Supplemental Information

Alpha/beta Chimeric Peptide Molecular Brush Eradicating MRSA Biofilms and Persister Cells to Mitigate Antimicrobial Resistance

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Supplementary methods

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay

Bacteria were cultured in Luria-Bertani (LB) medium at 37 °C for 9 hours under shaking at 200 rpm. After centrifugation at 4000 rpm for 5 min, the cells were diluted in Mueller-Hinton (MH) medium to a cell density at 2×10^5 CFU mL^{-1} to give the working suspension. A serial 2-fold diluted solution of 50 μL of antibacterial agents in MH medium was added into each well of a 96-well plate with the concentration ranging from 400 to 3.13 μg mL^{-1}. After added equal volumes of bacterial cells working suspension (50 μL) into each well. The 96-well plate was incubated at 37 °C for 9 hours, and then the optical density (OD) value of each well was collected at 600 nm on a SpectraMax M2 plate reader. Wells containing MH medium only and wells containing cells in MH medium without polymer were included in the same plate as blank and positive control respectively. The percentage of bacterial cells survival was calculated as follow:

$$\left(\%\ \text{cell growth} = \frac{A_{600}^{\text{polymer}} - A_{600}^{\text{blank}}}{A_{600}^{\text{control}} - A_{600}^{\text{blank}}} \times 100\right)$$

The MIC was defined as the minimum concentration of antibacterial agents to completely inhibit bacterial growth.

For the minimum bactericidal concentration (MBC) test, an aliquot of 3.5 μL bacterial suspension from each well of the above final mixture in the MIC study was

transferred to a LB agar Petri dish. After incubating the plate at 37 °C for 14 h, the MBC value was determined by visually checking the bacterial growth.

The MIC values of α/β CPMB and norfloxacin against *S. aureus* ATCC6538 are 12.5 μg/mL and 0.2 μg/mL, respectively. The MBC values of α/β CPMB and norfloxacin against *S. aureus* ATCC6538 are 12.5 μg/mL and 0.39 μg/mL, respectively.

Inhibition of biofilm formation

S. aureus USA300 was inoculated and cultured at 37 °C for 6 hours in LB medium and then bacterial cells were diluted in MH medium (containing 1% glucose) at 2 × 10^6 CFU mL^{-1} to give the working suspension. Two-fold serial dilution of vancomycin and α/β CPMB were performed in a tissue culture treated 96-well plate using MH medium (containing 1% glucose) to provide each well of 50 μL compound solution. An aliquot of 50 μL bacterial working suspension was added to each well, followed by gentle shaking of the plate for 10 seconds. The plate was incubated at 37 °C for 24 hours, and then the medium in each well was removed followed by adding 10 μL MTT (5 mg mL^{-1}) and 90 μL PBS to each well for 4 hours further incubation at 37 °C. After old medium was removed, 150 μL DMSO was added to each well followed by gentle shaking for 15 min to dissolve the purple solid. The OD value of solution in each well was collected at 570 nm using a Molecular Devices SpectraMax M2 plate reader. Wells containing MH medium (containing 1% glucose) only and wells containing cells in MH medium (containing 1% glucose) without polymer were included in the same plate as the blank and positive control respectively. The percentage of cell viability in

each well to evaluate the ability of these antimicrobial agents in prohibiting biofilm formation was calculated as follow:

$$\text{(}\ \%\ \text{cell growth} = \frac{A_{570}^{polymer} - A_{570}^{blank}}{A_{570}^{control} - A_{570}^{blank}} \times\ 100\text{)}$$

The experiment was reproduced independently three times.

