

Supporting Information

Bacterial Acidity-Triggered Antimicrobial Activity of Self-assembling Peptide Nanofibers

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Experiment section

Materials and methods

1. Materials

Fmoc-protected amino acids, 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU), MBHA rink amide resin, were purchased from Novabiochem. Piperidine, diisopropylethylamine (DIPEA) 5(6)-Carboxyfluorescein (FAM), 5(6)-carboxy-tetramethyl-rhodamine, Mueller Hinton Broth (MHB), MTT assay kit were purchased from Sigma-Aldrich. LIVE/DEAD™ BacLight™ Bacterial Viability Kit, Centrifugation filters with molecular weight cutoff at 10 kDa and 30 kDa, Agar, Triton™ X-100, Blood agar (TSA with 5% sheep blood) were purchased from Fisher Scientific. Dulbecco's modified Eagle medium (DMEM) culture medium was purchased from Life Technologies. Fetal Bovine Serum (FBS) was purchased from VWR. TEM staining reagent, uranium acetate dihydrate and TEM grid were purchased from TED PELLA, INC. *Escherichia coli* (ATCC 25922), *Bacteroides fragilis* (ATCC 25285) and *Staphylococcus aureus* (ATCC 29213) were purchased from ATCC.

2. Synthesis and purification of peptides

Multidomain peptides were synthesized on a *Prelude*® peptide synthesizer using standard FMOC-solid phase peptide synthesis procedures. Fmoc groups were deprotected by 20% (V/V) piperidine in N, N-dimethylformamide (DMF) for 5 min (2 times). HCTU was used as the coupling reagent and mixed Fmoc protected amino acids in the presence of DIPEA with a molar ratio of 1:1:2.5 (amino acid: HCTU: DIPEA). Upon the completion of the synthesis, the N-terminus of the peptides were acetylated in the presence acetic anhydride and DIPEA in DMF. The acetylated peptides were cleaved from the resin using a mixture of trifluoroacetic acid (TFA) / triisopropanolsilane (TIS) / H₂O (95/2.5/2.5 by volume) for 3 hours. The cleavage solution was collected through filtration and neat TFA was used to wash the resin twice. TFA solution was evaporated under moderate air flow. The residual peptide solution was precipitated in cold diethyl ether, followed by centrifugation and washing with cold diethyl ether for four times. The crude peptide was dried under vacuum overnight for HPLC

purification. The peptide was purified using a preparative reversed phase C4 column with a linear gradient of water/acetonitrile containing 0.05% TFA. Elution was monitored at 230 nm and 280 nm. The mass of the three peptides were confirmed by MALDI. WH₅: expected [M+H]⁺: 2634, observed [M+H]⁺: 2634; WH₇: expected [M+H]⁺: 2908, observed [M+H]⁺: 2908; WH₉: expected [M+H]⁺: 3182, observed [M+H]⁺: 3182. Fluorescein and rhodamine terminated peptides were synthesized as follows. After final deprotection of the peptide, the N-terminus was coupled with 4 equivalents of 5(6)-carboxyl fluorescein or 5(6)-carboxy-tetramethyl-rhodamine using a combination of 4 equivalents of HCTU and 8 equivalents of DIPEA in DMF. The reaction mixture was stirred overnight. The completion of the coupling reaction was confirmed by the Kaiser test. If necessary, the coupling of 5-(6)-carboxyl-fluorescein or 5(6)-carboxy-tetramethyl-rhodamine was repeated once. The cleavage and purification procedure followed the same procedure as described for the nonlabelled peptides. The molecular weight was confirmed by MALDI. FITC-WH₉: expected [M+H]⁺: 3499, observed [M+H]⁺: 3500; Rho-WH₉: expected [M+H]⁺: 3553, observed [M+H]⁺: 3554.

3. Structural Characterization

3.1 Circular Dichroism (CD) Spectroscopy

Samples were prepared by dilution from the peptide stock solution to a concentration at 50 μM in either Tris buffer (pH 7.4, 20 mM) or MES buffer (pH 5.7, 20 mM). The samples were incubated at 4°C overnight. Data were collected from 250 nm to 190 nm at room temperature (RT) using a 1 mm cuvette, a bandwidth at 1 nm, scan rate at 100 nm/min and a response time of 2 sec. Each spectrum was averaged from three scans. The mDeg of rotation was converted to molar residual ellipticity via the formula $\theta = (\text{mDeg} * 1000) / (c * n * l)$, where c is the concentration of the peptide solution expressed in mM, n is the number of amino acids in the peptide sequence and l is the path length of the cell used in mm.

3.2 Transmission Electron Microscopy (TEM)

Sample preparation was the same as that used in the CD experiment. Peptide solution (10 μL) was dropped onto a holey carbon grid (TED PELLA 01824). After 2 minutes, excess solution was carefully removed with filter paper. 10 μL of 2 wt % uranyl acetate aqueous solution was dropped onto the grid for negative staining. After 2 minutes, excess staining solution was

removed and the TEM samples were dried for overnight before imaging.

3.3 Critical aggregation concentration (CAC) measurement

Peptide solution (160 μ M) was added in either 200 μ L Tris buffer (20 mM, pH 7.4) or 200 μ L MES buffer (20 mM, pH 5.7) with an increment of 2 μ L each time. Fluorescence spectra were acquired after each peptide addition by monitoring the emission of peptides from 295 nm to 440 nm using an excitation wavelength at 280 nm. Fluorescence intensity at 350 nm was plotted as a function of the peptide concentrations. The CAC was determined to be the concentration at which nonlinearity started to develop as shown in **Figure S2**.

