Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supporting Information

A Light-Activated Nanotherapeutic with Broad-Spectrum Bacterial Recognition to Eliminate Drug-resistant Pathogens

Xiaosong Wei, Yingchao Gao, Yuqing Hu, Yufei Zhang and Xinge Zhang*

Key Laboratory of Functional Polymer Materials of Ministry Education, Institute of Polymer Chemistry, College of Chemistry, Nankai University, Tianjin 300071, China

* Corresponding author.

E-mail: zhangxinge@nankai.edu.cn

2. Process and Methods

2.1 Raw Materials

Sodium sulfide hydrate ($Na_2S \cdot 9H_2O$) and cupper chloride dehydrates were procured from Tianjin Bohua Reagent Co., Ltd (Tianjin, China) and used without further dispose. 3-Aminophenylboronic acid monohydrate as a precursor for the preparation of 3-acrylamidophenylboroniccid (AAPBA) was supported by Nanjing Chemlin Co., Ltd (Nanjing, China). Furthermore, β-D-galactose pentaacetate as a cornerstone in the construction of hydrophilic monomers was offered by HEOWNS Co., Ltd (Tianjin, China). As for hydroxyethyl methacrylate and methacryloylchloride, Aladdin Biochemical Technology Co., Ltd (Shanghai, China) and HEOWNS Co., Ltd (Tianjin, China) were selected as suppliers, respectively. Culture medium such as fetal bovine serum (PBS), penicillin & streptomycin solution, trypsin and Dulbecco's modified Eagle's medium (DMEM) were all provided from Gibco (CA, USA). Several common organic solvents, including dichloromethane (DCM), trimethylamine (TEA), dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) were all of analytical grade and used as such.

2.2 Synthesis of Hydrophilic Monomer

The glycosyl-based 2-(2', 3', 4', 6'-tetra-O-acetyl-β-D-glucosyloxy)ethyl acrylate (AcGEA) was synthesized from β-D-galactose pentaacetate and hydroxyethyl methylacrylate (HEMA) in one step.[1] Concisely, 3.9 g of β-D-galactose pentaacetate was dispersed in 20 mL of dichloromethane which has been managed with dehydration in advance. Afterwards, hydroxyethyl methylacrylate (HEMA) was

introduced to participate in the reaction. The reaction solution was occurred at 0℃ in the presence of a catalyst boron trifluoride diethyl ether and stirred under nitrogen atmosphere for 0.5 h. After further 3 h of reaction at 25℃, the cessation reaction was achieved by adding 30 mL of trichloromethane. The resultant mixture was washed with 100 mL of ultrapure water for three times, followed by saturated sodium bicarbonate solution for three times and eventually with saturated sodium chloride solution. The collected organic phase was dried with desiccant overnight. After filtrating out anhydrous magnesium sulfate, the filtrate was evaporated under pressure to afford the crude product. For acetoxy group protected glycosyl-based molecules, silica column was introduced to isolate pure AcGEA from crude product with ethyl acetate/hexane (1/6) eluent. The ^{1}H NMR data of AcGEA was delineated in Figure S2 .

2.3 Synthesis of Hydrophobic monomer

3-acrylamidophenylboroniccid (AAPBA) was synthesized from 3-aminophenylboronic acid monohydrate and methacryl chloride according to a published procedure.[2] Curtly, a solution of 3-aminophenylboronic acid monohydrate (1.0 g, 6.44 mmol) and sodium bicarbonate (1.0 g, 11.9 mmol) in THF/H_2O (2:1) was cooled to 0℃ and kept stirring. Next, 1 mL of methcryl chloride was added dropwise and then the mixture was allowed to transfer to room temperature and stir for further 2 h. The resultant solution was extracted with 100 mL of ethyl acetate for three times. The isolated organic phase was condensed to afford a light brown solid. This crude product was performed with recrystallization from hot water, filtered and dried to yield 0.66 g (50%) of 3-acrylamidophenylboroniccid as an acicular solid. The

chemical structure of AAPBA was validated by ^{1}H NMR and outlined in Figure S3.

