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Supporting Information

Cyclic topology enhances killing activities of polycation against planktonic and biofilm bacteria

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Experimental Section

Materials

Rhodanine (99%), tetrabutylammonium chloride (TBAC, 99%) were purchased from Alfa Aesar. 2-((allyloxy)methyl)thiirane (PEMT)[1] and S,S'-bis(α,α'-dimethyl-α''-acetic acid)-trithiocarbonate (BDMAT)[2] was synthesized as reported before. Benzoin dimethyl ether (DMPA, 98%) was purchased from Energy Chemical. Chloroform-d ($CDCl_3$, 99.8 atom % D), DMSO-d_6 (99.8 atom % D) were purchased from Sigma-Aldrich. Petroleum ether (60~90, AR) was purchased from Chengdu Chron Chemicals Co., Ltd. N-Methylpyrrolidone (NMP, AR>99.0%, GC) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Ethanol (AR), N,N-Dimethylformamide (DMF, AR) were purchased from Sinopharm Chemical Reagent Co., Ltd. Sterile trypsin soy broth (TSB), Mueller–Hinton (MH) medium and agar powder were purchased from Qingdao Hope Bio-Technology Co., Ltd. All reagents were used as received unless otherwise stated.

General analytical information

All ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectra were recorded on a Bruker NMR spectrometer (AVANCE III) with resonance frequency of 400 MHz. Before test, the samples (5.0~10.0 mg) were dissolved in chloroform-d or DMSO-d_6 (0.6 mL) with tetramethylsilane (TMS) as internal reference. Zeta potential measurement was conducted in Dynamic light scattering (DLS, Brookhaven, NanoBrook 90PlusPALS) at 25 °C (3 Cycles, 3 Runs, 5 s Inter Cycle Delay) with palladium (Pd) as electrode (SR-691) and the obtained data were managed in BIC Particle Solutions v. 3.5 software. The bacteria were cultured in intelligent thermostatic oscillator (HNY-100D, Tianjin Honour Instrument Co., LTD) at 37 °C with 100 rpm/min cyclotron oscillation. Visible spectrophotometer (V-T3, Shanghai Yipu Instrument Co., LTD) was running at room temperature to determine the bacterial growth period and the samples were loaded in 400 μL glass cuvette. The optical density (OD) was read on a microplate reader (Thermo SCIENTIFIC, VARIOSKAN FLASH) at room temperature after 5 min of shaking. Bacterial Morphology assay was record by scanning electron microscope (SEM, SU8220, Hitachi) at an accelerating voltage of 3.0 KV.

Polymer synthesis and characterization

The cyclic polymer C-P(PEMT)$_{60}$ was obtained through rhodanine-initiated anionic ring-opening polymerization. In brief, rhodamine (99.0 mg, 0.75 mmol), PEMT (5.85 g, 45 mmol), TBAC (625.0 mg, 2.25 mmol) were dissolved in NMP to obtain 15 mL solution and transferred into a transparent glass tube. After three freeze-pump-thaw cycles to remove oxygen, the tube was sealed and reaction was conducted in an oil bath at 75 °C for 24 h. Afterwards, 10 mg of result solution was dissolved in 0.6 mL of $CDCl_3$ and the ^{1}H NMR spectra were recorded to character the monomer conversion rate. After that, the result solution was precipitated into petroleum ether/ethanol (v/v, 5/1) several times and the product as brown viscous solid was obtained after being centrifuged and dried in vacuum. C-P(PEMT)$_{100}$ was synthesized through adjusting the proportion of reactants. The linear polymer L-P(PEMT)$_{60}$ and L-P(PEMT)$_{100}$ were synthesized through BDMAT- initiated polymerization with similar experimental operation.

The polymer was functionalized with trialkyl amine by thiol-ene click reaction[3]. Briefly, 0.5 g of C-P(PEMT)$_n$, 1.0 g of 2-(diethylamino)ethyl mercaptan hydrochloride and 20.0 mg of photoinitiator DMPA were dissolved in DMF in a silica tube. The reaction was stirred under argon atmosphere and subjected to UV irradiation for 24 h. Afterwards, the result solution was dialyzed against ethanol (MCOW: 1000) and precipitated into petroleum ether. Orange red viscous solid was obtained after being centrifuged and dried in vacuum. The linear analogues were functionalized in the same way. The ^{1}H NMR spectra were recorded to character the obtained polymers. Each spectrum was recorded in duplicate.

