Supporting Information

Screening of a short chain antimicrobial peptide-FWKFK and its application in wound healing

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Experimental procedure

Material

TEOS, C_2H_5OH, $NH_3 \cdot H_2O$, formaldehyde aqueous solution (37%), urea, DMF, piperidine, and methanol were produced from Shanghai Sinophosphoric Chemical Reagent Co., LTD. PyBoP, HoBT, DIEA, PBS, triisopropyl silane, potato dextrose agar/broth (PDA), Bertani agar / broth (LB), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrabromide (MTT) were purchased from Macklin. *S. aureus* (ATCC 25923), *S. epidermidis* (CMCC(B)26069) standard strain (ATCC12228), *B. subtilis* standard strain (CMCC(B)50094), *P. aeruginosa* (CMCC(B)10104) standard strain (ATCC9027), *E. coli* (ATCC 8739) were from Beijing Biological Collection Center.

Preparation of silica microsphere stationary phase

Preparation of sol-gel self-made silica sol

Take 6 mL ultrapure water, 350 mL anhydrous ethanol, and 16 mL ammonia, adjust the water bath temperature, and magnetic stirring for 1 hour. Take 15 mL of TEOS, quickly added to the flask, stirring vigorously for 30 minutes, stirring slowly for 3 hours to obtain a slightly blue transparent liquid, and then distilled ethanol under reduced pressure to obtain a blue-white liquid.

Preparation of microspheres

Take 10 g of urea added to the silica sol solution, magnetic stirring dissolved, adjust the solution pH to 1.2, according to the reaction molar ratio quickly added formaldehyde solution, so that the microspheres quickly cross-linked. When the

solution gradually changes from transparent solution to white turbid liquid, a large amount of ultrapure water is added to terminate the reaction and static overnight stratification.

Post-treatment of microspheres

Wash the lower layer of the white precipitate with ultrapure water, the layered silica gel microspheres slowly remove the supernatant, re-add a large amount of water, slowly stir until the system is uniform, stand for 12 hours, pour out the supernatant, repeat the above operation 2 times, add 150 mL of anhydrous ethanol, continue to stir, stand for 12 hours, pour out the supernatant, repeat 2 times, suction filter out the white precipitate, vacuum drying, into the muffle furnace to remove the organic resin.

Preparation of bacterial cell membrane

The 25 mL bacterial solution was centrifuged to remove the culture medium. The bacteria obtained by centrifugation were dissolved in 25 mL of normal saline, and then crushed by a cell grinder, and then centrifuged to collect the cell membrane.

Preparation of stationary phase

Since the direct use of silica microspheres to connect bacterial membranes is not strong, we prepared a diazo resin (DR) as a linker to connect microspheres and bacterial membranes according to previous literature[1]. 0.5 g of silica microspheres were placed in an aqueous solution of 5 mg / mL DR, stirred in the dark for 30 minutes, centrifuged and collected, washed three times with ultrapure water, placed in the bacterial membrane aqueous solution, stirred in the dark for 30 minutes, centrifuged and

collected, and then placed in a 365 nm UV lamp for 15 minutes to convert the ionic bond at the junction into a covalent bond.

Synthesis of Peptide Library by One Bead One Compound Method

According to previous reports[2], we found that antimicrobial peptides are composed of positively charged amino acids and hydrophobic amino acids, so we designed a peptide library based on this principle. The peptide library was synthesized by Fmoc solid phase synthesis. Firstly, the dichloride resin was swollen with dichloromethane for 2 hours, and the first amino acid and DIEA were added for 2 hours. Then, methanol and DIEA were added to block the unreacted sites on the dichloride resin. Subsequently, piperidine (20% piperidine + 80% DMF) was added to remove the Fmoc protecting group on the amino group. Then, add amino acids, PyBoP, HoBT, and DIEA and react for two hours, then remove the protective group. Cycle this step until the desired peptide library is synthesized.