4. Minimum inhibitory concentration (MIC) determination

For the MIC test in the aerobic condition, *E.coli* was cultured in MHB media under constant shaking at 100 rpm at 37 °C to reach the mid-exponential growth phase. The bacterial solution was plated on an agar plate for colony forming unit (CFU) counting. Bacterial suspensions were diluted to approximately 2×10^5 CFU/mL in MHB media at either pH 7.4 or 5.7. Peptide solutions at various concentration (80, 40, 20, 10, 5, 2.5 μ M) were prepared in either Tris buffer (pH 7.4, 20 mM) or MES buffer (pH 5.7, 20 mM) through filter sterilization and exposed under UV light for at least 30 mins. 50 μ L of each peptide solution was mixed with 50 μ L of bacterial solution in a 96-well plate and the experiments were performed in triplicates. The plates were incubated at 37 °C under constant shaking at 100 rpm for 18 hrs and the optical density (OD) at 600 nm was measured on a plate reader. The MIC was determined at the peptide concentration in which OD reading is below 0.06 and no cloudiness was visible to naked eyes.

For the MIC test in the anaerobic condition, *E. coli* (ATCC 25922), *B. fragilis* (ATCC 25285) and *S. aureus* (ATCC 29213) inocula were prepared using the BBL Prompt Inoculation System to generate an approximate 1.5×10^8 CFU/mL that was further diluted to generate an inoculum at 1.0×10^6 CFU/mL in MHB media at pH 7.4. Peptide solutions with various concentration (80, 40, 20, 10, 5, 2.5 μ M) were prepared in Tris buffer (pH 7.4, 20 mM). All the reagents for the anaerobic test were reduced under anaerobic conditions for at least 2 hrs prior to the initiation of testing and care was taken to minimize all bacterial strains to oxygen exposure. 50 μ L of each peptide solution was mixed with 50 μ L of bacterial solution in a 96-well plate and the experiments were performed in triplicates. The plates were incubated 48 hrs

under constant shaking at 100 rpm. The MIC was determined at the peptide concentration in which OD reading is below 0.06 and no cloudiness was visible to naked eyes. For all MIC tests, bacterial culture without peptides (with equal volume mixing of Tris buffer) was used as a negative control. Gentamicin was used as the positive control that helps validate the MIC assay. The aerobic control plates only and against the control drugs (Gentamicin and Moxifloxacin) have MIC values within the CLSI control ranges and MBCs consistent with known bactericidal modes of action. Gentamicin is efficacious against *E.coli* and *S. aureus* although the MIC is shifted higher in anaerobic versus aerobic conditions.¹

5. Scanning electron microscopy to examine the morphology of the bacterial membrane

400 μ L bacterial suspensions (*E.coli*, 10^8 CFU/mL) were added to a 24-well plate with a cover glass (d=12 mm) placed on the bottom of each well. After 24 hrs of incubation, bacterial suspension was removed and the plates were washed with PBS buffer (pH 7.4) to remove any non-adherent bacteria. 100 μ L of fresh MHB media (pH 5.7) and 100 μ L of 40 μ M peptide solution in MES buffer (pH 5.7, 20 mM) were mixed and added in each well and incubated at 37 °C for 1 hr. The media were removed and the cover glasses were washed with Tris buffer for three times. Bacteria were fixed using 4% glutaraldehyde solution for overnight. The cover glasses were further dehydrated using a series of graded ethanol solutions from 35, 50, 75, 90, 95 and 100% (contents of ethanol volume). Samples were placed on a carbon tape and further coated with a 5 nm-thick gold layer. The morphology of the bacteria with and without peptide treatments were observed using a field emission scanning electron microscope operated at an accelerating voltage of 1.0 kV and a working distance of 5.8 mm.

6. Live and dead bacterial assay

400 μ L of bacterial suspensions (*E.coli*, 10^8 CFU/mL) was added to confocal dish and incubated at 37 °C for 24 hrs. Bacterial suspensions were removed from the confocal dish and washed with PBS buffer (pH 7.4) for three times to remove any non-adherent bacteria. 100 μ L of fresh MHB media and 100 μ L of 40 μ M peptide solution in either Tris buffer (pH 7.4, 20 mM) or MES buffer (pH 5.7, 20 mM) was added sequentially in the confocal dish. After incubation at 37 °C for 3 hrs, the culture media were removed and washed with PBS buffer (pH 7.4) for three times. Bacteria were stained with live/dead bacteria assay kit solution at room

temperature for 15 min. Finally, bacteria were washed with PBS buffer (pH 7.4) for three times. Images were captured with epifluorescence and processed with ImageJ software.

7. Membrane localization assay

Bacterial suspensions (*E.coli*, 10^8 CFU/mL) were added to a confocal dish. After 24 hrs of incubation, bacterial suspensions were removed and confocal dish was washed with PBS buffer (pH 7.4) for three times to remove any non-adherent bacteria. Next, 100 μ L of MHB media and 100 μ L of 7% FITC-labeled peptides were added to confocal dish to reach a concentration at 40 μ M. After 3 hrs of incubation, bacteria were washed with PBS (pH 7.4) for three times. Bacteria were stained with PI at room temperature for 15 min. Finally, bacteria were washed with PBS buffer (pH 7.4) for three times. Images were captured using a fluorescence microscope and processed with ImageJ software.

8. Hemolytic activity test

Human red blood cells (RBCs) were donated from a volunteer and 4% of human RBCs were prepared in PBS buffer (pH 7.4). 20 μ L of peptide solution at various concentrations (1600, 800, 400, 200, 100, 50, and 25 μ M) were prepared in PBS buffer (pH 7.4). Peptides were mixed with 180 μ L of RBC suspensions in a 1.5 mL Eppendorf tube. The mixtures were incubated at 37 °C for 1 hr, followed by centrifugation at 3000 g for 5 mins. 100 μ L of the supernatant was taken out and transferred to a 96-well plate. Hemoglobin release was determined by measuring the absorbance of the supernatant at 540 nm on a microplate reader (Vitor2 1420 Multilabel Counter, PerkinElmer). RBCs treated with 1% Triton-X served as positive controls and untreated RBCs served as a negative control group. Each sample was tested in three replicates. The percentage of hemolysis remained is calculated using the following equation

$$\% \text{ hemolysis} = (A_{\text{peptide}} - A_{\text{negative control}}) / (A_{\text{Triton X}} - A_{\text{negative control}}) \times 100$$

in which the negative control group contains RBC suspension mixed with PBS buffer without peptides.

