Electronic Supplementary Material (ESI) for ChemComm.

Supporting information

Immobilized random peptide mixtures exhibit broadantimicrobial activity with high selectivity

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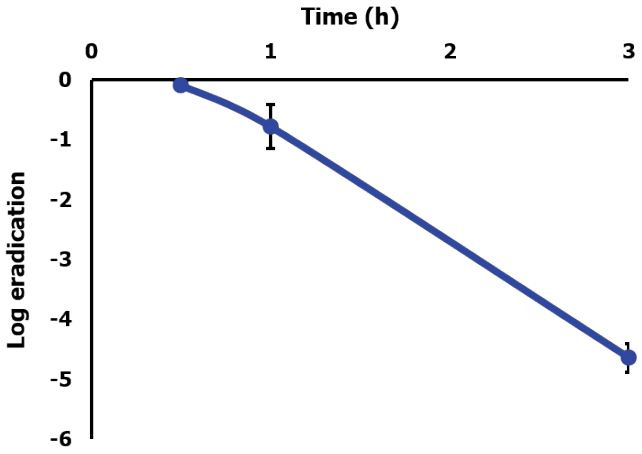

Figure S1: *E. coli* rp eradication by LK 20-mer bioactive beads over time. Survival assays in PBS containing initial bacterial load of 10^7 CFU mL^{-1} bacteria and 4 µmol mL^{-1} immobilized LK 20-mer RPM were performed at 37°C. Log eradication = Log $CFU/mL_{(Treatment)}$ – Log $CFU/mL_{(Control)}$.

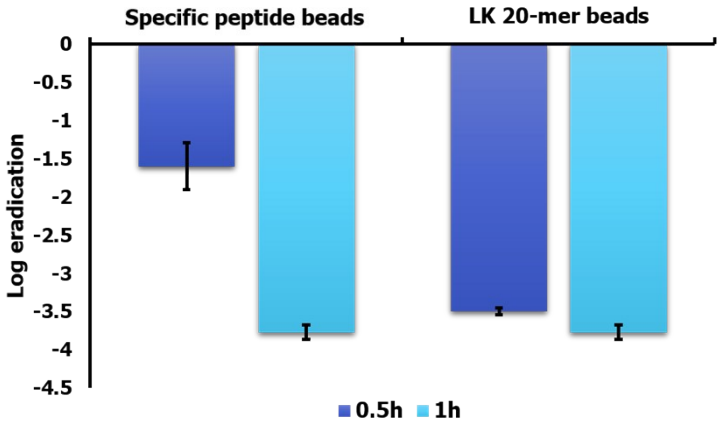

Figure S2: *E. coli* rp eradication by LK 20-mer bioactive beads compared to specific peptide beads. Eradication was determined by survival assays on 10^4 CFU mL^{-1} *E. coli* rp in PBS containing 10 µmol mL^{-1} immobilized LK 20-mer RPM or LKLLKKLLKLLKKL at 37°C. Log eradication = Log $CFU/mL_{(Treatment)}$ – Log $CFU/mL_{(Control)}$.

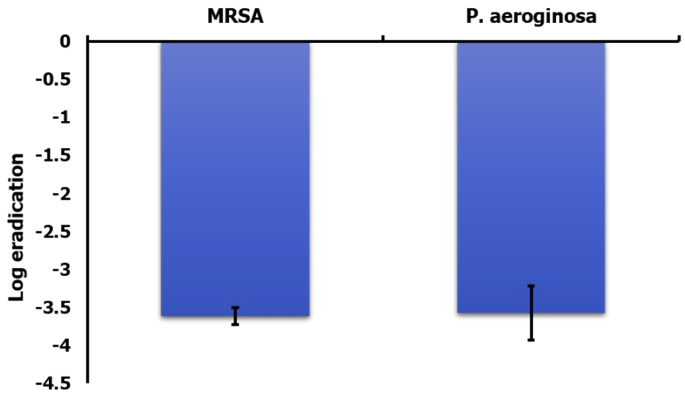

Figure S3: Eradication of pathogenic bacteria by LK 20-mer bioactive beads. Incubation of 10^4 CFU mL^{-1} Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* PAO1 was carried out in PBS at 37°C for 1 hour, with 10 µmol mL^{-1} immobilized LK 20-mer RPM. Log eradication = Log $CFU/mL_{(Treatment)}$ – Log $CFU/mL_{(Control)}$.

Table S1: *E. coli* WT and mutant strains that were used to examine the role of LPS in the bioactive beads mechanism of action against *E. coli*, the function of deletion of specific genes in each mutant and the LPS structure of each bacterium.