Screening peptide chain

We screened the peptide chain by cell membrane chromatography. Through the stationary phase prepared above, the stationary phase was filled into the chromatographic column, and the peptide library was separated by a chromatographic column. By comparing the peak area, the peptide chain with the effect of adsorbing bacteria was screened.

Inhibition zone test

The effect of antimicrobial peptides was verified by an inhibition zone experiment.

The experimental steps are as follows: Before using all bacteria, they were incubated on LB plates at 37°C for 24 hours to observe the growth of bacteria. The LB plate with good bacterial growth was selected, and a single colony on the LB plate was inoculated into the LB broth, and then the inoculated LB broth was placed in a bacterial incubator (37°C, 300rpm) until the logarithm was reached. Before the experiment, the medium (0.25 g broth, 0.14 g agar, 10 mL deionized water) was prepared, and the medium and petri dish was placed in a sterilization pot for sterilization for 2 hours. After the sterilization was completed, the medium temperature was cooled to room temperature, and bacteria (1×10^{12} CFU/mL) were added, fully mixed, and poured into the petri dish. After cooling to solidify, a circular hole with a diameter of 1 cm was cut out, and the pre-prepared antimicrobial peptide solution was added to the hole, and then incubated in the incubator for 24 hours (37°C, 300rpm).

Minimum inhibitory concentration test (MIC)

Minimum inhibitory concentration is an important test index of antibacterial inhibitors. We tested the minimum inhibitory concentration of antimicrobial peptides by agar plate method. Before the start of the experiment, the same as the inhibition zone experiment, the medium and petri dish were sterilized, and then the bacterial solution was diluted with a certain concentration (1×10^{9} CFU/mL) and used as a solvent to configure a series of antibacterial peptide solutions with different concentrations. Put them into the bacterial incubator for 2 hours (37°C, 300rpm). After sterilization, 10 mL of culture medium was poured into a petri dish, shaken, and cooled to complete coagulation. 100 μL of antimicrobial peptide solution was taken and evenly applied to

the culture medium, and then incubated in an incubator (37°C, 24 hours). After incubation, colonies were observed and counted. The experiment was performed in triplicate and MIC results were calculated using the following formula.

$$\text{kill}(\%) = \frac{(\text{CFU \& negative control} - \text{CFU \& AMP})}{\text{CFU \& negative control}} \quad (1)$$

Testing antimicrobial resistance of antimicrobial peptides to bacteria

The MIC of *E. coli* and *S. aureus* was configured, and the MIC of gentamicin sulfate was compared. Cultured in a bacterial incubator (37°C), passaged every two hours, sampled every two hours during the period, and evenly smeared on the LB culture plate, incubated for 24 hours, and counted the colonies for photographing.

In order to further explore the antimicrobial resistance of antimicrobial peptides to bacteria, it is necessary to determine the changes in the MIC of antimicrobial peptides and antibiotics. The specific experimental design is as follows:

(1) The bacteria were placed in culture medium and incubated at 37℃ for 24 hours.

(2) Sterile PBS buffer solution was used for gradient dilution of antibacterial peptides and antibiotics, and then the bacterial concentration was diluted to 10^6 CFU/mL. The diluted antibacterial peptide solution and antibiotic solution were mixed with the bacterial solution in equal volume, respectively, and incubated at 37℃ for 4 hours.

(3) 100 μL of the mixed solution at each concentration was placed in 96-well plates, sterile PBS buffer solution was used as the control group, and their optical density values were measured with an enzyme label under a specific wavelength (600nm) and a line chart was made. The intersection of the experimental group and the

control group was MIC, which was denoted as MIC_1.

(4) The group of bacterial solution with half concentration of MIC_1 was placed in a new culture medium and incubated at 37℃ for 24 hours. Step (2) and step (3) were repeated, and the MIC of this test was denoted as MIC_2. After that, the group of bacterial solution with half concentration of MIC_2 was placed in a new culture medium and steps (2) and (3) were repeated.

(5) The operation was repeated 11 times, and the MIC values of antimicrobial peptides and antibiotics at each stage were recorded, and plotted to observe the changing trend.