9. Cytotoxicity measurement

NIH/3T3 cells were seeded onto a 96-well plate at a density of 10^4 cells/well and incubated for 24 hrs at 37 °C in an incubator with 5% of CO₂. After 24 hrs, the culture medium was removed. 10 μ L of peptide solution at various concentrations (800, 400, 200, 100, 50, 25, 12.5 μ M) was

mixed with 90 μ L fresh culture medium in a 96-well plate. After 24 hrs of incubation, the MTT assay was performed to quantify the cell viability by monitoring the UV absorbance at 490 nm. Cell culture without peptides were used as a negative control. All the experiments were performed in four replicates.

10. Measurement of the microenvironmental pH in bacterial colonies

The pH value of the *B. fragilis* living milieu was measured using a pH ratiometric fluorescence imaging probe based on our previous publication with minor modification.² First, probes in PBS (0.5mg/mL) with different pH values were dropped on surface of the blank TSAB (Trptic Soy Agar with 5% sheep blood) plate, imaged with an *in vivo* Kodak imager (Ex 630nm, Em 700 nm, Exposure time 10s; Ex 760nm, Em 830 nm, Exposure time 10s). The results were analyzed to acquire a correlation curve between fluorescence ratio and indicated pH value and further plotted as a standard curve. Secondly, probes suspended in DI water (0.5mg/mL) were dropped on individual *B. fragilis* colonies on a TSAB plate and the plate without bacteria (as a control) and were imaged with the same protocol. The pH around bacterial colonies was determined based on the standard curve established above.

11. Statistical analysis

Student's *t*-test was used to analyze the cell viability and hemolytic activity of peptides at different concentrations. Experiments were performed in three replicates. A *p*-value of less than 0.05 was considered to be statistically significant.

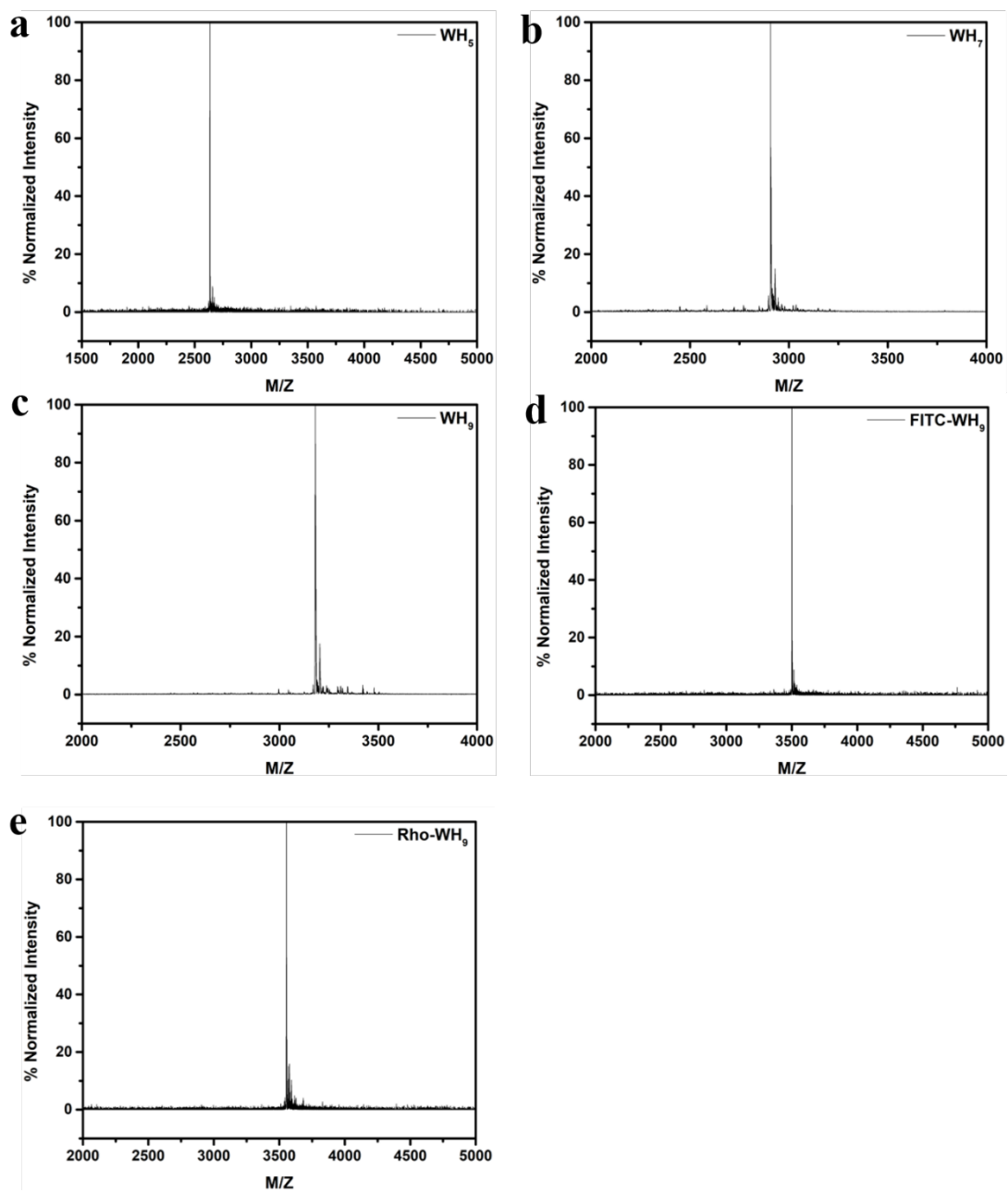


Figure S1. MALDI spectra of WH₅ (a), WH₇ (b), WH₉ (c), FITC-WH₉ (d) and Rho-WH₉ (e).

