Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information

Polymyxin B-modified conjugated oligomer nanoparticle for targeted identification and enhanced photodynamic antimicrobial therapy

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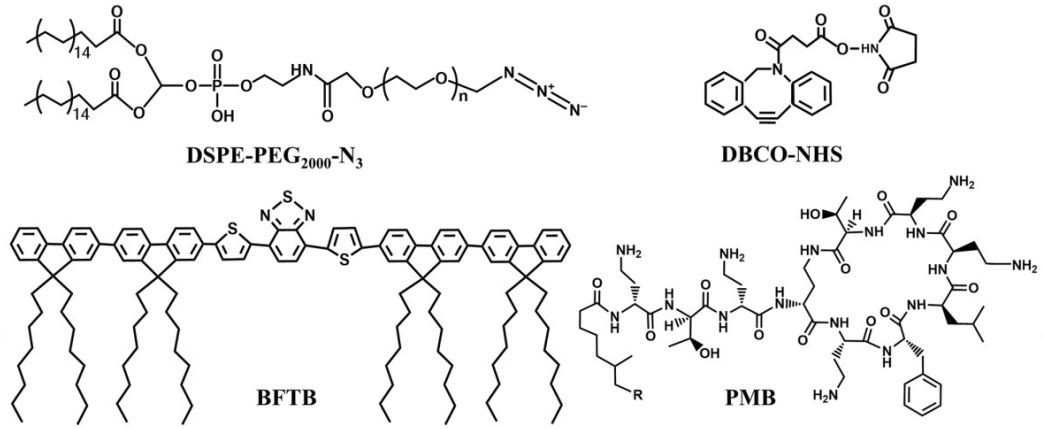
1. Experimental section

Materials and instruments. DBCO-NHS, DSPE-PEG_{2000}-N_3 and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Polymyxin B sulfate (PMB) and tetrahydrofuran (THF, 99.5%) were purchased from J&K. Anti-*E. coli* O + *E. coli* K antibody was obtained from Abcam. DCFH-DA and SYTOX Green dyes were purchased from Thermo Fisher. Phosphate buffered saline (PBS) was obtained from Sangon Biotech (Shanghai, China). The *Escherichia coli* (BL21) with kanamycin resistance (kana^r *E. coli*) was obtained by transfecting kanamycin-resistant plasmids of pET28a-PE66. *Staphylococcus aureus* (*S. aureus*) ATCC 6538 was obtained from the China General Microbiological Culture Collection Center. The conjugated oligomer BFTB was synthesized according to the literature reported previously.[1] The structures of materials were shown in Scheme S1.



Scheme S1 The structures of materials.

The UV-vis spectra of nanoparticles and the optical densities of bacteria were measured using a TU-1901 UV-vis spectrophotometer (Purkinje, China) and a Microplate Reader SpectraMax M2 (Molecular Devices), respectively. Fluorescence spectra of the solution were detected on a Hitachi F-7000 spectrofluorometer (Tokyo, Japan) equipped with a xenon lamp. CLSM experiments were performed with a FV1000-IX81 confocal laser scanning microscope (Olympus). Light irradiation tests were performed with the MVL-210 white light source (400-800 nm) equipped with a metal halogen lamp (Mejiro Genossen, Japan). Plate counting photographs were taken by a Nikon D90 digital camera.

Synthesis of conjugated oligomer nanoparticle (CON). The BFTB-loaded conjugated oligomer nanoparticle (CON) was prepared using the reprecipitation method. 450 μL BFTB (1 mg mL^{-1}) and 200 μL DSPE-PEG_{2000}-N_3 (1 mg mL^{-1}) in THF stock solution were added into 4350 μL THF and

mixed under ultrasonication for 2 min. Then, the mixture was injected directly into 10 mL H_2O under ultrasonication for 5 min. THF was removed through a nitrogen flow on a 90 °C hotplate, and the solution was concentrated to 5 mL. The resulting CON solution was filtered through a 0.22 μm membrane filter to remove any aggregates formed during preparation. Then, the CON was filtered by a 100 KD centrifugal filter (Millipore, USA) and diluted to 5 mL where the concentration was about 90 μg mL^{-1}. And the stock solution was stored at 4 °C for use.