2.4 Synthesis of macro chain transfer agent p(AcGEA)

p(AcGEA-*b*-AAPBA) was synthesized through reversible-addition-fragmentation chain transfer (RAFT) technique with p(AcGEA) and 2, 2-azobisisobutyronitrile (AIBN) as macromolecular chain transfer agent and initiator, respectively. Accordingly, monomers AcGEA were supposed to convert to macromolecular chain transfer agent primarily. In particular, 3.28 mg of recrystallized AIBN and 11.28 mg of CPADB were transferred into a reaction vessel. Then, presupposed amounts of AcGEA were dissolved in ultra-dried dimethylformamide (DMF). The mixture solution was managed with vacuum degassing purification by nitrogen for 40 min and occurred in an oil bath at 70℃ to start polymerization. After 24 h, routine operations were performed for cessation reaction. The expected polymer p(AcGEA) was extracted by precipitation in cold diethyl ether for three times, centrifuged and dried to afford a pale pink solid (called macro-CPADB). It should be noted that RAFT agent CPADB was synthesized referring to a published literature and used directly without further elaboration. As for other macro-CPADB endowed with different molecular weights were all prepared in the same method but changing initial monomer AcGEA to CPADB molar ratio. All details about the identity of p(AcGEA) were exhibited in Table S1.

2.5 Synthesis of Glycosylated copolymers p(GEA-b-AAPBA)

A sequence of glycosylated polymers such as p(GEA_{15}-*b*-$AAPBA_{10}$), p(GEA_{20}-*b*-$AAPBA_{10}$) and p(GEA_{25}-*b*-$AAPBA_{10}$) were synthesized from three macro-CPADB

and AAPBA, followed by deprotection. A typical procedure was delineated as follows. Primarily, 84 mg of AAPBA monomer was dissolved in the solution of macro-CPADB p(AcGEA) and then taken to a dry reactor. The supplementary addition of AIBN (3.28 mg, 0.02 mmol) fulfilled the necessary preparations for the following polymerization. The reaction mixture was soaked in nitrogen atmosphere to exclude the oxygen inside the reactor, followed by initiating polymerization in oil at 70℃. Once polymerization was accomplished, the resultant mixture was executed to dialyze against distilled water for three days and then lyophilized to yield the acetoxy group protected p(AcGEA-*b*-AAPBA). For deprotection, 200 mg of p(AcGEA-*b*-AAPBA) was dissolved in DMSO/H_2O in 4:1 ratio, the calculated hydrazine hydrate solution was added dropwise within 15 min under nitrogen atmosphere. After that, the deprotection was initiated at 25℃ and kept for 24 h. Acetone's accession to this reaction solution meant that the deprotection had been achieved. A new round of dialysis was performed, the final product p(GEA-*b*-AAPBA) was collected by freezing drying as snowflake solid. The identity information of p(GEA-*b*-AAPBA) was confirmed through ^{1}H NMR (Figure S4).

2.6 Preparation of p(GEA-b-AAPBA)@CuS Nanoparticles

To prepare p(GEA-*b*-AAPBA)@CuS nanoparticles, the operation was carried out according to many relative references but with a little modifying. Specifically, p(GEA_{15}-*b*-$AAPBA_{10}$)@CuS served as an illustration was outlined as follows. 1.9 mg of p(GEA_{15}-*b*-$AAPBA_{10}$) was dissolved in DMSO/H_2O (4:1) in advance. After that, 21 mg of $CuCl_2 \cdot 2H_2O$ combined with a solution of p(GEA_{15}-*b*-$AAPBA_{10}$) were

uniformly dispersed in 50 mL of distilled water under vigorous stirring. Until a pellucid solution was obtained, 24 mg of $Na_2S·9H_2O$ was introduced to participate in the following reaction. Eventually, the solution was immersed in a 60℃ water bath for a further 2 h and the color of this mixture changed from dark brown to dark green in this process. The resultant solution was performed with natural cooling and then converted to a dialysis bag for purification. Similar steps were followed to prepare p(GEA_{20}-*b*-$AAPBA_{10}$)@CuS as well as p(GEA_{25}-*b*-$AAPBA_{10}$)@CuS nanoparticles.