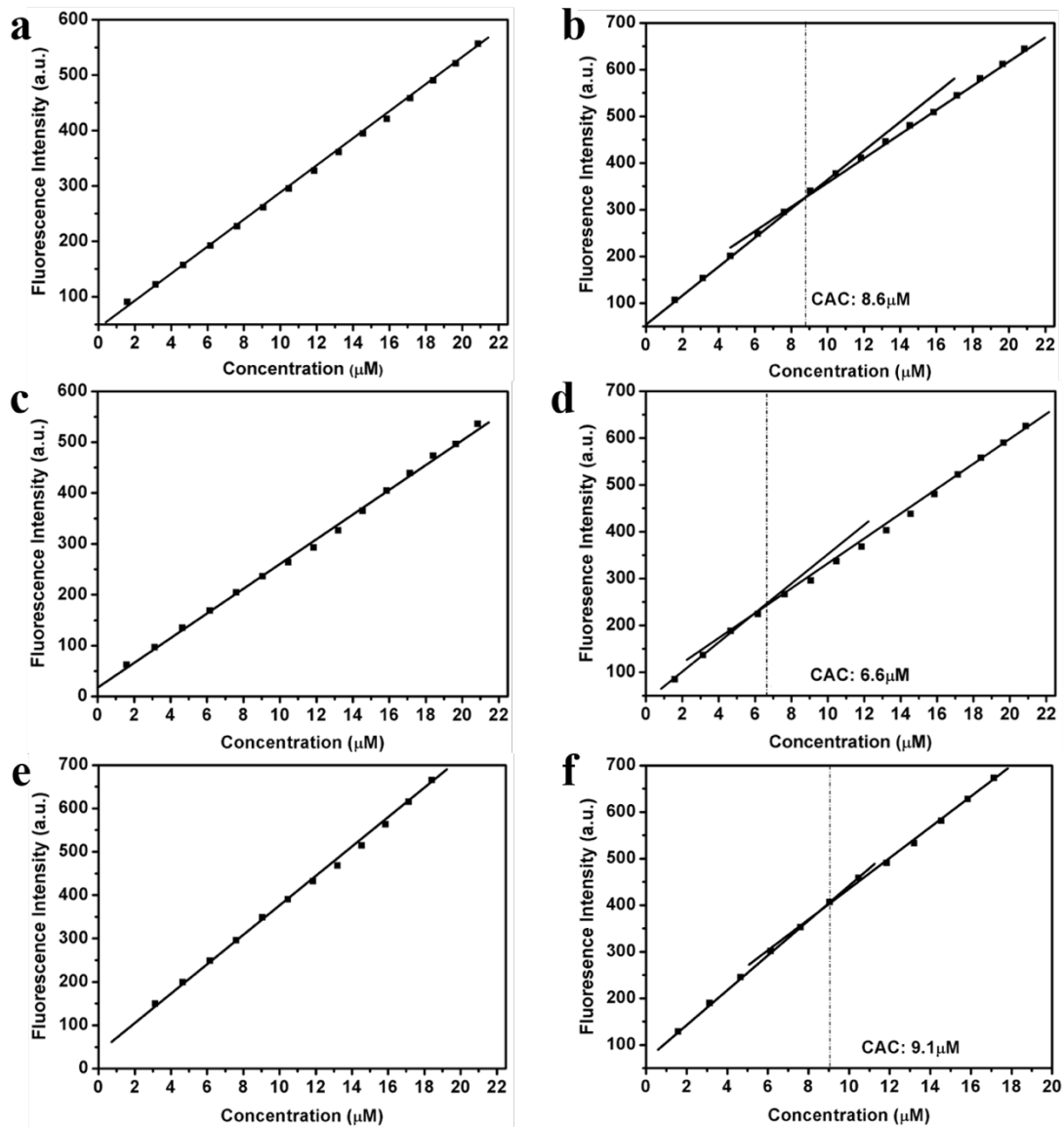


Figure S2. CAC determination by monitoring the tryptophan fluorescence at various peptide concentrations at pH 7.4 and pH 5.7. WH₅ at (a) and pH 5.7 (b) pH 7.4; WH₇ at (c) pH 5.7 and (d) pH 7.4; WH₉ at (e) pH 5.7 and (f) pH 7.4.