Preparation of PMB-CON nanoparticles. PMB-CON was prepared by using the DBCO-NHS to crosslink azide nanoparticle CON and the amino of PMB. In detail, 50 μL PMB (5 mg mL^{-1}) and 2 μL DBCO-NHS (5 mg mL^{-1}) were added into 449 μL 1×PBS and shaken for 30 min at 25 °C. Subsequently, 500 μL CON (90 μg mL^{-1}) were added into the above mixture, and the mixture was shaken for 2 h at 4 °C. Then, the mixture was filtered by a 100 KD centrifugal filter and diluted to an optical density OD = 1 at 375 nm (1 cm optical path). The solution was stored at 4 °C for use. CON-N_3 conjugated Ab (Ab-CON) was obtained by the same method as PMB-CON. First, sodium azide in the antibody was extracted by centrifugal ultrafiltration with 100 KD centrifugal filter. 5 μL Ab (0.2 mg mL^{-1}), 2 μL DBCO-NHS (5 mg mL^{-1}) and 300 μL CON-N_3 (90 μg mL^{-1}) were added into 693 μL 1×PBS. Then the mixture was shaken for 4 h at 4 °C. Ab-CON mixture was filtered by a 100 KD centrifugal filter and stored at 4 °C for use.

Characterization of PMB-CON by TEM and DLS. The morphology and Zeta potential were examined by transmission electronic microscopy (TEM, Tecnai G2 F20 S-TWIN, FEI, USA) and a dynamic light-scattering instrument (ZEN3700, Malvern, UK), respectively. For TEM, the PMB-CON solution is diluted with water to a suitable concentration and dispersed ultrasonically for 20 min. 10 μL of PMB-CON solution was dropped onto the copper grid and allowed to dry naturally. Then the nanoparticles were stain with 10 μL of 2% phosphotungstic acid and washed three times with water. Finally, the sample is dried by a vacuum freeze dryer.

For DLS, CON (50 μg mL^{-1}) and PMB-CON were diluted to 0.5 μg mL^{-1} by sterile water. The concentrations of CON and PMB-CON were quantified according to the content of BFTB. Then take 1 mL of the solution and determine its hydrated particle size and Zeta potential by DLS.

Bacterial culture. A $kana^r$ gram-negative *E. coli* (BL21) was chosen as the research model. Bacteria were seeded and cultured in suspension using Luria-Bertani (LB) medium. For $kana^r$ *E. coli*, a single

colony was inoculated in 10 mL LB medium with kanamycin (50 μg mL^{-1}) at 37 °C and 180 rpm for about 6 h, and an optical density of 600 nm is about 0.8 (OD_{600} = 0.8). After centrifugation (8000 rpm) for 3 min and 1×PBS washing three times, the bacterial cells were diluted to the desired concentration in LB medium.

Bacterial Detection by PMB-CON. 950 μL washed bacteria were diluted to the appropriate concentration by LB medium in a 1.5 mL centrifuge tube. Then, 50 μL PMB-CON (100 μg mL^{-1}) was added into the above tube, and the mixture was shaken at 120 rpm for 20 min at 37 °C. After centrifugation (8000 rpm) for 5 min and 1×PBS washing two times, the precipitation was diluted by 200 μL 1×PBS. The suspension was mixed by ultrasound for 10 min, followed by using it for fluorescence detection.

CLSM Imaging of PMB-CON for kana^r *E. coli* and *S. aureus*. The kana^r *E. coli* and *S. aureus* was treated with PMB-CON (1.0 μg mL^{-1}), and untreated bacteria were used as control. After incubating for 20 min at room temperature, the solution was centrifuged with 8000 rpm for 3 min and washed with 1×PBS for one time. Then, the precipitations were suspended with 0.5% glutaraldehyde for 30 min and subsequently washed two times with 1×PBS. Then, the bacterial pellets were suspended with 1×PBS. Finally, 10 μL bacterial suspensions were transferred to a glass slide for CLSM imaging. The CLSM imaging of *S. aureus* was obtained by the same method as kana^r *E. coli*.

Characterization of reactive oxygen species (ROS) generation. ROS detection in vitro: 20 μL PMB-CON (or CON-N_3, 80 μg mL^{-1}) and 40 μL DCFH (40 μmol L^{-1}) were added to 140 μL 1×PBS. As the control group, 40 μL DCFH (40 μmol L^{-1}) is added to 160 μL 1×PBS. After irradiated for different times by the white light source (MVL-210), the fluorescence signal of DCF was collected by an F-7000 fluorimeter (Hitachi). The light irradiation condition is 40 mW cm^{-2}, and the excitation wavelength of the fluorimeter is 480 nm. Each sample is prepared in three groups in parallel.