2.7 Characterizations

The chemical structures of monomers and designed polymers were characterized by ^1H NMR (AVANCE III 400MHZ, Bruker). The molecular weights and corresponding polydispersity indices of macro-CPADB ($pAcEGA_{20}$, $pAcGEA_{30}$ and $pAcGEA_{40}$) were determined by GPC (Waters 1525, Waters). The mean particle size and zeta potential of resultant nanoparticles were tested by DLS (ZetaSizer Nano ZS90, Malvern). The morphology of nanoparticles was observed by TEM (Tecnai G2 F20, FEI). UV-vis spectra of p(GEA-*b*-AAPBA)@CuS were corroborated by an ultraviolet spectrophotometer (UV-2550).

2.8 Evaluation of in Vitro Photothermal Effect

To evaluate the photothermal performance of p(GEA-*b*-AAPBA)@CuS nanoparticles in aqueous solution, a digital thermometer was introduced to monitor the temperature shifts under exposure of an 808 nm laser every 1 min. Primarily, a range of samples with different concentrations were acquired by the 2-fold serial dilution method. Once the preparatory work was accomplished, the resultant samples

were irradiated with an 808 nm laser at the power density of 2.0 W/cm^2 for 5 min. PBS served as blank sample was also performed in the same manner. And it was notable that a thermal camera was equipped to capture photographs for each sample, synchronously.

2.9 In Vitro Antibacterial Performance

The antibacterial activity of p(GEA-*b*-AAPBA)@CuS nanoparticles was investigated through a battery of reliable testing methods such as colony counting method, Live/Dead imaging and SEM assay. Importantly, drug-resistant *pseudomonas aeruginosa* and *staphylococcus aureus* worked as model pathogens were involved in these projects. Additionally, preparatory work was implemented analogously for each detection means and delineated as follows. Primarily, the bacterial cells were revived and cultured in LB medium at 37℃ overnight to afford a fresh bacterial suspension. After that, cleaning operations of the resultant bacterial suspension was essential. Eventually, the acquired bacteria were re-diluted with sterile PBS until the optical density at 600 nm (OD_{600}) reached approximately 1.0 and reserved for the following applications.

2.9.1 Colony-forming Unit (CFU) Counting

Hence, in detection 1, 1 mL of purified bacteria was managed with 5 min of centrifugation at 5000 rpm and then re-suspended in p(GEA_{15}-*b*-$AAPBA_{10}$)@CuS, p(GEA_{20}-*b*-$AAPBA_{10}$)@CuS and p(GEA_{25}-*b*-$AAPBA_{10}$)@CuS, respectively. After further 4 h co-cultivation at 37℃ silently, three groups of experimental samples combined with PBS group were exposed under an 808 nm laser (2 W/cm^2) for

constant 5 min. Both experimental and control samples became massively diluted to afford 10^{-4} bacterial suspension. Afterwards, the agar plates were vaccinated with 40 μL of dilution from each sample. Ultimately, the resultant plates were transferred to an incubator with a constant temperature of 37℃ for 24 h. The average of colonies generated from survival was recorded to evaluate the viability. Each sample was performed in triplicate and the statistical result was expressed as CFU/mL.

2.9.2 Live/Dead Staining Analysis

In detection 2, Live/Dead staining also played an important role in confirming and supplied with a direct and reliable observing images for assessment. Thus, absolute bacterial strains were segregated from overnight cultured bacterial suspension via the same manner as mentioned before. After that, quondam PBS medium was replaced by p(GEA_{15}-*b*-$AAPBA_{10}$)@CuS, p(GEA_{20}-*b*-$AAPBA_{10}$)@CuS or p(GEA_{25}-*b*-$AAPBA_{10}$)@CuS nanoparticle solutions. The harvested experimental samples were disposed with 4 h cultivation away from light, followed by managing with laser-induced phtothermal therapy. Once operations were completed, biological dye acridine orange (AO) combined with ethidium bromide (EB) was added into four groups of samples. Staining process lasted 15 min based on light interception, which enabled live bacteria to be stained with AO and dead bacteria to be stained with EB. The stained samples were washed with sterile PBS for many times to remove the redundant fluorescent dyes. At the end of specimen preparation period, a drop of glycerol was added into each centrifuge tube to support bacteria. As-prepared samples were imaged using confocal laser scanning microscopy (CLSM) on a Leica TCS SP8

microscope through dual channels simultaneously. For receiving signals, 488 nm laser and 561 nm laser were allocated for AO and EB, respectively.

