Supporting Information

Targeted recognition, fluorescent tracking and augmented killing of multi-bacterial infections via synergizing a magnetic bead-armored phage cocktail with enzyme-activated AIE probes

Zhenyue Su,[a] Ling-Hong Xiong,[b] Jing Zhang,[a] Ben Zhong Tang*[c] and Xuewen He*[a]*

[a]State Key Laboratory of Bioinspired Interfacial Materials Science, The Key Lab of Health Chemistry and Molecular Diagnosis of Suzhou, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou 215123, China.

[b]School of Public Health, Suzhou Medical College of Soochow University, Soochow University, Suzhou 215123, China.

[c]School of Science and Engineering, Guangdong Basic Research Center of Excellence for Aggregate Science, Shenzhen Institute of Aggregate Science and Technology, Shenzhen Institute of Aggregate Science and Technology, The Chinese University of Hong Kong, Shenzhen (CUHK-Shenzhen), Guangdong 518172, China.

*E-mail: xionglinghong@suda.edu.cn; tangbenz@cuhk.edu.cn; xheao@suda.edu.cn

Experimental section

Materials

Alkaline phosphatase (ALP), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 2′,7′-dichlorodihydrofluorescein diacetate, lysozyme, trypsin, luciferase, transferrin, peroxidase, glucose oxidase, and ATP colorimetric/fluorometric assay kit were purchased from Sigma-Aldrich. The superparamagnetic carboxyl-adembeads (500 nm in diameter) was purchased from Ademtech SA. Live/dead bacterial staining kit (DMAO/PI), alkaline phosphatase assay kit, and crystal violet staining solution were purchased from Beyotime Biotechnology Co., Ltd. 3,3'-Dipropylthiadicarbocyanine iodide (DiSC3(5)) was purchased from Aladdin. GelRed nucleic acid stain and Hoechst were purchased from Sangon Biotech. Bacterial strains including *S. aureus* (SA, ATCC 25923), *S. xylosus* (SX, ATCC29971)**,** *S. enterica* (SE, ATCC 14028)**,** and *E. coli* (EC, ATCC 15597) were purchased from American Type Culture Collection (ATCC). Ultrapure water from a Milli-Q water purification system was used in all of the experiments (18.2 MΩ cm).

Bacteriophage Screening and Purification

A 200 mL liquid sample was collected from domestic sewage and centrifuged at 4 °C at 5000 rpm for 15 min. The resulting supernatant was filtered through a 0.22 μm sterile filter to remove bacteria. Subsequently, 10 mL of the filtrate was mixed with bacterial suspensions of *S. aureus* (SA), *S. xylosus* (SX), *S. enterica* (SE) and *E. coli* (EC), each cultured to the logarithmic growth phase. The mixtures were incubated at 37 °C for 12 h with shaking at 180 rpm. After incubation, the cultures were centrifuged at 10,000 g for 15 min at 4 °C, and the supernatant was filtered through a 0.22 μm sterile filter. Phages were isolated using the double-layer plate method. This process was repeated five times until clear and uniform plaques with consistent shape and size appeared on the double-layer plates. These steps successfully yielded bacteria-specific virulent phages targeting SA, SX, SE and EC. The morphologies of phages were characterized by negative-stained transmission electron microscopy (HITACHI, HT7700).

Preparation of Phage-Functionlized Magnetic Beads (MNs@phage)

To prepare phage-functionalized magnetic beads (MNs@phage), 40 μL of magnetic beads (MNs) (50 mg/mL) were incubated with 50 mM EDC and 50 mM NHS in 1 mL of MES buffer (0.1 M MES, 0.15 M NaCl, pH 6.5) at 37 °C for 30 min. The activated MNs were then separated and washed three times with 1× PBS buffer (pH 7.4). Next, the activated MNs were covalently coupled with 1 mL of 1×10^{12} PFU/mL of SA phages, SX phages, SE phages or EC phages,

respectively, by incubating for 4 h at 37 °C. This process resulted in the formation of MNs@SA phage, MNs@SX phage, MNs@SE phage, and MNs@EC phage. The resulting MNs@phage complexes, at a concentration of 2 mg/mL, were suspended in 1 mL of 1 × PBS buffer (pH 7.4) and stored at 4 °C for further use.

Optical Properties and ROS generation of ALP-Responsive TPE-APP

Experiments were conducted to investigate the relationship between ALP concentration and the FL intensity of TPE-APP. TPE-APP (2 μM) was incubated with ALP (200 U/L) in Tris–HCl buffer (10 mM, pH 8.0) at 37°C for 35 min. The FL spectra were recorded using an FLS1000 photoluminescence spectrophotometer. Additionally, a kinetic study of the enzymatic hydrolysis process was performed using a microplate reader (Infinite M200 Pro, Tecan) to monitor the reaction dynamics.

DCFH was employed to evaluate the ROS generation under the following conditions. For the ROS generation of TPE-DMA products upon white light irradiation, a DCFH stock solution (10 μM) was added to the TPE-DMA (10 μM) in 10 mM Tris–HCl (pH 8.0). The mixture was exposed to white light irradiation (5 $mW \cdot cm^{-2}$) for 300 s. FL measurements were conducted using an FLS1000 photoluminescence spectrophotometer, with an excitation wavelength of 488 nm. The emission maximum intensities at 525 nm were used to track the ROS generation. To assess ROS generation from the quinone methide (QM) intermediate products resulting from the interaction of TPE-APP with ALP in the dark, 10 μM DCFH, 10 μM TPE-APP and 200 U/L ALP were incubated in 100 μL of Tris–HCl buffer (10 mM, pH 8.0) at 37 °C for 300 s. The FL spectrum of the reaction mixture was recorded using an FLS1000 photoluminescence spectrophotometer with 488 nm as the excitation light source. For control measurements, DCFH (10 μM) was incubated with TPE-APP (10 μM), ALP (200 U/L), or DCFH alone in 100 μL of Tris–HCl buffer (10 mM, pH 8.0) for 300 s. The fluorescence spectra of these control samples were also recorded under the same conditions to ensure the specificity of ROS detection.

Analysis of Bacterial ALP Levels within Four Types of Bacteria

To analyze intracellular ALP levels, SA, SX, SE and EC cells (1 × 10^9 CFU each) were subjected to three freeze-thaw cycles at -80 °C. The bacteria were then incubated with 10 mg/mL lysozyme at 37 °C for 1 h. Following this, RIPA lysis buffer was added, and the samples were lysed on ice for 40 min. The lysates were centrifuged at 12,000 rpm for 10 min, and the

supernatant was collected. ALP activity in the supernatants was determined using an enzymatic activity assay kit according to the manufacturer's instructions.

Bacteria Culture

SA, SX, SE and EC were cultured in LB medium at 37 °C with shaking at 180 rpm. The bacteria were collected by centrifugation at 6000 rpm for 5 min to thoroughly remove the culture medium, then resuspended in 1 × PBS buffer (pH 7.4) for subsequent experiments. The concentrations of the bacterial suspensions were determined by measuring the absorbance at 600 nm using an optical density meter (MAPADA PV1 visible spectrophotometer).

Bacterial ALP Response toward the TPE-APP Probes

Four types of bacterial suspensions, each with a concentration of 5×10^7 CFU/mL, were incubated with TPE-APP (120 μM) in Tris-HCl buffer (10 mM, pH 8.0) at 37 °C for varying time intervals. In the inhibited group, the bacterial suspensions were pretreated with sodium orthovanadate (Na_3VO_4) for 60 min, followed by treatment with TPE-APP (120 μM) for different time periods. Fluorescence images were then captured using an IX71 fluorescence microscope (Olympus). Time-dependent fluorescence spectra of the bacteria after the addition of 120 μM TPE-APP at different time points were recorded using an FLS1000 photoluminescence spectrophotometer.

Multiple Bacterial Detection based on FL Assay Combined Armed Phage Cocktail

Bacterial concentrations were determined using a plaque formation assay. The prepared MNs@phage were blocked with blocking buffer (1% BSA in 1 × PBS buffer, pH 7.4) for 2 h at 37 °C with shaking at 180 rpm. After blocking, the MNs@phage were washed twice with 1 × PBS buffer. Different amounts of bacteria were then added to the blocked MNs@phage (final concentration: 0.15 mg/mL) in 1 × PBS buffer (pH 7.4) to a final volume of 200 μL, and the mixture was incubated for 40 min at 37 °C with shaking at 180 rpm. After washing three times with PBS, 200 μL of 120 μM TPE-APP was added to the MNs@phage@bacteria mixture and incubated for 2 h at 37 °C. The detection solution was then collected for fluorescence spectral measurements using an FLS1000 fluorescence spectrometer (Edinburgh Instruments). To assess the specificity of this fluorescence assay, several other bacterial species were used as negative controls, and PBS was used as a reagent blank control. The concentration of the negative control bacteria was 10 times higher than that of the positive bacteria, and the same procedure was followed for these controls. For simultaneous detection of multiple bacterial

species, a mixture of four bacterial types, each at a concentration of 5×10^7 CFU/mL, was prepared and processed following the optimized method described above. Subsequently, 0.15 mg/mL of pre-prepared and blocked MNs@phage was added to the bacterial mixture and incubated at 37 °C with shaking at 180 rpm for 40 min. After incubation, magnetic separation was performed to remove unbound components. Next, 120 μM TPE-APP probe was added, and the mixture was incubated at 37 °C for an additional 2 h. Finally, magnetic separation and washing steps were carried out, and the fluorescence intensity of each sample was measured.