ROS detection *in vivo*: 800 μL *E. coli* and 2 μL DCFH-DA (10 mmol L^{-1}) were added to 198 μL LB medium and incubated at 37 ℃, 180 rpm for 20 min after mixing. Then, the bacterial solution was centrifuged at 8000 rpm for 2 min to remove the excess DCFH-DA and washed twice with 1×PBS. The bacteria were resuspended in LB medium. 800 μL bacteria, 100 μL 1×PBS and 100 μL PMB-CON (16 μg mL^{-1}) were taken into a centrifuge tube and incubated at 37 ℃, 80 rpm for 20 min in a shaking table. After 4 min of illumination under white light (40 mW cm^{-2}), the bacteria solution was

centrifuged at 8000 rpm for 3 mins and removed the supernatant. Then 200 μL of 0.5% glutaraldehyde aqueous solution was used to resuspend the bacteria and wash once by centrifugation after fixation for 30 mins at room temperature. Finally, resuspend the pellet with 20 μL 1×PBS and take 10 μL for CLSM (488 nm excitation) to observe the fluorescence of DCF.

Antibacterial activity test by colony counting. The kana^r *E. coli* were grown in liquid LB medium with shaking at 180 rpm at 37 °C to reach OD_{600}=0.8 approximately. First, the above bacterial solutions (0.8 OD) were diluted 40 times. Next, aliquots of 800 μL of the bacterial solution were transferred in 48-well plate. Then, PMB-CON with different concentrations were added to the above bacterial solution, and the final volume of each well is 1 mL. Four portions of bacterial solution which contained bacteria with CON, bacteria with CON under white light irradiation (40 mW cm^{-2}, 40 min), PMB-CON and bacteria with PMB-CON under white light irradiation (40 mW cm^{-2}, 40 min) were tested. Then, each sample was serially diluted a series of times. Subsequently, a 100 μL portion of the dilution bacteria was evenly spread on the solid LB agar plate. The plates were cultured at 37 ℃ for about 15 h. The number of colony forming units (CFUs) was counted.

Antibacterial effect of PMB-CON by SYTOX Green dye. 100 μL PMB-CON (8 μg mL^{-1}) and 99 μL 1×PBS were added into 800 μL *E. coli* and 100 μL PMB-CON (8 μg mL^{-1}) was replaced by 100 μL 1×PBS in control. Then the mixture was shaken at 800 rpm for 10 min at 37 °C. 1 μL SYTOX Green (1 mmol L^{-1}) was added to above solution and shaken at 800 rpm for 20 min at 37 °C after being treated with white light irradiation (40 mW cm^{-2}, 40 min). The solution was washed by 1×PBS twice and suspended with 200 μL 0.5% glutaraldehyde for 30 min. The above solution was washed twice with 1×PBS, and the precipitation was suspended with 1×PBS. Finally, 10 μL bacterial suspensions were transferred to a glass slide for CLSM imaging.

Minimum inhibitory concentration (MIC) of PMB-CON and Ab-CON. First, the above 0.8 OD bacterial solutions were diluted 40 times. Next, aliquots of 80 μL of the bacterial solution at a density of about 2×10^6 CFU $well^{-1}$ in LB medium were transferred to a 96-well plate. Then, PMB-CON or Ab-CON with different concentrations was added to the bacteria solution, and the final volume of each well was adjusted to 100 μL with LB medium. The concentrations of PMB-CON and Ab-CON were quantified according to the content of BFTB.Then, the 96-well plates were irradiated by a white

light source at different time. Afterward, the plates were cultured at 37 ℃ and 180 rpm overnight for about 18 h.

Finally, the absorbance of the solution at 600 nm was measured with a microplate reader, and the bacteria killing-efficiency was calculated according to the following equation: survival rate (%) = [(C-B)／(A-B)] × 100%, where A was the absorbance of the mixture of bacteria without the addition of PMB-CON, CON or Ab-CON as the control, B was the absorbance of the equivalent LB medium solution as background, and C was the absorbance of the mixture of bacteria with different concentration of PMB-CON, CON or Ab-CON as the sample.