Thermal stability test of antimicrobial peptides

To test the stability of antimicrobial peptides under extreme temperature conditions, we conducted a test of the thermal stability of antimicrobial peptides. Firstly, the antimicrobial peptide solution with a concentration of 1mg/mL was prepared. It was soaked in boiling water for 0, 15, 30, 45, 60, 75, 90, 105, 120 minutes and then analyzed by high-performance liquid chromatography. The content of antimicrobial peptides under various conditions was calculated by peak area analysis and normalization based on the blank control group. To show the thermal stability of antimicrobial peptides at high temperatures.

Antioxidant test experiment of antimicrobial peptides

To test the stability of antimicrobial peptides under strong oxidation conditions, 1mg/mL antimicrobial peptide solution was first prepared, then treated with different

concentrations of H_2O_2 solution, and then analyzed by high-performance liquid chromatography. Through peak area analysis and normalization based on the blank control group, the content of antimicrobial peptides under various conditions was calculated. To show the stability of antimicrobial peptides under strongly oxidizing conditions[3].

Stability test of antimicrobial peptides in artificial gastric juice

To test the stability of antimicrobial peptides in artificial gastric juice, 1mg/mL antimicrobial peptide solution was first prepared and then placed in artificial gastric juice for 0, 10, 20, 30, 40, 50, and 60 minutes, and then analyzed by high-performance liquid chromatography. The content of antimicrobial peptides under various conditions was calculated by peak area analysis and normalization based on the blank control group. To show the stability of antimicrobial peptides in artificial gastric juice.

pH stability test experiment of antibacterial peptides

To test the pH stability of antimicrobial peptides, 1mg/mL antimicrobial peptide solution was first prepared and then treated with buffers with pH of 2, 4, 6, 9, 10, and 12 respectively. After that, high-performance liquid chromatography was used for analysis. Through peak area analysis and normalization based on the blank control group, the content of antimicrobial peptides under various conditions was calculated. To show the stability of antimicrobial peptides at different pH[4,5].

Cytotoxicity assay

The cytotoxicity of antimicrobial peptides was tested by MTT assay and live/dead

staining assay using 3T3 cells as model cells. Before the formal experiment, a series of concentrations of antimicrobial peptide samples were prepared. The 3T3 cells were treated with trypsin for 3 minutes, centrifuged and collected, and then transferred to a 5mL cell culture medium (RPMI-1640), washed, and counted. Quantitative cells were taken and diluted to the required concentration. 100 μL of cell culture medium was taken to 96-well plates with a pipette gun so that there were 10000 cells per well. Subsequently, the 96-well plates were transferred to a cell incubator and cultured for 24 hours. Cell growth was observed under a microscope. The cell culture medium was used as the solvent to prepare the expected concentration of antimicrobial peptide solution. After fully mixing, 100 μL was added to the 96-well plate. One column was selected as the negative control group, that is, the cell culture medium without antimicrobial peptide was added, and then one group was selected as the positive control, that is, deionized water was added. Subsequently, 96-well plates were placed in a cell incubator and cultured for 24 hours. The 96-well plate was taken out, and the culture medium in the hole was sucked out with a pipette gun and washed with PBS. Subsequently, in the dark environment, the pre-configured MTT solution was added to the 96-well plate and placed in the cell incubator for 4 hours. The upper solution was sucked out with a pipet gun, washed with PBS, and then 100 μL of dimethyl sulfoxide (DMSO) was added to each well and shaken for 5 min. Subsequently, the absorbance was tested three times at a test wavelength of 490 nm using a microplate reader. According to the absorbance obtained from the test, the relative absorbance was calculated by the absorbance values of the test group and the negative control group,

and the positive control group.