Activity against mature MRSA biofilm

S. aureus USA300 was inoculated and cultured at 37 °C for 6 hours in LB medium and then bacterial cells were diluted in MH medium at 10^5 CFU mL^{-1} to give the working suspension. Then an aliquot of 100 µL working suspension was added to each well in a 96-well plate followed by 24 hours incubation at 37 °C. Two-fold serial dilution of vancomycin and α/β CPMB were performed in a separate 96-well plate using MH medium to provide each well of 125 µL stock solution. The old medium was removed from the biofilm plate followed by carefully transferring 100 µL of above antimicrobial stock solution to corresponding wells in the biofilm plate and incubated the plate for another 24 hours at 37 °C. Then, medium was removed and 10 µL MTT (5 mg mL^{-1}) and 90 µL PBS were added to each well followed by further incubation at 37 °C for 4 hours. After old medium was removed from each well, 150 µL DMSO was added to each well followed by gentle shaking for 15 min to dissolve the purple solid. The OD value of solution in each well was collected at 570 nm using a Molecular Devices SpectraMax M2 plate reader. Wells containing MH medium only and wells containing cells in MH medium without polymer were included in the same plate as blank and positive control respectively. The percentage of cell viability in each well to

evaluate the ability of the antimicrobial agents in killing sessile MRSA cells within mature biofilms was calculated as follow:

$$(\%\ \text{cell growth} = \frac{A_{570}^{\text{polymer}} - A_{570}^{\text{blank}}}{A_{570}^{\text{control}} - A_{570}^{\text{blank}}} \times 100)$$

The experiment was reproduced independently three times.

Confocal laser scanning microscopy study

Using confocal dish, not 96-well plate, to perform the anti-biofilm study. Before microimaging, the biofilms were stained by 50 μL SYTO 9 (3 μM) and 50 μL propidium iodide (PI) (15 μM) for 10 min. Then the biofilms were used for confocal laser scanning microscopy (CLSM) study directly. The excitation maximum and emission maximum of SYTO 9 is at 483 nm and 503 nm, respectively. The excitation maximum and emission maximum of PI is at 493 nm and 636 nm, respectively.

MRSA persister cell killing kinetics

Log-phase *S. aureus* USA300 was diluted in MH medium at a final concentration of 10^8 CFU mL^{-1}. Ciprofloxacin was added to 1 mL of bacterial suspension to obtain a desired final concentration of 10 × MIC. The mixtures were incubated under shaking at 37 °C for 18 hours. 500 μL bacterial suspension was washed to remove antibiotics and treated with α/β CPMB at 4 × MIC in MH medium. The left 500 μL bacterial suspension was washed to remove antibiotics and treated with ciprofloxacin as a control. At desired time points, aliquots of each sample were serial diluted in PBS, then plated on LB agar plates for colony-forming unit (CFU) determination.

Antimicrobial resistance test

S. aureus ATCC6538 was inoculated and cultured in LB medium at 37 °C for 10 hours. The cells were diluted 400-fold with MH medium and added α/β CPMB or norfloxacin hydrochloride into the work suspension at a final concentration at 0.5 × MBC. The mixtures were cultured at 37 °C for 24 hours. Then the cell suspension was diluted 400-fold with MH medium and subjected to above cell-drug incubation cycle. This cell-drug incubation cycle was repeated every 24 hours and the MIC/MBC values were tested every 4 days. MIC/MBC tests were repeated at least twice at different time.

Bacteria growth kinetics

S. aureus ATCC6538 was inoculated and cultured at 37 °C for 10 hours in LB medium and then bacterial cells were diluted in MH medium to generate the working suspension at 2×10^6 CFU mL^{-1}. The bacteria cells were treated with α/β CPMB and norfloxacin hydrochloride in 0.5 × MBC concentration. At various time point of incubation, the bacterial cells were sampled and diluted for plating on LB Agar plate for incubation overnight at 37 °C. The colony-forming units were counted.

***In vitro* bactericidal killing kinetics**

Bacteria were prepared according to the MIC assay described above to obtain a cell density at 2×10^5 CFU mL^{-1} as the working suspension. The bacteria were treated with α/β CPMB at a final concentration of 2 × MIC and 4 × MIC respectively, the mixture was incubated at 37 °C. At predetermined time points, the diluted bacterial

solution was plated on LB agar plates, followed by incubation at 37 °C overnight, then the CFUs were counted.

