Supporting Information

Combating Pseudomonas aeruginosa Biofilms by a Chitosan-PEG-Peptide Conjugate via Changes in Assembled Structure

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Experimental Section

Materials

Chitosan (Mw = 50 kDa, degree of deacetylation > 95%) was supplied from Chengdu Yuannuo Tiancheng Technology CO., LTD. in Chengdu, China. Carboxyl and azide-terminated polyethylene glycol (COOH-PEG-N_3, M_W = 1 kDa) was purchased from Tuoyang Biotechnology CO., LTD. in Shanghai, China. The artificially designed peptide with an alkynyl group at its C-terminus (LKLLKKLLKKLKK-

propargylglycine, LK_{13} peptide) was supplied from GL Biochem Ltd. in Shanghai, China. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl, Aladdin) and N-Hydroxysuccinimide (NHS, Aladdin) were used for the PEGylation of chitosan. 2-(N-Morpholino)ethanesulfonic acid monohydrate ($MES·H_2O$, Aladdin), aminoguanidine (Sigma-Aldrich), ascorbic acid sodium (Sigma-Aldrich), copper (II) sulfate pentahydrate ($CuSO_4·5H_2O$, Beijing Chemical Works), ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) were used for conjugating the LK_{13} peptide onto the PEGylated chitosan.

Sodium dodecyl sulfate (SDS, Aladdin), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) sodium salt (POPG, ApexBio) and 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (DOPC, Avanti) were used for mimicking the membrane structure in the circular dichroism (CD) test.

Tryptic soy broth (TSB), nutrient broth (NB), Luria-Bertani (LB, Beijing Aoboxing Bio-Tech CO., LTD.), Mueller-Hinton agar (MH agar, OXOID) were used for the bacteria culture. Resazurin (Aladdin), propidium iodide (PI) (Invitrogen Detection Technologies), 8-anilinonapthalene-1-sulfonic acid (ANS, Aladdin) and Live/Dead BacLight Bacterial Viability Kits-L7012 (Invitrogen Detection Technologies) were used for the characterization of antibacterial efficiency and antibacterial mechanism.

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin (P/S) were purchased from Life Technologies and used for cell culture. Cell Counting Kit-8 (CCK-8, DOjinDO Molecular Technologies, ING) was used for the cell growth inhibition assessment.

Synthesis of CS-PEG-LK_{13}

The CS-PEG-LK_{13} was synthesized in two steps (Fig. S1). Firstly, the chitosan was PEGylated through an EDC/NHS activated reaction between the amino groups of chitosan and the carboxyl groups of the COOH-PEG-N_3[1]. In brief, 100 mg chitosan was dissolved in 10 mL 25 mM pH 5.0 MES buffer to form chitosan solution. Then 100 μL dilute hydrochloric acid (1 M) was added to promote chitosan dissolution. Meanwhile, 0.62 g COOH-PEG-N_3 (1 eq per amino groups of chitosan) was dissolved in 10 mL 25 mM pH 5.0 MES buffer in another bottle. 0.48 g EDC (4 eq per amino groups of chitosan) and 0.29 g NHS (4 eq per amino groups of chitosan) were further added to activate carboxyl group. The activation reaction solution was stirred at room temperature for 30 min, and then was added into the chitosan solution for a further 48-

h reaction with stirring at room temperature. The obtained PEGylated chitosan (CS-PEG) was purified by dialysis (MWCO = 3,500 Da) in deionized water and then obtained by lyophilization.

Secondly, the LK_{13} peptide was conjugated onto the azido groups of CS-PEG through a click reaction. The click chemistry of azido and alkynyl groups was referred to the previously reported and slightly modified[2]. 6.9 mg CS-PEG (5.92 μmol) and 10 mg LK_{13} peptide (5.92 μmol) were dissolved in 583 μL 150 mM pH 6.0 MES buffer to form solution 1. 118 μL 0.5 M $CuSO_4$ aqueous solution was mixed with 118 μL 1 M sodium ascorbate aqueous solution under ice bath conditions to obtain the yellow precipitate Cu(I) solution, and then was mixed with 118 μL 1 M aminoguanidine aqueous solution, to obtain the white precipitate solution 2. Add solution 2 to solution 1, then the mixture was kept stirring at 4 °C for 24 h. After the reaction was completed, the mixture was in green color. EDTA/Na (pH ≈ 8, 100 mM, 2.96 mL) was added and further stirred for another 1 h. The resulted CS-PEG-LK_{13} was purified by dialysis (MWCO = 3,500 Da) in deionized water and then obtained by lyophilization.

Characterization of CS-PEG-LK_{13}

An Excalibur 3100 fourier transform infrared spectrometer (FTIR, Varian, America) and an Avance 400 nuclear magnetic resonance spectrometer (NMR, Bruker, Germany) were applied to investigate the molecular structure of CS-PEG-LK_{13}. The mass ratio of the grafted peptides to chitosan was determined by the integrals of peptide and chitosan ^1H NMR signals. Zeta potentials were measured by a Zetasizer nano ZSP (Malvern). The morphology of the CS-PEG-LK_{13} assembly was assessed through an HT7700 transmission electron microscope (TEM, Hitachi, Japan). The sample was negatively stained with uranyl acetate. The radius of hydration was measured by a Dybapro NanoStar Dynamic Light Scattering (DLS). The secondary conformation was analyzed through a CD spectropolarimeter (JASCO, J-815) at room temperature. The path length of the quartz cuvette was 1.0 mm, and the spectral scanning range was 190-250 nm. Each sample was scanned for 3 times at a scanning speed of 100 nm/min. The CD spectra were obtained by removing the solvent background.

