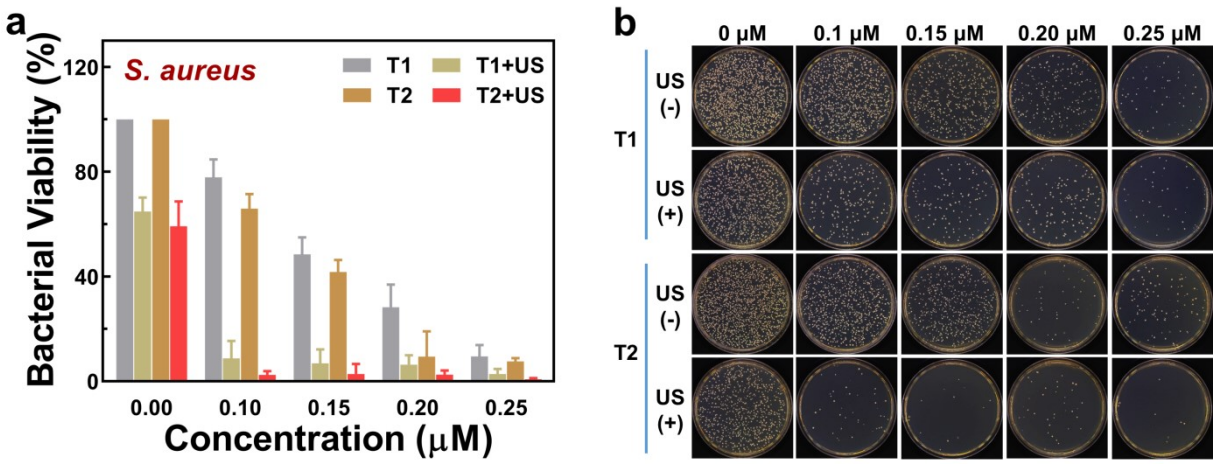


Figure S19. (a) The *S. aureus* viability after treatment of higher concentrations of sonosensitizers with or without ultrasound and (b) corresponding photographs of colony formed on agar plate.

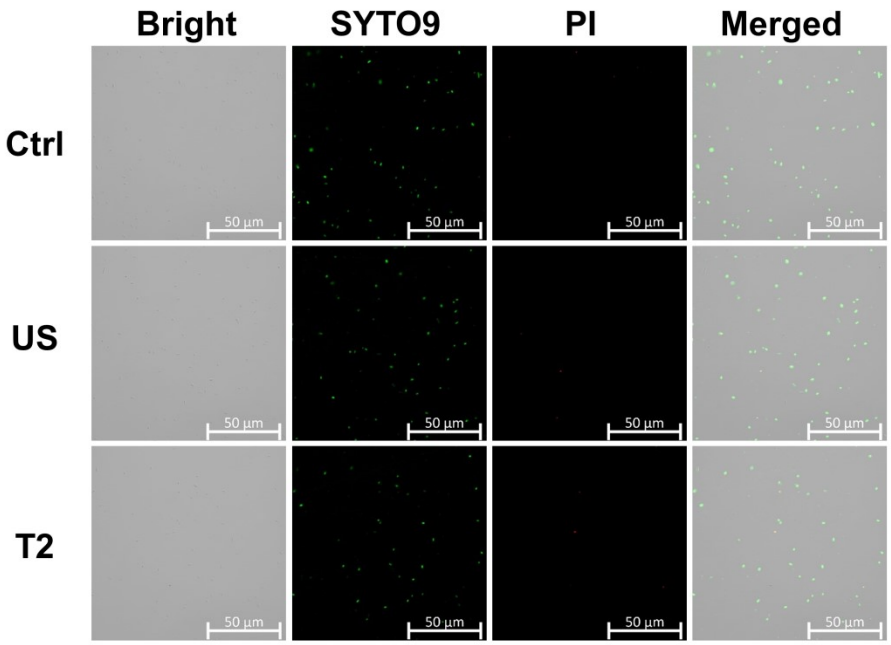


Figure S20. CLSM images of *E. coli* stained by live/dead dyes after different treatments.

