Supporting Information for

Designed Supramolecular Filamentous Cationic Peptides: Balance of Nanostructure, Cytotoxicity and Antimicrobial Activity

Dawei Xu,^a Linhai Jiang,^a Anju Singh,^b Derek Dustin,^a Miao Yang,^a Ling Liu,^c Reidar Lund,^d Timothy J. Sellati,^b He Dong^{*,a}

^a Department of Chemistry & Biomolecular Science, Clarkson University, Potsdam, NY 13699

^b Trudeau Institute, Saranac Lake, NY, 12983

^c Department of Mechanical and Aerospace Engineering, Utah State University, Logan, UT, 84322

^d Department of Chemistry, University of Oslo, 0315 Oslo, Norway

Contents

1. General methods
2. Synthesis and purification of peptides
3. Structural characterization
3.1 Circular dichroism (CD) Spectrum
3.2 TEM
4. Critical assembly concentration (CAC)
5. Small angle x-ray scattering experiment and fitting
5.1. Synchrontron experiment6
5.2. Theoretical modeling
6. Cytotoxicity assay
6.1 Isolation and differentiation of mouse bone marrow-derived monocytes (BMDMs)8
6.2 Lactate dehydrogenase (LDH) cytotoxicity assay8
6.3 Confocal microscopy for quantitative analysis of the effect of peptides on BMDMs9
7. Proteolytic stability measurement 10
8. Bacterial culture
9. Minimum inhibitory concentration (MIC) measurements 11
10. Killing efficiency test 11

1. General Methods.

Fmoc-protected amino acids, 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3tetramethylaminium hexafluorophosphate (HCTU), 2-(1H-Benzotriazole-1-yl)-1,1,3,3tetramethyluronium hexafluo-rophosphate (HBTU) were purchased from Novabiochem. 5(6)-Carboxyfluorescein (FAM), piperidine, trypsin and alpha-chymotrysin were purchased from Sigma-Aldrich. All other reagents and solvents were purchased from Fisher Scientific and used as received. Circular dichroism (CD) spectrum was acquired with Jasco-J715 Spectropolarimeter using a quartz cell with 1 mm path length. Transmission electron microscopy (TEM) images were obtained from JEOL 2010 highresolution transmission electron microscope. Emission intensities of 5(6)carboxyfluorescein terminated multi-domain peptide were obtained from Varian Cary Eclipse fluorescence spectrophotometer using a quartz cell with 1 mm path length. Reversed-phase analytical HPLC was performed using HITACHI L-7100 pump, Waters 717 plus auto-sampler and Higgins analytical column (proto 300 C4 10 µm, 250*10 mm). The identity and purity of the peptides were verified by MALDI-TOF mass spectrometry using α -cyano-4-hydroxycinnamic acid matrix. Mass spectra were recorded on an Applied BioSystems Voyager-DE Pro. Scanning electron microscopy images of bacteria were acquired with JEOL 7400 high-resolution field emission electron microscope. Minimum inhibitory concentration measurements were performed on a micro-plate reader (Vitor² 1420 Multilabel Counter, PerkinElmer).

2. Synthesis and Purification of Peptides

Multidomain peptides, MDPs, were synthesized on a PS3 peptide synthesizer using standard FMOC-solid phase peptide synthesis. 20% (V/V) piperidine in N,N-dimethylformamide (DMF) was used to deprotect Fmoc groups for 5 min (2 times). HCTU and DIPEA were used as amino acid coupling reagents in a molar ratio of 1:1:2.5 (amino acid: HCTU: DIPEA). Fmoc protected amino acids were added in four equivalents of the resin. The N-terminus was acetylated in the presence of 50 equiv of

acetic anhydride and 6 equiv of DIPEA in DMF. Then the peptide was cleaved from the resin in a mixture of TFA / triisopropanolsilane (TIS) / H₂O (95/2.5/2.5 by volume) for 3 hours. The TFA solution was collected and then the resin was rinsed twice with neat TFA. After evaporation of the combined TFA solutions, the residual peptide solution was triturated with cold diethyl ether. The resulting precipitate was centrifuged and washed for three times with cold diethyl ether. The crude peptide was then dried under vacuum overnight for further HPLC purification. The peptide was purified using a preparative reversed phase C18 column with a linear gradient water/acetonitrile containing 0.05% TFA. Elution was monitored at 230nm and 280 nm. The masses of the three peptides were confirmed by MALDI. MDP-1: expected $[M+H]^+$: 2205.3, observed $[M+H]^+$: 2333.9, observed $[M+H]^+$: 2334.0;