Bacteria culture

Bacterial strains were purchased from China General Microbiological Culture Collection Center (CGMCC). They are all derived from American Type Culture

Collection (ATCC). The bacterial strains in this study are Pseudomonas aeruginosa (*P. aeruginosa*, ATCC 15442), Escherichia coli (*E. coli*, ATCC 25922) and Staphylococcus aureus (*S. aureus*, ATCC 6538). P. aeruginosa was incubated in NB medium. E. coli and S. aureus were incubated in TSB medium. They were all cultured in an oscillating incubator at a speed of 200 rpm, at 37 °C for 18-24 h.

Minimum inhibitory concentration (MIC) measurement

The antibacterial test for planktonic bacteria was performed by a modified micro broth dilution method[3]. The bacterial suspension was cultured overnight and diluted to a concentration of 10^5 CFU/mL in broth. 100 μL of LK_{13} peptide or CS-PEG-LK_{13} assembly solutions at different concentration were added in advance in a sterilized 96-well plate. Then 100 μL of bacterial suspension was added to each well. 200 μL broth was used as a negative control, and 100 μL broth plus 100 μL bacterial suspension was used as a positive control. Then the plate was placed in an incubator and cultured for 12-16 h. 20 μL resazurin aqueous solution (0.625 mg/mL) was added in each well. After another culture for 2-4 h, the MIC was determined according to the color change. A pink color means that there are bacteria existing. And a purple color means that no bacteria are existing.

Hemolysis assay

5 mL of fresh rabbit blood (Solarbio Science & Technology CO., LTD.) was centrifuged at 1000 g for 10 min to obtain red blood cells. The red blood cells was washed three times with Tris buffer (10 mM Tris, 150 mM NaCl, pH = 7.2) until the supernatant is clear. 100 μL of samples at different concentrations in Tris buffer and 100 μL of red blood cells were added to centrifuge tubes and incubated at 37 °C for 1 h. After centrifuging the mixture at 1000 g for 10 min, 50 μL of the supernatant and 50 μL Tris buffer were transferred to a 96-well microplate. The absorbance at 540 nm was measured by using a microplate reader (MULTISKAN FC, Thermo Fisher Scientific). Pure water was used as a positive control, and Tris buffer was used as a negative control. The hemolysis rate was calculated according to the following formula:

$$\text{Hemolysis} = \frac{H - H_n}{H_p - H_n} \times 100\%$$

Where H, H_n and H_p represent the absorbance for the sample group, negative control group and positive control group, respectively.

Cytotoxicity assay

L929 cells (mouse fibroblasts) were cultured in a DMEM medium supplemented with 10% FBS and 1% P/S, at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were firstly seeded at a 96-well plate at a concentration of 5,000-8,000 cells/well, and cultured overnight. Then the old culture medium was replaced by the new culture mediums containing samples at different concentrations. The pure medium without samples was used as a control. After 24 h of incubation, the previous medium was replaced with the medium containing 10% (v/v) CCK-8. After another culture for 2-4 h, the absorbance at 450 nm was measured with a microplate reader (MULTISKAN FC, Thermo Fisher Scientific). The cell viability was obtained by the following formula:

$$\text{Cell viability} = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100\%$$

Where A_{sample} and A_{control} represent the absorbance of the sample group and the control group, respectively.

Scanning electron microscopy (SEM) for planktonic *P. aeruginosa*

10^9 CFU/mL *P. aeruginosa* suspensions without any treatment or treated with 1 mg peptide/mL samples were centrifuged, washed twice, and then resuspended in a 2.5% glutaraldehyde solution in PBS at 4 °C overnight for fixation. Then the fixed cells were washed twice with PBS, and dehydrated by ethanol at a gradient concentration (20%, 50%, 60%,70%, 80%, 90%, 95% once, 100% ethanol 3 times, 10 min/time). Finally, the bacterial suspension was dropped on a silicon wafer, sprayed with gold. And the bacteria morphology was observed on an S-4800 SEM (Hitachi, Japan).

Ultrathin section TEM for planktonic *P. aeruginosa*

10^9 CFU/mL *P. aeruginosa* suspensions without any treatment or treated with 1 mg peptide/mL samples were centrifuged, washed twice, and then resuspended in a 2.5% glutaraldehyde solution in PBS at 4 °C overnight for fixation. After 3 times of washing with 0.1 M pH 7.4 KH_2PO_4-K_2HPO_4 buffer, the bacterial suspension was fixed in the ice box with special osmic acid (1% osmic acid + 1.5% potassium ferricyanide) for 1 h. And after 3 times of washing with ultrapure water, the bacterial suspension was then stained by 1% uranyl acetate at room temperature for 1 h. Then the bacterial suspension was washed with ultrapure water for 3 times and dehydrated in the ice box with gradient ethanol (30%, 50%, 70%, 80%, 90%, and 95% once, 100% ethanol 3 times,

15min/time). The dehydrated bacteria were embedded in Epon and hardened at 60 °C for 5 days. Finally, a Leica UC7 ultramicrotome was used to make ultrathin sections (70-80 nm) and a HT7700 TEM was applied to observe the detailed structure.

