

Supporting Information

Photo-Sensitizable Phage for Multidrug-Resistant *Acinetobacter* *Baumannii* Therapy and Biofilms Ablation

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Methods

1. Materials and reagents

Yeast extract, tryptone, and agar are purchased from OXOID (Shanghai). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is purchased from TCI Chemical. Live/dead biofilm viability kit and oxygen species assay kit are supplied by Beyotime biotechnology Co., Ltd. Crystal violet (CV) is supplied by Sinopharm Chemical Reagent Co., Ltd. All the other solvents and reagents used in this study are of analytical grade. Ultrapure water (Millipore, 18.25 MΩ cm) is used to prepare the solutions. Absorption and emission spectra for NB and APNB are performed with a Lambda 35 UV-visible spectrophotometer (PerkinElmer) and a VAEIAN CARY Eclipse fluorescence spectrophotometer (Serial No. FL0812-M018), respectively. Confocal laser scanning microscope (CLSM) images are performed on Olympus

FV1000-IX81 confocal laser scanning microscope.

2. Bacterial Culture

We pick *A.baumannii* and *P.aeruginosa* from a single colony and transfer them to LB medium (5 mL) and then incubate at 37 °C and 200 rpm overnight. Next, we use a Lambda 35 UV-visible spectrophotometer (PerkinElmer) to measure the OD_{600 nm} of the bacteria. Then, the bacterial solution is centrifugated at 5000 rpm for 5 min and supernatant is discarded. Finally, we wash the bacterial solution with PBS buffer three times and suspend it with PBS buffer for the following experiments.

3. Synthesis and Characterization of APNB conjugate

We mix the 4.0 µL NB solution (1.0 mM) with 996 µL ABP solution (1×10⁹ PFU/mL) for a 36 h reaction at 4 °C. Then, we purify the produced APNB with free NB molecules by chloroform extraction for three times. We use UV-vis spectrophotometer and fluorescence spectrophotometer to record UV-vis absorption and fluorescence spectra of APNB, respectively. The morphology of APB phage is characterized by transmission electron microscopy (TEM, FEI Tecnai F20).

4. Detection of ROS

We use ROS-sensitive probe (DCFDA) to measure the total ROS generated from the NB and APNB under light irradiation. NB (100 µL, 0.5 µM) or APNB (100 µL, 0.5 µM) and DCFDA (200 µL, 30 µM) solutions are added to 500 µL of PBS buffer. After the irradiation of 20 mW/cm² light, we the fluorescence spectra of DCF solution are recorded on an fluorescence spectrophotometer from 505 nm to 650 nm with the excitation wavelength of 480 nm.

A ROS assay kit (Beyotime) is chosen for intracellular detection of ROS. We dilute *Acinetobacter baumannii* (OD₆₀₀ = 0.8, 80 µL) solution with LB medium (919 µL) and then stain the solution with of DCFDA (5 mM, 2 µL). Following the incubation at 37 °C for 20 min, the solution is centrifuged with 5000 rpm for 5 min. After discarding the supernate, the precipitation is suspended with 1 mL of LB medium

with 0.5 μM APNB and NB, respectively. Then, the bacterial solutions are transferred to a 48-well plate. Subsequently, the 48-well plates are irradiated with 20 mW/cm^2 light for 15 min. Next, the bacterial solutions are transferred to two 1.5 mL centrifuge tubes and centrifuged at 8000 rpm for 3 min, respectively. After discarding the supernatants, the precipitations were resuspended with 50 μL of PBS buffer (1 \times). Finally, 10 μL of *A. baumannii* suspensions are transferred to a glass slide to be imaged with a FV1000-IX81 confocal laser scanning microscope (Olympus).

5. Cytotoxicity Evaluation

The COS-7 cells are used to evaluate the cytotoxicity of APNB by MTT assay. In brief, COS-7 cells are seeded in 96-well plates at a density of 1×10^4 cells per well and cultured overnight. The cells are then exposed to various concentrations of APNB (0.5, 0.25, 0.125, 0.06, 0.03, 0.01, and 0 μM) and cultured at 37 $^\circ\text{C}$ for 24 h followed by light irradiation (660 nm, 20 mW/cm^2) for 15 min. Then, MTT (5%, 10 mL) is added to each well and the medium is replaced by 150 μL DMSO after 4 h. The absorbance at 490 nm is measured by a microplate reader.