For 5(6)-carboxyfluorescein terminated multi-domain peptide (FAM-MDP) was used for the determination of the critical assembly concentration. After final deprotection, the free amino group at the N-terminus was coupled with 5(6)-carboxyl fluorescein using a combination of HBTU/DIPEA (3/5) as the coupling and activating reagents. The reactant mixture was stirred overnight at RT. The completion of the coupling reaction was confirmed by the Kaiser test. If necessary, coupling of 5-(6)-carboxyl fluorescein was repeated once. Cleavage, purification and MALDI characterization follow the same procedures used for the non-labeled MDPs. FAM-MDP-1: expected [M+H]⁺: 2521.6, observed [M+H]⁺: 2521.5; FAM-MDP-2: expected [M+H]⁺: 2649.7, observed [M+H]⁺: 2649.9.

3. Structural Characterization

3.1 Circular Dichroism (CD) Spectrum

All samples were freshly diluted to a concentration of 50 μ M in Tris buffer (pH 7.4, 20mM) and then examined on a CD spectrometer. All the data were collected from 250 nm to 190 nm at room temperature (RT) for 10 scans with each scan rate at 100 nm/min, a response time of 2 sec, and a bandwidth of 1 nm. The mDeg of rotation was converted to molar residual ellipticity via the formula θ =(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. The proportion of secondary structures was calculated according to the formula $\theta = x_{\alpha}\theta_{\alpha} + x_{\beta}\theta_{\beta} + x_{c}\theta_{c}$ $(x_{\alpha}+x_{\beta}+x_{c}=1)$ via least squares method, where x_{α} , x_{β} and x_{c} represent the proportion of α -helix, β -sheet, and random coil, respectively, θ_{α} , θ_{β} , and θ_{c} represent the molar residual ellipticity of pure α -helix, β -sheet, and random coil, respectively, and θ represents the experimental molar residual ellipticity for each peptide.





Peptide solution was prepared in Tris buffer (pH=7.4, 20 mM) to reach a final peptide concentration of 50μ M.

3.2 TEM

Lyophilized MDP powder was dissolved in Tris buffer (pH=7.4, 20mM) to reach a final concentration of 100 μ M. Peptide solution (10 μ l) was dropped onto a holey carbon grid (TED PELLA 01824). After 2 minutes, excess solution was carefully removed by using filter paper, the sample was dried for another 2 minutes, following by addition of 10 μ l of 2 wt% uranyl acetate aqueous solution, which is a negatively staining reagent. After 30 seconds, excess staining solution was removed and the TEM samples were allowed to dry for 5 hrs before imaging.

4. Critical assembly concentration (CAC)

5(6)-carboxyfluorescein terminated peptide (FAM-MDP) powder was dissolved in Tris buffer (pH=7.5, 20mM) and diluted to a concentration of 30 μ M. A serial 2-fold dilution was performed to obtain peptide solutions at concentrations of 15, 7.5, 3.8, 1.9, 0.94, 0.47, 0.23, 0.12 and 0.06 μ M. The solutions were incubated for 1 hr at RT for equilibration. Emission intensity of the FAM-MDP solution at different concentrations was acquired at λ_{ex} =494 and λ_{em} =521 nm. The emission intensity as a function of concentration was plotted as shown in Figure S1. The crossing point of the two fitting line was defined as the CAC.



5. Small angle x-ray scattering experiment and fitting

5.1. Synchrotron experiment

The SAXS experiments were performed on the automated BM29 beamline at the ESRF, Grenoble, France. For technical details we refer to Pernot et al. *J. Synchrotron Rad.* (2013). **20**, 660-664. The data were obtained using a wavelength of 1 Å and detector distance 2.87 m covering a Q-range (Q= $4\pi \sin (\theta/2)/\lambda$, λ is the wavelength, θ is the scattering angle) of about 0.0047 Å⁻¹< Q < 0.5 Å⁻¹.