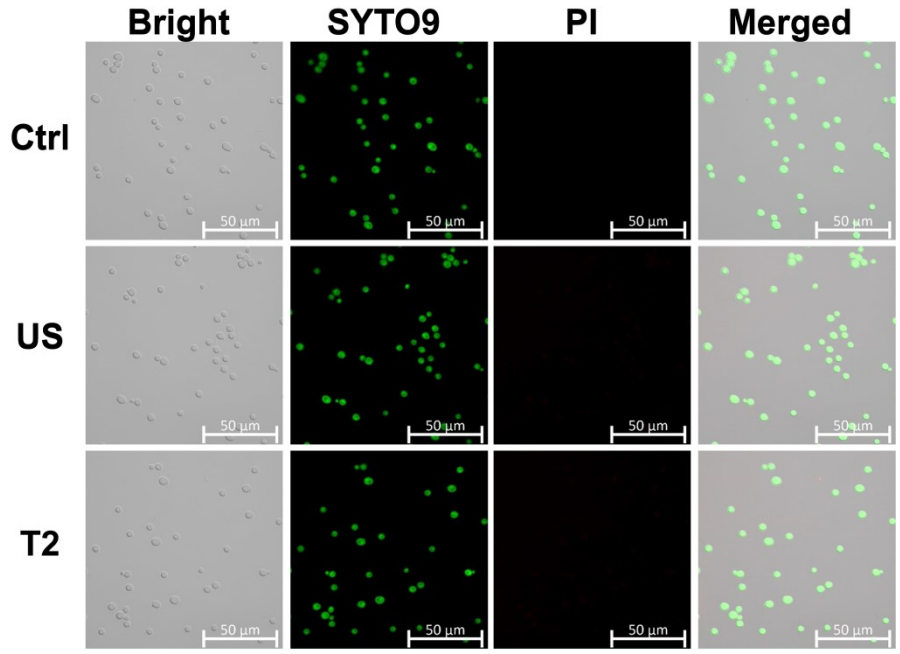


Figure S21. CLSM images of *C. albicans* stained by live/dead dyes after different treatments.

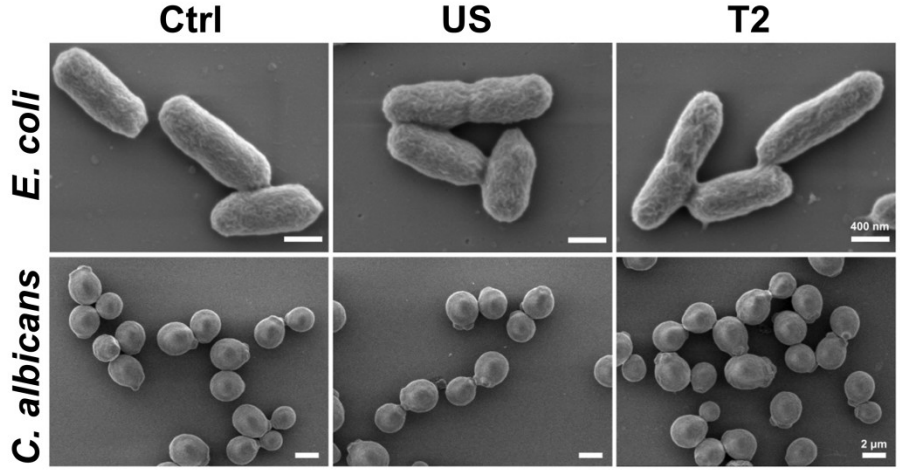


Figure S22. SEM images of *E. coli* and *C. albicans* after different treatments.

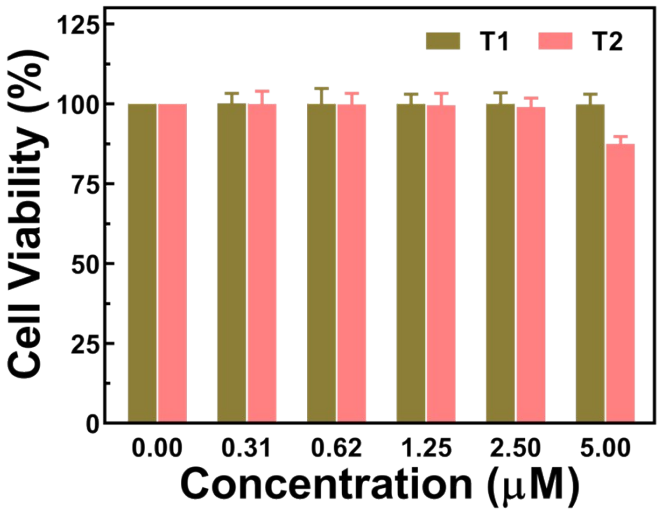


Figure S23. Cell viability of L929 cells after treatment of sonosensitizers with different concentrations.