2.9.3 Scanning Electron Microscope (SEM) Assay

In detection 3, the morphology of bacteria dealt with light exposure was visualized using scanning electron microscope (SEM) on a Zeiss Merlin Compact. And the involved samples were dealt with the same procedure but staining. After three cycles of washing operations, the harvested bacterial suspension was transferred to a clean glass slide and left to be static overnight. The supernatant on the glass slide was replaced by 2.5% glutaradehyde solution for 4 h of immobilization. Ultimately, serial concentrations of dehydrating agent were prepared from absolute ethyl alcohol, resulting in final concentrations of 30%, 50%, 70%，90% and 100%. Once four groups of samples were completely dried, SEM was involved in the detection of microscopic morphology after coating with gold.

2.10 In Vitro Biofilm Obliterating Efficiency

Growing evidences have suggested that bacteria were not the defectors of organization or working alone but evolving biofilms with complicated structure. Moreover, biofilm acted as a safe harbor for planktonic bacteria cause wide dissemination of resistance. Despite great achievements in killing planktonic bacteria, it doesn’t mean p(GEA-*b*-AAPBA)@CuS nanotherapeutics is good at eliminating biofilms. Therefore, it’s meaningful to evaluate the performance of obliterating biofilms. Here, reliable technique, CLSM imaging was invited to attend this experiment.

CLSM could visually witness the formation as well as eradication of biofilms which make it stand out among numerous advanced devices. Initially, a single colony of bacteria was transferred from ager plate into LB medium and routinely cultured at 37℃. The dense bacterial cells were diluted with fresh medium and the optical density at 600 nm of bacteria was adjusted to 0.02. Thereafter, preprocessed glass cover slips (10 × 10 mm) were placed into a 12-well plate, followed by adding 2 mL of inoculum in each well. Bacterial cells adhered to the surface of glass and presently evolved to biofilms during 24 h. The harvested mature biofilms were managed with p(GEA-*b*-AAPBA)@CuS nanoparticles at the highest concentration and irradiated continuously for 5 min. Upon sample preparation for CLSM assay, biofilms were fixed with 4% glutaradehyde diluent for 4 h and rinsed with PBS for three times. Subsequently, 10 μL of FITC-ConA and EB were added in biofilms and retained in dark for 15 min. In the end, redundant dyes were removed by rinsing with PBS for at least 5 times and resultant biofilms were visualized using a confocal laser scanning microscope. Biofilms treated with PBS were selected as control but dealt with the same process.

To quantify the biofilm survival percentages, the viable bacterial cells in biofilm were transformed into sterile PBS. After homodisperse, obtained bacterial suspension was diluted 10000-fold for a standard plate counting assay. Specifically, 50 μL of the sample suspension was inoculated onto ager plates and allowed to grow for 10 h. Finally, the colony-forming units were imaged and counted.

2.11. Cytotoxicity of p(GEA-b-AAPBA)@CuS Nanoparticles to Lung Cancer Cells (A549)

Biosafety of p(GEA-*b*-AAPBA)@CuS nanoparticles towards A549 cells was detected by using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. And lung cancer cells (A549) were kindly supported by Tianjin Medical University (Tianjin, China). Culture medium was acquired in such a recipe that 50 mL of Dulbecco's modified Eagle's medium (DMEM) contained 10% foetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin). As for cultivation environment, an incubator endowed with 95% humidity and 37℃ came to service. Hence, A549 cells were revived in an incubator firstly, and then fresh cell seeds were transferred into a 96-well plate with 5×10^3 cells/well and allowed to grow by static adherence for 24 h. Culture medium was abandoned and replaced by p(GEA-*b*-AAPBA)@CuS at serial concentrations (a range from 11 to 170 μg/mL). Additional 24 h was consumed, subsequently p(GEA-*b*-AAPBA)@CuS nanoparticles were removed by repeated rinse. Ultimately, MTT working solution was added to each well and incubated for additional 4 h to enable color development. A microplate reader was invited to record the absorbance at 540 nm and each concentration of p(GEA-*b*-AAPBA)@CuS was performed in three times. Similarly, A549 cells were treated with NIR irradiation for 5 min, the phototoxicity of the nanotherapeutic was also evaluated.