6. Evaluation of *In Vitro* Antibacterial Activity

The antibacterial activity of APNB against *A. baumannii* is assessed by the CFU counting method, and NB and ABP serve as control. Briefly, *A. baumannii* is first cultured in LB medium at 37 $^\circ\text{C}$ overnight, wash with PBS two times, and dilute to 10^4 , 10^5 and 10^6 CFU/mL. The diluted bacterial suspension (800 μL) is mixed with APNB (1 mg/mL, 200 μL) and incubate at 37 $^\circ\text{C}$ for 30 min. Then, the bacterial suspension is irradiated using a 660 nm light (20 mW/cm^2) for 15 min. Next, bacterial suspension (200 μL) is inoculated on LB plates and incubate at 37 $^\circ\text{C}$ for 24 h. Finally, the CFU is counted and the survival rate of bacterial is calculated.

We observe the morphology of bacteria after APNB treatment is by SEM. Bacterial suspension (10^8 CFU/mL, 1.6 mL) is first mixed with APNB (1 mg/mL, 400 μL) in 12-well plate and incubate at 37 $^\circ\text{C}$ for 30 min, then irradiate with 660 nm light (20 mW/cm^2) for 15 min. After that, the bacteria are collected and wash twice with

PBS, then fix with glutaraldehyde (2.5%) for 1 d, followed by dehydrated with graded ethanol (30, 50, 70, 80, 90, and 100%, v/v). Finally, the fixed bacteria are dried and sputtered with gold for SEM (SU8220, HITACHI, Japan) observation.

We perform Live/dead staining assay to test the antibacterial activity of APNB against *A. baumannii* and *P. aeruginosa*. NB and ABP serve as control. Bacterial suspension (10^8 CFU/mL, 800 μ L) is first mixed with of APNB (1 mg/mL, 200 μ L) in and incubate at 37 °C for 30 min, then irradiate with 660 nm light (20 mW/cm²) for 15 min. Then, the bacteria are collected after centrifugation, and stain the bacterial solutions using a BacLight bacterial viability kit for 30 min. Finally, the bacteria are imaged by FV1000-IX81 confocal laser scanning microscope (Olympus).

7. Ablation Effect of Bacterial Biofilm *In Vitro*

The ability to ablate the formed *A. baumannii* biofilm by APNB is performed by crystal violet (CV) staining. 0.9% NaCl, NB and ABP serve as control. First, *A. baumannii* is cultured in TSB medium overnight, then we seed bacterial suspension (100 μ L, 10^8 CFU/mL) onto a 24-well plate and incubate at 37 °C for 48 hours incubation. Then, we add 100 μ L of APNB (0.5, 0.25, 0.12 and 0.06 μ M) into the 24-well plates and culture the plates at 37 °C for 3 h, following the irradiation with a 660 nm light (20 mW/cm²) for 15 min. After 24 h incubation, we discard the medium, wash the residual biofilms three times with PBS, and add 100 μ L of 0.1% CV to each well and incubate at 37 °C for 0.5 h. After three times washing with PBS, we catch the photograph of residual biofilms using digital camera. To quantitatively analyze the residual biofilm, the biofilms are exposed to 150 μ L of 33% acetic acid and incubated at 37 °C for another 10 min, then the optical density of the solution is measured at 595 nm using an UV-vis spectrophotometer. The ablation rate of biofilms can be calculated by the following formula (**Formula 1**). OD₅₉₅(control) is the absorbance of the biofilms treated with 100 μ L of fresh TSB medium plus 100 μ L 0.9% NaCl.

$$\text{Biofilms ablation rate (\%)} = \frac{OD_{595}(\text{Control}) - OD_{595}(\text{Sample})}{OD_{595}(\text{Control})} \times 100\% \quad (1)$$

8. Inhibition Efficacy on Bacterial Biofilms Formation

The ability to inhibit the formation of *A. baumannii* biofilm by APNB is evaluated by crystal violet (CV) staining. 0.9% NaCl, NB and ABP phage serve as control. First, *A. baumannii* is cultured in TSB medium overnight, then we mix 100 μL of different concentrations of APNB (0.5, 0.25, 0.12 and 0.06 μM) with 100 μL of bacterial suspension (10^8 CFU/mL) in a 96-well plates at 37 $^{\circ}\text{C}$ for 30 min. Subsequently, the suspensions are exposed with 660 nm light (20 mW/cm²) for 15 min and incubate for 24 hours at 37 $^{\circ}\text{C}$. After that, we discard the media, wash the bacteria with PBS, and add 100 μL of 0.1% CV is to each well and incubate at 37 $^{\circ}\text{C}$ for 0.5 h. After three times of PBS washing, 200 μL of 33% acetic acid is added. Following the incubation for 10 min, we measure the optical density of the solution at 595 nm through UV-vis spectrophotometer. The biofilms inhibition rate of APNB is calculated by the following formula (**Formula 2**). $OD_{595}(\text{control})$ is the optical density of the solution at 595 nm for 0.9% NaCl-treated bacterial suspension.