Cytoplasmic membrane depolarization assay

Log-phase *S. aureus* USA300 was washed with HEPES buffer (5 mM HEPES, 20 mM glucose, pH=7.4) and suspended in HEPES buffer at 1×10^7 CFU mL^{-1}. The cell suspension was incubated with 0.4 μM $DiSC_3(5)$ until a stable fluorescence intensity was achieved (approximately 1 hour). Then KCl was added to a final concentration of 0.1 M to equilibrate the cytoplasmic and external K^+. An aliquot of 90 μL the cell suspension was placed in a 384 well plate. Changes in fluorescence were recorded on a SpectraMax M2 plate reader using an excitation wavelength of 622 nm and an emission wavelength of 670 nm. When fluorescence intensity almost keep steady, antimicrobial agent was added into the system (Time=0 s) and fluorescence signals was recorded continuously. Vancomycin was used as a control.

TEM characterization

S. aureus USA300 was inoculated and cultured in LB medium at 37 °C for 6 hours, then diluted in MH medium to 2×10^9 CFU mL^{-1} and incubated with 8 × MIC of α/β CPMB at 37 °C for 4 hours. The bacterial cells were collected through centrifugation and washed with PBS. Then bacterial cells were fixed by using 2.5% glutaraldehyde in PBS at 4 °C overnight. After washed with PBS for three times, the bacterial cells were fixed with 1% OsO4 in PBS for 2 hours. Then the samples were washed with PBS for three times and dehydrated with a gradient concentration of ethanol solutions (including 30%, 50%, 70%, 80%, 90%, 95%). Each concentration was treated for 15

min, and then treated with 100% ethanol for 20min. The dehydrated bacterial cells were treated with a mixture of embedding agent and acetone at 1:1 (v/v) and 3:1 ratio (v/v) for 1 hour and 3 hours respectively. Eventually, the samples were treated with pure embedding agent at 70 °C overnight to obtain slices at 70-90 nm thickness. The slices were stained with uranyl acetate and led citrate for 5 min and used for transmission electron microscopy characterization.

Intracellular ROS assay

The intracellular ROS level in *S. aureus* was conducted by following the previously reported literature with modifications.[1] Log-phase *S. aureus* USA300 was mixed with DCF-DA (40 mM) and incubated for 30 min at 37 °C. Then bacteria were collected and washed twice with PBS to remove the DCF-DA outside of cells and diluted to 1×10^8 CFU/mL. An aliquot of 90 μL cells suspension was placed in a 96 well plate, then 10 μL α/β CPMB, PBS, and the mixture of α/β CPMB and NAC (100 mM) was added to the cell suspension respectively to obtain a final polymer concentration at 10 × MIC. The DCF fluorescence intensity was recorded continuously on a SpectraMax M2 plate reader using an excitation wavelength of 488 nm and an emission wavelength of 530 nm. This experiment was conducted in triplicates and repeated twice.

DNA binding assay

DNA binding assay was conducted by following the previously reported literature.[1] Agarose gel electrophoresis was used to evaluate α/β CPMB binding to plasmid DNA. 1.0 μg/mL DNA and 0.5 mg/mL α/β CPMB were mixed at variable N:P ratios (0.1 : 1, 0.2 : 1, 0.5 : 1, 1 : 1, 2 : 1). N means the number of amine side groups in α/β CPMB, P

means the number of phosphate anions in the plasmid backbone. Then diluted to the final volume of 10 μL with DI water and added 2 μL DNA loading buffer. 5 min later the mixtures were analysed by electrophoresis using 1% agarose gel and ethidium bromide in Tris-borate-EDTA buffer at 120 V for 30 min. DNA bands were visualized using a Gel Documentation and Image Analysis System (SAGECREATION, China). Native loading buffer was used, containing 10 mM Tris-HCl (pH=7.5), 40% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol.