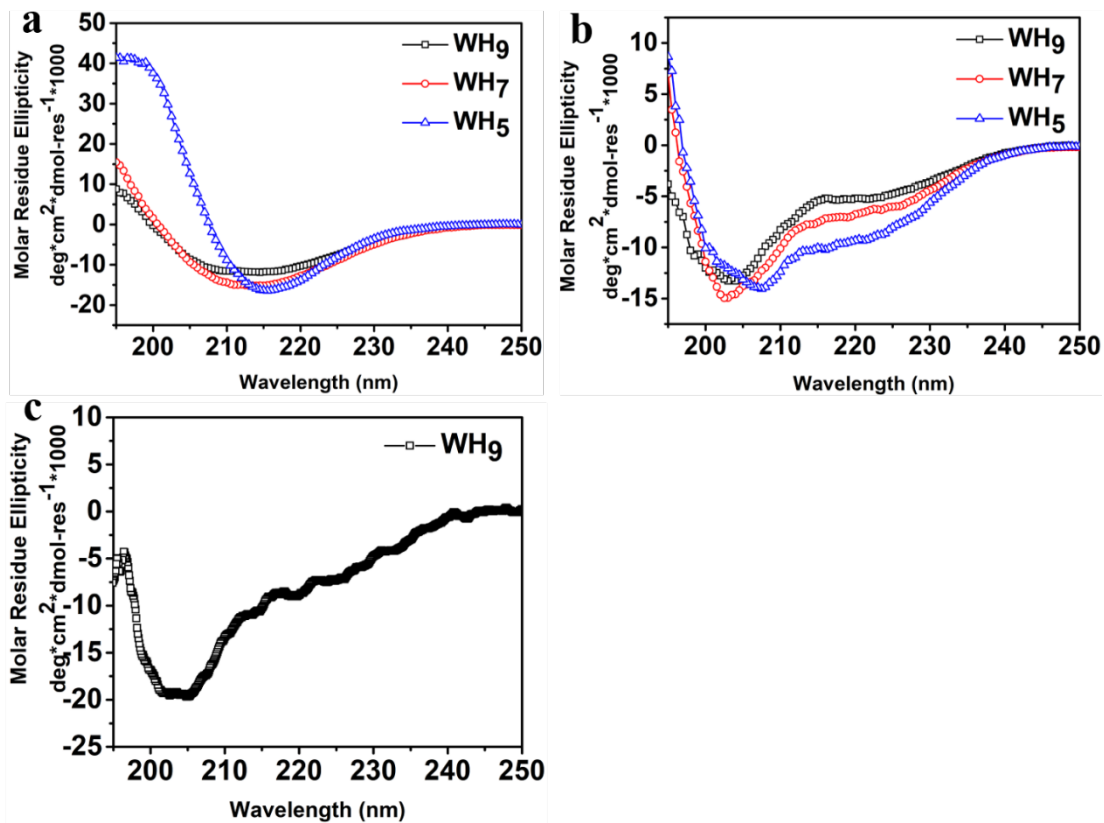


Figure S3. pH-dependent peptide secondary structures by CD spectroscopy at RT. (a) CD spectra of peptides showing predominant β -sheet secondary structures in Tris buffer (pH 7.4, 20 mM); (b) CD spectra of peptides in MES buffer (pH 5.7, 20 mM) showing weak helices/random coils; (c) CD spectrum of the filtrate of WH₉ in MES buffer (pH 5.7, 20 mM) showing a random coiled structure. Peptide concentration: 50 μM .

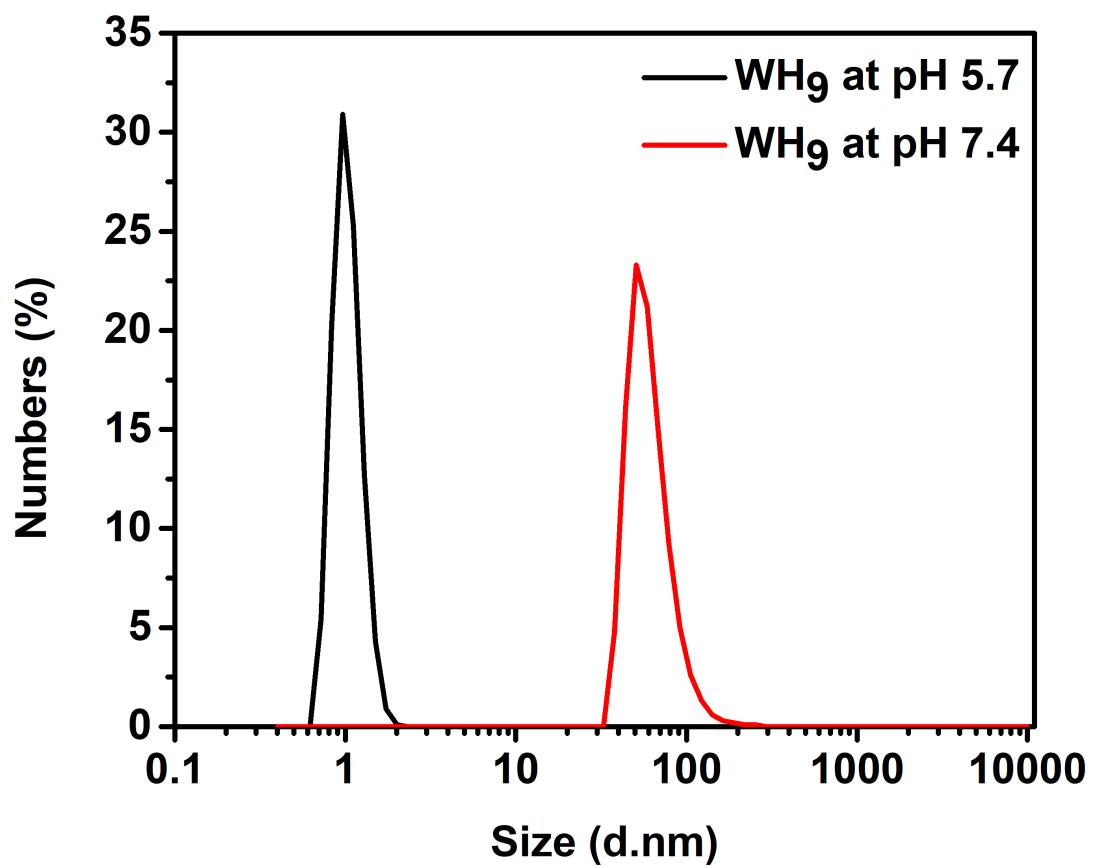


Figure S4. Particle size distribution by numbers (%) of WH₉ at neutral and acidic condition.