Culturing and harvesting of bacterial strains and bactericidal test

The polycations' bactericidal activities against *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) were evaluated by performing plate bacterial killing assays. Briefly, 3–5 individual colonies were inoculated into fresh TSB medium and incubated at 37 °C for 16–18 h to stationary phase. 40 μL of bacterial suspension was diluted with fresh TSB by 100-fold and regrown at 37 °C to logarithmic phase (OD_{600}=0.4-0.6). Afterwards, bacteria were centrifuged (10,000 × g for 5 min at 4 °C), harvested, washed and adjusted with sterile PBS to ~1.5×10^6 colony-forming units (CFU) per milliliter, and 50 μL of adjusted bacterial suspension was inoculated into 96-well microplate. 100 μL serial 2-fold dilutions of polycation solutions made with sterile PBS were added into each well in 96-well microplate to achieve 5×10^5 CFU/mL of bacterial suspension in each well (150 μL). The 96-well plate was then incubated at 37 °C for 3 h and 100-

fold dilutions were made with sterile PBS buffer, followed by plating the dilutions (20 μL) onto MH agar plates for overnight incubation at 37 °C to form visible colonies. Inoculum size was indicated by control samples containing bacteria treated uniformly but without polycation. The results were photo recorded and the number of bacteria was counted. Each test was performed in triplicate and the data was expressed as mean and standard deviation of 3 replicates.

Minimum inhibitory concentration (MIC) assay was performed to evaluate the inhibitory activity of polycations against *E. coli*. Bacteria in logarithmic phase of growth were centrifuged (10,000 × g for 5 min at 4 °C), harvested, washed and diluted in MH medium to 3×10^5 CFU/mL as the working suspension. The polycation aqueous solution was diluted to the desired concentration in MH medium by two-fold serial dilution and added into a 96-well plate. 100 μL bacterial suspension was added into each well, which was then incubated at 37 °C. The OD value was recorded at 600 nm on a microplate reader after 12 hours. MH medium without sample solution was used as control. The reported MIC values are defined as the minimum concentrations of antibiotics or polycations to inhibit 90% bacterial growth. Each test was performed in triplicate and the data was expressed as mean and standard deviation of 3 replicates.

Bacterial live/dead viability assays were conducted to confirm the membrane permeabilization of PAO1 with different polycations. The PAO1 in logarithmic phase of growth was centrifuged (10,000 × g for 5 min at 4 °C), harvested, washed and diluted with PBS to 1.5×10^8 CFU/mL. As-adjusted bacterial suspension was then diluted with different polycations solutions to 1.0×10^8 CFU/mL and a final polycations concentration was 100 μg/mL. After 3 hours incubation at 37 °C, the bacteria were centrifuged (10,000 × g for 5 min at 4 °C) and subsequently stained with propidium iodide (PI, 10 μg/mL in PBS, 500 μL) by incubation in the dark for 15 min. The resulting mixing suspension was centrifuged (10,000 × g for 5 min at 4 °C) to remove the supernatant and the bacteria were washed with PBS twice. Flow cytometry was than applied to measure the red fluorescence intensity of PAO1 bacteria. The test was performed in triplicate.

Bacterial morphology observation and surface potential characterization

Scanning electron microscopy (SEM) was used to observe the bacterial morphology after treatment with PBS and polycation solution to investigate the antibacterial mechanism. *E. coli* was inoculated, incubated, harvested, washed once with sterile PBS via centrifugation (10,000×g for 5 min at 4 °C) and adjusted with sterile PBS to ~1.5×10^8 CFU/mL. Polycation solution in sterile PBS buffer (84 μL) was added into 166 μL adjusted bacterial suspension, to achieve a final bacterial concentration of ~1.0×10^8 CFU/mL and a final polycation concentration of 50 μg/mL. After 3 h incubation at 37 °C, the bacteria were centrifuged (10,000 × g for 5 min at 4 °C), harvested and washed with PBS, followed by fixation with 4% glutaraldehyde at 4 °C for 3 h and dehydration successively with a series of graded ethanol solutions (30, 50, 70, 90, 95, and 100%) via centrifuge (10,000 × g for 5min). The resulting bacteria were then dispersed in 500 μL ethanol, and one drop of bacterial suspension was dropped on a silicon pellet and dried overnight. After gold sputtering, the sample was imaged under SEM (FEI Apreo, USA). Control group was those disposed uniformly but without polycations treatment. The SEM measurements were repeated twice to confirm that the observations were credible.

The Zeta potential of *E. coli* was measured to character the transformation of bacterial surface potential. As SEM measurements operation, after 3 h incubation with polycations, centrifugation and harvest, the *E. coli* was then dispersed in water and the Zeta potential was measured with DLS. The *E. coli* disposed with pure PBS was as control. The Zeta potential measurements were repeated twice to confirm that the results were credible.

Resistance test

The drug-resistance of polycations against *E. coli* was detected compared with polymyxin B. The *E. coli* was cultured in MH medium containing drugs with the previous generation 0.5 MIC concentration at 37 °C for 12 h. The MIC of *E. coli* after incubation with polycations and polymyxin B at each four generations were measured by the MIC assay procedure as described above. Each MIC test was performed in triplicate and the reported results are the averages of them.