The experimental steps of Live/Dead staining were roughly the same as MTT. Firstly, 3T3 cells were added to 96-well plates as needed, and then cultured for 24 hours. Then the plates were taken out, the upper liquid was sucked out, and the antibacterial peptide solution was added. At the same time, the negative control group and the positive control group were set up. The cells were cultured in an incubator for 24 hours. After that, the upper layer was aspirated and washed twice with PBS. The cells were stained with a pre-configured live-dead dye and placed in a cell incubator for 30 minutes. Then the 96-well plate was placed in an inverted fluorescence microscope to observe the cell status of each group.

Hemolytic test

We used methods reported in previous literature with minor modifications to assess the blood compatibility of the material[6-8]. First, 4mL of mouse tail blood was collected and centrifuged (1000rpm, 5min), and washed three times with normal saline (SPSS) to obtain red blood cells (RBC). Then, SPSS was added to the centrifuge tube to obtain a red blood cell suspension (volume 5%), and then 1mL of red blood cell suspension was placed in a 1.5mL centrifuge tube, centrifuged, the supernatant was sucked out, and a pre-configured series of concentrations of antimicrobial peptide solution was added to the centrifuge tube. At the same time, SPSS was added to a centrifuge tube as a negative control, and ultrapure water was added to another centrifuge tube as a positive control.

The centrifuge tube was placed in a cell incubator (37°C, 2h). After culture, the

mixture in the centrifuge tube was divided into two parts. Some are used for characterization under SEM and inverted fluorescence microscope. After the other part was centrifuged, the supernatant was placed in a 96-well plate and photographed. Then, the 96-well plate was placed in a microplate reader and the absorbance was measured at a wavelength of 570 nm. The experiment was carried out in triplicate. Use the following formula to calculate the percentage of hemolysis.

$$\text{hemolysis}(\%) = \frac{(A^0 - A^n)}{(A^P - A^n)} \times 100\% \quad (2)$$

A^0 is the absorbance of the sample, and A^n and A^p represent the absorbance of the negative and positive groups, respectively.

SEM characterization of mouse red blood cell morphology

We used SEM to characterize the morphology of red blood cells after hemolysis experiments. The red blood cells after the hemolysis test were immersed in a 2.5% glutaraldehyde phosphate buffer solution and placed in a refrigerator at 4°C overnight. After that, it was washed three times with PBS and dehydrated in 18%, 60%, 70%, 80%, 85%, 90%, 95%, 100% ethanol for 10 minutes. Subsequently, SEM characterization.

The antibacterial experiment on mouse epidermis

Based on literature research, we established a mouse model of local bacterial infection to test the antibacterial activity of antimicrobial peptides against *S. aureus*[9-10]. Experimental mice (8-week-old, 40±2g, purchased from Jinan Jielikang Biotechnology Co., Ltd., China) are maintained and processed according to the requirements of the

Institutional Animal Care and Use Committee (IACUC).

The mice were divided into four groups, three mice in each group. The same amount of sterile PBS, the minimum inhibitory concentration of antimicrobial peptide solution, *S. aureus* bacteria solution (10^9 CFU/mL), antimicrobial peptide, and *S. aureus* mixed solution were added. Each mouse was given free access to food and water throughout the experiment. On the backs of each group of mice, sterilized scissors were used to create wounds 1cm in diameter. Nodular at the bottom of the wound, no obvious bleeding. 50 μL sterile PBS was added to the wound of mice in the blank control group, and 50 μL antimicrobial peptide solution was added to the antimicrobial peptide control group. The remaining two groups were added with 50 μL *S. aureus* solution and 50 μL mixed solution of antimicrobial peptide and *S. aureus*, respectively. The wound area and body weight of mice were measured daily.