$$\text{Biofilms inhibition rate (\%)} = \frac{OD_{595}(\text{Control}) - OD_{595}(\text{Sample})}{OD_{595}(\text{Control})} \times 100\% \quad (2)$$

9. Evaluation of *In Vivo* Antibacterial Effect

All animal operations are in accordance with institutional animal use and care regulations approved by the Model Animal Research Center of Dalian Medical University (MARC). In order to build a wound infection model, we first create an artificial wounds on the back of the mouse, then infect the wounds 50 μL of *A. baumannii* (10^8 CFU/mL). 24 hours later, we expose the wounds to 20 μL of APNB (0.5 μM), ampicillin (100 mg/mL) and polymyxin B (100 mg/mL), the APNB group is treated by the irradiation with 660 nm NIR light at a dose of 20 mW/cm² for 15 min. To evaluate the antibacterial effect, all mouse received twice irradiation on day 1 and day 2, respectively. Besides, 24 h after the second irradiation, some mouse in each

group are sacrificed, and the wounds tissues are taken for bacterial counting.

For the bacteria counting, we weigh the obtained wound tissue, chop it up, then homogenize in 2 mL of 0.9 % NaCl with a homogenizer. After centrifugation at 4000 g for 6 min, we inoculate 100 μ L of supernatant onto an LB agar plate for CFU counting.

10. Statistical Analysis

All data are presented as mean \pm SD and analyzed by ANOVA, and analysis of variance is analyzed by the Student's test. A significant p-value indicates a significant difference where the probability is < 0.05 (* $p < 0.05$), 0.01 ($0.05 < **p < 0.01$), and 0.001 ($0.01 < ***p < 0.001$).