Membrane permeabilization assay

For the membrane permeabilization assessment, PI was added to a 10^8 CFU/mL *P. aeruginosa* suspension at a final concentration of 10 μM. The mixture was kept in dark at 37 °C for 40 min. Then the fluorescence intensity at 617 nm was monitored every minute at an excitation wavelength of 535 nm. After 5 min, CS-PEG-LK_{13} assembly, LK_{13} peptide or tobramycin were added into the *P. aeruginosa* suspension at a final concentration of 100 μg peptide/mL, 100 μg/mL and 0.8 μg/mL, respectively. The control group was treated with ultrapure water. The fluorescence intensity at 617 nm was measured continually for another 35 min at every minute.

For the outer membrane permeabilization assessment, ANS was added to a 10^8 CFU/mL *P. aeruginosa* suspension at a final concentration of 10 μM. The mixture was kept in dark at 37 °C for 20 min. CS-PEG-LK_{13} assembly, LK_{13} peptide and tobramycin were added into the mixture at a final concentration of 100 μg peptide/mL, 100 μg/mL and 0.8 μg/mL, respectively. The control group was treated with ultrapure water. The fluorescence intensity was monitored between 450-600 nm at an excitation wavelength of 380 nm.

***In vitro* antibacterial assessment in a biofilm**

The antibacterial efficiency in a biofilm is assessed referring to the previous literature with slight modification[4]. 100 μL of 10^7 CFU/mL *P. aeruginosa* suspension in LB broth containing 30 mM glucose was added to a 96-well plate (Nunc 269787). Then the plate was covered with a plate lid containing 96 pegs (Nunc 445497) and incubated in an incubator at 37 °C for 48 h to form biofilms. The peg lid was washed with PBS for twice, and transferred into a new 96-well plate containing 200 μL of samples in PBS at different concentrations. The pure PBS was set as a control group. After 24 h incubation at 37 °C, the peg lid was washed with PBS and transferred to a new 96-well plate containing 200 μL PBS. By sonication for 15 min, the biofilms were dispersed. Then the suspensions in the wells were diluted to a certain concentration and spread on agar plates for incubation overnight. The number of colonies were counted.

Visualization of antibacterial assessment in a biofilm

The glass slides (size: 0.8×0.8 cm^2) treated with 75% ethanol were placed in a sterile 24-well plate. 1 mL of 10^5 CFU/mL bacterial suspension (dispersed in LB broth containing 30 mM glucose) was added in the 24-well plate and incubated at 37 °C for 48 h. The old broth was replaced with a fresh one every 24 h. The glass slides with biofilm were washed twice with PBS, and then immersed into a 24-well plate containing 2 mL solutions containing CS-PEG-LK_{13} assembly, LK_{13} peptide, tobramycin or PBS. After 12 h of incubation, the glass slides were washed twice with PBS, and then immersed in a mixture solution of SYTO 9 and PI (1: 1). After 15 min staining in dark at room temperature, the Live/Dead assay was performed on a Nikon confocal laser scanning microscopy (CLSM). The wavelength for the excitation/emission maxima are about 480/500 nm for SYTO 9 stain and 490/635 nm for PI.

SEM measurements for biofilm

The glass slides with biofilm were prepared as described above. The biofilms without treatment or treated with CS-PEG-LK_{13} assembly were washed with PBS buffer (0.01 M, pH = 7.2-7.4) twice, and then immersed in 2.5% glutaraldehyde in PBS at 4 °C overnight for fixation. After being washed twice with PBS, the biofilms on glasses were dehydrated with ethanol at a gradient concentration (20%, 50%, 60%, 70%, 80%, 90%, 95% once, 100% ethanol 3 times, 10 min/time). Finally, the glass slides with biofilm were sprayed with gold, and observed on an S-4800 SEM.

***In vivo* antibacterial assessment in a biofilm**

The *in vivo* experiments were approved by the Animal Care and Use Committee of Technical Institute of Physics and Chemistry, Chinese Academy of Sciences.

The polydimethylsiloxane (PDMS) sheets (0.2×0.6 cm^2) were prepared according the suggested protocol of the sylgard 184 silicone elastomer kit (Dow Corning, USA). The biofilm formation on the PDMS sheets was the same as that on the glass slides.

Twelve Balb/c (female, 4-6 weeks) mice weighing 13-17 g were anesthetized by intraperitoneal injection of 4% chloral hydrate at 10 mL/kg 30 min before surgery. Then the back of mice was depilated with depilatory paste, and wiped with 75% ethanol for sterilization. About 0.5 cm incision was made on the back, and the PDMS sheets with *P. aeruginosa* biofilm on it was implanted into the subcutaneous area of the incision. The wound was closed with a surgical suture. After 24 h, mice were injected in situ

with 6.7 mg peptide/kg (12.5 MIC) CS-PEG-LK_{13}, 6.7 mg/kg (12.5 MIC) LK_{13} peptide, 6.7 mg/kg (1562 MIC) tobramycin, or PBS (control group) once a day. The wound appearance was recorded by taking photos every day. After 96 h, the mice were sacrificed, and all implants were transferred to 1 mL of sterile PBS. After sonication for 15 min in a water-bath sonicator to disperse the bacteria, the bacterial suspension was diluted to a certain concentration and plated on MH agar plates. After incubation at 37 °C for 24 h, numbers of the *P. aeruginosa* colonies were counted. The tissues of heart, liver, spleen, lung, kidney, skin and muscle of the mice were fixed in 4% paraformaldehyde solution after dissection. The pathology of the tissue was analyzed by H&E staining and observed with a fluorescence inverted microscope (Nikon eclipse Ti, Japan). The concentrations of TNF-α and IL-6 were measured by ELISA kits (Beyotime Biotechnology CO., LTD., China).