References

[1] Y. Liang, Z. Li and F. Li, *J. Colloid Interface Sci.*, 2000, **224**, 84-90

[2] Q. Guo, Z. Wu, X. Zhang, L. Sun and C. Li, *Soft Matter*, 2014, **10**, 911

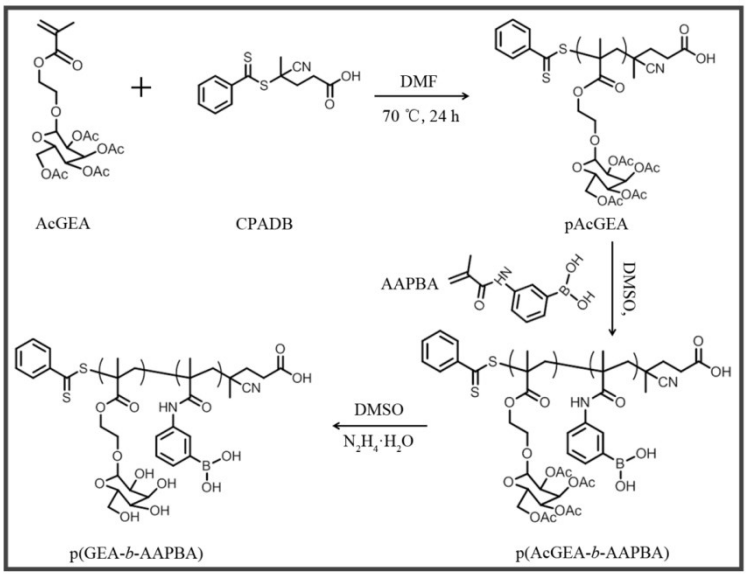

Figure S1. Synthetic route of pAcGEA, p(AcGEA-*b*-AAPBA) and p(GEA-*b*-AAPBA).

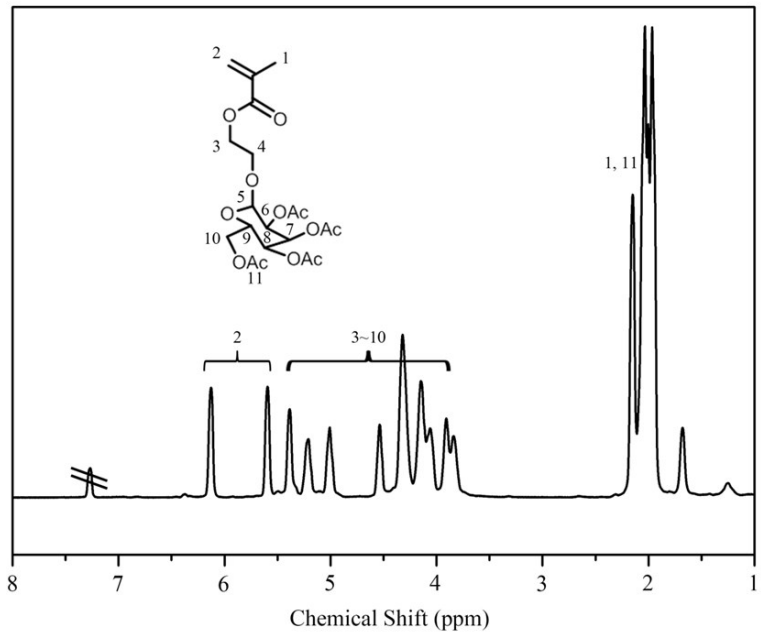

Figure S2. ^{1}H NMR spectrum of AcGEA in $CDCl_3$.