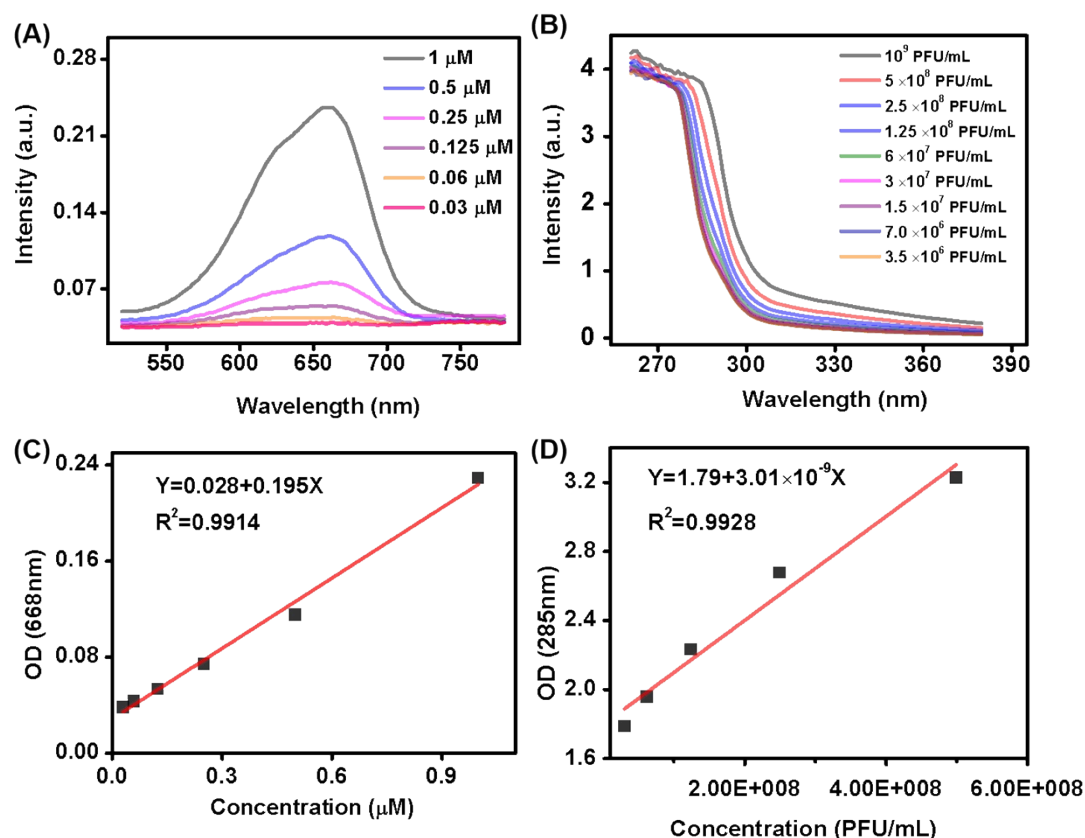


Figure S1. (A) UV-vis spectra of the aqueous solution of NB (1 μ M), and its titration by diluted 2 times, 4 times, 8 times, 16 times and 32 times, respectively. (B) UV-vis spectra of the aqueous solution of ABP (1×10^9 PFU/mL), and its titration by diluted 2

times, 4 times, 8 times, 16 times, 32 times, 64 times, 128 times and 256 times, respectively. (C) The standard absorbance curve of NB. $Abs_{668\text{ nm}}$ could be fitted linearly with their concentrations. (D) The standard absorbance curve of phage. $Abs_{285\text{ nm}}$ could be fitted linearly with their concentrations.

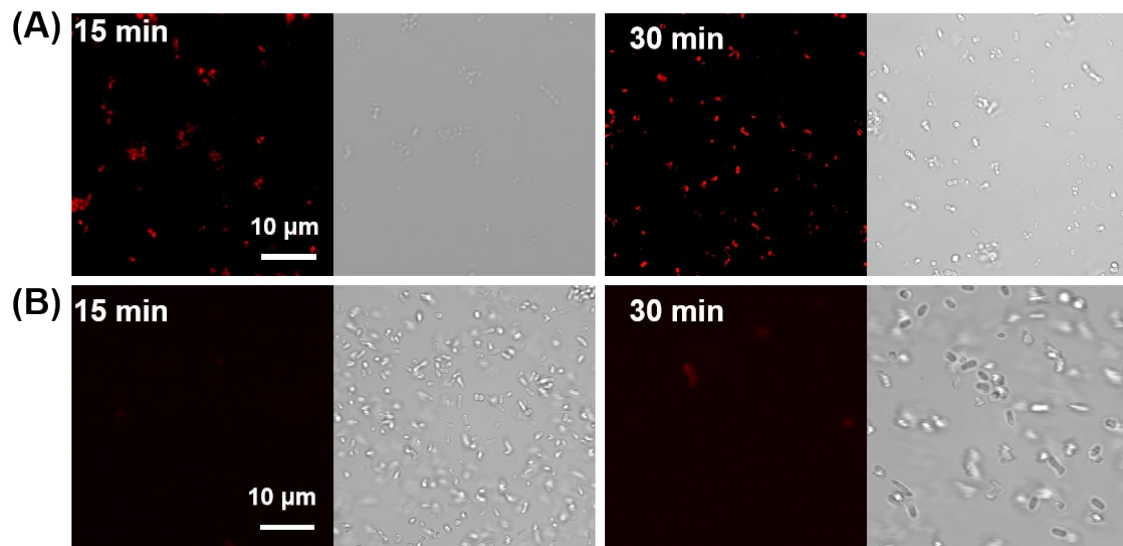


Figure S2 (A) Targeted bacterial imaging of APNB by fluorescence imaging of *A. baumannii* with APNB for 15 min and 30 min. (B) Targeted bacterial imaging of APNB by fluorescence imaging of *P. aeruginosa* with APNB for 15 min and 30 min. (scale bar = 10 μm). [APNB] = 0.5 μM. λ_{ex} =660 nm for APNB.