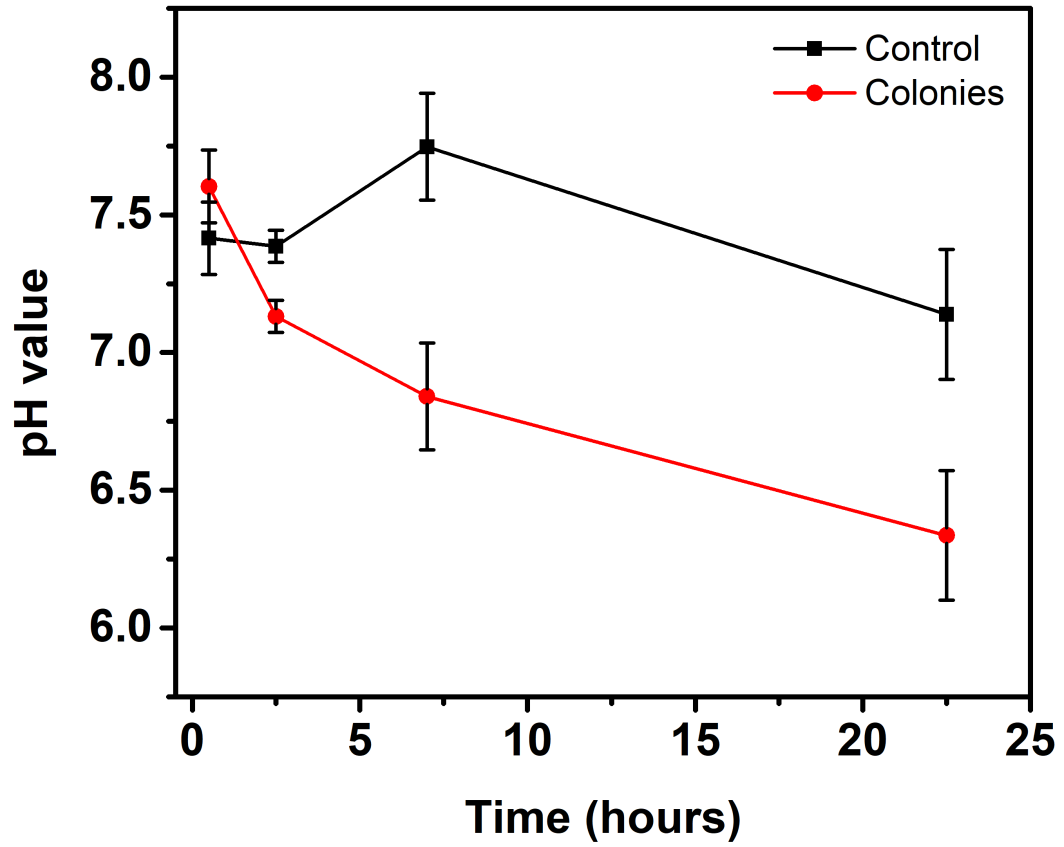


Figure S5. Time-dependent local pH of *B. fragilis* on the agar plate.

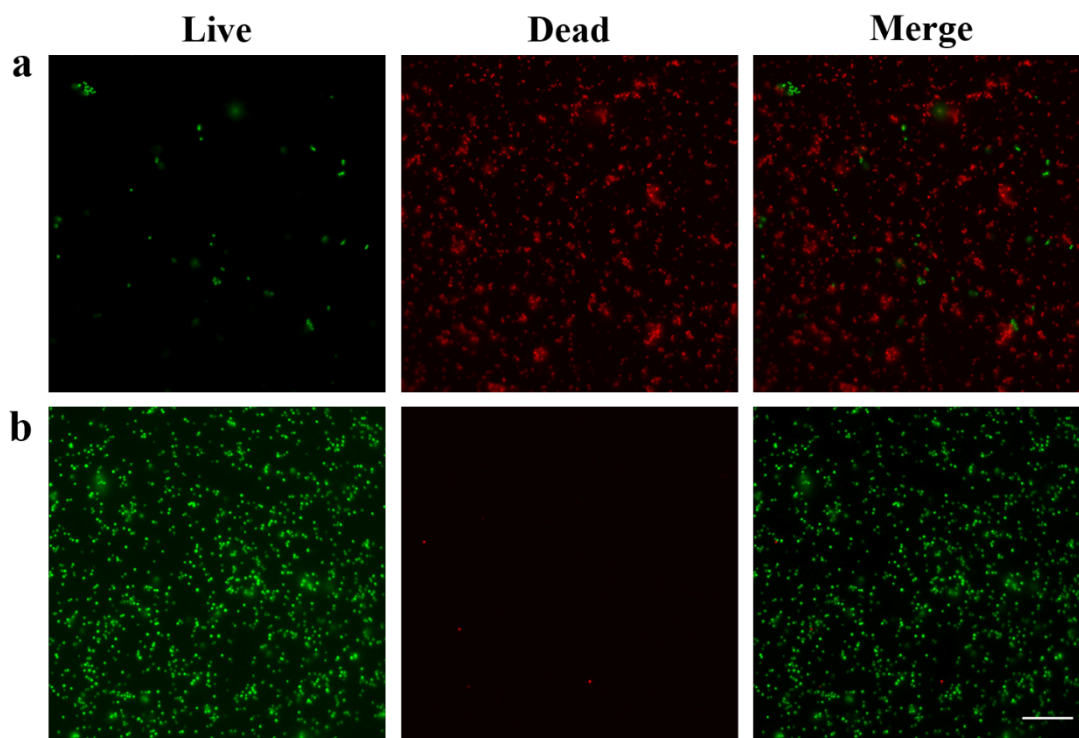


Figure S6. Fluorescence images of Live/dead bacterial assay results. Top panel: *S.aureus* treated with 10 μ M WH₉ at (a) pH 5.7 and (b) pH 7.4 for 3hrs. Live bacteria were stained with SYTO9 (green) and dead bacteria was stained with PI (red). Scale bar: 20 μ m.