In Vitro Antibacterial Assay

Bacterial suspensions (5×10^7 CFU/mL) were treated with TPE-APP probes at concentrations ranging from 0 to 180 μM. After incubation for 2 h, the bacteria were collected and washed twice with 1 × PBS buffer. The TPE-APP-treated bacteria were then exposed to white light (5 $mW \cdot cm^{-2}$) for 40 min. A separate group of bacteria was kept in the dark for the same duration. The bacterial suspension was diluted to an appropriate concentration, and 100 μL of the diluted bacterial solution was plated on 90 mm solid LB agar plates. The plates were incubated at 37 °C for 10 h to allow colony formation, and the number of colonies was counted to determine bacterial survival.

To further evaluate the antibacterial effect of phage lysis combined with photodynamic-chemodynamic synergistic therapy, the following procedure was performed: 120 μM TPE-APP was added to the MNs@phage@bacteria solution and incubated for 2 h. The complexes were then collected and washed by magnetic separation. After washing, the complexes were exposed to white light irradiation for varying durations (10, 20, 30 and 40 min). Following irradiation, the samples were diluted to appropriate concentrations, and 100 μL of the bacterial dilution was spread onto 90 mm LB solid agar plates. The plates were incubated at 37 °C for 10 h to allow bacterial colonies to form. The number of colonies was counted to assess bacterial survival.

ROS Generation in Four Types of Bacteria

Four types of bacteria, each at the same concentration (5×10^7 CFU/mL), were incubated with DCFH-DA (10 μM) for 30 min in the dark, followed by treatment with TPE-APP (120 μM) as described above. Fluorescence images were captured using an IX71 fluorescence microscope (Olympus).

Measurement of Bacterial Membrane Potential

The variations in membrane potential in four types of bacteria with different treatments were assessed using DiSC3(5) probes. Bacteria (5×10^7 CFU/mL) were treated with TPE-APP probes (120 μM) in the presence or absence of white light irradiation for 40 min. After treatment, the bacteria were collected and resuspended in PBS buffer. Next, 5 μM DiSC3(5) was added to the bacterial suspension, and the mixture was incubated in the dark for 30 min. After a centrifugation step at 5000 g for 5 min, the fluorescence intensity was measured using an FLS1000 FL spectrometer (Edinburgh Instruments).

Measurement of Intracellular and Extracellular ATP levels

Bacteria (5×10^7 CFU/mL) were treated with TPE-APP probes (120 μM) in the presence or absence of white light irradiation for 40 min. After treatment, the bacteria were centrifuged to separate the supernatant from the bacterial sediments. Both the supernatant and the bacterial sediments were further processed for ATP determination using the ATP Assay Kit. In parallel, 10 mg/mL lysozyme was used to lyse the bacterial sediments for measurement of intracellular ATP levels.

In Vitro Biofilm Targeting and Penetration Potential of Activated TPE-APP

To investigate the penetration potential of activated TPE-APP, 1×10^8 CFU/mL SA cells were seeded on a 8-chamber coverglass and incubated for 48 h at 37 °C to obtain the bacterial biofilm. Then, The bacteria supernatants were discarded and washed gently with 1 × PBS for twice. After that, TPE-APP probes (130 μM) was added to the chamber and incubated for 5 h in Tris-HCl buffer (10 mM, pH 8.0), and the supernatants were discarded and added 1 × PBS buffer (pH 7.4) to the plate, following by the darkness or white light irradiation (5 $mW \cdot cm^{-2}$) for 60 min. The bacterial biofilm-treated by TPE-APP was further incubated for 10 h at 37 °C. The bacterial biofilm-treated by TPE-APP was further incubated with Hoechst 33258 (5 μg/mL) at 37 °C for 30 min. The biofilm-treated was washed with 1 × PBS buffer (pH 7.4) for twice. Fluorescence images of the bacterial biofilms were captured by laser confocal microscopy (Leica TCS SP5) using a 63 × objective lens. The excitation and emission filters were as follows: Hoechst 33258: 405 nm excitation and 430−475 nm emission; TPE-DMA: 405 nm excitation and 500−550 nm emission.

To investigate the penetration potential of activated TPE-APP, 1×10^8 CFU/mL SA cells were seeded onto an 8-chamber coverglass and incubated for 48 h at 37 °C to form the bacterial biofilm. After incubation, the bacterial supernatant was discarded, and the biofilm was gently washed twice with 1 × PBS. Next, TPE-APP probes (130 μM) were added to the chamber and

incubated for 5 h in Tris-HCl buffer (10 mM, pH 8.0). Following incubation, the supernatants were discarded, and 1 × PBS buffer (pH 7.4) was added to the plate. The biofilm was then exposed to either darkness or white light irradiation (5 $mW \cdot cm^{-2}$) for 60 min. After irradiation, the bacterial biofilm treated with TPE-APP was further incubated for 10 h at 37 °C. Subsequently, the biofilm was incubated with Hoechst 33258 (5 μg/mL) at 37 °C for 30 min. The biofilm was then washed twice with 1 × PBS buffer (pH 7.4). Fluorescence images of the bacterial biofilms were captured using a laser confocal microscope (Leica TCS SP5) with a 63× objective lens. The excitation and emission filters were as follows: Hoechst 33258 (405 nm excitation and 430−475 nm emission); TPE-DMA (405 nm excitation and 500−550 nm emission).

For the evaluation of biofilm eradication, four types of bacteria, each at a concentration of 1×10^8 CFU/mL, were added to a 24-well plate and incubated for 48 h at 37 °C. After incubation, the bacterial supernatants were discarded. 130 μM TPE-APP in Tris-HCl buffer (10 mM, pH 8.0) was added to the plate and incubated for 5 h at 37 °C. After incubation, the supernatants were discarded, and 1 × PBS buffer (pH 7.4) was added to the plate, followed by exposure to either darkness or white light irradiation (5 $mW \cdot cm^{-2}$) for 60 min. After 24 h of further incubation, biofilm susceptibility was assessed using crystal violet staining. For this, the supernatants were discarded, and the biofilms were washed twice with PBS to remove planktonic bacteria. The fixed biofilms were then stained with 2% crystal violet for 30 min. Excess dye was removed by washing with 1 × PBS, and the dye was dissolved by adding 95% ethanol. The resulting supernatant was transferred to a new plate, and the absorbance was measured at 570 nm using a microplate reader.

In Vivo Multiple Bacterial Elimination

All procedures of animal experiments were approved by the Animal Care and Use committee of Soochow University and complied with all relevant ethical regulations. Female BALB/c mice (6−8 weeks old) were used as the animal model. To establish the infection model, 40 μL of mixed bacterial suspensions, including 1×10^7 CFU of SA, SX, SE, and EC, were applied to the back of the mice (diameter of the inoculated area, 12 mm). After 24 hours, the infected mice were treated starting on day 0 and were randomly divided into five groups, with five mice in each group. The treatment groups were as follows: 1 × PBS under white light irradiation, 2 mg/mL methicillin under white light irradiation, 10 mM TPE-APP in the dark, 10 mM TPE-APP under white light irradiation, and combined 1.15×10^{11} PFU/mL phage and 10 mM TPE-APP under white light irradiation.

For the groups exposed to white light irradiation, the infection wounds were subjected to continuous white light exposure (5 $mW \cdot cm^{-2}$) for 1 h at the 2 h treatment mark. The wound size and body weight of the mice were measured every two days. Additionally, the bacterial load in the wounds was determined using the established fluorescence assay. On day 10 of treatment, one mouse from each group was sacrificed, and wound tissue as well as major organs (heart, liver, spleen, lung, and kidney) were collected for hematoxylin and eosin (H&E) staining.