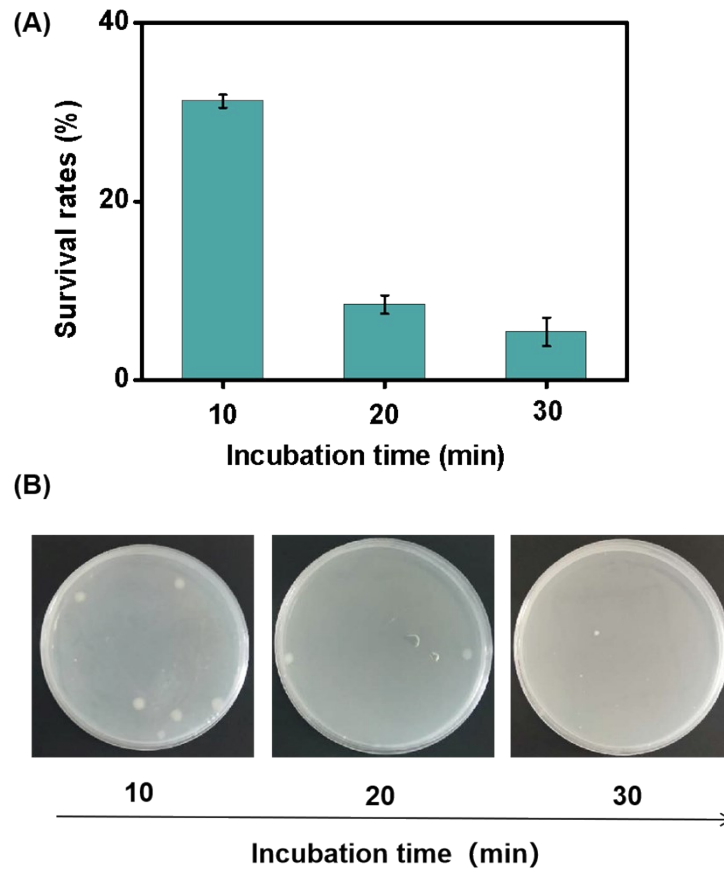


Figure S3. The optimization of incubation time by examining the survival rate of *A. baumannii*.

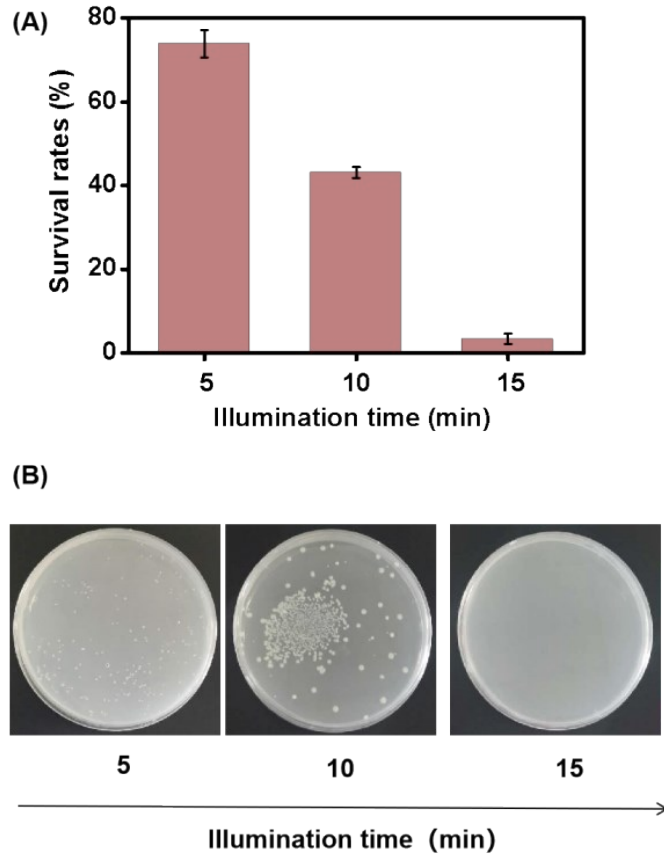


Figure S4. The optimization of light illumination time by examining the survival rate of *A. baumannii*.