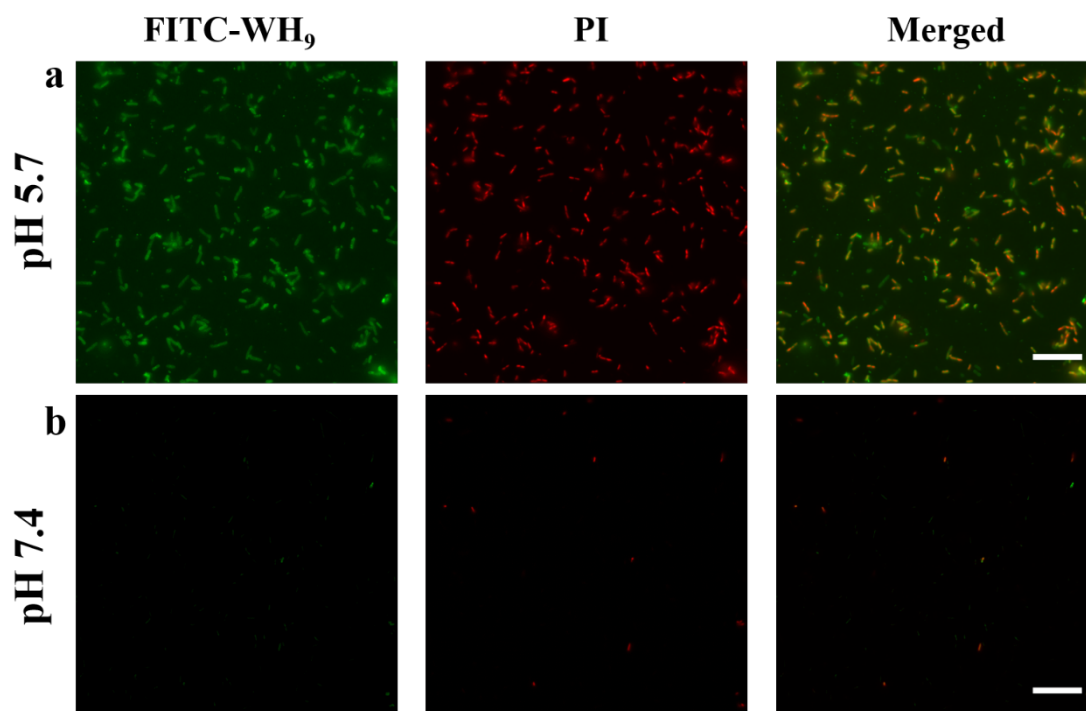


Figure S7. Fluorescence images of *E.coli* treated with FITC-WH₉ followed by PI staining in (a) acidic (pH 5.7) and (b) neutral culture condition (pH 7.4). FITC-WH₉ was found to attach on the bacterial membrane in the acidic condition, causing membrane disruption and bacterial death as stained by PI. Scale: 20 μm .

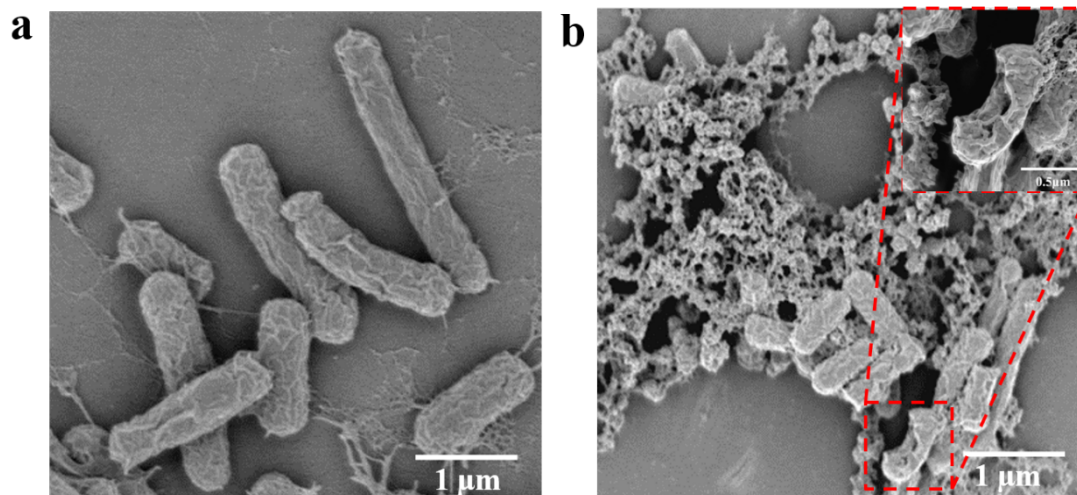


Figure S8. SEM images showing the morphological change of *E. coli* with and without peptide treatment in the acidic condition. **a)** *E. coli* without peptide treatment at pH 5.7. **b)** *E. coli* upon WH₉ incubation (2x MIC) for 1 hr at pH 5.7. The inset picture shows the damage of bacterial membrane upon peptide treatment.