Figures

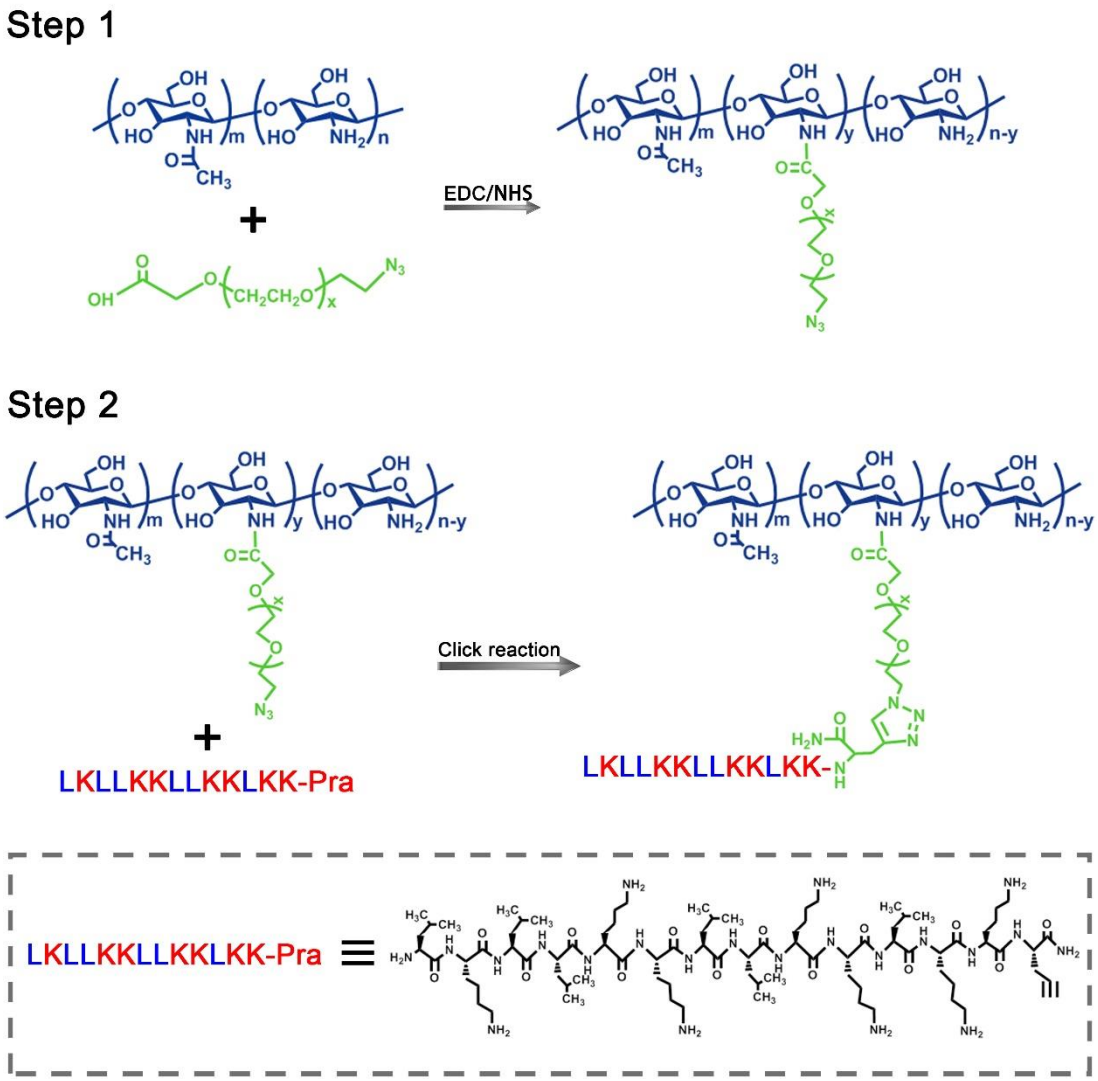

Figure S1. Schematic illustration for the synthesis of CS-PEG-LK_{13}.

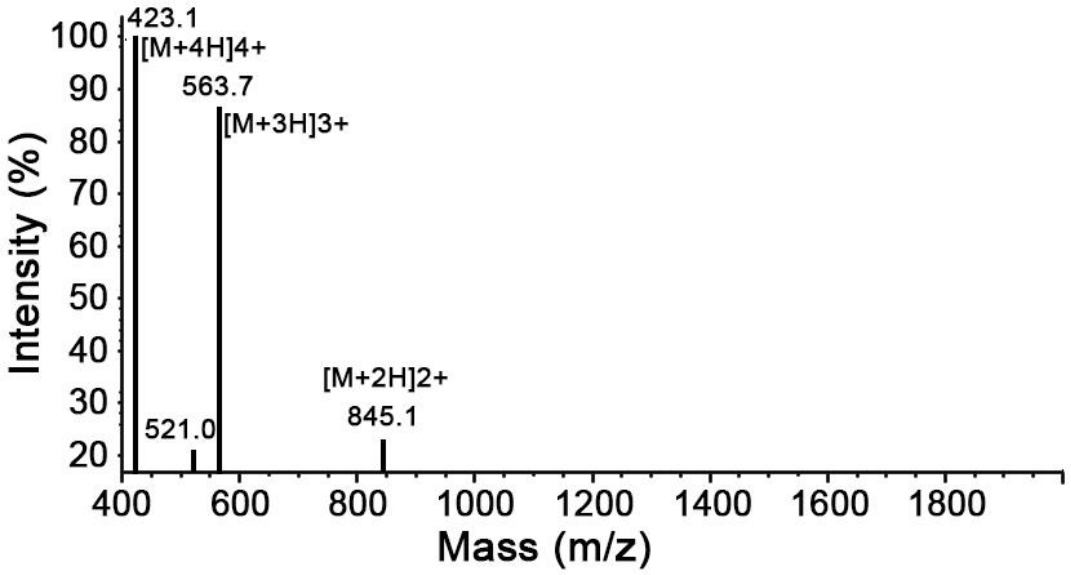

Figure S2. Mass spectrum of LK_{13} peptide.