General procedures for bacterial biofilm formation and harvesting

P. aeruginosa or PAO1 biofilm was used for different experiments. Briefly, MH medium (300 μL) and *P. aeruginosa* suspension (10^8 CFU/mL, 100 μL) were added into each well in a 24-well plate, which was placed in an incubator and cultured at 37 °C still. After 24 h incubation, the timeworn medium was replaced

with fresh MH medium gently, and the biofilms were cultured for another 24 h, which were used for further experiments.

Bactericidal test in biofilm

The killing activity against biofilm bacteria was firstly evaluated by colony forming unit assay. In brief, 200 μL of 50 μg/mL polycation aqueous solution was added into the *P. aeruginosa* biofilm in 24-well plate and equal sterile PBS was added as control. The 24-well plate was setting still at 37 °C for 4.0 h. After that, the biofilm was smashed and diluted into sterile PBS $10^6/10^7$ times. Colony forming unit assay as described in bactericidal test was conducted to count the bacterial number in the biofilm. The test was repeated 3 times and the data was expressed as mean and standard deviation of 3 replicates.

The inhibitory property of biofilm growth was evaluated. Briefly, the obtained *P. aeruginosa* biofilm was treated with polycation solution (80 μg/mL) in MH medium overnight. The biofilm treated with pure MH medium was the control. After smashed, the biofilm was diluted into sterile PBS $10^7/10^8$ times. The number of biofilm bacteria after 12 h incubation with cyclic/linear polycation or without polycation at MH medium were obtained using colony forming unit assay. The test was repeated 3 times and the data was expressed as mean and standard deviation of 3 replicates.

Biofilm was stained by PI after incubation with polycations to measure the bactericidal ability in biofilm. Briefly, the PAO1 biofilm was inoculated in a glass bottom cell culture dish (BS-20-GJM) with MH medium and washed with sterile PBS before used. The 1.0 mL of 200 μg/mL polycation solution was then added gently and 3 hours still incubation was conducted at 37 °C. After washed twice with sterile PBS, the biofilm was covered with 1.0 mL of 10 μg/mL PI/PBS solution and stained for 20 min under dark. After staining solution removed, the biofilm was washed by PBS twice for 3-dimensional fluorescence confocal laser scanning microscopy (CLSM) imaging. The fluorescence of green fluorescent protein (GFP) expressed by PAO1 and PI attached to dead in the biofilm were collected at 500−535 nm (GFP) and 583−688 nm (PI) respectively. The test was repeated 3 times.

Hemolytic evaluation

The hemolytic property of polycation was evaluated using the fresh human blood. The fresh blood was resuspended to 20% (v/v) in sterile PBS. Polycation was prepared in sterile PBS and diluted with sterile PBS to different concentration. Then, the equal volumes of blood suspension (100 μL) were added to the polycation/PBS solution (400 μL) and the final concentration of polycation was 2, 4, 8, 16, 32, 64, 128 μg/mL. The mixture was incubated at 37 °C for 1 h. PBS was used as the blank, and human red blood cells suspension in sterile deionized water was used as 100% hemolysis. After centrifugation, the supernatant (100 μL) in each tube was transferred to a new 96-well plate. The OD values was detected at a wavelength of 576 nm on a microplate reader. The percentage of hemolysis was calculated by the equation: Hemolysis (%)=$[(OD_{sample}-OD_{blank})/(OD_{100\%\ hemolysis}-OD_{blank})]\times100$. The tests were repeated three times and the data was expressed as mean and standard deviation of 3 replicates.

Cytotoxicity assay

Cytotoxicity of polycation against 293T cells was valued using methyl thiazolyl tetrazolium (MTT) assay. About 2.0×10^4 293T cells were seeded into each well of 96-well plate in H-DMEM (Hyclone) medium with 10% fetal bovine serum (Hyclone) and cultured for 24 h in a humidified 5% CO_2 in air incubator. Then, 100 μL of polycation solution in H-DMEM was diluted to the desired concentration, and replaced the original medium in 96-well plate. The plate was incubated for 12 h in a humidified 5% CO_2 in air incubator. 10 μL MTT solution in PBS (5 mg/mL) was added to each well of 96-well plate and the plate was incubated for 4 h in air incubator. After aspirating original solution, 150 μL of DMSO was added into each well. The plate was shaken for 10 min in order to completely dissolve the purple solid. Cells in H-DMEM without polycation were used as the positive control. The OD values was detected at a wavelength of 490 nm on a microplate reader. The percentage of cell viability was calculated by the equation: Cell viability (%) = $[(OD_{sample} - OD_{blank})/OD_{positive\ control}-OD_{blank})]\times100$. The tests were repeated three times and the data was expressed as mean and standard deviation of 3 replicates.