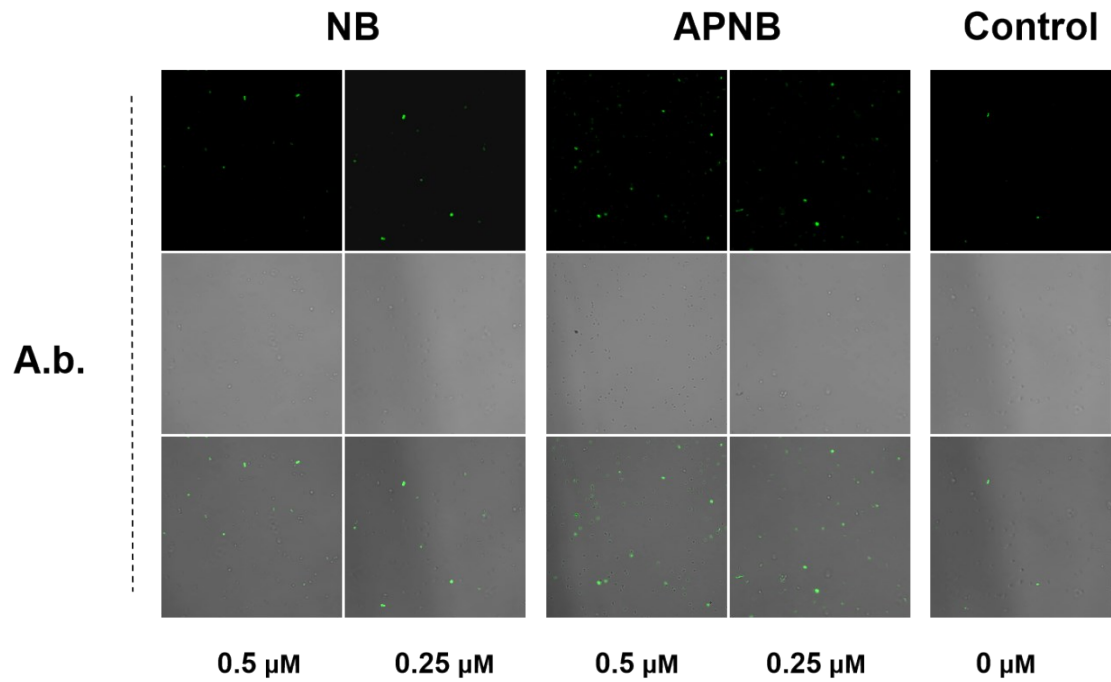


Figure S5. Confocal fluorescence images of *A. baumannii* treated with different concentration of APNB and NB, and followed by incubation with DCFH for 30 min then irradiated with 660 nm light source at a power density of 20 mW/cm² for 15 min.

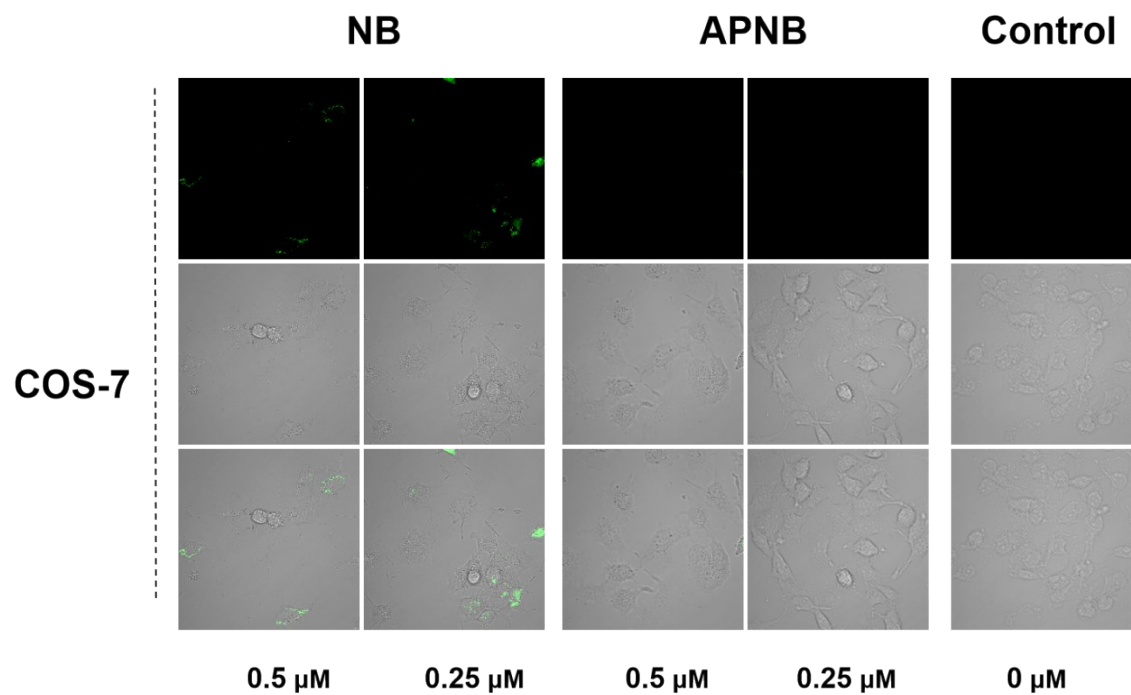


Figure S6. Confocal fluorescence images of COS-7 cells treated with different concentration of APNB and NB, and followed by incubation with DCFH for 30 min then irradiated with 660 nm light source at a power density of 20 mW/cm² for 15 min.