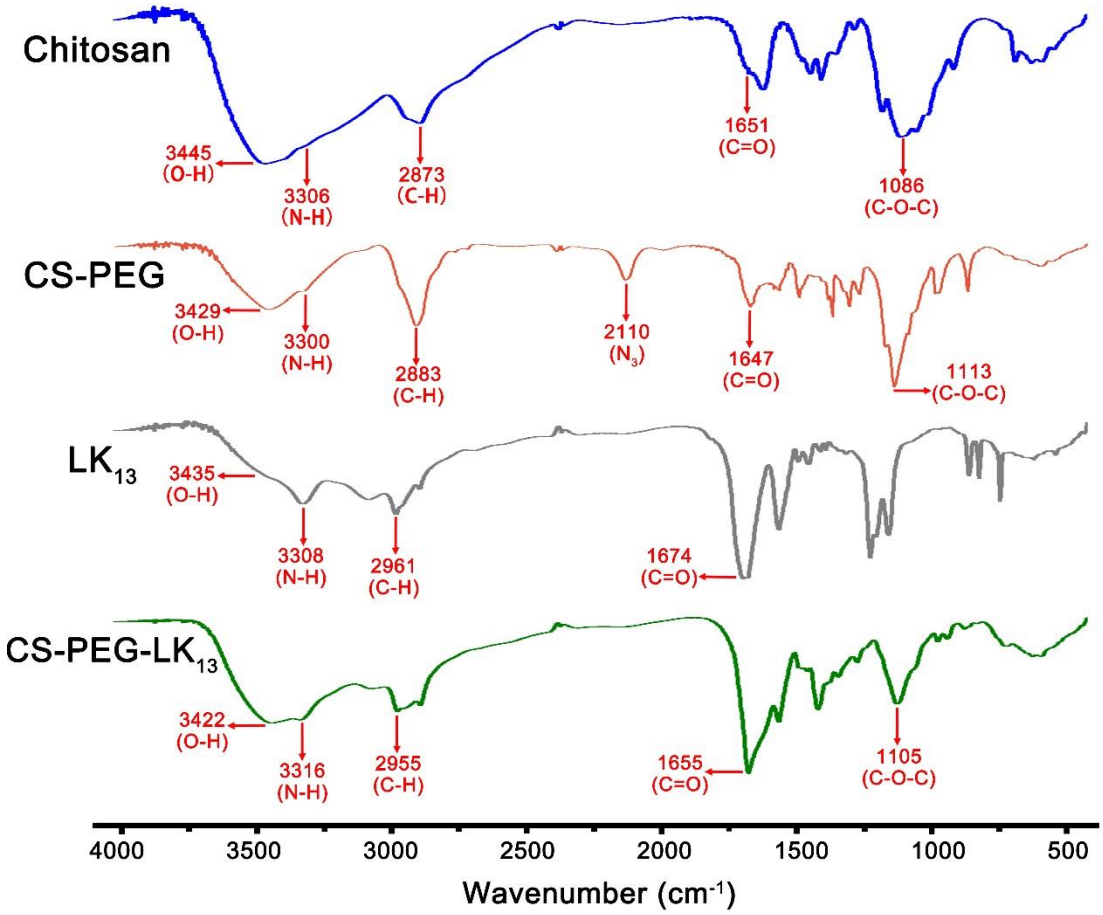

Figure S3. FTIR spectra of chitosan, CS-PEG, LK_{13} peptide, and CS-PEG-LK_{13}.

