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Experimental Section

Materials

PL-Rink amide resin was purchased from Polymer Laboratories. Fmoc-protected amino acids were purchased from Synpep. 1H-Benzotriazolium 1-[*bis*(dimethylamino) methylene]-5chloro-hexafluorophosphate (1-),3-oxide (HCTU) was purchased from Peptides International. Trifluoroacetic acid (TFA), thioanisole, ethanedithiol, anisole, and tris(hydroxymethyl)aminomethane hydrochloride (Tris) were purchased from Acros. Diethyl ether, sodium hydroxide and acetonitrile were purchased from Fisher. Dulbecco's Modified Eagle's Medium (DMEM) and Gentamicin were purchased from Sigma. Trypsin (0.25%) and L-Glutamine were obtained from Cellgro. NIH 3T3 fibroblasts were obtained from American Type Culture Collection (ATCC; CRL-1658). Calf serum was purchased from Colorado Serum Company. NIH3T3 fibroblast cell growth conditions are 90% DMEM with 50μg/mL Gentamicin, 10% calf serum and incubation at 37°C with 5% CO_2. Light scattering (OD_{625nm}) was measured on a Hewlett Packard 8453 UV-Visible Spectrophotometer employing a 1 cm pathlength cell.

Peptide Synthesis

MAX 1 was prepared on PL-Rink amide resin via automated Fmoc-based peptide synthesis employing an ABI 433A peptide synthesizer and HCTU activation. Resin-bound peptide was cleaved and side chains deprotected using a cleavage cocktail of TFA/thioanisole/ethanedithiol /anisole (90:5:3:2) for 2 hr under an N_2 atmosphere. The resin was filtered and the peptide precipitated from the supernatant using cold diethyl ether. Crude peptide was purified by RP-HPLC (preparative Vydac C4 peptide/protein column) employing a linear gradient from 14% to 100% B over 179 min, where solvent A is 0.1% TFA in water and solvent B is 90% acetonitrile, 10% water, and 0.1% TFA. MAX1 elutes at 31 min. The resulting peptidic solution was frozen via N_2(l) and lyophilized to afford pure MAX1 as the TFA salt which was used in all assays.

Antibacterial Assays

Hydrogels for the antibacterial assays were prepared in separate wells of 96-well tissue culture-treated polystyrene plates (Costar 3595). For a given well, 35 μL of a MAX 1 stock solution (4 wt%; 35 mg MAX 1 in 875 μL sterile filtered H_2O) was introduced, followed by the addition of 35 μL of serum-free Dulbeccos Modified Eagles Medium (DMEM; Sigma D6546) to initiate gelation. The resulting 2 wt% hydrogels (70 μL final volume; diameter = 8 mm; thickness = 2 mm) were allowed to incubate at 37ºC for 2 hours. Following incubation, gels were equilibrated with 200 μL DMEM overnight at 37ºC. Prior to the start of the assay this media was removed from the top of all of the hydrogels.

Bacterial stock solutions were prepared from powder bacteria supplied in separate vials which were purchased from Becton Dickinson (BD Bactrol™ Plus 237916). Bacteria strains from each vial were suspended in 1mL Tryptic Soy Broth (TSB; Bacto 211824) and quadrant streaked on a Trypticase™ Soy Agar plate with 5% sheep blood (BBL 221239), followed by incubation at 37ºC overnight. After which time, colonies from the fourth quadrant were transferred to a fresh agar plate and quadrant streaked followed by incubation for 24 hours. For each bacterial strain, one colony of bacteria from the fourth quadrant of the second agar plate was suspended in 1 mL of TSB in a 1

mL ependorf tube. The optical density of this suspension was adjusted to OD_{625nm} = 0.1 AU by the addition of TSB, resulting in a 10^8 colony forming units (CFU)/mL bacterial stock solution.