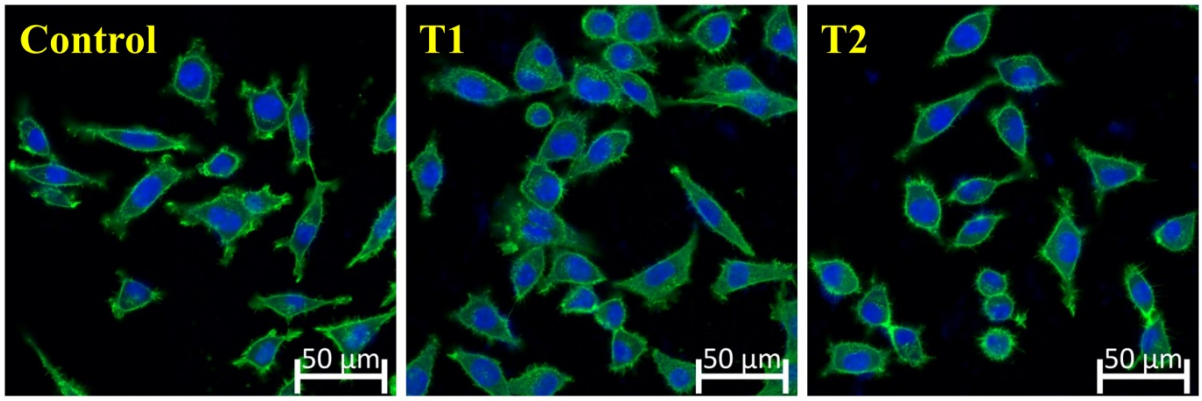


Figure S24. The cell morphology of L929 cells after different treatments.

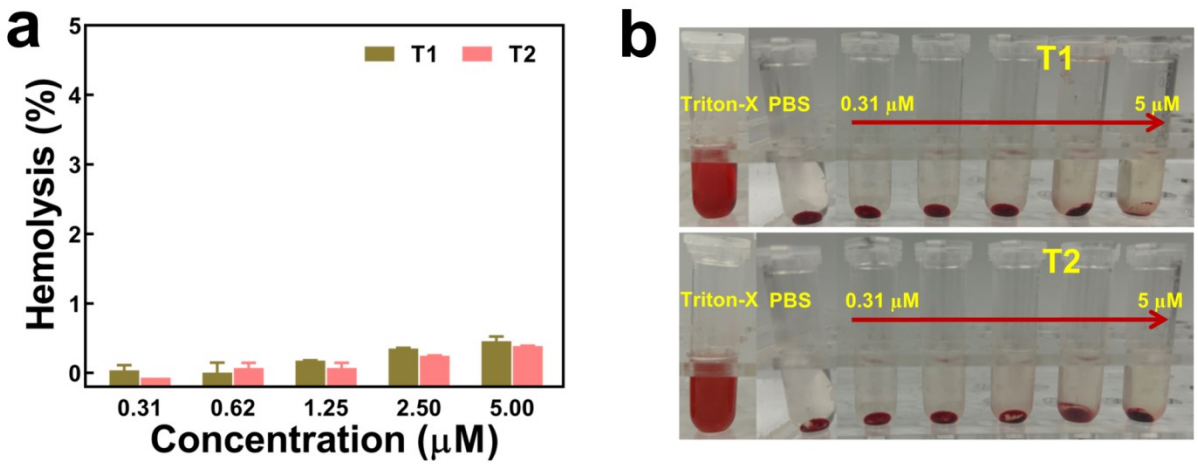


Figure S25. (a) Hemolysis ratio of T1 and T2, and (b) corresponding photographs of red bloods suspension after centrifugation.