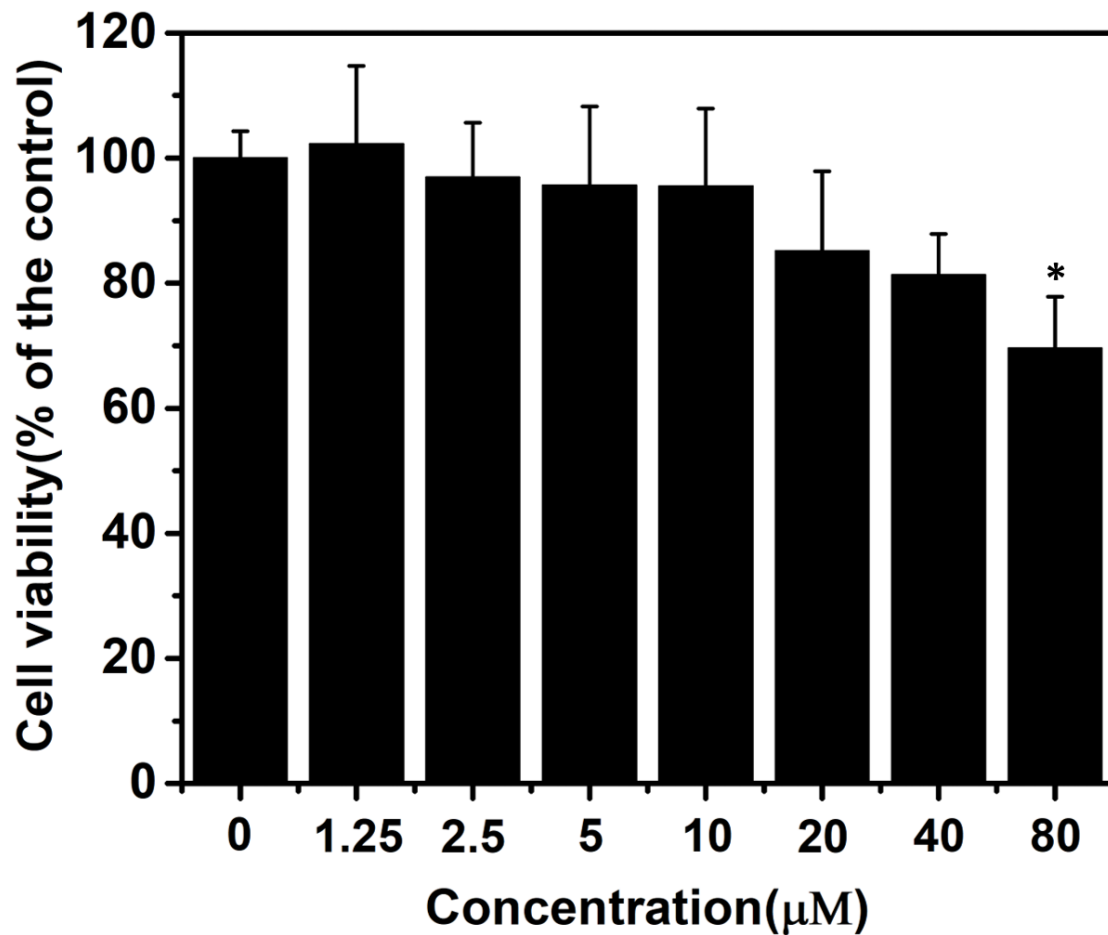


Figure S9. NIH/3T3 cell viability of peptide-treated cells in relative to the control group without peptides after 24 hrs of incubation with WH₉ at various concentrations. Statistic significant difference are indicated by * $p < 0.05$.

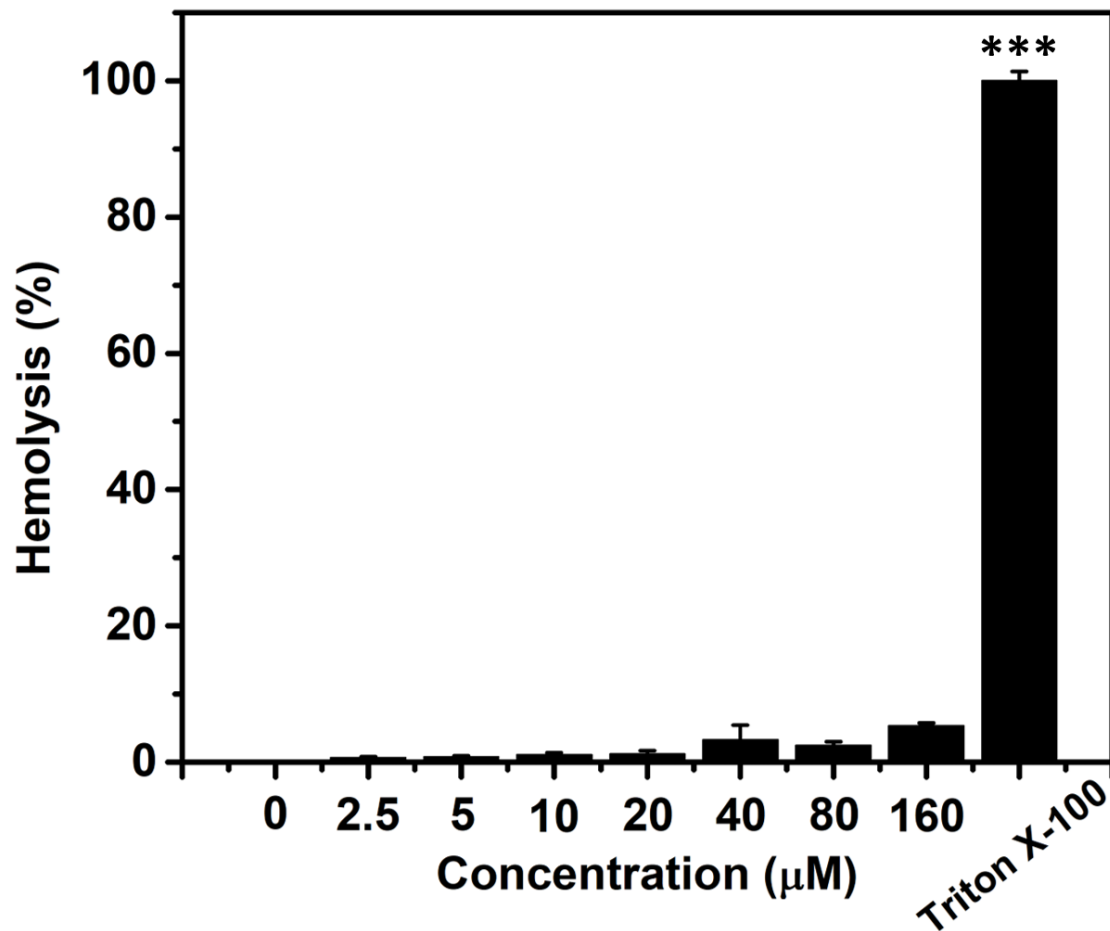


Figure S10. The percentage of hemolysis induced by WH₉ at various peptide concentrations. Statistic significant difference are indicated by *** $p < 0.001$.

- 1 (a) Z. DeMars, S. Biswas, R. G. Amachawadi, D. G. Renter and V. V. Volkova, *PLOS ONE* 2016, **11**, e0155599;
 (b) L. J. Harrell and J. B. Evans, *Antimicrob. Agents Chemother.* 1978, **14**, 927.
- 2 Y.-T. Tsai, J. Zhou, H. Weng, J. Shen, L. Tang and W.-J. Hu, *Adv. Healthc. Mater.* 2014, **3**, 221.