For each assay, 100 μL of bacteria-free TSB was introduced onto the surface of the hydrogels. Next, 100 μL of the 10^8 CFU/mL bacterial stock solution was introduced to the surface of a given hydrogel and serial 1:10 dilutions were performed across the plate, resulting in final bacterial concentrations of 2 x 10^3, 2 x 10^4, 2 x 10^5, 2 x 10^6, 2 x 10^7, 2 x 10^8 and 2 x 10^9 CFU/dm^2 respectively, for each of seven wells. Controls were carried out on tissue culture-treated polystyrene (TCTP) surfaces. Bacteria were incubated for 48 h on control and MAX 1 hydrogel surfaces at 37ºC (initial pH = 7.0). After which time, bacterial growth was monitored by measuring OD_{625nm} of the liquid above the gel by adding 100 μL of bacteria-free TSB to the surfaces, gently mixing and transferring the supernatant to a cuvette. Corrected OD_{625nm} were calculated to correct for dilution and to normalize for scattering that occurs for each individual bacterial strain (see Supporting Information for equation). Each assay reported in Figures 2 and 3 represent triplicate experiments for each bacterial strain. For each strain, at least four additional experiments were performed (each in triplicate) that showed reproducible results, consistent with those shown.

Confocal Microscopy

MAX 1 hydrogels (2 wt%, 80 μL final volume) were prepared directly in separate chambers of an 8-chambered borosilicate confocal plate (Fisher 155411) via the same methodology as described above. Stock bacterial solutions (10^2 and 10^8 CFU/mL) of *E. coli* and *S. pyogenes* were prepared as described above. 200 μL of the 10^2 and 10^8 CFU/mL bacterial suspensions were introduced to separate hydrogel surfaces resulting in 2.5 x 10^3 and 2.5 x 10^9 CFU/dm^2 respectively. Following 24 h of incubation at 37°C, 200 μL of LIVE/DEAD *Bac*Light (Molecular Probes L13152) commercial solution (prepared according to the insert directions) was added to each well resulting in a final concentration of 6 μM SYTO9 and 30 μM propidium iodide. Following incubation for 15 min, cells were imaged using 20 X magnification on a Zeiss 510 Laser Scanning Confocal Microscope. When excited at 488 nm with an Ar/Kr laser, bacteria with intact membranes display green fluorescence (Em = 500 nm) and bacteria with comprised membranes fluoresce red (Em = 635 nm).

Membrane Permeabilization Assay

Cytoplasmic β-galactosidase activity was measured using o-nitrophenyl-β-galactopyranoside (Fluka, 73660) as the substrate. *Escherichia coli* ML-35 was obtained as a gift from the laboratory of Robert E.W. Hancock, University of British Columbia. One colony from an overnight incubated Mueller Hinton agar plate was suspended in 5 mL of TSB and incubated overnight at 37ºC. From this overnight culture, 1 mL of the bacterial suspension was transferred to a 1 mL ependorf tube and centrifuged at 2,400 RPM for 5 minutes. The TSB supernatant was removed and to the resultant cell pellet, 1 mL of sodium phosphate buffer (10 mM, 100 mM NaCl, pH 7.4) was added and bacteria were resuspended, followed by centrifugation at 2,400rpm for 5 minutes. This process was repeated for a total of three washes. Following washes, the optical density of this

suspension was adjusted to OD_{625nm} = 0.1 AU by the addition of sodium phosphate buffer (10 mM, 100 mM NaCl, pH 7.4), resulting in a 10^8 CFU/mL bacterial stock solution. MAX1 hydrogels (2wt%, 70 μl final volume) were prepared as previously described in a 96-well cell culture plate. To a 2wt% MAX1 hydrogel, 200 μL of the 10^8 CFU/mL *E. coli* was introduced to the surface of three individual hydrogels resulting in bacterial concentrations of 4 x 10^9 CFU/dm^2 and allowed to sit at room temperature for 2 hours after which, the supernatant was removed and placed in a 1 mL eppendorf, (600 μL final volume), and centrifuged at 10,000 RPM for 1 minute. From the centrifuged supernatant removed from the hydrogel, 300 μL was transferred to a clean 1 mL eppendorf and 10 μL of 45 mM ONPG in sodium phosphate buffer (10 mM, 100 mM NaCl, pH 7.4) was added to obtain a 1.5 mM ONPG final concentration. Absorbance at 420 nm was monitored over 70 min. The positive control (100% lysis) was obtained by sonicating cells (4 x 10^9 CFU/dm^2, 3 intervals of 15s) using an Aquasonic Model 50HT water bath (VWR Scientific) and represents full β-galactosidase activity. Negative controls correspond to bacterial stock solution on TCTP control surfaces.