Result and discussion

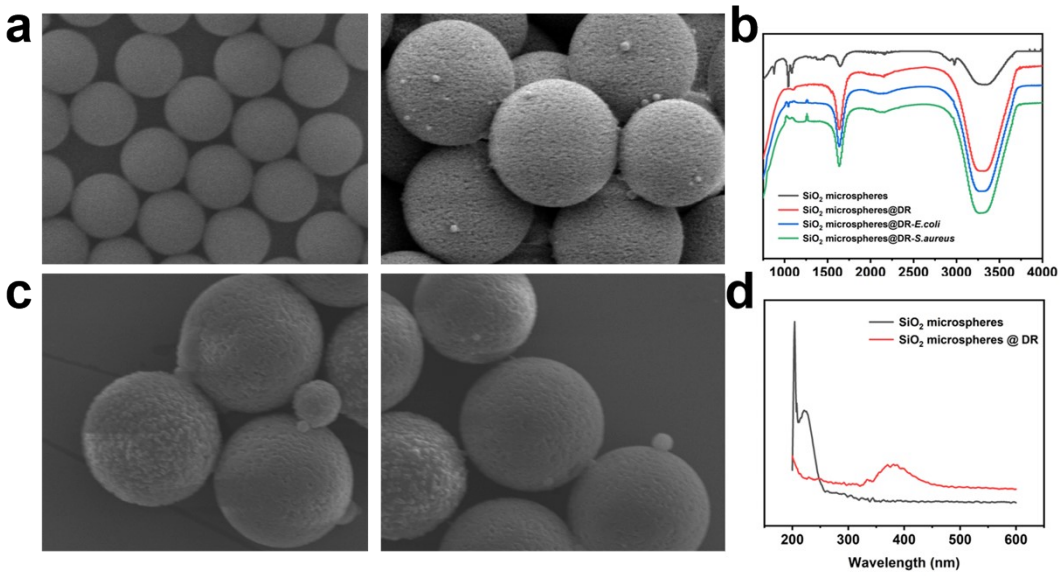

Figure S1 Characterization of membrane chromatographic fillers: (a) Scanning electron microscopic view of 4 μm silicon dioxide microsphere; (b) FT-IR spectrum of SiO_2 microspheres, SiO_2 microspheres@DR, SiO_2 microspheres@DR-*E. coli*, SiO_2 microspheres@DR-*S. aureus*; (c) Scanning electron microscopic view of SiO_2 microspheres@DR-*E. coli*, SiO_2 microspheres@DR-*S. aureus*; (d) The UV-Visible spectra of SiO_2 microspheres, SiO_2 microspheres@DR.

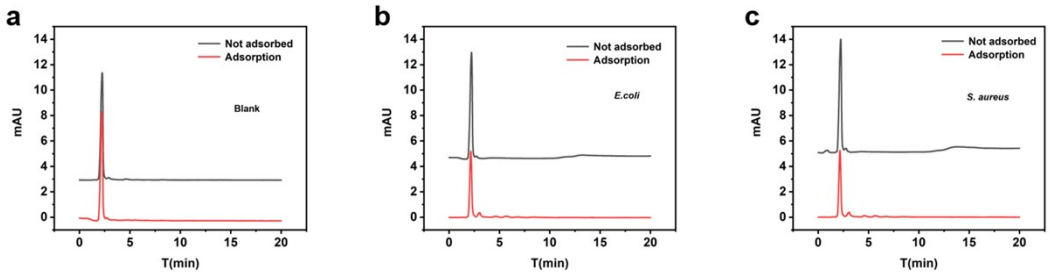

Figure S2 Liquid chromatogram of antimicrobial peptide FWKFK adsorbed by cell membrane chromatography: (a) Chromatograms of antimicrobial peptides separated using stationary phases of unadsorbed bacterial membranes and stationary phases of adsorbed water (blank control); (b) Chromatograms of antimicrobial peptides separated using stationary phases of unadsorbed and adsorbed *E. coli* bacterial membranes; (c) Chromatograms of antimicrobial peptides separated using stationary phases of unadsorbed bacterial membranes and stationary phases of adsorbed *S. aureus* membranes.