Antibacterial activity test *in vivo*

The therapeutic efficacy on wound healing of cyclic and linear polycation was evaluated using *E. coli* infection model on mouse skin. BALB/c female mice (6 weeks, 15-20 g) were obtained from Animal Experiment Centre of Anhui Medical University. Before experiment, the mice were cultured in quarantine for acclimatization and detection for 1-2 weeks. After depilation on the back one day in advance, the mice were anesthetized with 1% pentobarbital sodium/PBS solution at a dose of 100 μL, and wound (10 × 10

mm) was created on the back of mice using biopsy punch. To establish a *E. coli* infection model, 20 μL pre-cultured bacterial suspension (OD_{600}=0.5, in PBS) added on the wound and covered with a sterile dressing to continue the incubation for 24 h. Afterwards, all the mice were divided into four groups and each group contained at least five mice. 50 μL samples solution (PBS, L-P(PEMT-DEA)$_{100}$, C-P(PEMT-DEA)$_{100}$, polymyxin B, 100 μg/mL) was dropped on the wound, respectively. The wound was photographed and measured every day. The presented wound size was the average wound size of five mice in each group. After 9 days recovery, the wounds were harvested, three of which were then homogenized and colony forming unit assay was conducted to evaluate the number of the remaining bacteria in the wounds. The average of the remaining bacteria was reported. The H&E and Masson staining of another two wounds were applied and photographed and one of the results were represented here. H&E staining tissue sections from mouse was conducted to evaluate the toxicity of polycation *in vivo*.

Supporting figures

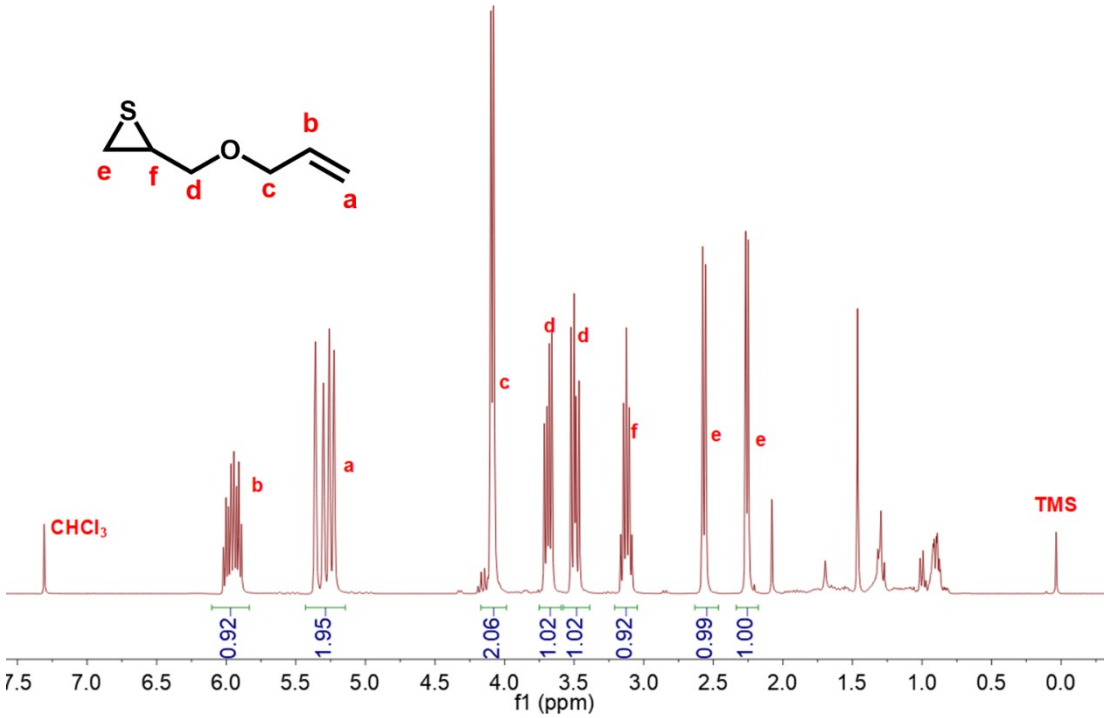

Figure. S1. The structure and corresponding ^{1}H NMR spectrum of PEMT.