Proliferation of *E. coli* and *S. aureus* in the presence of MAX1

The growth of *E. coli* and *S. aureus*, gram negative and gram positive strains respectively, was measured using a broth dilution method. A 10^8 CFU/mL stock solution in TSB was prepared as stated above. For each assay, 100 μL of bacteria-free TSB was introduced onto the TCTP control surface. Next, 100 μL of the 10^8 CFU/mL bacterial stock solution was introduced to the surface of a given well of the TCTP control surface and serial 1:10 dilutions were performed across the plate, resulting in final bacterial concentrations of 2 x 10^3, 2 x 10^4, 2 x 10^5, 2 x 10^6, 2 x 10^7, 2 x 10^8 and 2 x 10^9 CFU/dm^2 respectively, for each of seven wells. To each well, 10 μl of a 1 mM stock solution of MAX1 in H_2O was introduced, resulting in a final concentration of 100 μM MAX1. Samples were allowed to incubate at 37ºC for 24 hours, after which time, bacterial growth was monitored by measuring OD_{625nm} of the broth in each individual well by adding 100 μL of bacteria-free TSB to the surfaces, gently mixing and transferring the supernatant to a cuvette. Data in Figure 6 displays only data for the proliferation of 2 x 10^6 CFU/dm^2 *E. coli* and *S. aureus* respectively, in the presence of 100 μM MAX1. The same effect is seen for all other cell concentrations.

Proliferation of *E. coli* and *S. aureus* in the presence of Trifluoracetate

When 70 μL of a 2 wt% gel is prepared the maximal amount of TFA present is 3.87 x 10^{-3} mmoles. Adding 100 μL of tryptic soy broth to the gel results in a final volume of 170 μL and a final TFA concentration of 22.8 mM. The growth of *E. coli* and *S. aureus*, gram negative and gram positive strains respectively, was measured using a broth dilution method that is nearly identical to that described above for soluble MAX1 except that the tryptic soy broth was supplemented with 3.87 x 10^{-3} mmoles of trifluoroacetic acid. Since no gel is present, the total volume is 100 μL, resulting in a final TFA concentration of 38.7 mM, a 1.7 fold excess of TFA with respect to the theoretical maximal amount of TFA counter ion that could be present during the antibacterial assays shown in Figures 2 and 3. The pH of the resulting broth was adjusted to pH 7 with aqueous NaOH before bacteria were introduced. Again, the data in Figure 6 displays only data for the proliferation of 2 x 10^6 CFU/dm^2 *E. coli* and *S. aureus* respectively, in

the presence of TFA. The same effect is seen for all other cell concentrations. Since different lots of bacteria were used for the soluble MAX1 experiments and the TFA experiments shown in figure 6, independent control experiments (e.g. –TFA) for the new lots of gram positive and gram negative bacteria were performed and the OD_{625nm} values for the cultures containing TFA adjusted accordingly. This allows the TFA- and MAX1-containing cultures to be directly compared independent of bacterial lot.

NIH 3T3 Fibroblasts – (*Achromobacter xylosoxidans (xylosoxidans)* and *Stenotrophomonas maltophilia*) Co-culture

MAX1 hydrogels were prepared as stated above (2 wt%, 150 μL final volume) in a 48-well cell culture plate. The resultant hydrogels were placed in an incubator at 37ºC and 5% CO_2 for 2 h after which 200 μL of serum-free DMEM was added to the top of the hydrogels and allowed to equilibrate overnight. Stock NIH 3T3 cells were maintained in T150 flasks and infected with *Achromobacter xylosoxidans (xylosoxidans)* and *Stenotrophomonas maltophilia*, which were isolated from the cell culture facility ventilation system and independently verified by Microbial ID, Inc. (Newark, DE). Fibroblasts in co-culture were trypsinized and counted using a hemacytometer. The resulting co-culture containing fibroblasts, *Achromobacter xylosoxidans (xylosoxidans)* and *Stenotrophomonas maltophilia* was diluted with DMEM (containing 10% calf serum), and 200 μL containing 20,000 fibroblasts/cm^2 was introduced to both control and hydrogel surfaces. Co-cultures were incubated at 37°C and 5% CO_2. Images of fibroblasts in the presence of *Achromobacter xylosoxidans (xylosoxidans)* and *Stenotrophomonas maltophilia* were recorded at T = 25, 32 and 47 h using 10 x magnification on a Nikon Eclipse TE2000U microscope. The data in Figure 5 was recorded at T = 32 h.