Antibacterial activity of PMB-CON *in vivo*. The antibacterial efficacy of PMB-CON *in vivo* was assessed by bacterial counting, wound recovery, and histological analysis of kana^r *E. coli* infection model. The 20 6-week-old ICR mice were in four groups (Group 1, Group 2, Group 3 and Group 4). The mice were anesthetized with 5% chloral hydrate solution (0.001 mL g^{-1}). The wounds (a diameter of 1 cm) were created on the back of mice and then infected with kana^r *E. coli*. One day later, the wounds of mice in Group 1 and Group 2 were treated with PBS buffer and PMB-CON (2.0 μg mL^{-1} in PBS buffer), respectively. The wounds of mice in Group 3 and Group 4 were illuminated for 30 min under white light (40 mW cm^{-2}) after anesthetized and treated with PBS buffer and PMB-CON (2.0 μg mL^{-1} in PBS buffer). The wound areas were photographed every day for 8 days. After 8 days of treatment, the bacteria on infected tissues were obtained for bacterial counting on agar plates. And the skin tissues around the wounds were collected and fixed with 4% glutaraldehyde for 24 h at room temperature, followed by making tissue slices for hematoxylin and eosin (H&E) staining.

2. Spectral and morphological characterization of CON and PMB-CON

The UV-visible absorption and fluorescence spectra of the prepared CON and PMB-CON are shown in Fig. S1a. The CON (solid black line) and PMB-CON (red dot line) have the same two absorption peaks at 370 nm and 520 nm. Both CON (solid blue line) and PMB-CON (green dot line) have the same maximum fluorescence emission at 634 nm. The results indicate that PMB modification doesn't affect the absorption and fluorescence spectra of CON.

The morphology of PMB-CON was characterized by TEM image. As shown in Fig. S1b, PMB-CON is a spherical morphology with ~ 50 nm diameters. The size of the PMB-CON was further characterized by Dynamic light scattering (DLS) characterization showed that the hydrodynamic

diameter of PMB-CON is about 55 nm in an aqueous solution (Fig. S1c), which is consistent with TEM results.

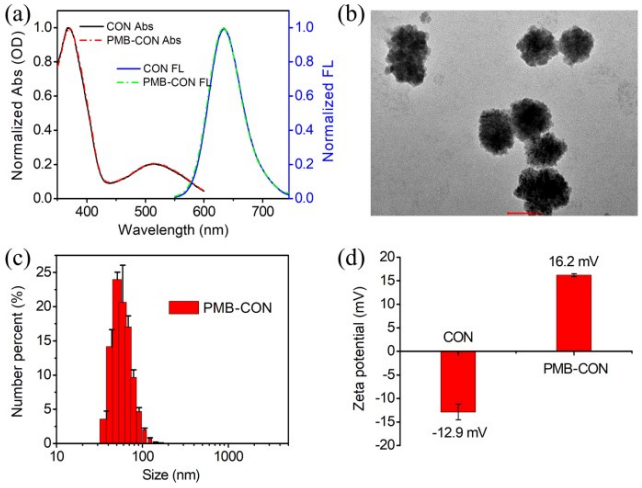


Fig. S1 Characterization of CON and PMB-CON. (a) The absorption and fluorescence spectra of CON and PMB-CON. (b) TEM imaging of PMB-CON. Scale bar: 50 nm. (c) Size of PMB-CON by DLS. (d) Zeta potential of CON and PMB-CON, respectively.

Moreover, we investigated the Zeta potential of CON and PMB-CON. As shown in Fig. S1d, the Zeta potential of PMB-CON is 16.2 mV, which is more positive than that of CON as -12.9 mV. This indicates that the positively charged PMB is successfully coupled to the surface of the negatively charged CON, resulting in an increase in charge.