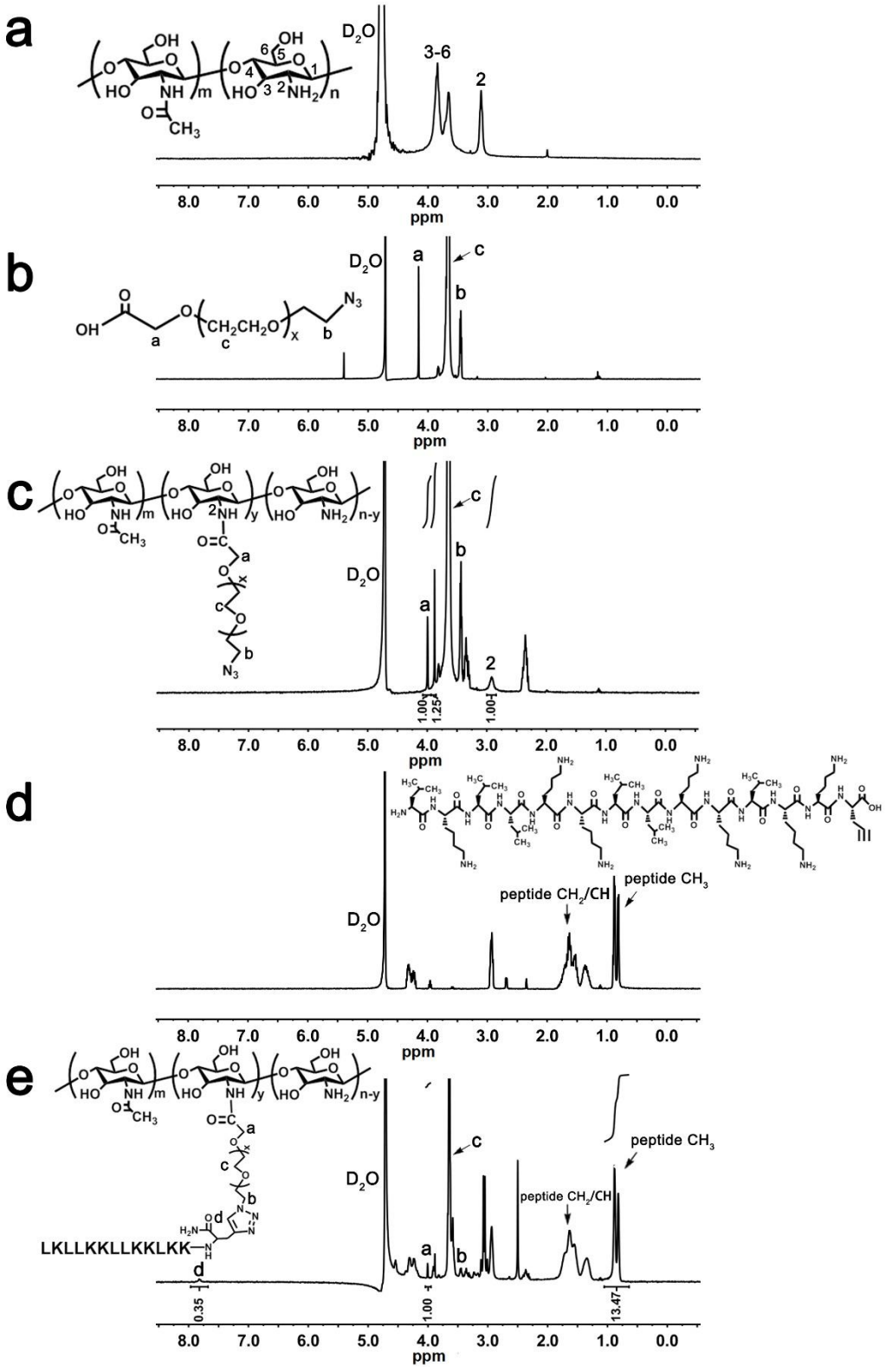

Figure S4. ^{1}H NMR spectra of (a) chitosan, (b) PEG, (c) CS-PEG, (d) LK_{13} peptide and (e) CS-PEG-LK_{13}.

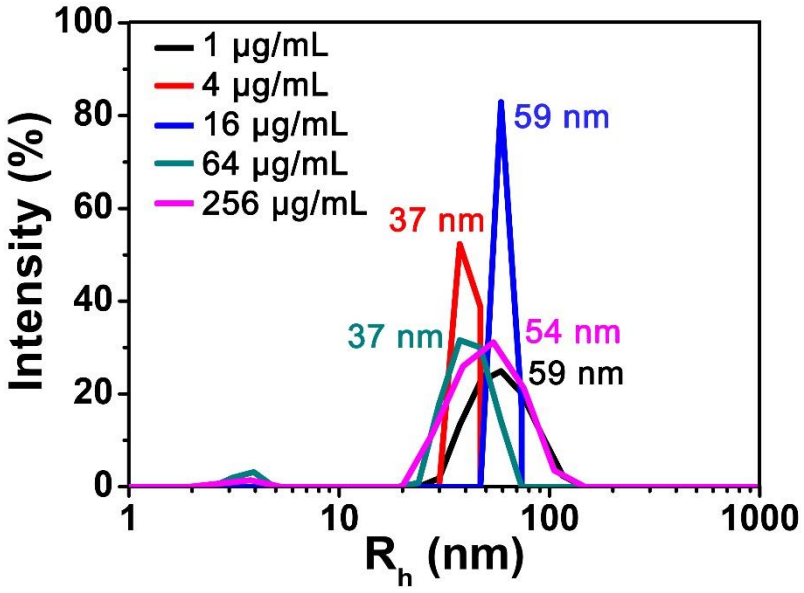

Figure S5. DLS of CS-PEG-LK_{13} assembly at a concentration of 1, 4, 16, 64, 256 μg peptide/mL in 0.01 M pH 5.5 PBS buffer.