For the in vivo biosafety evaluation, a mixture of four types of phages (1.15×10^{11} PFU/mL) combined with TPE-APP (10 mM) was intraperitoneally injected into healthy mice. 20 min post-injection, the mice were exposed to whole-body white light irradiation (5 $mW \cdot cm^{-2}$) for 1 h. Healthy mice injected with 1× PBS served as the control group. Each group included three mice (n = 3). The body weights of the mice were recorded every two days over a period of 10 days. At day 3 and 10 post-injection, blood samples were collected from the euthanized mice to assess hematological parameters, including red blood cells (RBCs) and white blood cells (WBCs), as well as plasma biochemical markers indicative of liver and kidney function. These markers included alanine transaminase (ALT), aspartate aminotransferase (AST), albumin (ALB), blood urea nitrogen (BUN) and creatinine (CRE). In addition, major organs (heart, liver, spleen, lung and kidney) were harvested on day 10 for histopathological analysis via H&E staining.

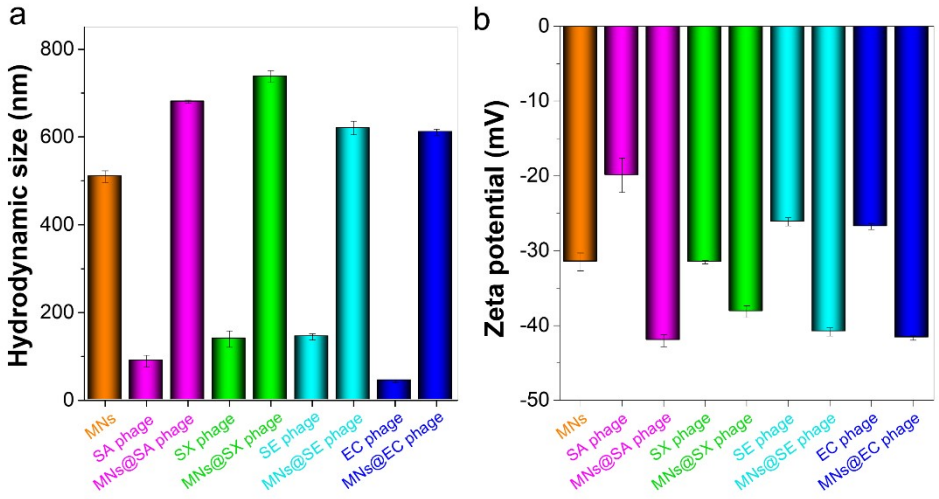


Figure S1. Hydrodynamic diameter (a) and zeta potential (b) measurements of four types of phages before and after modification with magnetic beads.

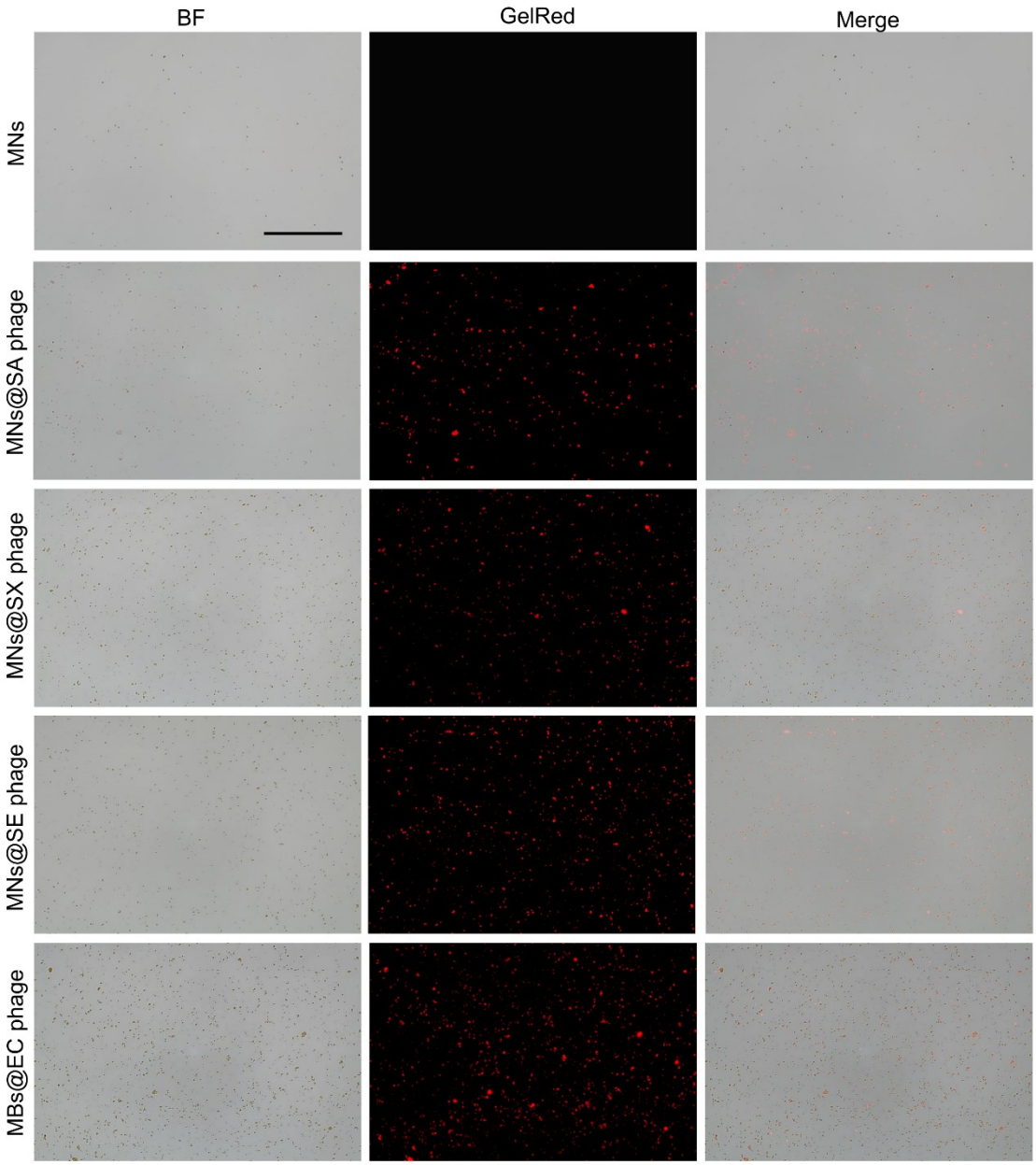


Figure S2. Nucleic acid staining (GelRed) results for MNs and MNs@phage. SA phage, SX phage, SE phage and EC phage were conjugated onto magnetic nanobeads and stained with GelRed, confirming the successful binding of phages to the magnetic beads. Scale bar: 50 μm.

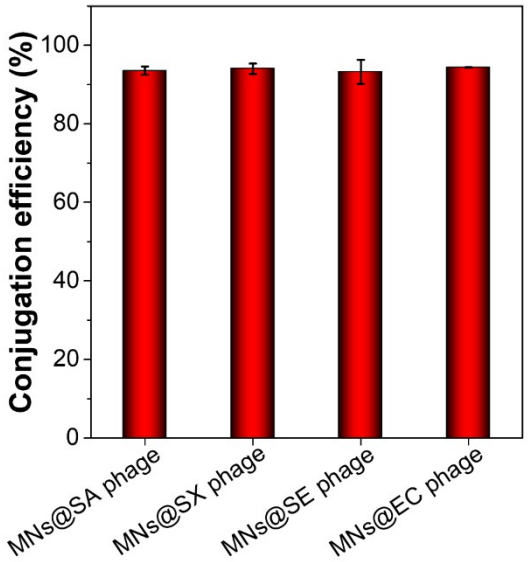
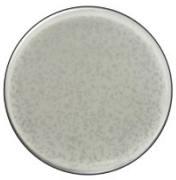
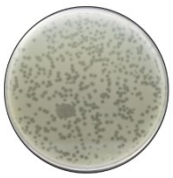
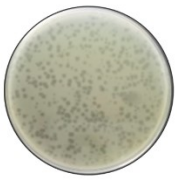
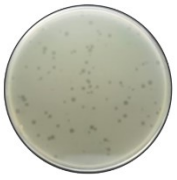


Figure S3. Conjugation efficiencies of SA phage, SX phage, SE phage and EC phage with magnetic beads, as determined by GelRed fluorescence colocalization with magnetic beads in the bright field.

Table S1. Number of phages conjugated onto a single magnetic bead. The inset images display double-layer plaque assays used for phage quantification.