Hemolysis assay

The hemolysis of α/β CPMB was conducted by following the literature with moderate modification using fresh human red blood cells (hRBCs).[2] Briefly, fresh human blood were washed 3 times with Tris-buffered saline (TBS, pH = 7.2), then was diluted to a 5% (v/v) hRBCs working suspension after centrifugation at 4000 rpm. The α/β CPMB were diluted to concentrations ranging from 3.13 μg/mL to 200 μg/mL in TBS by a serial 2-fold dilution. After mixing an equal amount of 50 μL of hRBCs suspension and α/β CPMB solution, the 96-well plate was incubated at 37°C for 1 h. Wells containing hRBCs in TBS only and wells containing hRBCs treated with 0.1% Triton X-100 were included in the same plate as blank and positive control respectively. After centrifugation at 3700 rpm for 5 min, 80 μL of the supernatant in each well was transferred to another 96-well plate and the OD values were collected to calculate the percentage of hemolysis from the equation:

$$\%\ \text{hemolysis} = \frac{A_{405}^{\text{polymer}} - A_{405}^{\text{blank}}}{A_{405}^{\text{control}} - A_{405}^{\text{blank}}} \times 100)$$

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Statistics analysis

Data were expressed as mean ± standard deviation which was indicated by the error bars, Statistical analysis of the data was conducted using One-Way ANOVA and Tukey test.

Supplementary figure

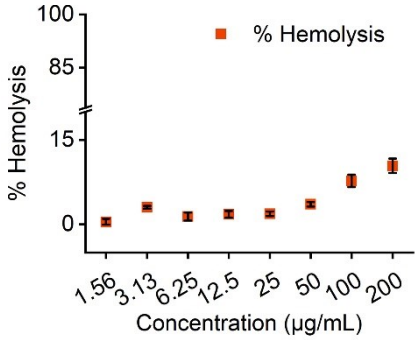

Fig. S1 Hemolysis of α/β CPMB toward human red blood cells.

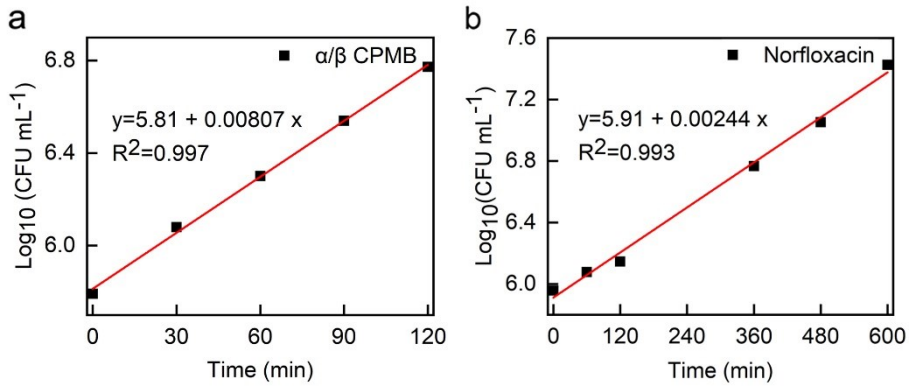

Fig. S2 Rate of bacteria growth in the presence of (a) α/β CPMB and (b) norfloxacin.

1. M. Zhou, Y. Qian, J. Xie, W. Zhang, W. Jiang, X. Xiao, S. Chen, C. Dai, Z. Cong, Z. Ji, N. Shao, L. Liu, Y. Wu and R. Liu, *Angew. Chem. Int. Ed.*, 2020, **59**, 6412-6419.
2. W. Jiang, X. Xiao, Y. Wu, W. Zhang, Z. Cong, J. Liu, S. Chen, H. Zhang, J. Xie, S. Deng, M. Chen, Y. Wang, X. Shao, Y. Dai, Y. Sun, J. Fei and R. Liu, *Biomater Sci*, 2020, **8**, 739-745.