5.2. Theoretical Modeling

In order to analyze the data we considered a simple platelet model with dimensions a < b < c and polydispersity in a modeled Gaussian distribution function. The total volume of the filament was therefore V = $\langle a \rangle \cdot b \cdot c = PV$ In addition we added Gaussian peak function and a constant background to take into account internal density fluctuations visible in the data at high *Q*. We also allowed for coexistence between freely dissolved peptide chains and supramolecular filaments by assuming superposition of the scattering from a simple chain form factor and platelets. The data were modeled on an absolute intensity scale using the following total intensity:

$$I(Q) = \frac{\phi}{\langle a \rangle \cdot b \cdot c} (\rho_p - \rho_0)^2 \cdot (I(Q)_{\text{fil}} \cdot (1 - f_{\text{chain}}) + f_{\text{chain}} I(Q)_{\text{chain}}) + I_{int}(Q) + bcg \quad (1)$$

where f_{chain} is the fraction of free chains, *bcg* is a constant background, ρ_p and ρ_0 are the scattering length densities of the peptide and solvent, respectively. For the peptide we find a $\rho_p = 1.10 \cdot 10^{11}$ cm⁻² based on a density of 1.2 g/mL whereas for the solvent, $\rho_0 = 9.43 \cdot 10^{11}$ cm⁻²

$$I(Q)_{\rm fil} = P_c(Q) \cdot b \cdot c \cdot \int_0^{2\pi} \mathrm{d}\beta \int_0^\infty f(a) \, a^2 A(Q,\beta,a,b)^2 \mathrm{d}a \tag{2}$$

$$A(Q,\beta,a,b) = \frac{\sin(Q\,b\cos(\beta)/2)}{Q\,b\cos(\beta)/2} \frac{\sin(Q\,a\sin(\beta)/2)}{Q\,a\sin(\beta)/2}$$
(3)

and where f(a) describes a polydispersity in *a* through a Gaussian distribution:

$$f(a) = \frac{1}{\sqrt{2\pi\sigma}} \exp\left(-\frac{(a-\langle a \rangle)^2}{2\sigma^2}\right)$$
(4)

It turned out that all MDPs, in particular $WK_2(QL)_6K_2$ and $WK_3(QL)_6K_2$, exhibited an excess scattering at the highest Q in the form of a prominent peak at $Q_{max} \approx 0.3 \text{ Å}^{-1}$. This indicates an internal structure not captured by the homogeneous prism. This contribution was included ad hoc by adding a Gaussian peak function:

$$I(Q)_{int.} = C \cdot \frac{1}{\sqrt{2\pi\sigma_Q}} \exp\left(-\frac{(Q-Q_{max})^2}{2\sigma_Q^2}\right)$$
(5)

Where Q_{max} is the peak maximum which we can approximately relate to characteristic domain size by $d = 2\pi/Q_{max}$. C is a numerical prefactor.

The scattering from single chains was taken from a general polymer-like expression (Beaucage, G. Small-Angle Scattering From Polymeric Mass Fractals of Arbitrary Mass-

$$P(Q)_{\text{chain}} = \exp(-Q^2 R_g^2/3) + (d_f/R_g^{d_f})\Gamma(d_f/2) \Big(\frac{(\operatorname{erf}(Q \, k \, R_g/\sqrt{6})^3}{Q}\Big)^{d_f} \quad (6)$$

Fractal Dimension. J. Appl. Cryst. 1996, 29, 134–146.)

Where R_g is the radius of gyration, d_f is the fractal dimension and Erf(x) is the error function.



Figure S3. SAXS data (BM29, ESRF, Grenoble) of MDPs (a) $WK_2(QL)_6K_2$ (b) $WK_3(QL)_6K_2$ (c) $K_3W(QL)_6K_2$. Peptide solutions were prepared in Tris buffer (pH=7.4, 20mM) with a final peptide concentration at ~ 5 mg/ml. Solid lines display fits to long platelets (prisms) with polydisperse short edges and a contribution from internal correlations presumably between the alternating leucine and glutamic acid units internally along the *c* axis. (Detailed fitting models are described in SI). Dotted lines display the same model without internal scattering contributions.

6. Cytotoxicity assay

6.1 Isolation and differentiation of mouse bone marrow-derived monocytes (BMDMs)

Bone marrow progenitor cells were isolated from femurs of C57BL/6 mice to enrich for BMDMs as previously described.¹ Briefly, progenitor cells were recovered from the bone marrow of six to eight week-old mice by flushing femurs and tibia with DMEM medium. After centrifugation, the cells were suspended in BMDM medium (DMEM supplemented with 10% fetal bovine serum, 20% L292-cell conditioned media, 0.01% HEPES, 0.01% sodium pyruvate, and 0.01% L-glutamine) and incubated in tissue culture-treated 25cm²-flasks (BD Falcon, BD Biosciences, San Jose, CA) overnight at 37°C with 5% CO₂ to eliminate adherent fibroblasts and granulocytes. The following day, 1×10^{7} suspension cells were maintained in 10-cm² bacteriological Petri dishes (BD-Falcon) in BMDM medium for 3-4 days. Cell monolayers were recovered using ice-cold PBS and scraping. Single cell suspensions were used immediately or frozen in liquid nitrogen with 90% FBS and 10% DMSO for use in future experiments.