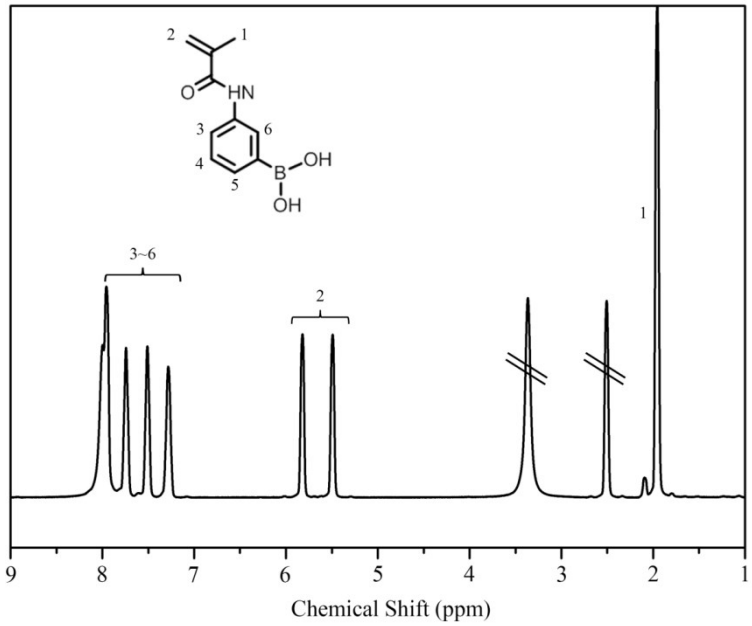

Figure S3. ^{1}H NMR spectrum of AAPBA in DMSO-d_6。

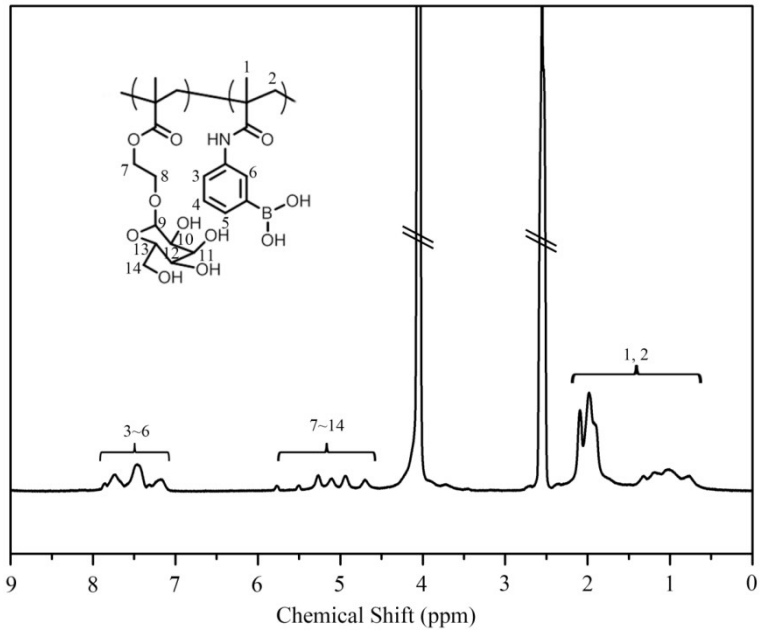

Figure S4. ^{1}H NMR spectrum of p(GEA-*b*-AAPBA) in DMSO-d_6.

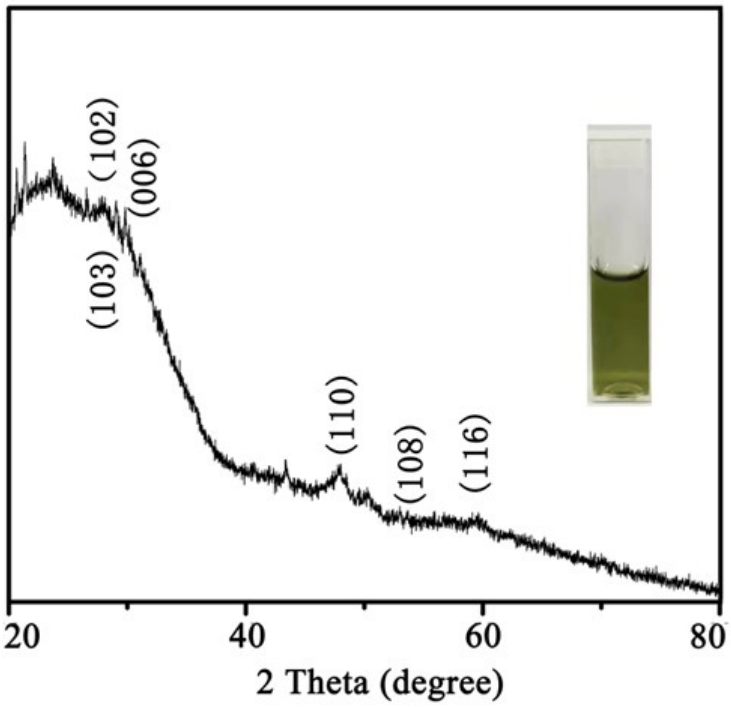

Figure S5. XRD pattern of the nanotherapeutic.