Strain	Gene deletion	Function of deleted gene	Character of LPS polysaccharide core
E. coli K12 BW25113	WT (no deletion)	-	Intact core
	rfaP	Phosphate groups addition to hepI of the inner core of the LPS	Lacking of HepIII and phosphate groups of the inner core
	rfaG	Involved in the addition of the first glucose of the LPS outer core	Lacking outer core
	rfaC	Addition of the first heptose of the LPS inner core	Lacking outer core and the heptoses of the inner core

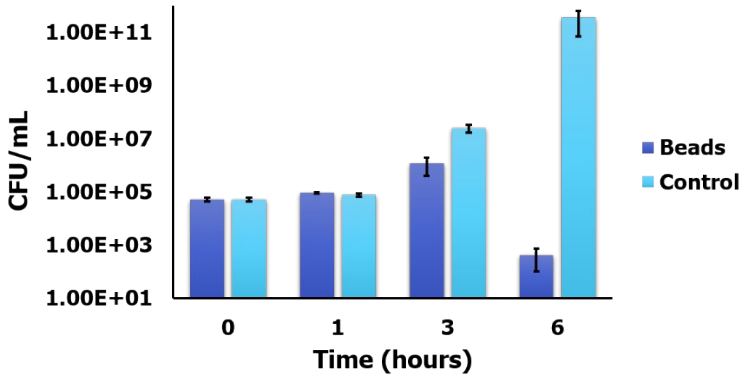

Figure S4: Antimicrobial activity against *E. coli* in lysogeny broth (LB) growth media. Incubation of *E. coli* rp with 10 µmol mL^{-1} immobilized LK 20-mer RPM was carried out in LB at 37°C.

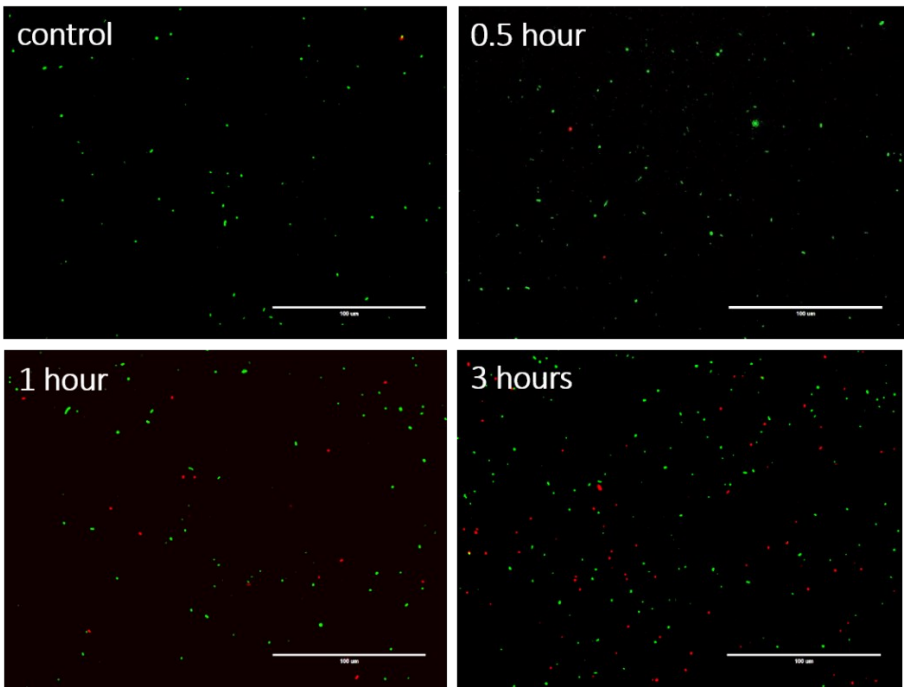

Figure S5: Live/Dead staining assay of *E. coli* rp in the supernatant during incubation with LK 20-mer beads in PBS at 37°C. Live and dead bacteria appear in green and red, respectively.

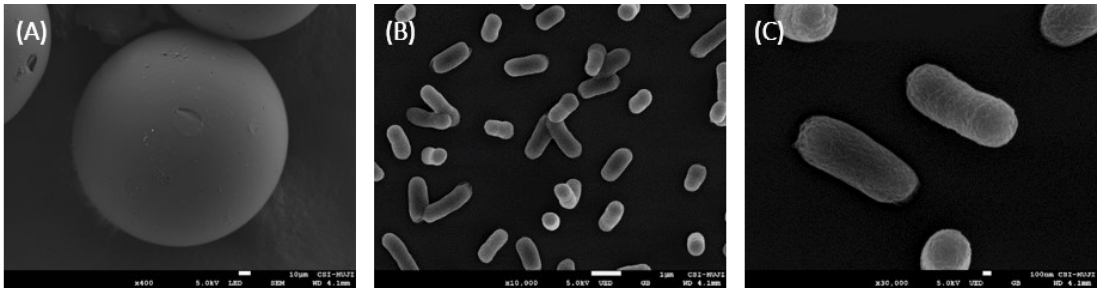

Figure S6: Representative Scanning Electron Microscopy images of: (A) LK 20-mer beads, (B) *E. coli* bacteria control (X10,000) and (C) *E. coli* bacteria control (X30,000). Images were taken after incubation of 10^7 CFU mL^{-1} *E. coli* rp with the beads for 1 hour in PBS at 37°C and cell fixation.