	SA phage	SX phage	SE phage	EC phage
Before modification				
After modification				
Phage numbers per one MN (PFU)	118	123	124	168

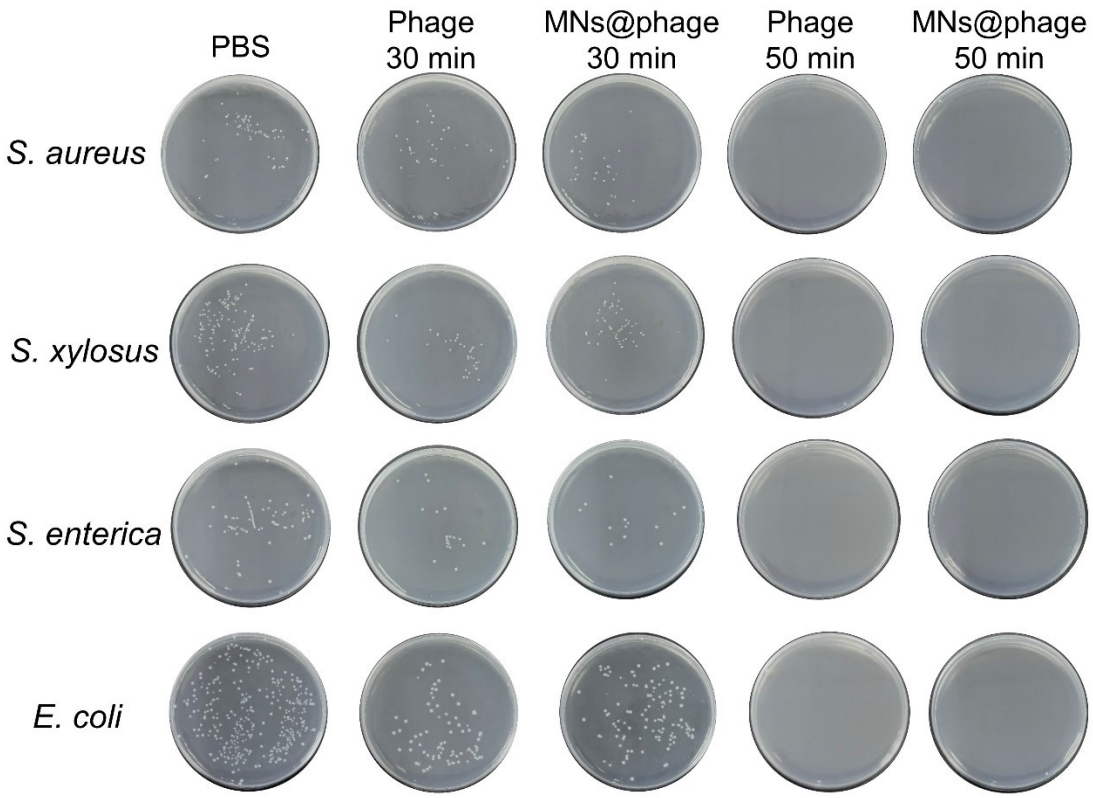


Figure S4. Lytic capability of SA phage, SX phage, SE phage, and EC phage against SA, SX, SE and EC, respectively, after conjugation with magnetic beads and treatment for different durations.

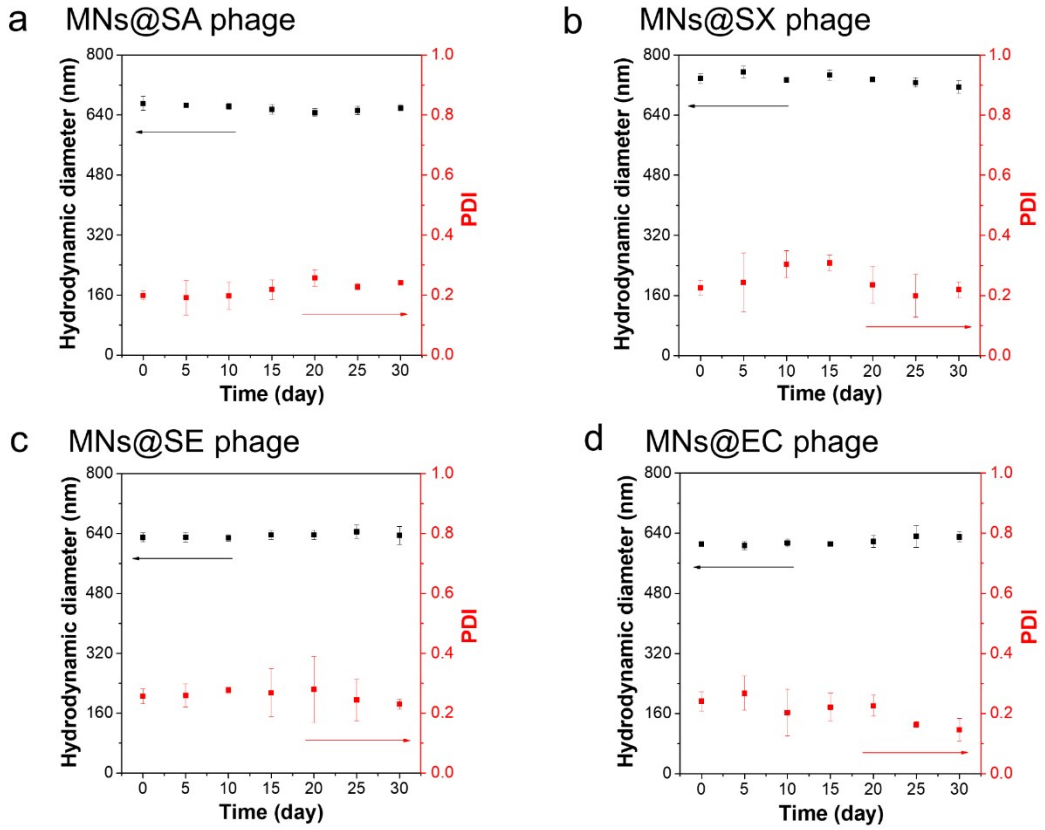


Figure S5. Colloidal stability test of MNs@phage. The hydrated particle size of MNs@SA phage (a), MNs@SX phage (b), MNs@SE phage (c) and MNs@EC phage (d) over a storage period of 30 days.

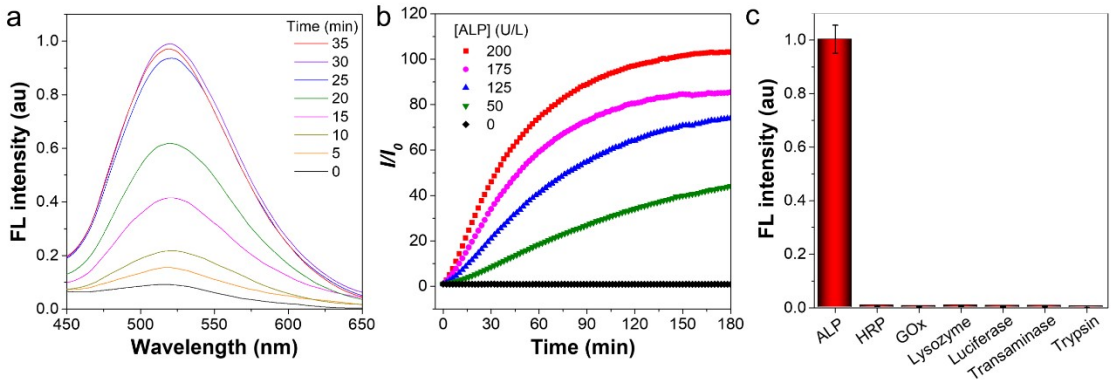


Figure S6. (a) Fluorescence spectra evolution of the mixture of TPE-APP (2 μM) and ALP (200 U/L) during a 35-min incubation. (b) Fluorescence intensity as a function of incubation time at different ALP concentrations. (c) Specificity test of TPE-APP for ALP detection.

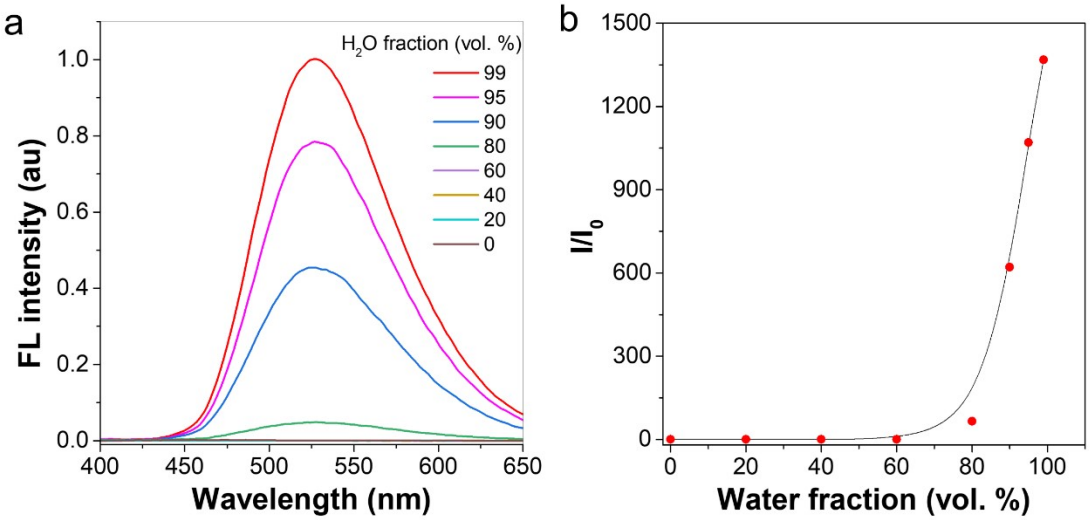


Figure S7. AIE property of TPE-DMA. (a) FL spectra of TPE-DMA (100 μM) in H_2O/THF mixture with varying H_2O fractions. (b) Scatter plot of relative FL intensity versus the composition of the aqueous mixture of TPE-DMA.