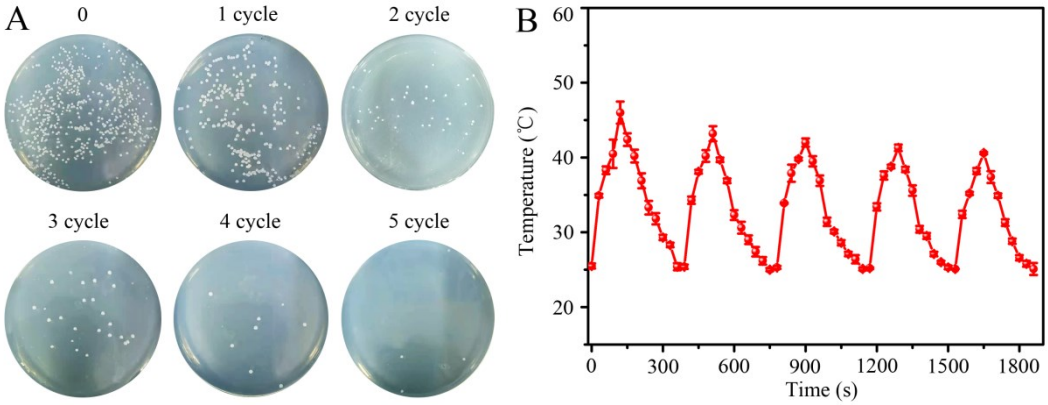

Figure S6. Photographs of bacterial colony formed bacteria after the various treatments by 0, 1, 2, 3, 4 and 5 cycles for irradiation (A). Temperature change of nanotherapeutic under NIR irradiation in five cycles (B).

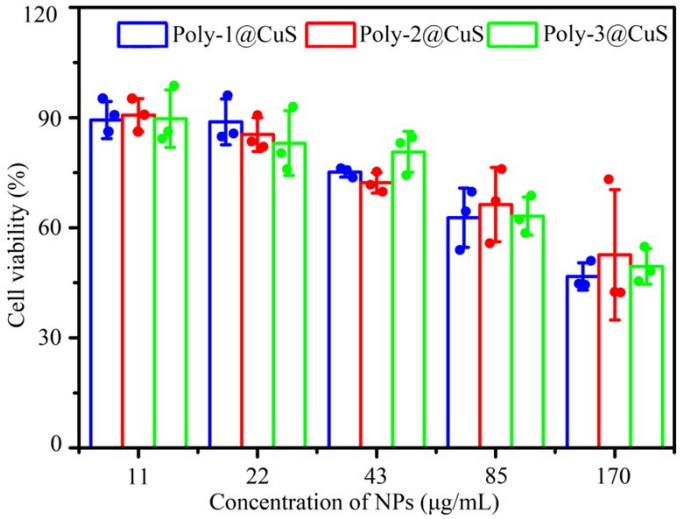

Figure S7. Cell viability of A549 incubated with different nanoparticles after 5-min NIR irradiation.

