

SUPPORTING INFORMATION

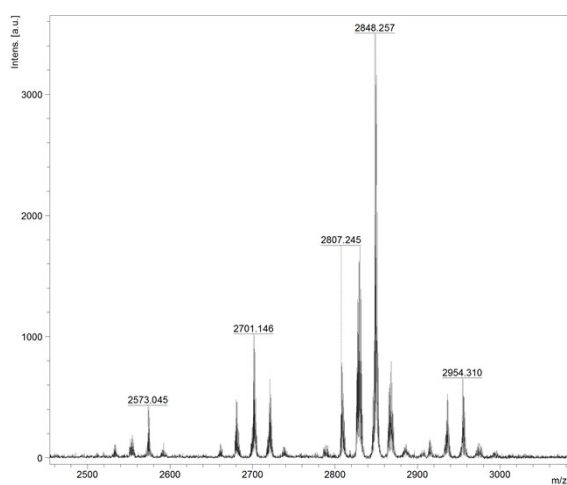
Random Peptide mixtures Inhibit and Eradicate Methicillin-Resistant *Staphylococcus aureus* Biofilm

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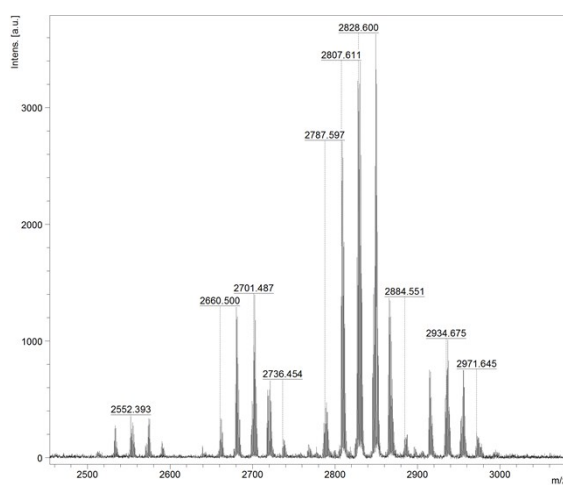
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Chemical and biological characterization of random peptide mixtures that were used in this study:

A.



B.



C.

D.

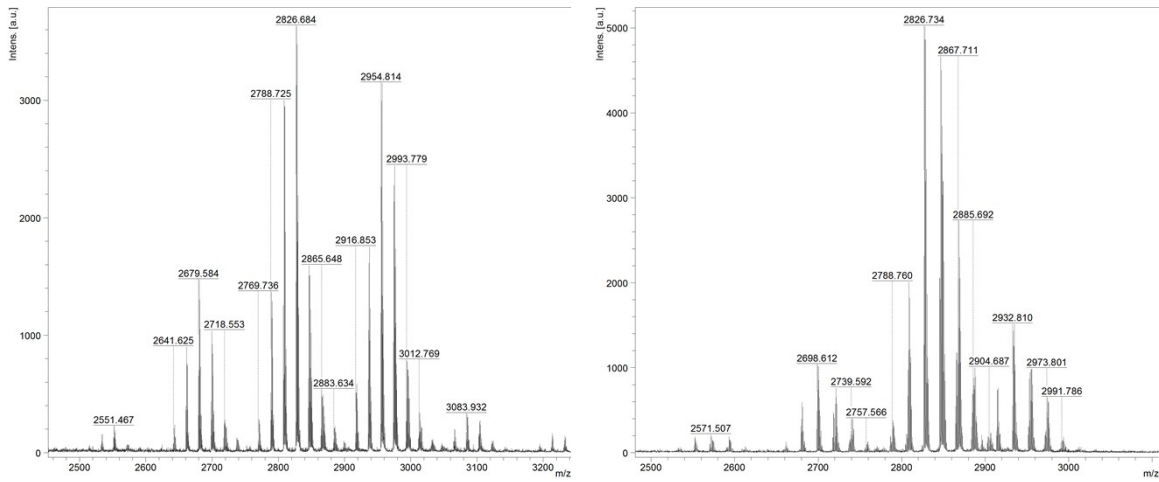


Figure S1: MALDI TOF MS representative spectra of two independent syntheses of L^{FLK} (A, B) and L^{FDK} (C, D).

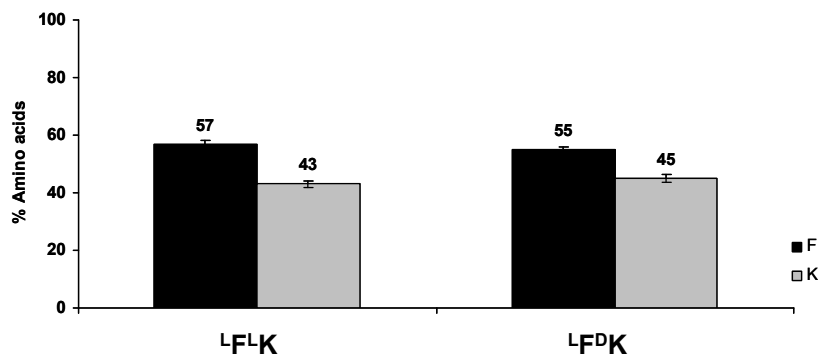


Figure S2: Amino acids analysis results of 3 different batches of L^{FLK} and L^{FDK} random peptide mixtures.