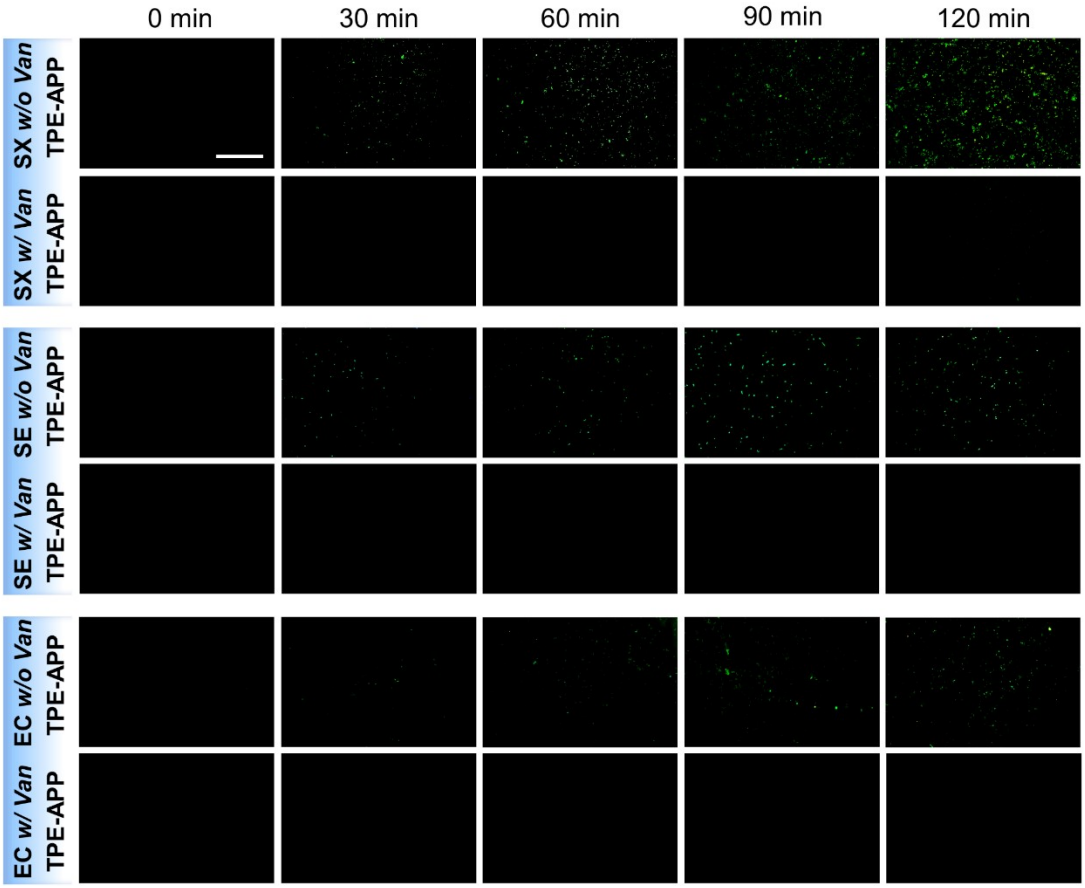


Figure S8. FL imaging of SX, SE, and EC (5×10^7 CFU/mL) without (w/o) Na_3VO_4 (Van) for 60 min, and with (w/) Van (100 μM) for 60 min, followed by incubation with TPE-APP (120 μM) for 0, 30, 60, 90 and 120 min, respectively. Scale bar: 50 μm.

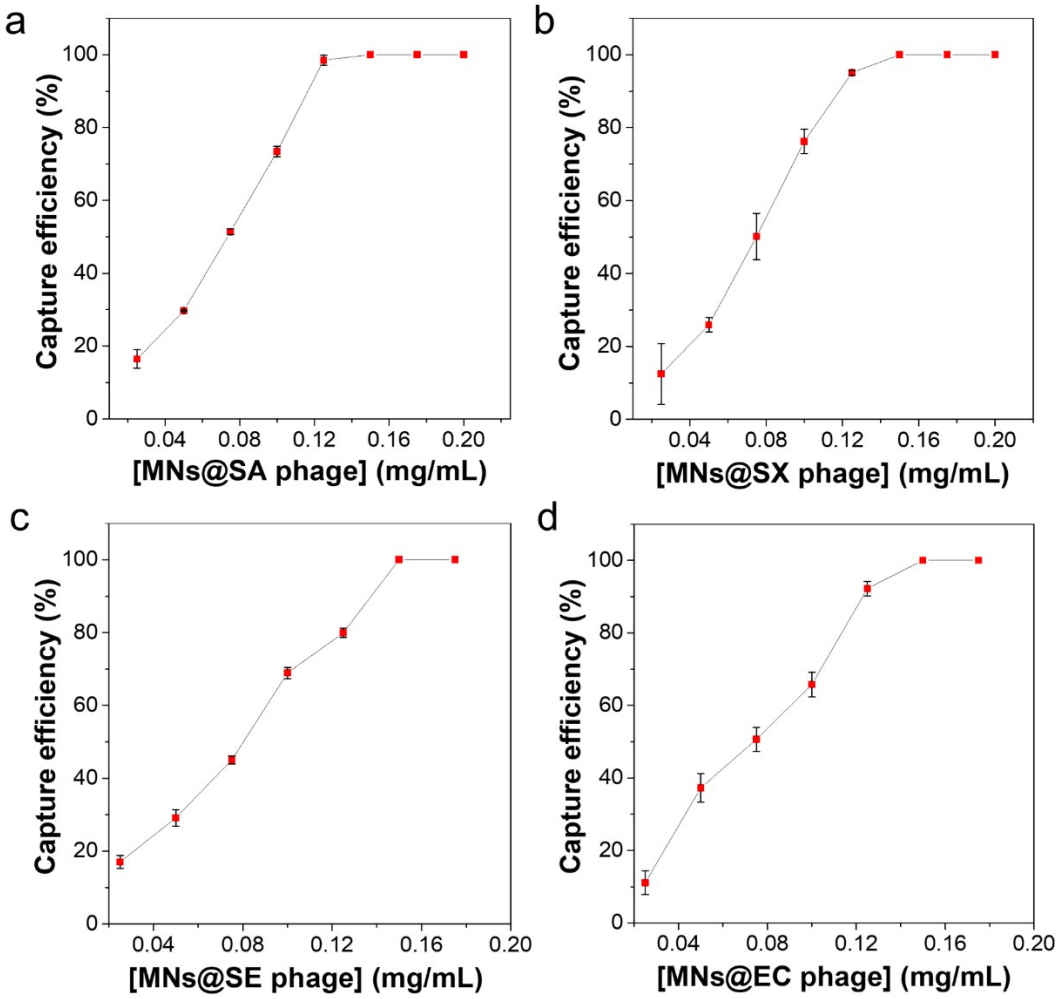


Figure S9. Optimization of the amounts of MNs@SA phage (a), MNs@SX phage (b), MNs@SE phage (c) and MNs@EC phage (d) for capturing different bacteria at a constant concentration of 1×10^7 CFU/mL.