Hemolytic Assays

MAX 1 hydrogels (2 wt%, 70 μL final volume) were prepared in a 96-well TCTP plate as described above. Hemolytic assays were performed using a modified protocol originally reported by DeGrado et al..[1] Fresh human blood was drawn into a 3.0 mL BD Vacutainer™ Plus tube containing spray-dried K_2EDTA(5.4mg). Blood was transferred to a 15 mL conical tube and centrifuged at 3,458 RPMs for ten minutes. Following centrifugation, serum and white blood cell supernatant was removed and pelleted human red blood cells (hRBCs) were resuspended in 10 mL of Tris buffer (10 mM, pH 7.4, 150 mM NaCl) and recentrifuged at 3,458 RPMs for ten minutes. This process was repeated until the supernatant displayed a negligible absorbance. Then, 30 μL of pelleted red blood cells were suspended in 12 mL of Tris buffer (10 mM, pH 7.4, 150 mM NaCl), resulting in a 0.25% (v/v) suspension of hRBCs. 80 μL and 160 μL of the 0.25% (v/v) hRBC stock solution was introduced to individual TCTP control and MAX 1 hydrogel surfaces, resulting in 6.9 x 10^5 and 1.4 x 10^6 hRBCs respectively. Red blood cell count was calculated using a packed cell volume of 4.6 x 10^{12} hRBCs/L.[2] Additional Tris buffer was added to each well to adjust the final buffer volume to 300 μL to ensure even distribution of hRBCs with respect to the surface being assayed. Samples were incubated at 37ºC under dynamic conditions using a rocker platform (Bellco Biotechnology, Vineland, NJ) for 1 h, after which time, the supernatant was removed and centrifuged at 14, 000 RPMs for 10 min. Hemoglobin released, as a result of hRBC lysis, was assessed

by measuring the Abs_{415nm}. 100% hemolysis (complete lysis) was defined by adding a 0.1% TRITON-X-100 (Fluka GA11820) solution to hRBCs on the control surface. Assays were performed in triplicate. Image of red blood cells was obtained using 20 X magnification on a Nikon Eclipse TE2000 inverted microscope.

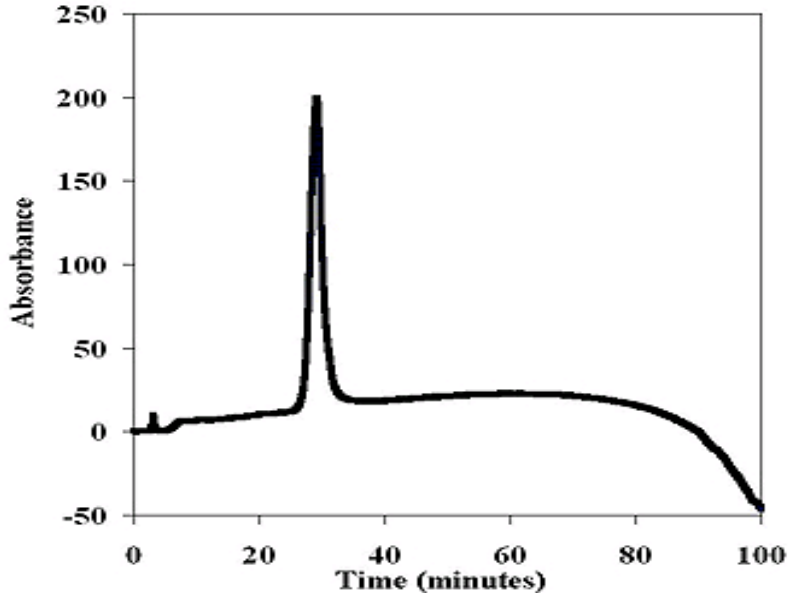
References:

(1) Liu, D. H.; DeGrado, W. F. *J. Amer. Chem. Soc.* **2001**, *123*, 7553-7559.
(2) Van Hove, L.; Schisano, T.; Brace, L. *Lab. Hematol.* **2000**, *6*, 93-108.