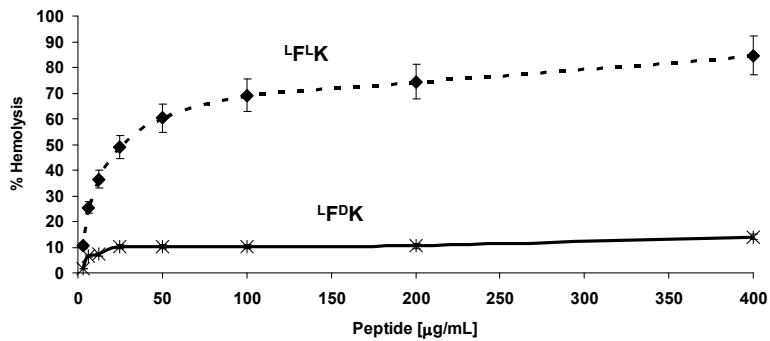


Figure S3: Hemolytic activities of L^{FLK} and L^{FDK} towards human red blood cells under several concentrations.

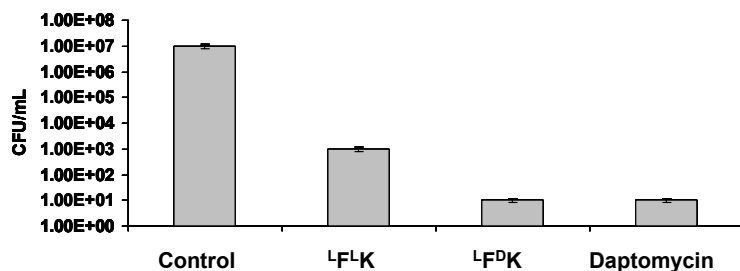


Figure S4: Bacterial cell counting after biofilm treatment with the random peptides mixtures L^FL^K and L^FD^K and with daptomycin as a positive control.

Materials and methods:

Chemicals:

Fmoc-protected L/D- α -amino acids with acid-labile side-chain protecting groups and rink Amide resin were purchased from Chemimpex. N-hydroxybenzotriazole (HOBt), N,N-dimethylformamide (DMF), and N,N-diisopropylethylamine (DIEA) were purchased from Biolab, Israel. 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was purchased from Sigma. Dehydrated LB culture medium (244610) was obtained from BD (Franklin Lakes, NJ). All other chemicals were purchased from Sigma Aldrich and used without purification.

Synthesis of sequence-random peptide mixtures:

Random peptide mixtures were synthesized using microwave irradiation on Rink Amide resin (Substitution 0.2 mmol/gr, 25 μ mol) in Alltech filter tubes as was described previously¹. Briefly, coupling reactions were conducted with binary combinations of L/D- protected α -amino acids, with a freshly prepared stock solution that contained the protected amino acids in 1:1 molar ratio and stereochemistry, which was used for each coupling step. Before each coupling step, an aliquot containing 4 equiv. (100 μ mol) of the amino acid mixture was activated with 4 equiv. of HBTU, 4 equiv. of HOBt, and 8 equiv. of DIEA, in DMF. After the activated amino acid solution was added to the resin, the reaction mixture was heated to 70 °C in a MARS VI (CEM, USA) multimode microwave (2 minute ramp to 70 °C, 4 minute hold 70°C) with stirring. Fmoc

deprotection reactions used 20 % piperidine in DMF. Reaction solutions were heated to 80 °C in the microwave (2 minute ramp to 80 °C, 2 minute hold 80 °C) with stirring. After each coupling/deprotection cycle the resin was washed 3 times with DMF. Upon completion of the synthesis, the peptide mixture was cleaved from the resin by stirring the resin in a solution containing 95 % trifluoroacetic acid (TFA), 2.5 % water, and 2.5 % triisopropylsilane for 3 hours. The peptide mixture was precipitated from the TFA solution by the addition of cold ether. The precipitated peptide mixture was collected by centrifugation. Ether was removed, and the pellet was dried under a stream of nitrogen, frozen in dry ice and lyophilized.