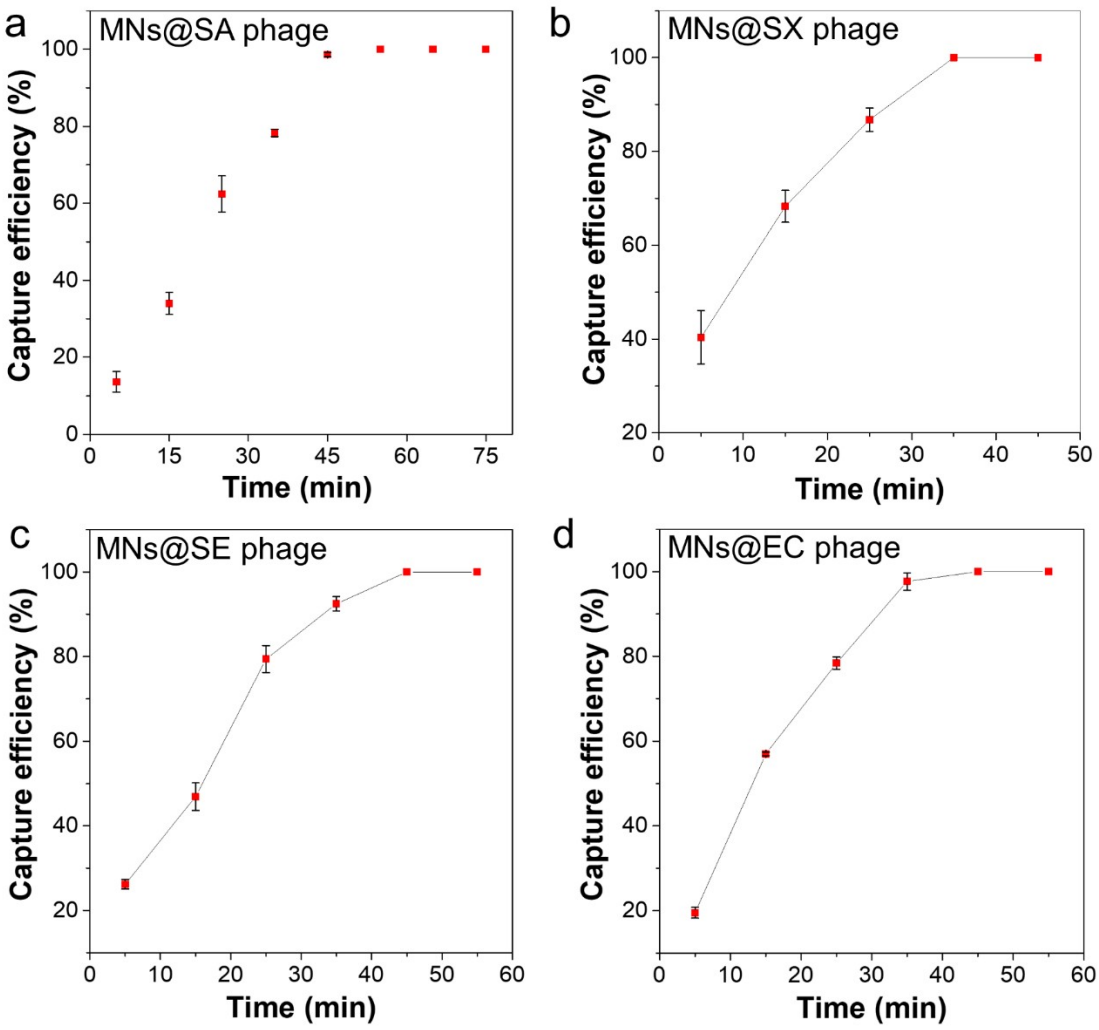


Figure S10. Optimization of capture time for MNs@SA phage (a), MNs@SX phage (b), MNs@SE phage (c) and MNs@EC phage (d) in capturing different bacteria at a constant concentration of 1×10^7 CFU/mL.

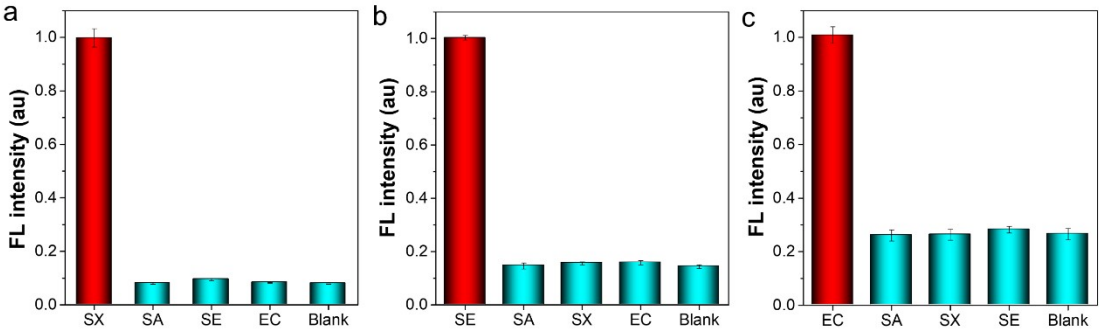


Figure S11. Specificity analysis of the assay for SX, SE and EC detection. The concentration of the target bacteria was 5×10^6 CFU/mL, while the concentrations of the negative control bacteria were 10 times higher than that of the target bacteria.

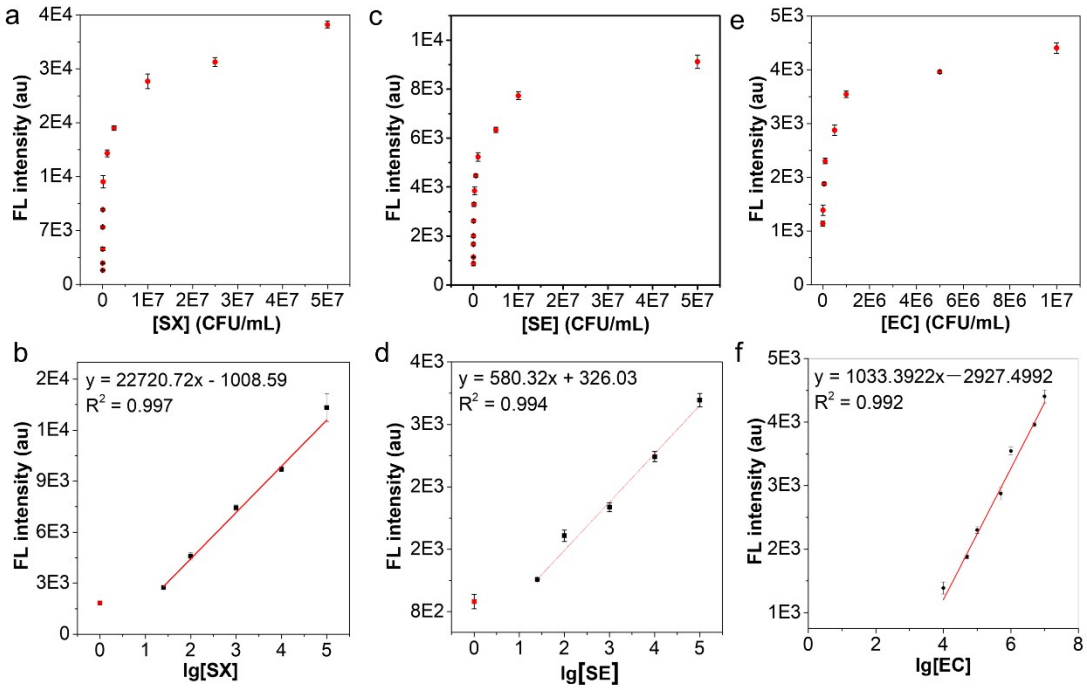


Figure S12. Sensitivity analysis of the assay for SX, SE and EC detection. FL intensity is plotted against SX concentration (a), SE concentration (c) and EC concentration (e), with the corresponding linear detection range shown in (b), (d) and (f), respectively.

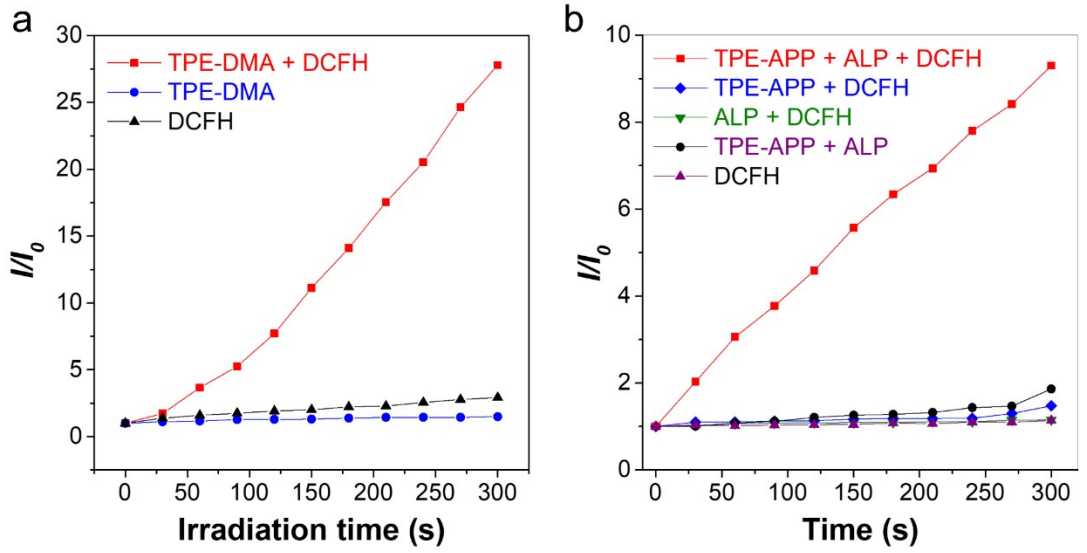


Figure S13. (a) Total ROS generation of TPE-DMA upon white light irradiation (5 mW·cm^{-2}). [TPE-DMA] = [DCFH] = 10 μM. (b) Chemodynamic activity characterization by monitoring the activation rate of DCFH at the emission maximum. [TPE-APP] = [DCFH] = 10 μM, [ALP] = 200 U/L.