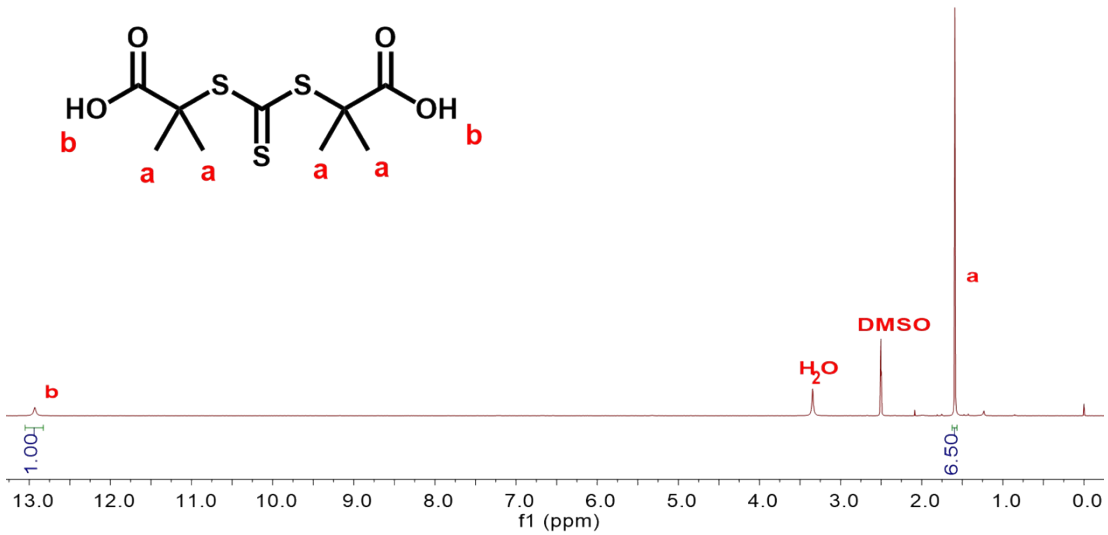

Figure. S2. The structure and corresponding ^{1}H NMR spectrum of BDMAT.

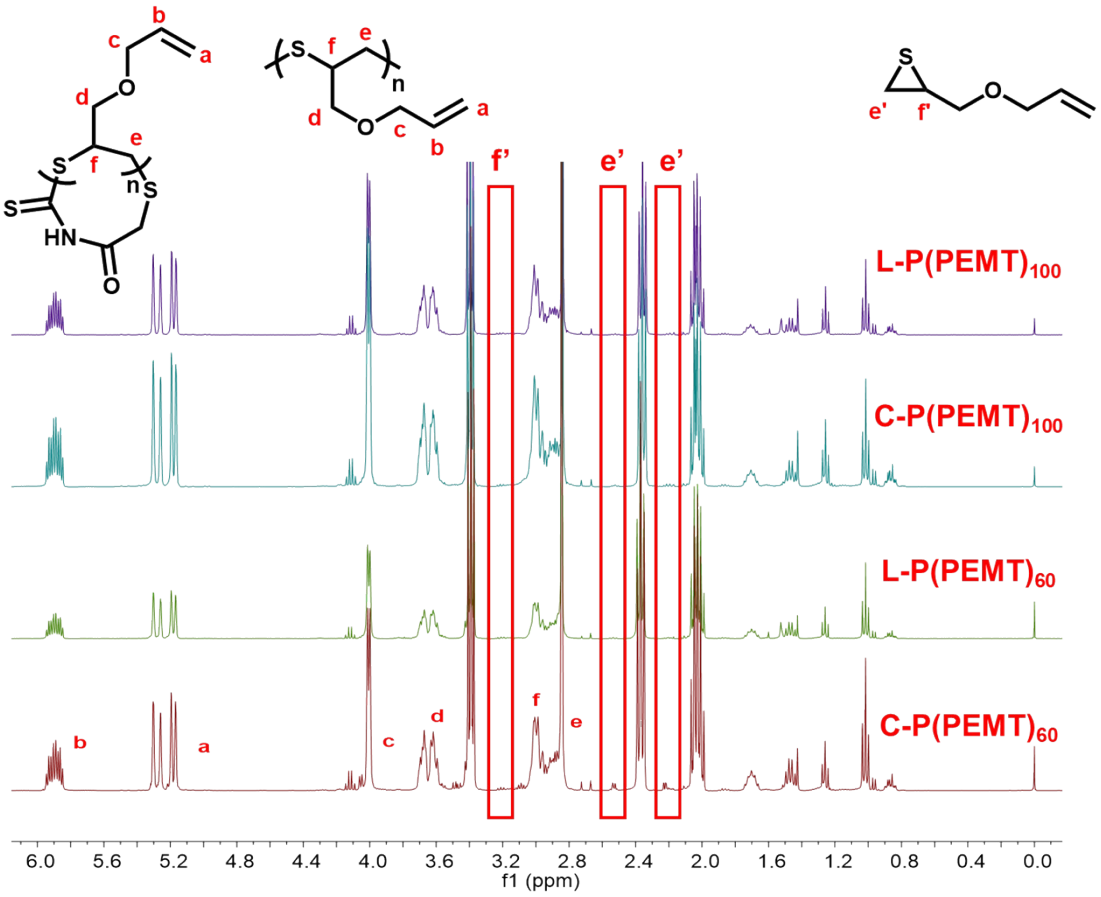

Figure. S3. The structure and corresponding ^{1}H NMR spectra of the result solution after the synthesis of L-P(PEMT)$_{60}$, C-P(PEMT)$_{60}$, L-P(PEMT)$_{100}$ and C-P(PEMT)$_{100}$, from which the monomer conversion rate could be figured out.