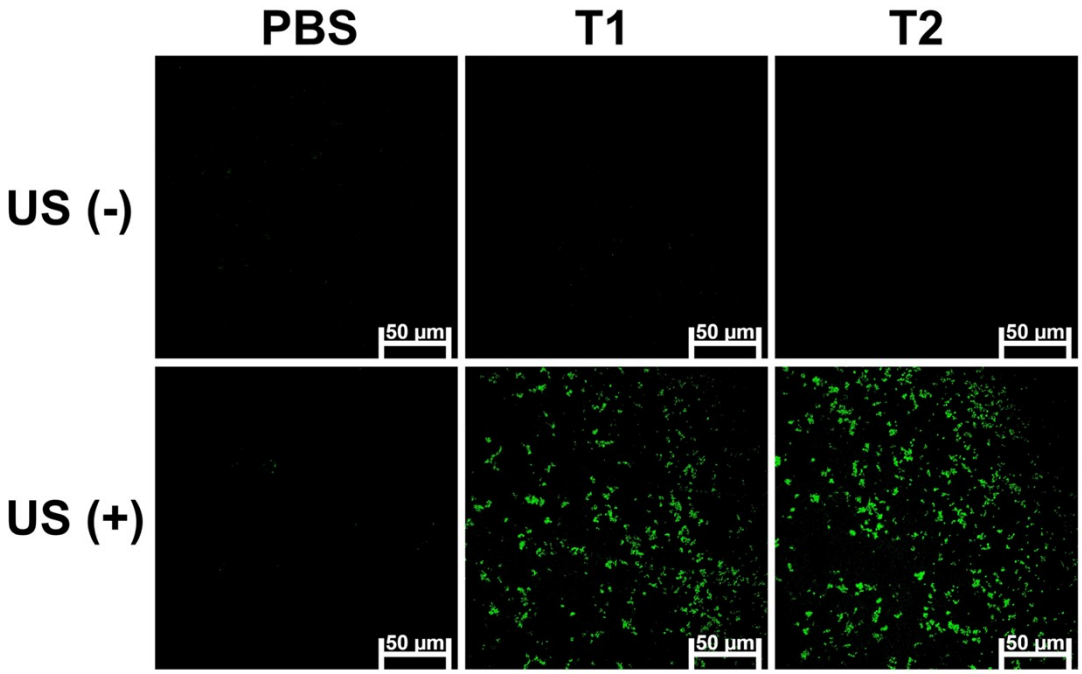


Figure S26. Intracellar ROS detection using DCFH-DA of *S. aureus*.

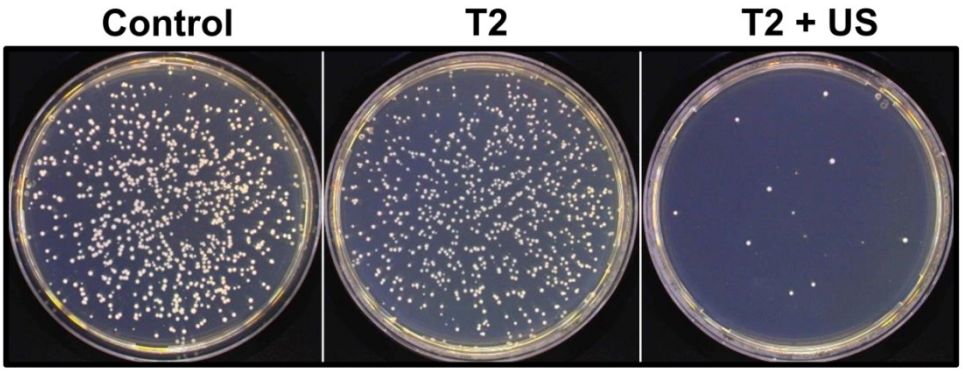


Figure S27. Images of bacterial colonies in different groups on agar plates.

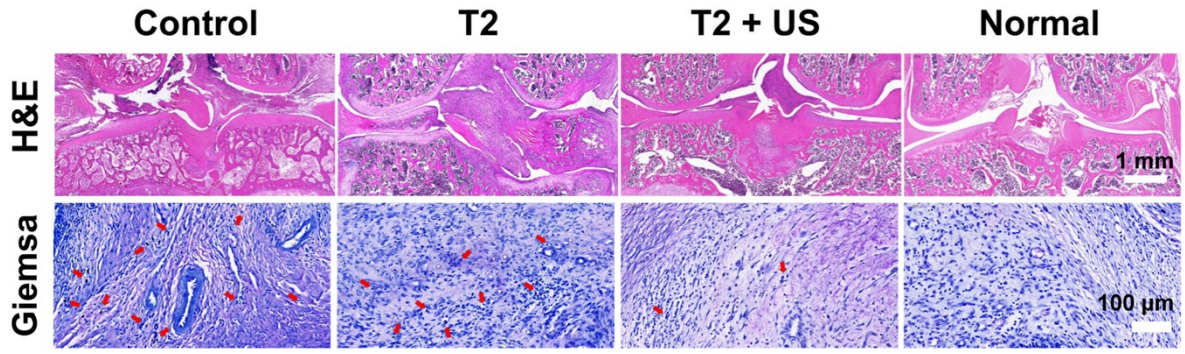


Figure S28. H&E and Giemsa staining of joint tissues extrated from rats in different groups.