6.2 Lactate dehydrogenase (LDH) cytotoxicity assay

BMDMs were plated on a 48-well plate $(1.25 \times 10^5 \text{ cells}/0.25 \text{ml/well})$ and pre-incubated for 18 hrs. Cells were treated with various concentrations of peptides and incubated at 37°C with 5% CO₂ for 24 hrs. The release of LDH from BMDMs was used to detect cytotoxicity at 24 hrs. The leakage of LDH from the cytoplasm of the BMDMs into the medium is associated with secondary necrosis and is a means of measuring the membrane integrity of the cells. LDH activity was determined by the production of NADH during the conversion of lactate to pyruvate by using a CytoTox96 LDH-release kit (Promega Corp., Madison, WI). The culture medium was centrifuged at 10,000 rpm for 5 min at 4°C to ensure removal of cell residue. Cell-free supernatants were used to measure the LDH activity by following the manufacturer's protocol. The optical density was measured using an ELISA plate reader at a wavelength of 490 nm. The percent cytotoxicity was calculated relative to the manufacturer-provided positive cell lysis control. Release of LDH from untreated BMDMs served as a negative control and this amount was subtracted from the positive control and peptide-treated groups.

6.3 Confocal Microscopy for quantitative analysis of the effect of peptides on BMDMs.

BMDMs were seeded into 35 mm petri dishes with a 20 mm microwell in the center (MatTek Corporation, Cat no. P35G-1.5-20-C, Ashland, MA). Cells were seeded at a concentration of 5×10^5 cells /1 ml /well and allowed to adhere overnight. The following day, cells were treated with various concentrations of peptides and incubated at 37° C with 5% CO₂ for 24h. Differential Interference Contrast (DIC) Imaging imaging was performed using a Leica TCS SP5 Laser scanning confocal microscope operating with the software LAS AF version 2.6.0.7266. Images were acquired using a 63×1.4 NA oil immersion objective. Image processing and analysis was performed using ImageJ (NIH, 1.49g). The results shown in Figure 2c represents a compilation of the total number of cells counted in 6 random fields of view per peptide / experiment and was combined from two separate experiments (n = 12 total)

7. Proteolytic Stability Measurement

Proteolytic stability of MDP-1, 2 and 3 was monitored by reserved phase analytical HPLC. Peptide stock solution was prepared at a concentration of 1mg/ml in Tris buffer (pH=7.4, 20mM). Trypsin and α -chymotrypsin solution were prepared in Tris buffer (pH=7.4, 20mM) at a concentration of 0.1 mg/ml. For each peptide, three solutions were prepared and the structural integrity of the peptides was monitored by HPLC. Sample 1: 0.15 mg/ml peptide in Tris buffer (pH=7.4, 20mM). Sample 2: 0.15 mg/ml peptide and 0.005 mg/ml trypsin in Tris buffer (pH=7.4, 20mM). Sample 3: 0.15 mg/ml peptide and 0.005 mg/ml α -chymotrypsin in Tris buffer (pH=7.4, 20mM). All the samples were incubated at RT for 18hrs before HPLC analysis. The degraded percentage of peptide by trypsin or α -chymotrypsin is calculated according to the formula D.P.=1-A.P./A.C., where A.P. is the peak area integrated for the intact peptide after 18 hrs incubation with enzymes, A.C. is the peak area of the intact peptide in the control sample without enzymatic treatments, and D.P. represent the degradation percentage.

Peptide Sequence	Percentage of peptide degraded by protease after 18hrs treatment	
	Trypsin	α-Chymotrypsin
WK ₂ (QL) ₆ K ₂	40.8%	33.8%
$WK_3(QL)_6K_2$	80.8%	100%
$K_3W(QL)_6K_2$	58.2%	66.7%

 Table S1. Quantitative result of the degradation percentage

8. Bacterial culture

Four bacteria were used in this work including *E.coli* (25922) and *S. epidermidis* (12228) from ATCC and *P. aeruginosa* (15442) *S. aureus* (6538) ordered from Presque Isle Cultures. All bacteria were cultured in MHB (Fisher) under constant shaking at 100 rpm at 37°C. All bacteria were used when they reached their mid-exponential growth phase.