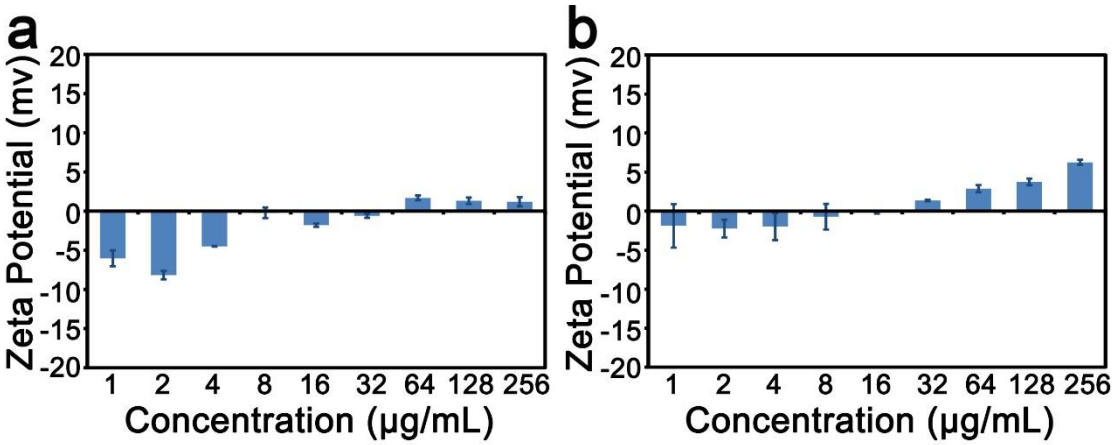

Figure S6. Zeta potentials of CS-PEG-LK_{13} assembly at a concentration of 1, 2, 4, 8, 16, 32, 64, 128, 256 μg peptide/mL (a) in 0.01 M pH 7.4 PBS buffer and (b) in 0.01 M pH 5.5 PBS buffer. Data are shown as mean ± SD (n = 3). Error bars represent the SD of 3 replicates.

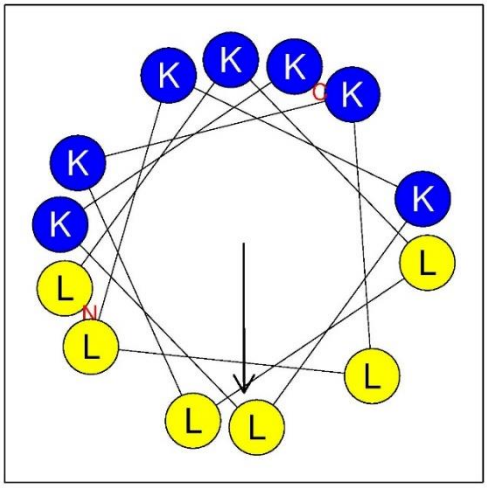

Figure S7. Helical wheel diagram of designed LK_{13} peptide generated by HeliQuest (http://heliquest.ipmc.cnrs.fr/)[5]. The hydrophobic residues are yellow and positively charged hydrophilic residues are blue. The arrows represent the helical hydrophobic moment.

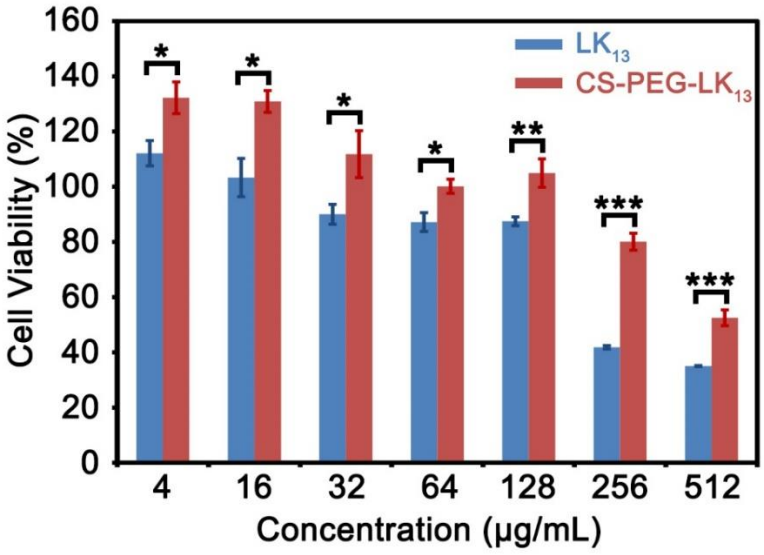

Figure S8. Cell growth inhibition of LK_{13} peptide and CS-PEG-LK_{13} assembly on L929 cells for 24 h. Data are shown as mean ± SD (n = 3). Error bars represent the SD of 3 replicates. *P < 0.05; **P < 0.01; ***P < 0.001.

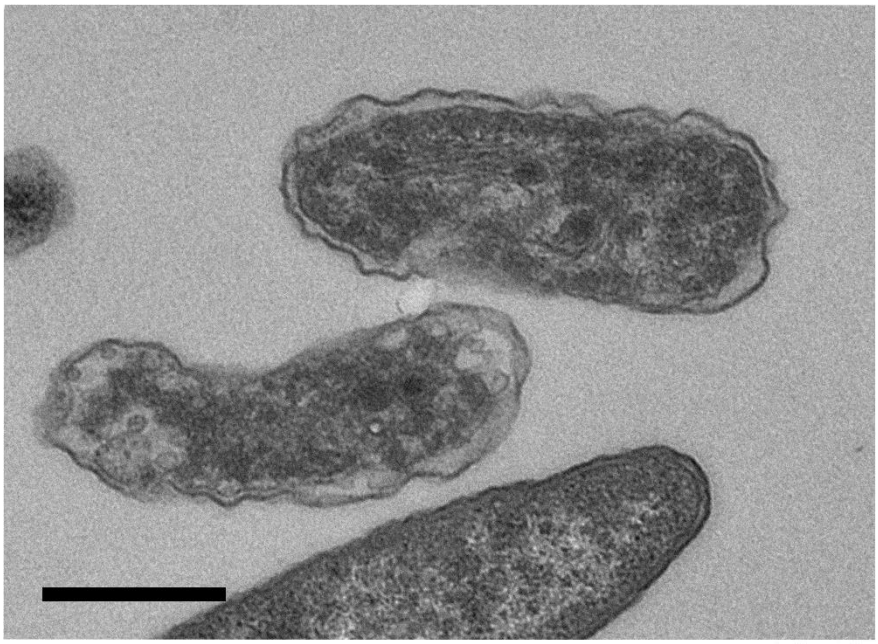

Figure S9. TEM images of *P. aeruginosa* ultrathin sections treated with LK_{13} peptide (scale bar: 500 nm).