3. The targeted identification and specific fluorescence imaging of kana^r *E. coli* by PMB-CON

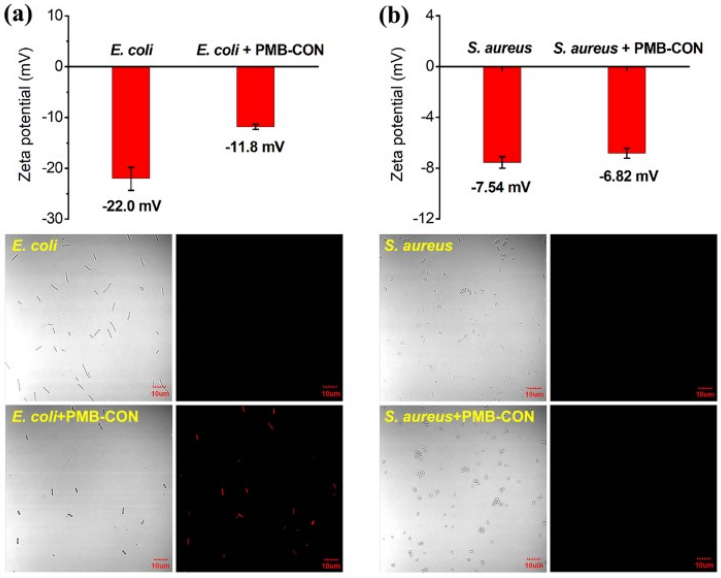


Fig. S2 The Zeta potential and CLSM of kana^r *E. coli* (a) and *S. aureus* (b) treated without and with PMB-CON, respectively. The scale bar of CLSM is 10 μm.

To demonstrate targeted identification and specific imaging capabilities of the PMB-CON, a gram-negative bacterium, kana^r *E. coli* and a gram-positive bacterium, *S. aureus*, are selected as the research model. PMB-CON can specifically bind to kana^r *E. coli* based on the targeted identification ability of PMB to gram-negative bacteria. Zeta potential measurement showed that the Zeta potential of kana^r *E. coli* treated without and with PMB-CON are -22.0 mV and -11.8 mV, respectively (Fig. S2a). The increase of Zeta potential in kana^r *E. coli* indicates that PMB-CON binds to kana^r *E. coli*. Moreover, we used confocal laser scanning microscope (CLSM) to observe the binding of PMB-CON to kana^r *E. coli* directly. As shown in Fig. S2a, there is no fluorescence signal in kana^r *E. coli* untreated with PMB-CON. By comparison, the strong red fluorescence emission from CON is obviously observed in kana^r *E. coli* treated with PMB-CON. However, the Zeta potential of *S. aureus* treated without PMB-CON and with PMB-CON has little change (Fig. S2b). And no red fluorescence signals are observed in *S. aureus* treated with and without PMB-CON. These results suggest that PMB-CON can selectively identify and image gram-negative bacteria.

4. Detection of ROS by DCFH-DA

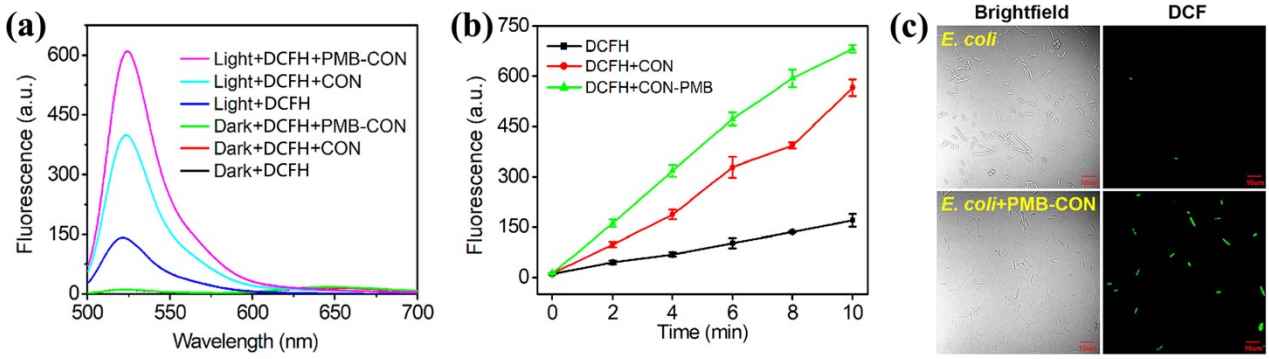


Fig. S3. Detection of ROS from CON and PMB-CON by the fluorescence dye DCFH-DA. (a) Fluorescence spectra of DCF with CON and PMB-CON in light and dark at 6 min, respectively. The concentration of PMB-CON is 8 μg mL^{-1} and the optical density is 40 mW cm^{-2}. (b) Detection of ROS generation of PMB-CON in different time by using DCFH. (c) CLSM imaging of ROS produced from kana^r *E. coli* treated without and with PMB-CON under light irradiation. Scale bar: 10 μm.