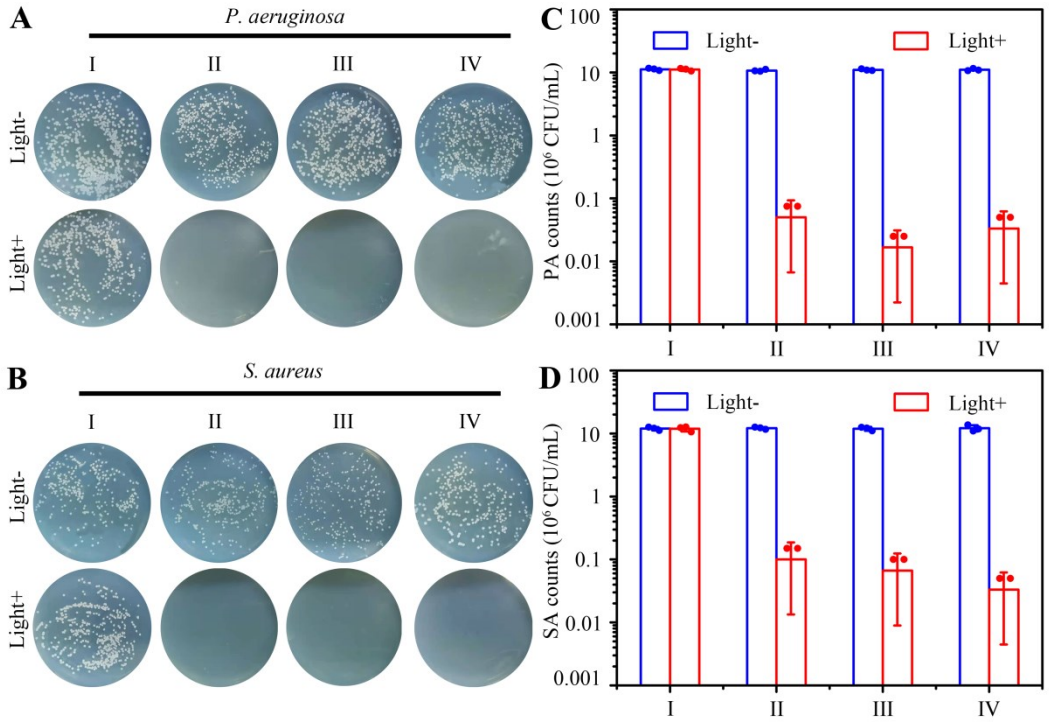

Figure S8. Photographs of bacterial colonies formed by *P. aeruginosa* (A) and *S. aureus* (B) after the various treatments by I: PBS, II: Poly-1@CuS, III: Poly-2@CuS and IV: Poly-3@CuS with and without light irradiation. Relative bacteria survival rates of *P. aeruginosa* (C) and *S. aureus* (D) after different treatments with or without irradiation for 10 min.

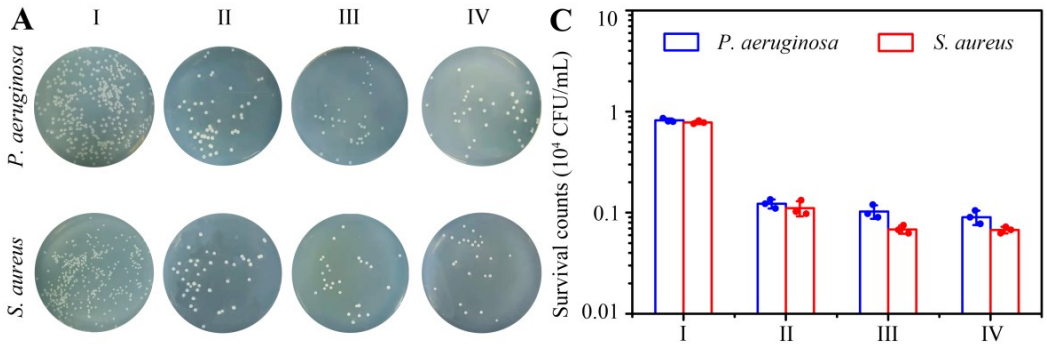

Figure S9. *P. aeruginosa* and *S. aureus* colonies detached from biofilms after different treatments by I: PBS, II: Poly-1@CuS, III: Poly-2@CuS and IV: Poly-3@CuS (A) and corresponding survival counts (B).

Table S1. Characterizations of amphiphilic block copolymers.

Samples	feeding[a]	product[b]	Mn[b]	Mn[c]	Mw/Mn[c]
$pAcGEA_{15}$	15	12	9.7	7.1	1.15
$pAcGEA_{20}$	20	16	14.3	8.5	1.17
$pAcGEA_{25}$	25	20	21.9	9.7	1.18
$p(GEA_{15}$-*b*-$AAPBA_{10})$	15/10	12/9	8.3	--	--
$p(GEA_{20}$-*b*-$AAPBA_{10})$	20/10	16/9	11.8	--	--
$p(GEA_{25}$-*b*-$AAPBA_{10})$	25/10	20/9	14.5	--	--

[a]The theoretical values. [b]Calculated from ^{1}H NMR. [c]Determined by GPC.