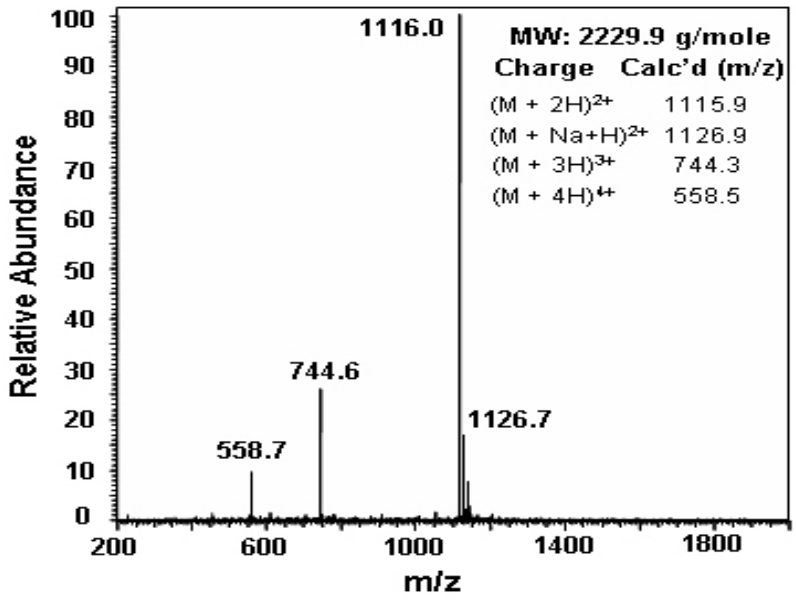
HPLC Solvent A: (0.1% TFA in water)
HPLC Solvent B: (90% acetonitrile, 10% water, 0.1% TFA)

A. **Analytical HPLC (Vydac C18) of Purified MAX 1**

0% to 100% B over 100 minutes; 100 µL injection



B. **ESI (+) Mass Spectrum of Purified MAX 1**



C. For all bacteria assays utilizing UV-Vis spectroscopy to monitor bacterial proliferation, data are reported as a corrected optical density at 625nm. Spectral fluctuations due to scattering, caused by the large particle diameter of bacteria, is normalized for by using multi wavelength measurements at 625 nm and 1100 nm. Therefore, the corrected OD_{625nm} was calculated as follows:

$$\text{Corrected OD}_{625nm} = 2\left(\text{Observed OD}_{625nm} - \Delta\text{OD}_{1100nm}\right),$$

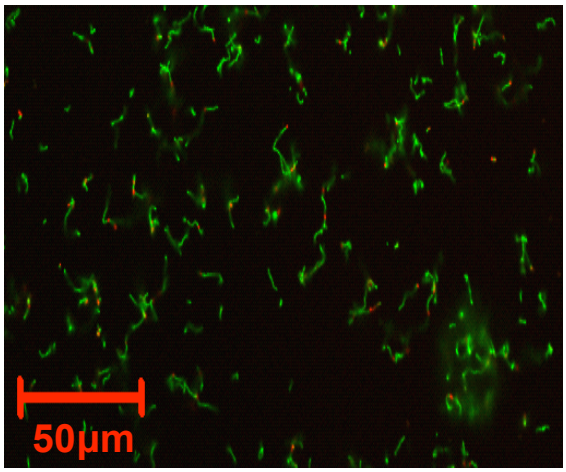
where the multiplication factor of 2 accounts for dilution and

$$\Delta\text{OD}_{1100nm} = \text{Observed OD}_{1100nm} - \text{Expected OD}_{1100nm}.$$