Bacterial growth inhibition assays (MIC)²⁴:

The bacteria used in these assays was methicillin resistant *Staphylococcus aureus* 1206². Antibacterial activities were determined in sterile 96-well plates (BD Falcon 353072 tissue culture plates). Bacterial cells were grown overnight at 37 °C on agar, after which a bacterial suspension of approximately 2×10^6 CFU/mL in Luria Bertani (LB) growth medium was prepared. Samples (50 µL) of this suspension were added to 50 µL of medium containing the random peptide mixture in 2-fold serial dilutions for a total volume of 100 µL in each well. The plates were then incubated at 37 °C for 6 or 24 hours. Bacterial growth was determined by measuring the optical density (OD) at 650 nm (Tecan Safire plate reader). The positive control was LB without addition of peptide mixture. The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which complete inhibition of bacterial growth was observed (no increase in OD over the course of the experiment).

Biofilm assay:

A clinical isolate strain of *S. aureus* (1206) was used. Bacterial cells were grown overnight at 37 °C on agar, after which a bacterial suspension of approximately 2×10^6 CFU/mL in LB medium was prepared. 100 µL of the bacterial suspension was then inoculated in several wells of a 96 well plate and incubated overnight at 37 °C. The supernatant liquid was discarded and the wells were washed with milli-Q water three times. 100µL of LB medium containing the random peptide mixture or antibiotic in 2-

fold serial dilutions were added to the wells containing the biofilm and the plates were incubated overnight at 37 °C (in the biofilm biomass assay for daptomycin, all antibiotic/biofilm mixture at varied daptomycin concentration contained 1mM CaCl₂). The supernatant liquid was discarded and the wells washed with milli-Q water three times.

Crystal violet assay for biomass quantification:

The wells were treated with 125µL of 0.1% crystal violet and incubated for 15 min at 37 °C³. Excess crystal violet was washed off thoroughly with milli-Q water three times. 125µL of 30% acetic acid was added to each well and the solution was transferred to a new 96-well plate, and absorbance was measured at 550 nm (Tecan plate reader) using 30% acetic acid in water as blank. The results were expressed as a percentage of biomass in the control biofilm, which was grown without any treatment.

XTT assay for cell viability within biofilm:

Cell viability of the biofilm was quantified after incubation with peptides or antibiotic for 24 hours by an XTT assay ²⁷. XTT (1 mg/mL) and phenazine methosulfate in the ratio 3:1 were freshly prepared in 1X PBS buffer. Following the washing step of the biofilm after treatment, 100 µL of this solution was added to the wells and the plates were incubated at 37 °C for 3 hours in the dark. An aliquot of 90 µL was taken and transferred to a 96-well plate and OD was measured at 490 nm (Tecan plate reader) using the XTT solution as blank. The results were expressed as a percentage of cell viability relative to the control biofilm without any treatment.

SEM study:

Bacteria cells were grown overnight in LB at 37°C, 180RPM, diluted with LB to OD_{600nm} 0.1A. Sterile glass slides in 96 well plates were covered with 100 µL of bacteria suspension and incubated at 37 °C for 24 hours for biofilm growth. 100µL of LB medium containing the random peptide mixture in 2-fold serial dilutions were added to the wells containing the biofilm and the plate was incubated at 37 °C for another 24 hours. The slides were washed with deionized water three times. 200 µL glutaraldehyde

4% in PBS was added to slides and incubated for 1 hour at room temperature. Rinsing was with PBS 10 minutes, five times. Dehydration was done in increasing ethanol concentrations (25, 50, 75, 95, 100% ethanol in deionized water), 10 minutes twice for each concentration. Drying was performed with a "critical point dryer" (CPD BAL-TEC CPD-030) and gold coating was performed with Gold Sputter Coater (Polaron SEM Coating Unit). Jeol JSM 5410 LV - A Low Vacuum SEM was used for observing the influence of ^LF^LK and ^LF^DK on MRSA biofilm.

Confocal microscopy:

Bacteria cells were grown overnight in LB at 37 °C, 180RPM, diluted with LB to OD_{600nm} 0.1A. Sterile glass slides were covered with 1 mL of bacteria suspension and incubated in 37°C 24h. Bacteria suspension was discarded and slides were washed gently with 1 mL LB. 5/6 Carboxyfluorescein labeled peptides ^LF^LK or ^LF^DK were dissolved in LB at a concentration of 200 µg/mL and added to slides for incubation of 90 minutes at 37°C. Slides were washed gently with sterile PBS*1 three times and observed by a Leica SP8 Confocal Laser Scanning Microscope with water objective 63. UV vis 488: 1.5% laser intensity, PMT emission 500-540.

References:

- ¹ Z. Hayouka, S. Chakraborty, R. Liu, M. D. Boersma, B. Weisblum, and S. H. Gellman, *J Am Chem Soc*, 2013, **135**, 11748-51.
- ² B. Weisblum and V. Demohn, *J Bacteriol*, 1969, **98**, 447.
- ³ G. A. O'Toole and R. Kolter, *Mol Microbiol*, 1998, **30**, 295.