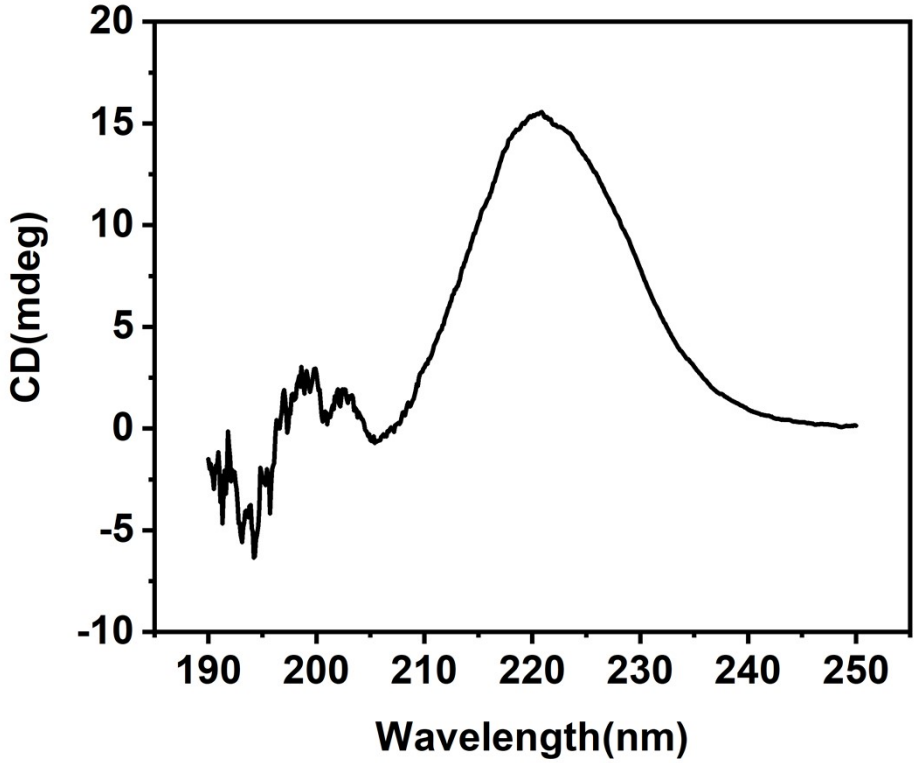

Figure S3 The secondary structure of antimicrobial peptides was determined by circular dichroic spectroscopy.

Figure S4 The inhibition zone of *E. coli* and *S. aureus*.

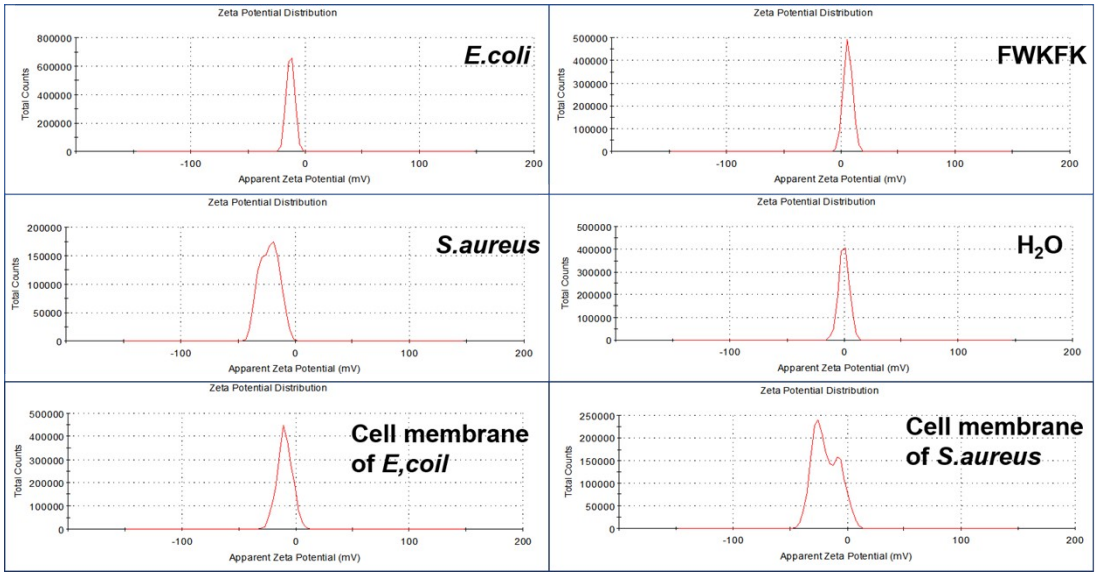

Figure S5 *E. coli*, antimicrobial peptides FWKFK, *S. aureus*, water, the cell membrane of *E. coli*, the cell membrane of *S. aureus* potential diagram.