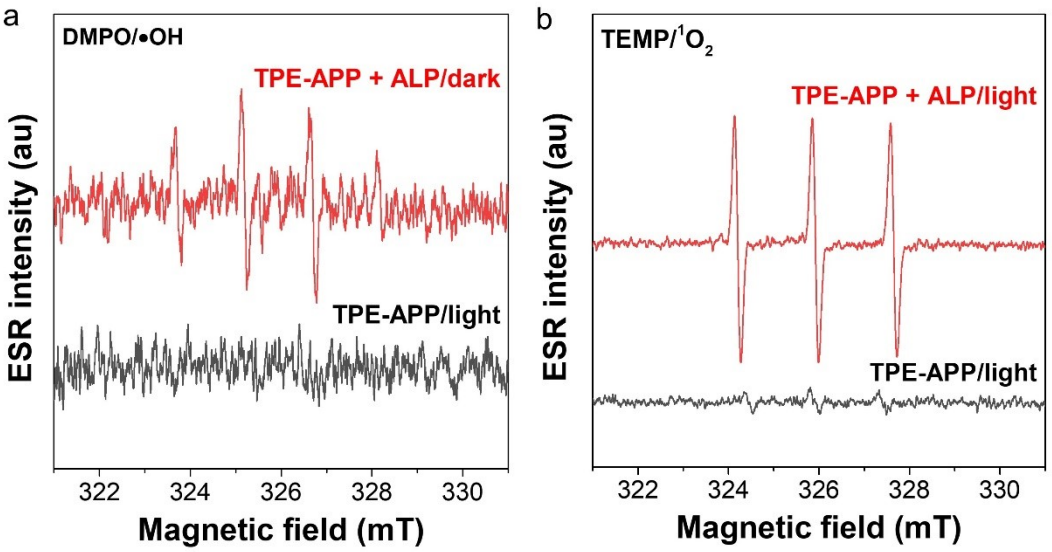


Figure S14. ESR spectra of activated TPE-APP probes using DMPO and TEMP as •OH and $^1O_2$ under darkness or upon the white light irradiation (30 min, 5 mW·cm^{-2}), respectively.

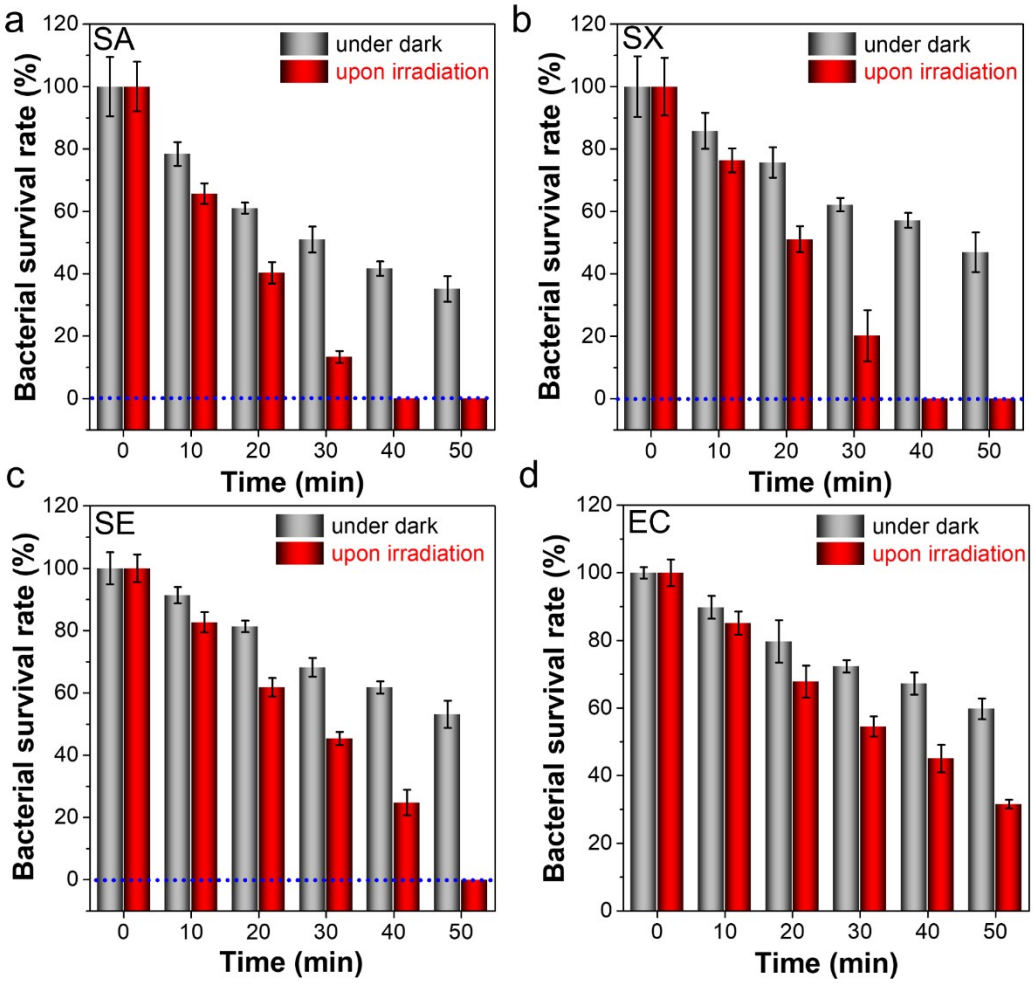


Figure S15. Bacterial survival rate of SA (a), SX (b), SE (c) and EC (d) treated with TPE-APP probes (120 μM) for different durations under darkness or light exposure.

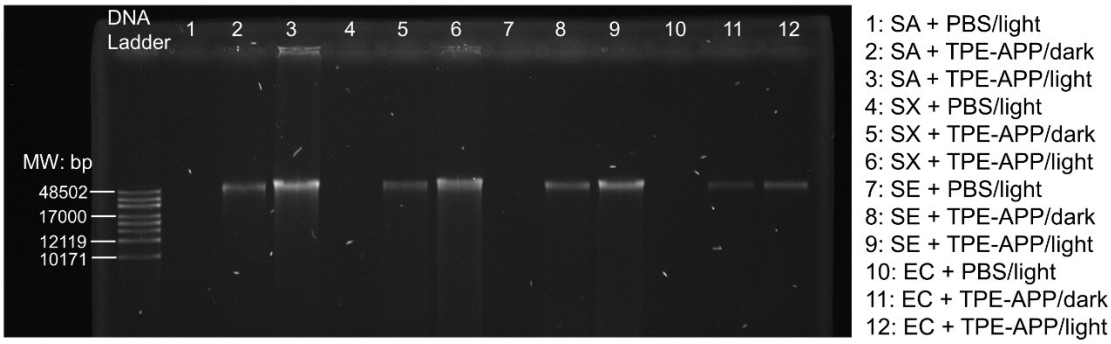


Figure S16. Agarose gel electrophoresis of free DNA in the culture supernatant of SA, SX, SE and EC under different treatments.