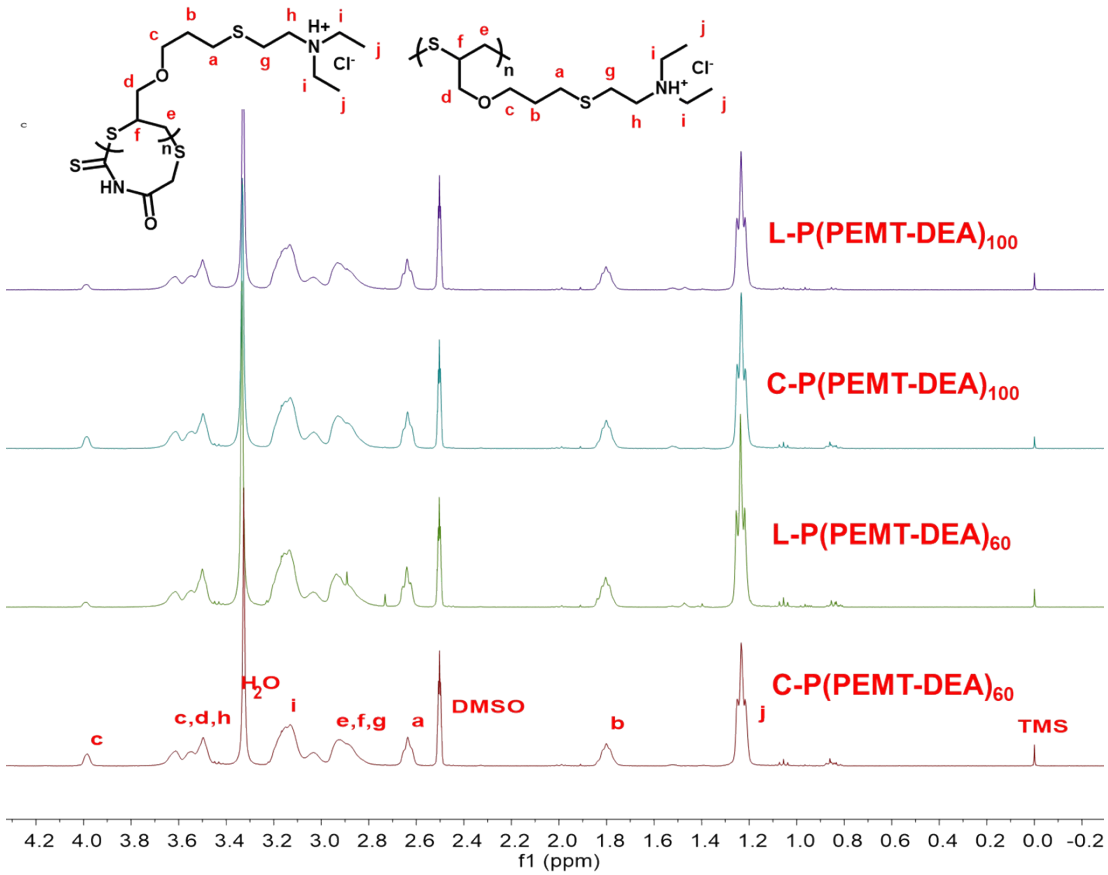

Figure. S4. The structure and corresponding ^{1}H NMR spectra of L-P(PEMT-DEA)$_{60}$, C-P(PEMT-DEA)$_{60}$, L-P(PEMT-DEA)$_{100}$ and C-P(PEMT-DEA)$_{100}$.

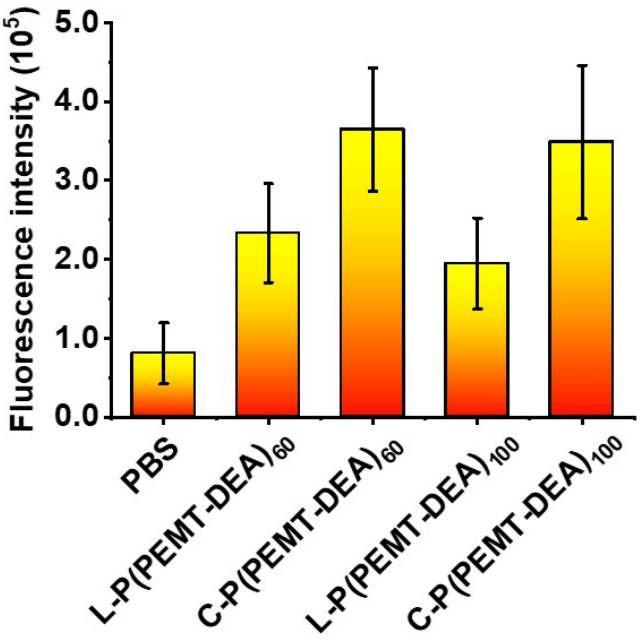

Figure. S5. Red fluorescence intensity of PAO1 stained with PI after incubated with PBS, L-P(PEMT-DEA)$_{60}$, C-P(PEMT-DEA)$_{60}$, L-P(PEMT-DEA)$_{100}$ and C-P(PEMT-DEA)$_{100}$, which was detected by flow cytometry.