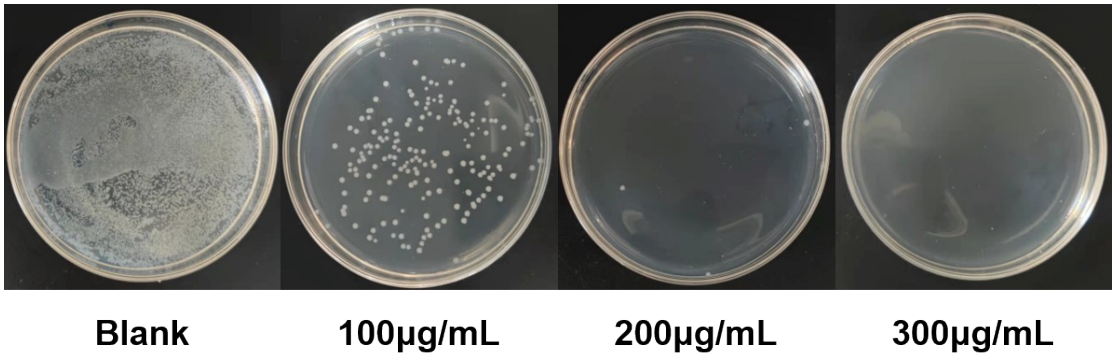

Figure S6 Minimum inhibitory concentration of *P. aeruginosa*.

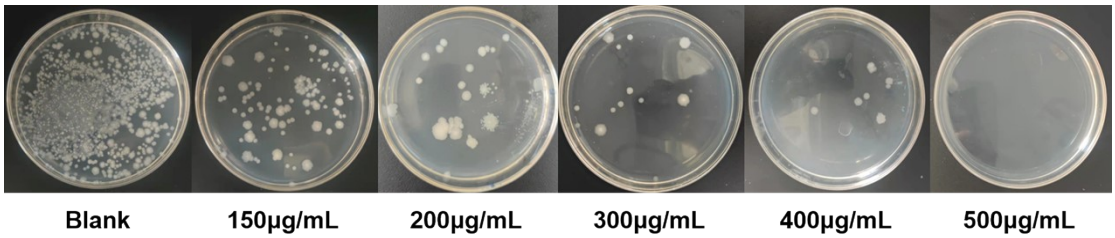

Figure S7 Minimum inhibitory concentration of *B. subtilis*.

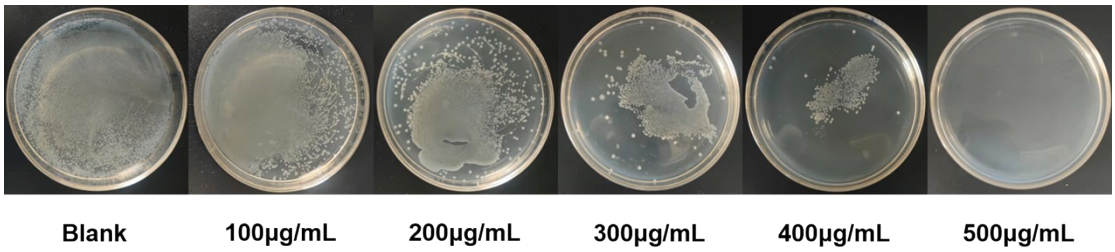

Figure S8 Minimum inhibitory concentration of *S. epidermidis*.