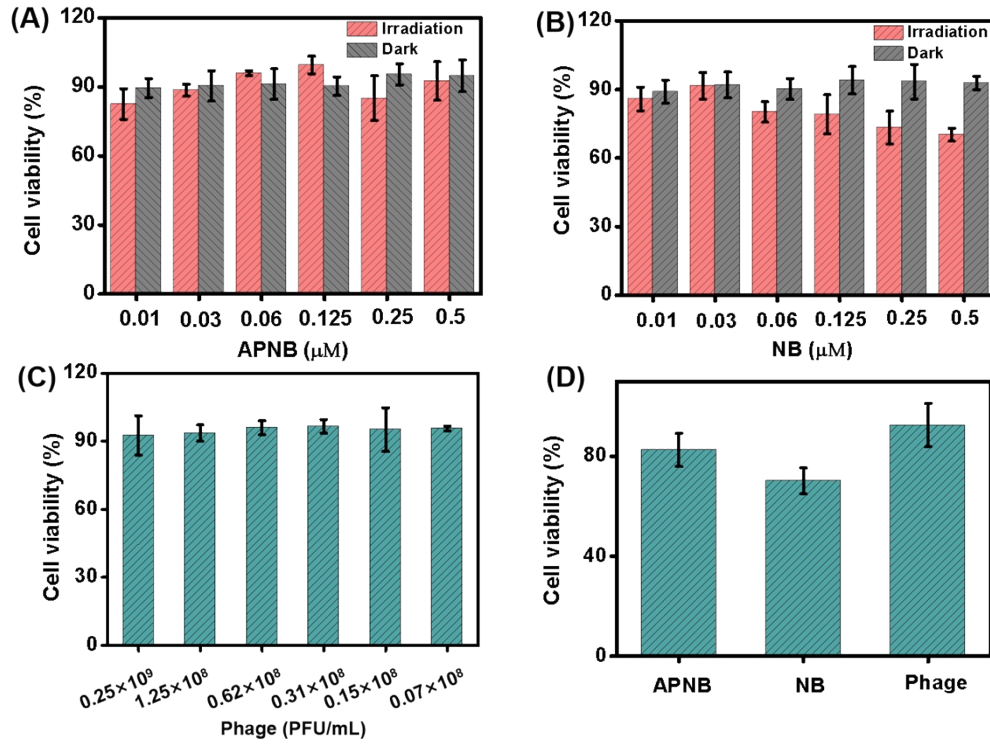


Figure S7. Cell viability of COS-7 cells after treatment with different dosages of APNB, NB and APNB for 24 h with and without light illumination (660 nm, 20 mW/cm²) for 15 min.

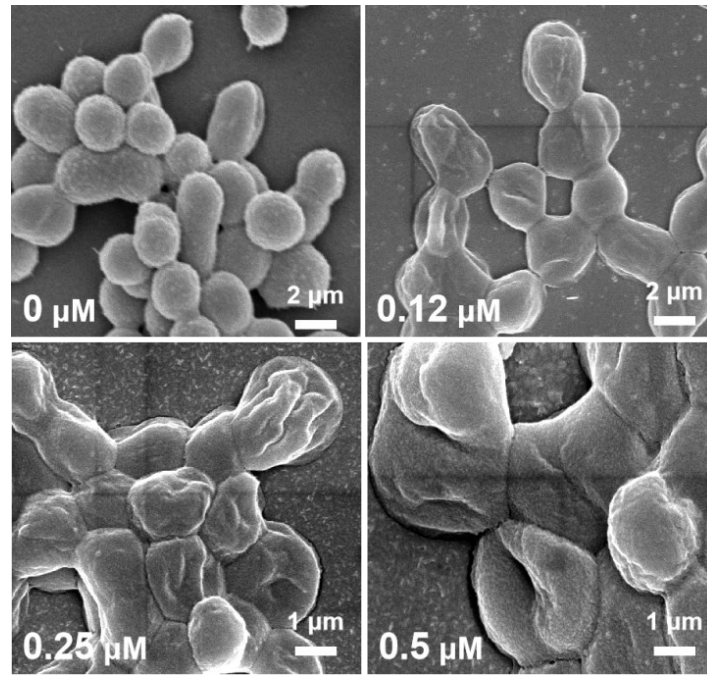


Figure S8. SEM images of *A. baumannii* after treatment with different dosages of APNB.