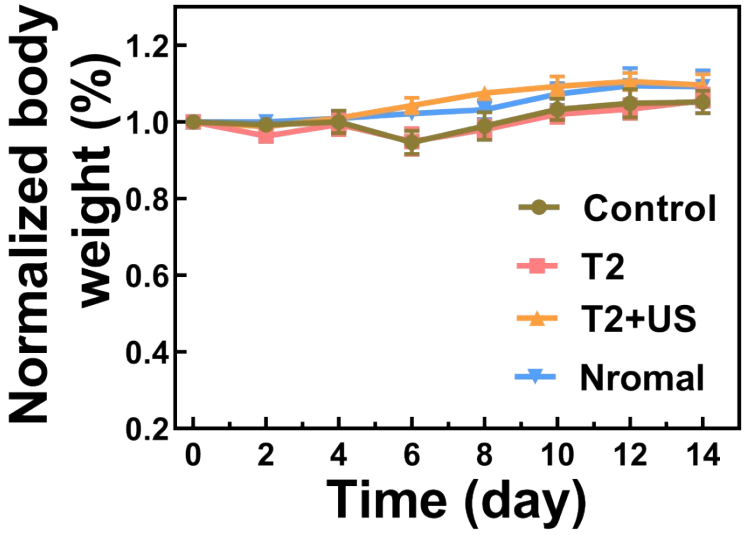


Figure S29. Body weight changes of rats in different groups.

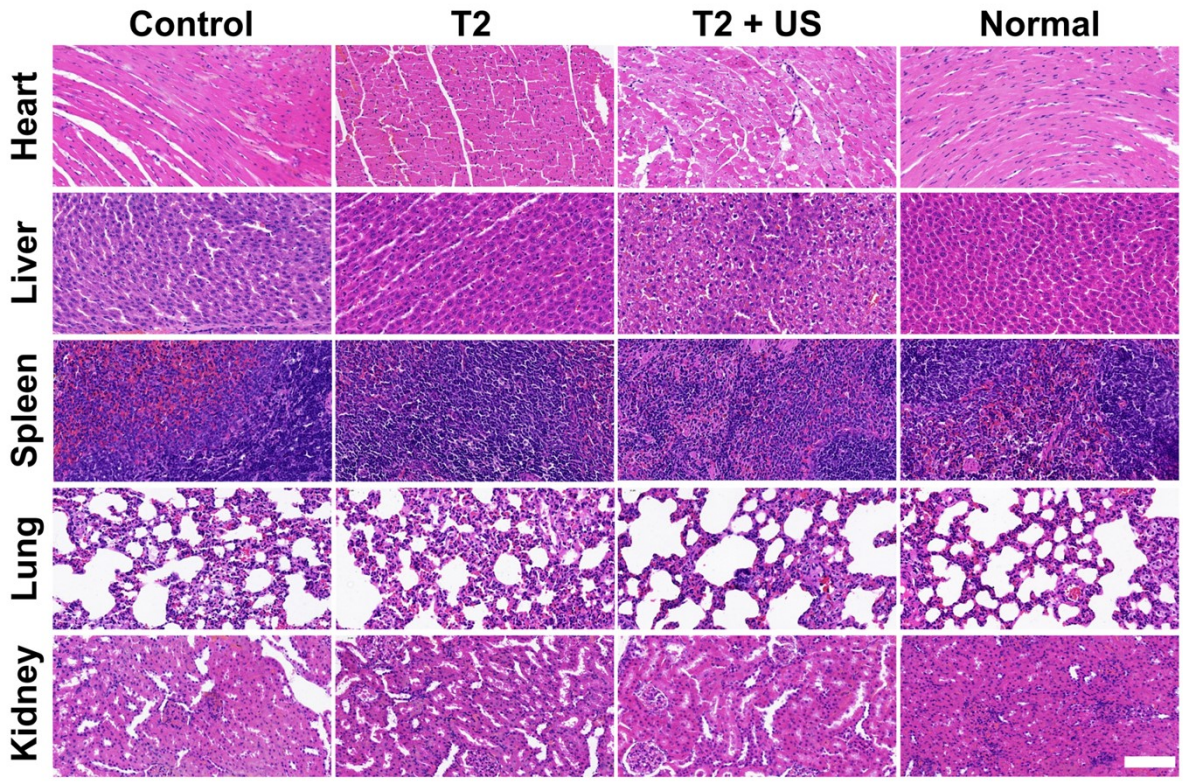


Figure S30. H&E images of major organs from rats in different groups. Scale bar: 100 μm.