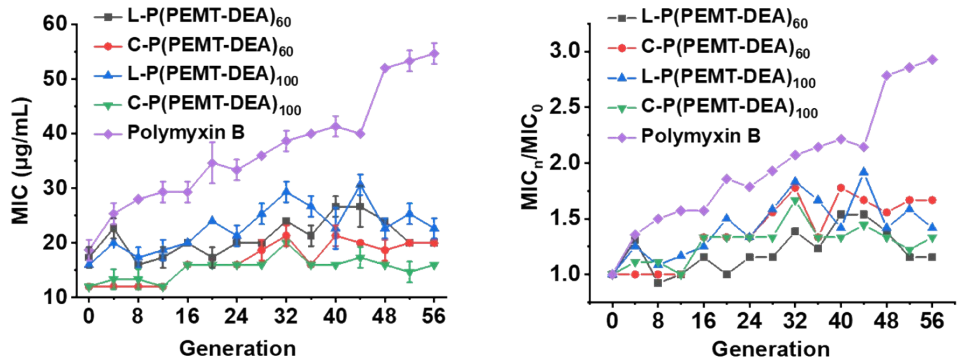

Figure. S6. The MIC of L-P(PEMT-DEA)$_{60}$, C-P(PEMT-DEA)$_{60}$, L-P(PEMT-DEA)$_{100}$, C-P(PEMT-DEA)$_{100}$ and polymyxin B against *E. coli* after the treatment of them repeatedly at a 0.5 × MIC concentration.

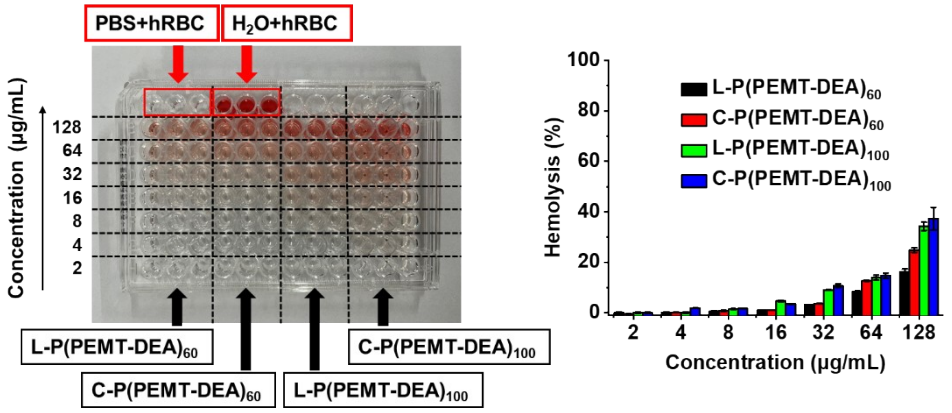

Figure. S7. Hemolytic properties of L-P(PEMT-DEA)$_{60}$, C-P(PEMT-DEA)$_{60}$, L-P(PEMT-DEA)$_{100}$ and C-P(PEMT-DEA)$_{100}$.

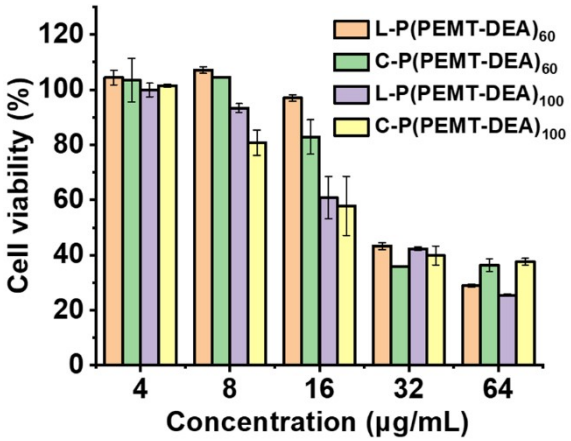

Figure. S8. Cell viability of 293T after the treatment of L-P(PEMT-DEA)$_{60}$, C-P(PEMT-DEA)$_{60}$, L-P(PEMT-DEA)$_{100}$ and C-P(PEMT-DEA)$_{100}$.