5. Optimization of the experimental conditions of kana^r *E. coli* killing

As shown in Fig. S4a, CON and PMB-CON from 0.4 to 1.2 μg mL^{-1} have no damage to kana^r *E. coli* without light irradiation. As shown in Fig. S4b and S4c, the viability of kana^r *E. coli* treated with CON almost unchanged under the light irradiation time from 20 to 40 min. However, the viability of

kana^r *E. coli* treated with PMB-CON gradually decreased with the increase of the light irradiation time from 20 to 40 min. Fig. S4b shows that the viability of kana^r *E. coli* treated with PMB-CON significantly decreased with the increase of the optical density as 30, 40, and 50 mW cm^{-2}. With the increase of the light irradiation time and optical density, the photodynamic antibacterial effect of PMB-CON was enhanced significantly. Therefore, we selected the optimal light irradiation time as 40 min and optical density as 40 mW cm^{-2} for the study on the photodynamic killing of PMB-CON against kana^r *E. coli*.

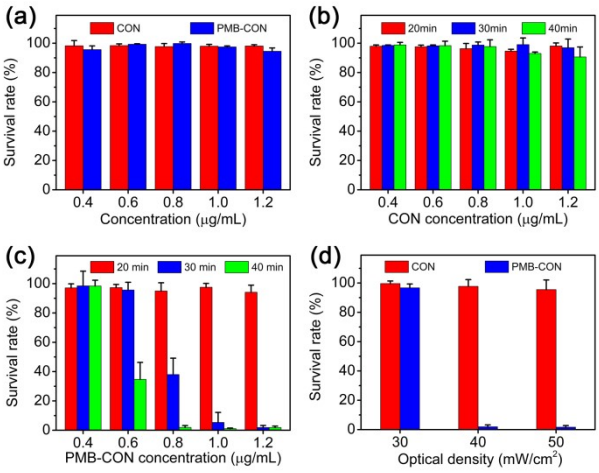


Fig. S4. The influence of light irradiation time and optical density on the viability of kana^r *E. coli*. The viability of kana^r *E. coli* treated by CON and PMB-CON without light-irradiation (a). The viability of kana^r *E. coli* treated by CON (b) and PMB-CON (c) with different light irradiation time. Optical density: 40 mW cm^{-2}. (d) The viability of kana^r *E. coli* treated by CON and PMB-CON with different optical density: 30, 40, 50 mW cm^{-2}. The concentration of CON and PMB-CON were 0.8 μg mL^{-1} and the light irradiation time was 40 min. Error bars represent standard deviation from three repeated measurements.

6. The viability of kana^r *E. coli* treated with Ab-CON and PMB-CON in dark

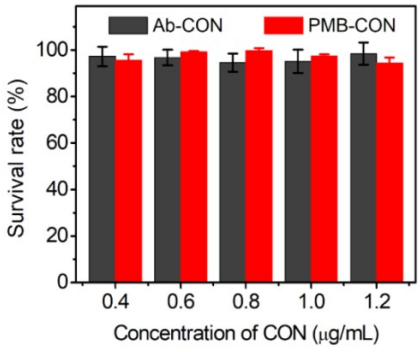


Fig. S5. The viability of kana^r *E. coli* treated with Ab-CON and PMB-CON without light irradiation.

7. Measuring bacterial counts in LB agar plates harvested from infectious tissues by colony forming unit

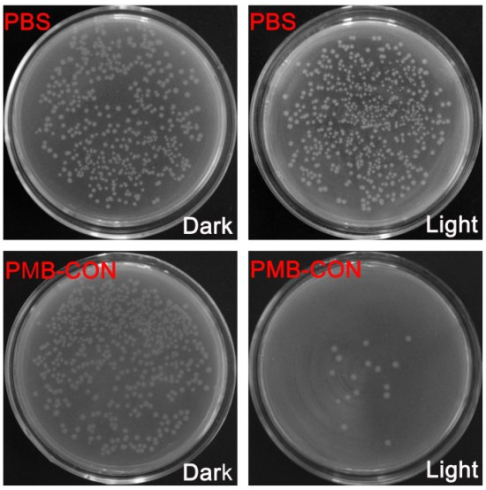


Fig. S6. Corresponding photographs of kana^r *E. coli* colonies on the LB agar plates which were derived from the skin tissue of the infected sites.

8. Animal welfare statement

The animal experiments were performed in compliance with the policy of Animal Welfare and Ethical Committee of Hebei University on animal use and ethics (Approval number: IACUC-2020XG044).

9. References

1 B. Li, J. Li, Y. Fu and Z. Bo, *J. Am. Chem. Soc.* 2004, **126**, 3430.