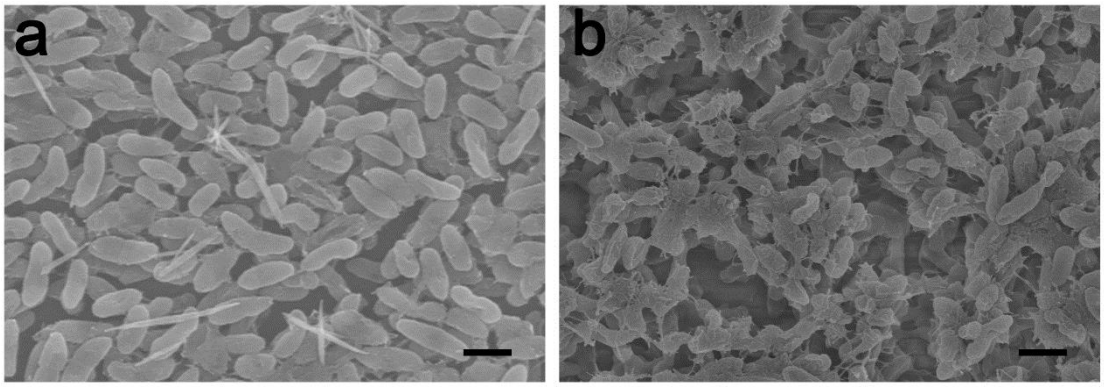

Figure S10. SEM images of the *P. aeruginosa*. biofilm (a) without any treatment and (b) treated with CS-PEG-LK_{13} assembly (scale bar: 1 μm).

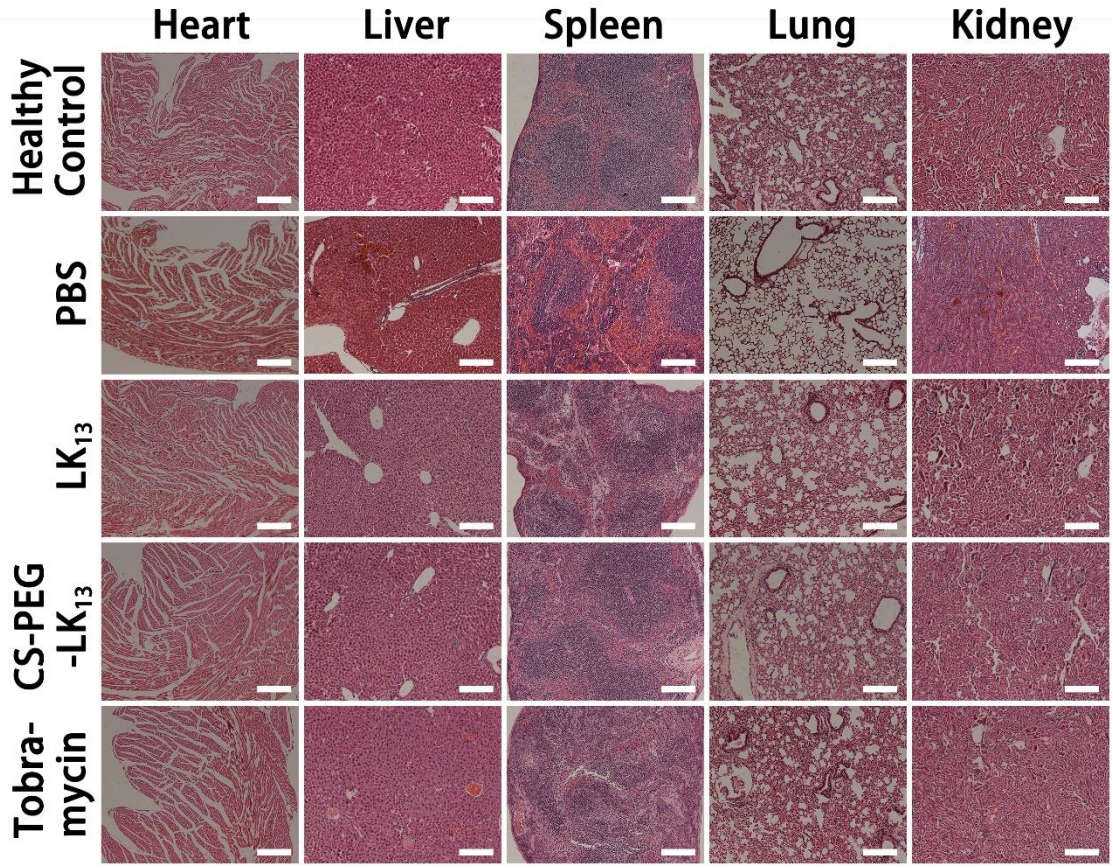

Figure S11. H&E staining of various organs of infected mice treated with PBS, LK_{13} peptide, CS-PEG-LK_{13} assembly and tobramycin in vivo (scale bar: 200 μm).

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