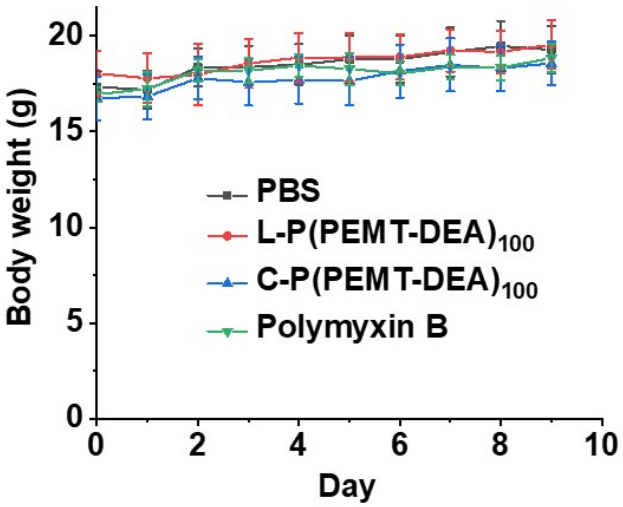

Figure. S9. The weight changes of each group during the wound repairing experiment.

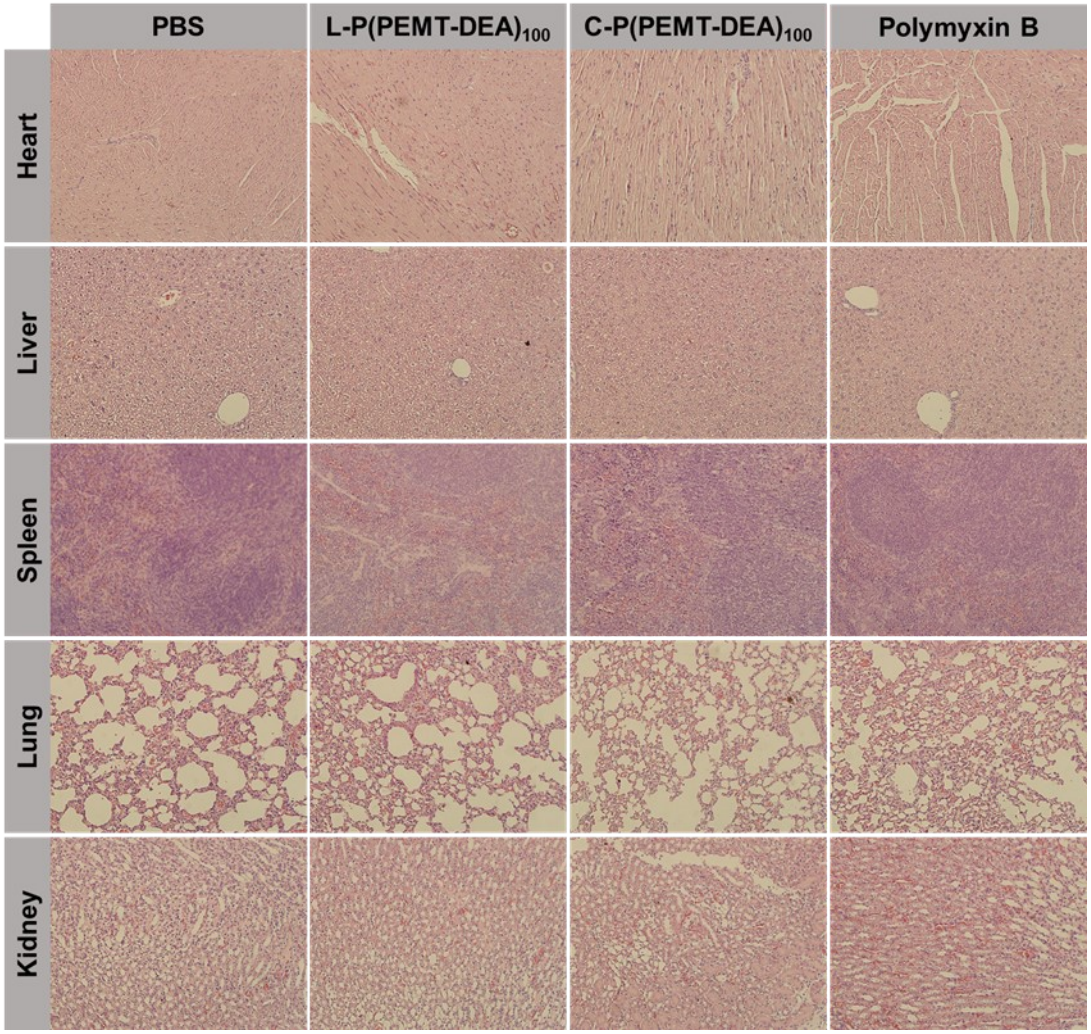

Figure. S10. H&E staining tissue sections from mouse after treated with PBS, L-P(PEMT-DEA)$_{100}$, C-P(PEMT-DEA)$_{100}$ and polymyxin B.

Notes and references

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