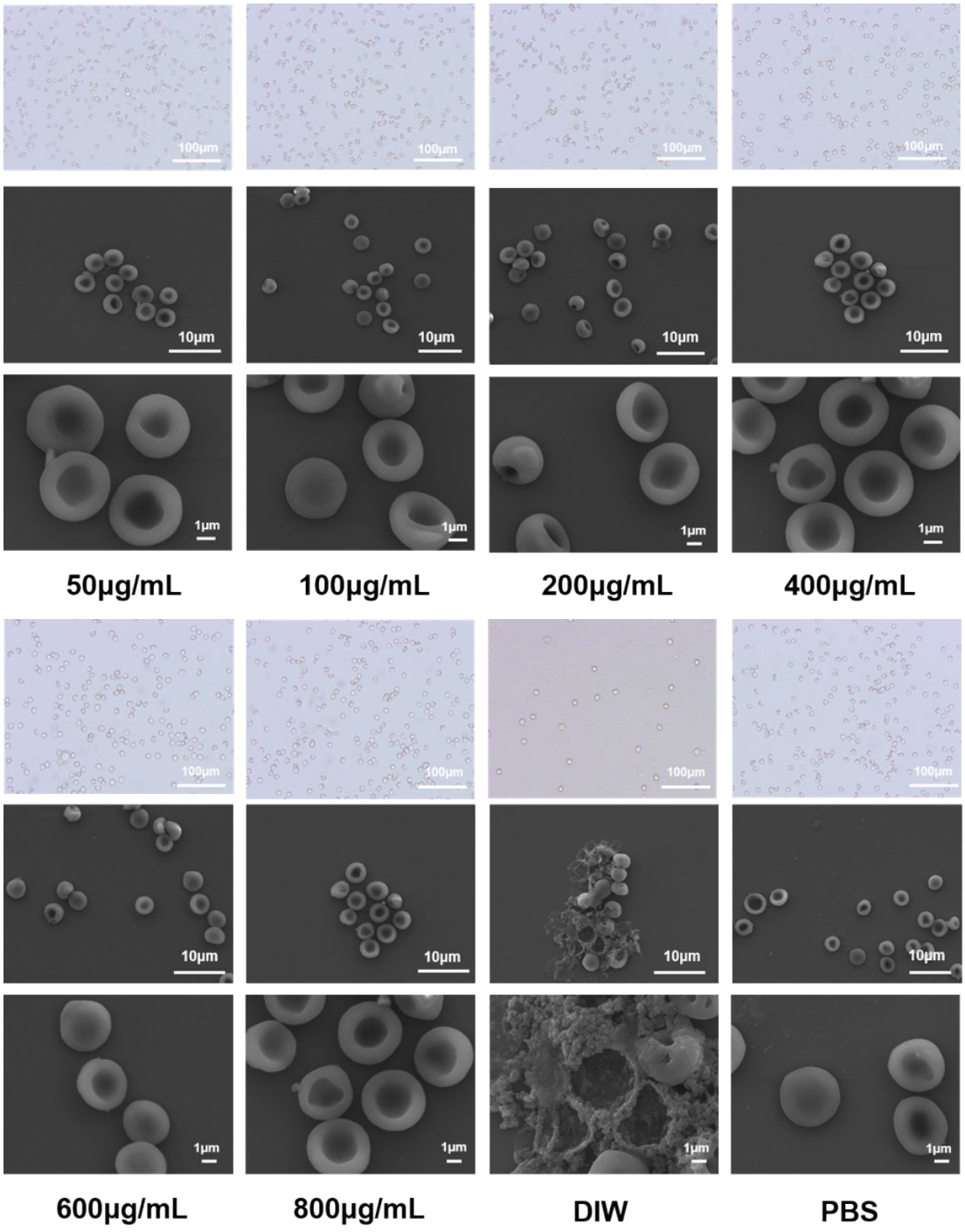

Figure S9 Inverted fluorescence microscope and scanning electron microscope images of mouse red blood cell suspensions fully cultured with different concentrations of antimicrobial peptides, DIW and PBS.

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