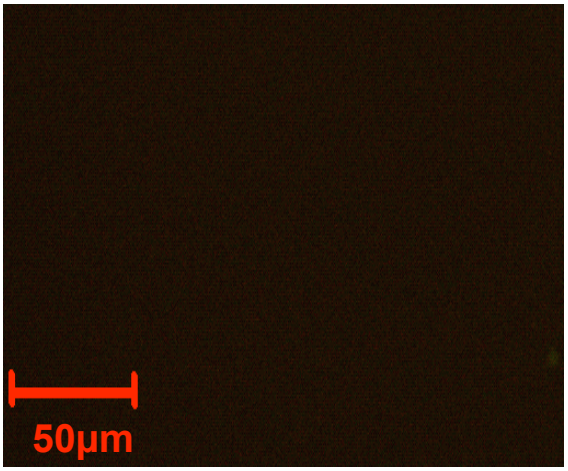
$$\text{The Expected OD}_{1100nm} = \frac{\text{Observed OD}_{625nm}}{\left(\frac{\text{OD}_{625nm_{\text{Control}}}}{\text{OD}_{1100nm_{\text{Control}}}}\right)_{avg}}, \text{ where}$$

$\left(\frac{\text{OD}_{625nm_{Control}}}{\text{OD}_{1100nm_{Control}}}\right)_{avg}$ is constant and is dependent on bacterial species, (*Escherichia coli* = 3.37 ± 0.05; *Klebsiella Pneumoniae* = 3.13 ± 0.05; *Staphylococcus Aureus* = 3.19 ± 0.14; *Staphylococcus Epidermidis* = 3.08 ± 0.08; *Streptococcus Pyogenes* = 2.96 ± 0.12). Averages were obtained using independent lots for each species.

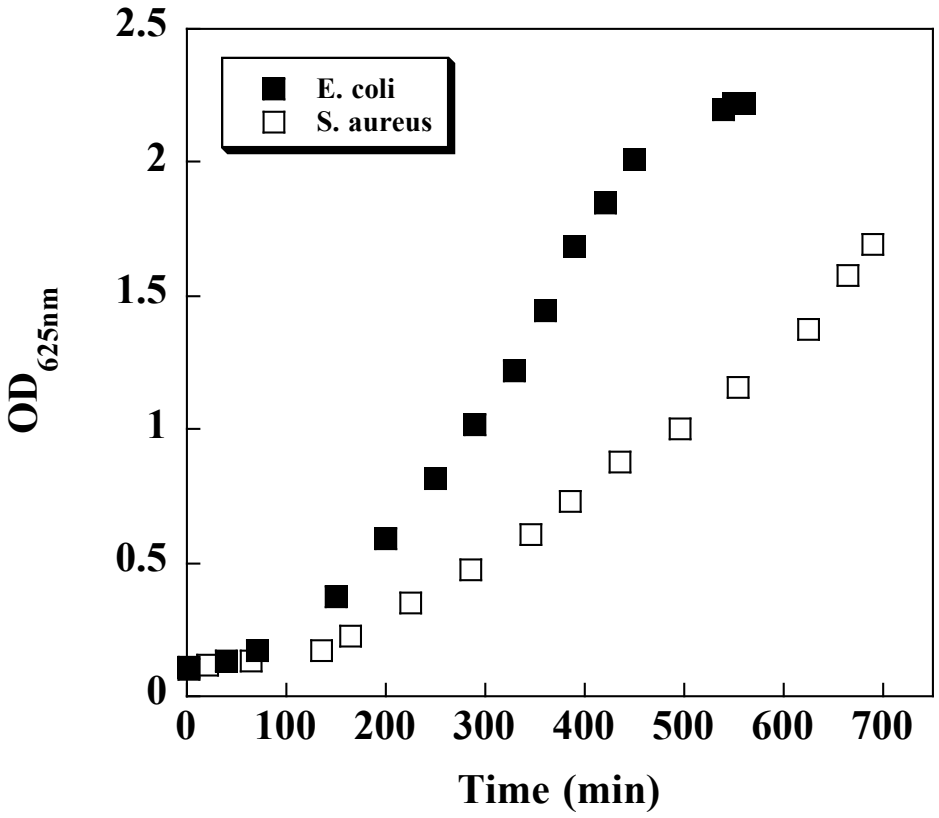
D.



E.



F.


E. coli
S. aureus
OD625nm
Time (min)
0
0.5
1
1.5
2
2.5
0
100
200
300
400
500
600
700