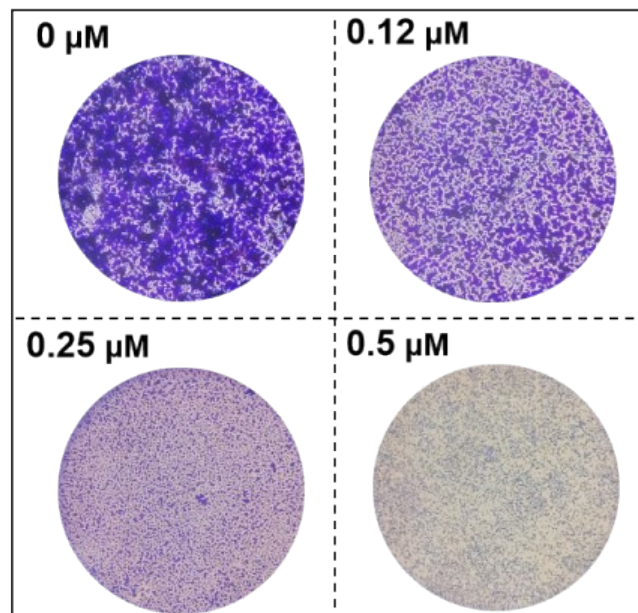


Figure S9. (A) Optical microscope photographs of the remaining biofilms after the treatment of the different concentrations of APNB.

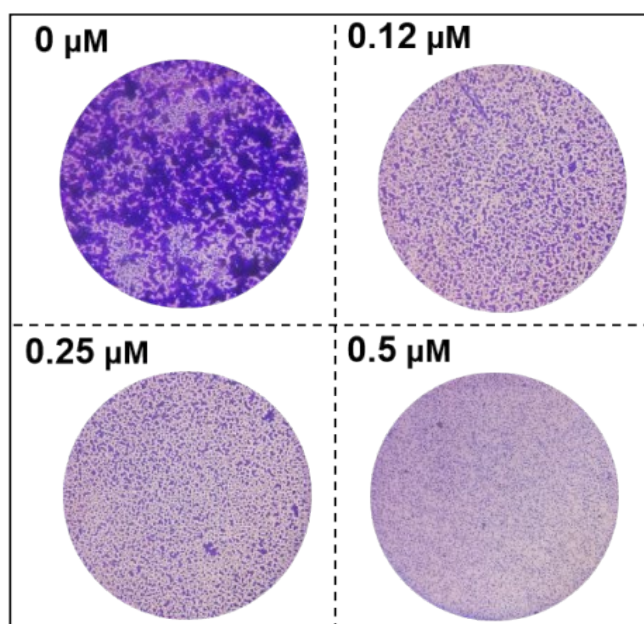


Figure S10. (A) Optical microscope photographs of the formed biofilms after the treatment of the different concentrations of APNB.

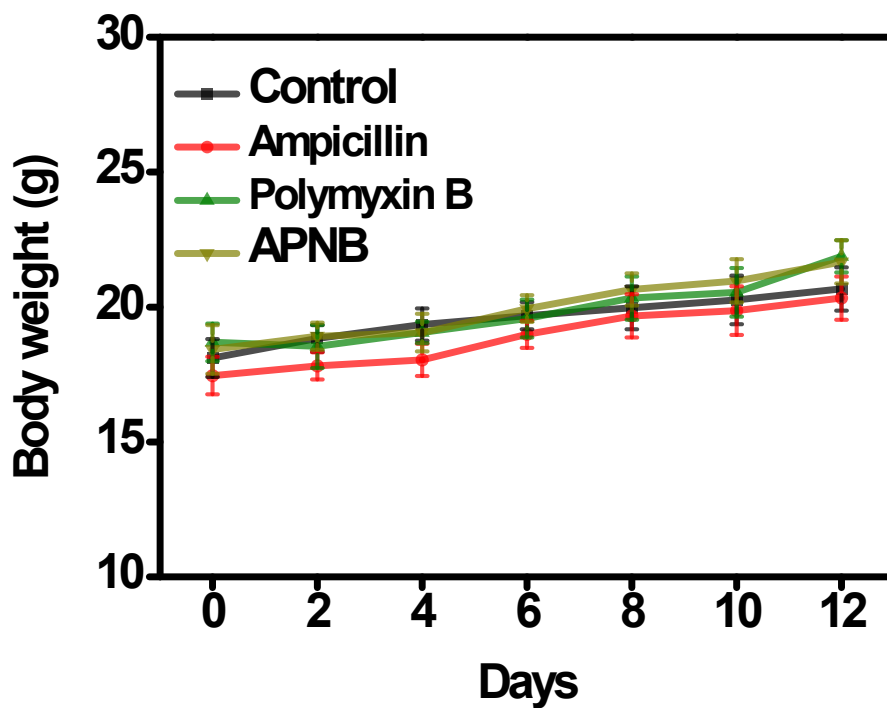


Figure S11 Changes of body weight during 12 days after different treatments (saline,

ampicillin, polymyxin B and APNB with 660 nm light at 20 mW/cm² for 15 min.

Error bars represent standard error of mean.