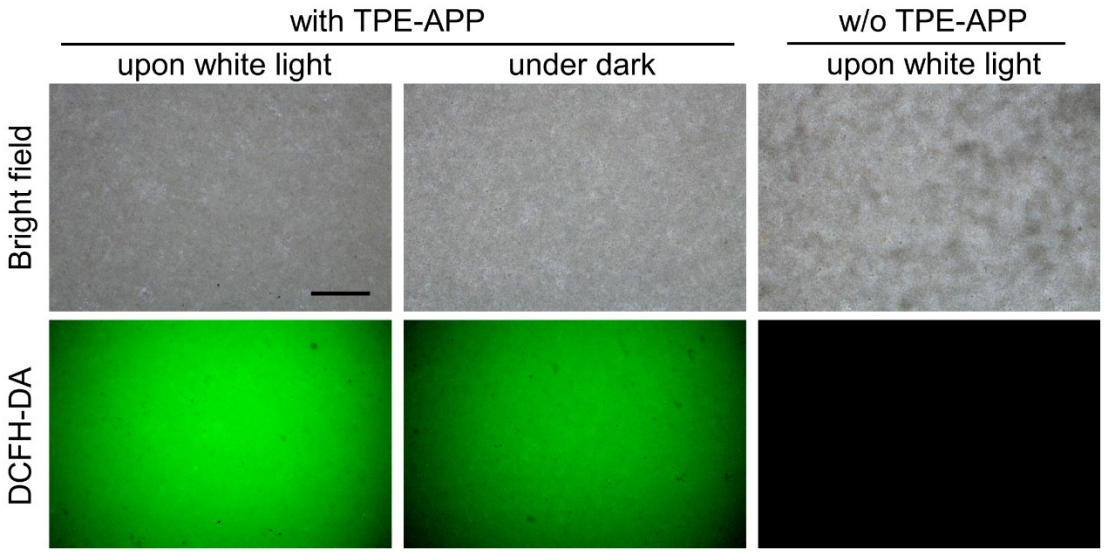


Figure S17. ROS generation in SA biofilm after treatment with TPE-APP (130 μM) for 5 h, followed by either dark condition or white light irradiation (60 min, 5 $mW \cdot cm^{-2}$), measured using DCFH-DA (20 μM) as an indicator. Scale bar: 100 μm.

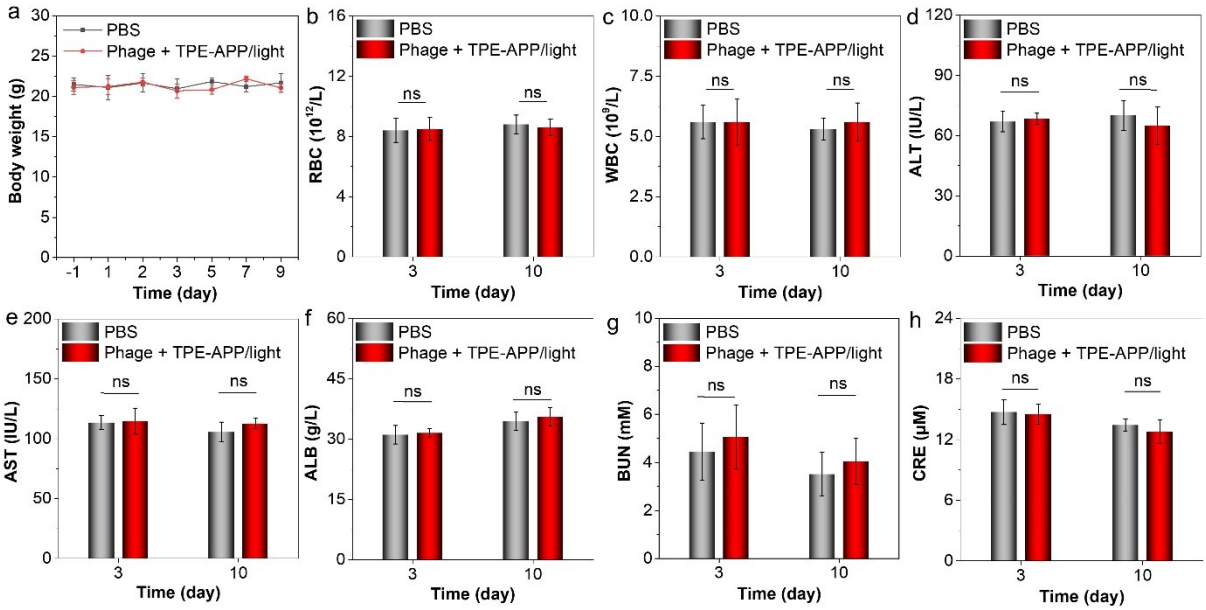


Figure S18. (a) Body weight measurement of healthy mice following intraperitoneal injection of four types of phages (1.15×10^{11}) PFU/mL and TPE-APP (10 mM) or PBS. (b–h) Hematology analysis and blood biochemistry of mice at day 3 and day 10 post-injection. ns: no significance.

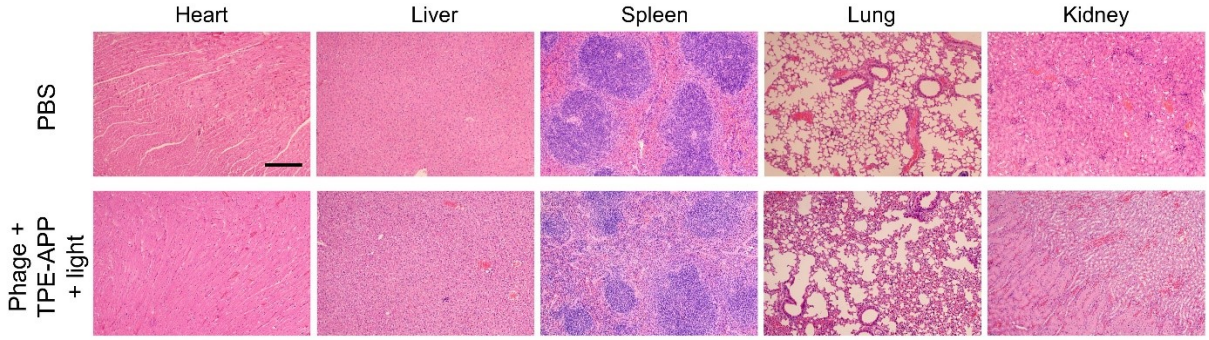


Figure S19. H&E staining of vital organs harvested from healthy mice after 10 days of treatment. Scale bar: 200 μm.

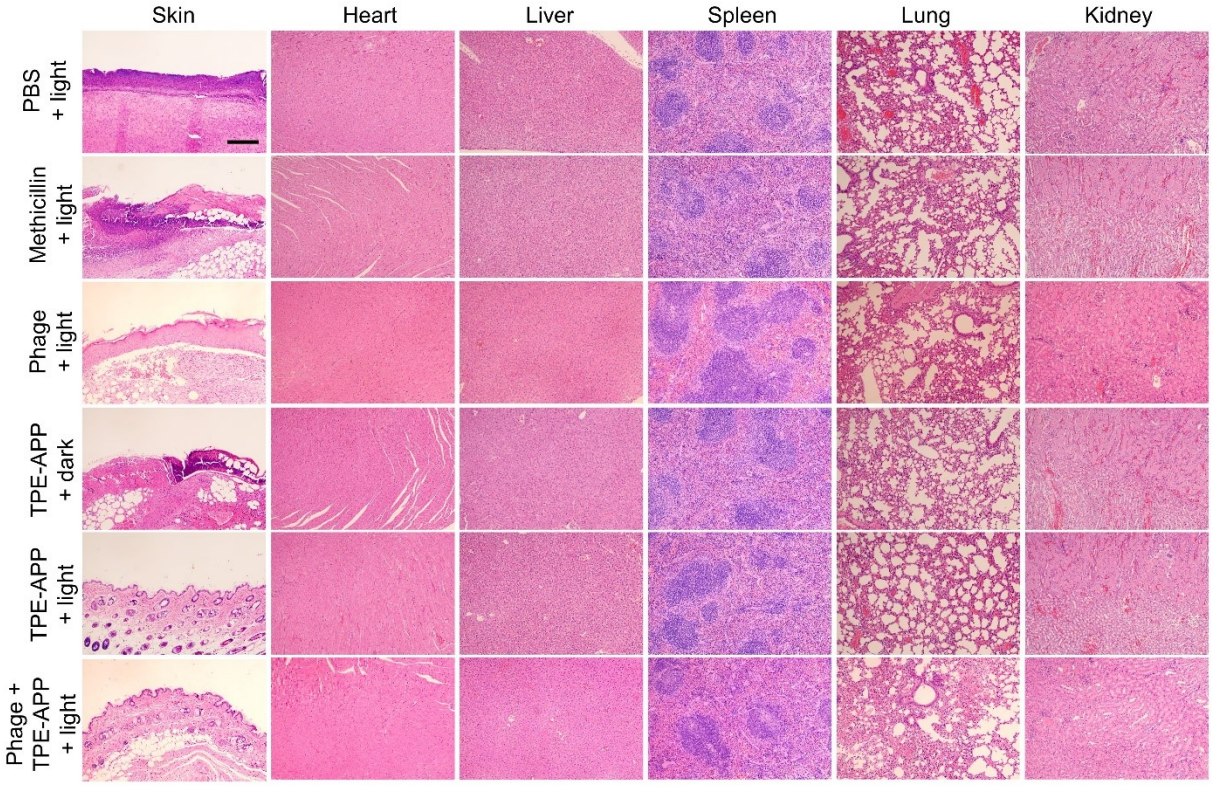


Figure S20. H&E staining of various tissues from mice with multiple bacterial-infected wounds after different treatments. Scale bar: 200 μm.