9. Minimum inhibitory concentration (MIC) measurements

MIC of each peptide against different bacteria was measured using the broth microdilution method. A bacterial suspension was diluted to approximately 10^5 CFU/mL in MHB medium. Peptides at varying concentrations (800, 400, 200, 100, 50, 25, and 12.5 μ M) were prepared by serial dilution and 10 μ L of peptide solution was added to 90 μ L bacterial solution in a 96-well microtiter plate. The UV absorbance at 600 nm of the bacterial suspensions was measured after 18 hrs of incubation for *E. coli* and *P. aeruginosa*, and 36 hrs of incubation for *S. aureus* and *S. epidermidis*. The MIC was reported as the minimum concentration of the peptides required to completely inhibit the growth of microbial cells after incubation. In order to ensure aseptic handling environment, a negative control of pure broth without bacteria accompanied each MIC test. Each MIC test was reproduced 3 times, using 2 replicates in each experiment.

10. Killing efficiency test

Peptide solution with concentrations corresponding to MIC was first prepared and added to an equal volume of bacterial suspension at 10^5 CFU/mL in each well of a 96-well microtiter plate. The plates were incubated at 37°C for 18 h for *E. coli* and *P. aeruginosa*, and 36 h for *S. aureus* and *S. epidermidis*. Then they were serially diluted in nutrient broth for the determination of viable counts. Diluted samples were plated onto agar plates and total bacterial counts were determined after 24 hrs of incubation at 37°C.



Figure S4. Killing efficiency of MDPs. The killing efficiency was measured in bacterial cultures after incubation with peptides at their MIC. For MDP-1 effects on *E. coli*, *P. aeruginosa* and *S. aureus* 80 uM was used.

11. Scanning electron microscopy (SEM) analysis of bacterial cells

Bacterial suspensions (10^{8} CFU/mL) were added to a 24-well plate with a 12mm round cover glass fitted on the bottom of each well. After 12 hrs of incubation, the bacterial suspension was withdrawn, and the plates was nd washed with PBS to remove any non-adherent bacteria. Next, 180µL of fresh media to each well and plates were incubated at 37° C for 1 hr. Media was removed and the cover glasses were washed with PBS twice. The bacteria were fixed with 4% glutaraldehyde solution for overnight. The cover glasses were further washed with PBS, followed by dehydration using a series of ethanol solutions with different volume contents (35, 50, 75, 90, 95 and 100%). The sample was placed on a carbon tape, which was further coated with a 5nm-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 6.0 mm.



Figure S5. SEM images of *E. coli* (top panel) and *S. aureus* (bottom panel) in the absence and presence of MDPs. Peptides are co-incubated with bacteria for 1 hrs followed by fixation and ethanol dehydration for SEM imaging. Scale bar: 1 μ m. Peptide conc: 40 μ M.



Figure S6. The residual amounts of peptides upon 18 hrs of incubation with 10% FBS as quantified by HPLC. Peptide concentration: 0.1mg/ml (~50 uM).



Figure S7. Summary of MDP beta sheet content, protease resistance, bacterial killing activity and cytotoxicity toward BMDMs. The data showed a significant correlation between the molecular structure, fiber stability, and biological activity of the MDPs. The cytotoxicity and bactericidal activity against *S. aureus* was measured at a peptide concentration of 20 uM. Y-axis represents the percentage of the beta sheet content (green), percentage of residual intact peptides upon enzymatic treatment (blue), relative counts of bacterial cells being killed by MDPs compared to the control without the addition of peptides (blue), and the percent of cytotoxicity measurement by LDH assay described in the manuscript and Figure 2a (red).

1. Singh A, Rahman T, Malik M, Hickey AJ, Leifer CA, Hazlett KR, et al. Discordant results obtained with *Francisella tularensis* during in vitro and in vivo immunological studies are attributable to compromised bacterial structural integrity. PLoS One 2013; 8:e58513; PMID: 23554897.

2. Rydell-Tormanen, K., L. Uller, and J. S. Erjefalt. 2006. Direct evidence of secondary necrosis of neutrophils during intense lung inflammation. *Eur. espir. J.* 28: 268-274.