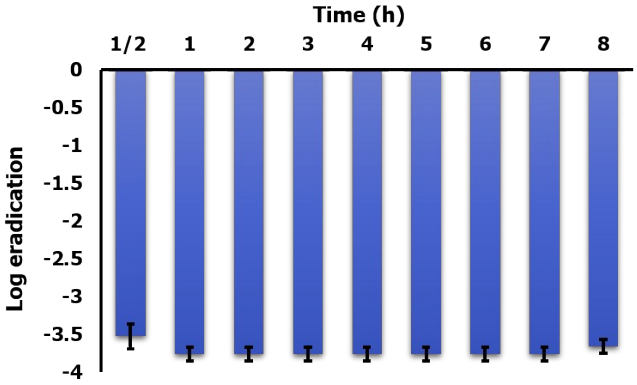

Fig. S7: *E. coli* rp eradication by LK 20-mer bioactive beads column over time. PBS inoculated with 10^4 CFU mL^{-1} *E. coli* rp was pumped in a constant flow rate of 2.5 mL min^{-1} through a column containing 5% bioactive beads in sand. Log eradication = Log $CFU/mL_{(Treatment)}$ – Log $CFU/mL_{(Control)}$.

Table S2: Results of kinetic analysis model of bacterial filtration in LK 20-mer bioactive beads column for initial bacterial load (C_0) of $3.4 \cdot 10^6$ CFU mL^{-1}. T – Time in hours, V – total pumped volume in liters, C – viable bacterial cells in the emerging water in CFU mL^{-1}.

T (h)	V (L)	Active layer length (cm)	C/C_0	C (CFU mL^{-1})
1/2	0.075	4	$2.39 \cdot 10^{-4}$	$8.12 \cdot 10^2$
		10	$1.01 \cdot 10^{-9}$	$3.43 \cdot 10^{-3}$
20	3	4	$2.4 \cdot 10^{-4}$	$8.16 \cdot 10^2$
		10	$1.02 \cdot 10^{-9}$	$3.47 \cdot 10^{-3}$
40	6	4	$2.4 \cdot 10^{-4}$	$8.16 \cdot 10^2$
		10	$1.02 \cdot 10^{-9}$	$3.47 \cdot 10^{-3}$
336 (2 weeks)	50.4	4	$2.4 \cdot 10^{-4}$	$8.16 \cdot 10^2$
		10	$1.02 \cdot 10^{-9}$	$3.47 \cdot 10^{-3}$

Materials and methods:

Materials

Fmoc - rink amide (RAM) resin (loading 0.53 mmol g^{-1}), Fmoc protected α-amino acids with acid-labile side-chain protecting groups, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and Ethyl (2Z)-2-cyano-2-hydroxyiminoacetate (oxyma) were purchased from ChemImpex. Tert-Butoxycarbonyl (Boc) - Glycine - phenylacetamidomethyl (PAM) resin (outer diameter 100-200 µm, loading 1.1 mmol g^{-1}, 100-200 mesh) was purchased from Rapp Polymere. Triisopropylsilane (TIPS) and N,N-Diisopropylcarbodiimide (DIC) were purchased from Sigma-Aldrich. N,N-dimethylformamide (DMF), N,N-diisopropylethylamine (DIEA), Piperidine, Diethyl ether, Trifluoroacetic acid (TFA) and Dichloromethane (DCM) were purchased from BioLab. Sand (purified by acid, 40-100 mesh) was purchased from BDH.

Synthesis of bioactive beads

Bioactive beads were synthesized on PAM resin by random peptide mixtures (RPMs) synthesis procedure, as described previously[1], with minor modifications. For all experiments except hemolysis assay, mutants' experiments and column assay, synthesis was carried out in filter

tubes (Silicol, Israel) in MARS VI multimode microwave. For hemolysis assay, mutants experiments and column assay the synthesis of the peptides was conducted by a peptide synthesizer (Liberty Blue). Removal of Boc groups on PAM-Gly-Boc resin was performed by agitation in a solution containing 50% TFA and 50% DCM for 10 minutes. The resin was then filtered and washed with DCM and DMF. Each coupling step was carried out by using 1:1 molar ratio mixture of protected α-amino acids: Fmoc-L-Lysine-Boc with Fmoc-L-Leucine/Fmoc-L-Phenylalanine/Fmoc-L-Tryptophan. Prior to coupling execution, 4 equivalents of amino acids were activated in DMF. Four equiv of HBTU and 8 equiv. DIEA for microwave synthesis or 4 equiv. DIC and 8 equiv. Oxyma for synthesis by the peptide synthesizer. Activated amino acids were added to the resin. Temperature was raised to 70°C for 2 minutes and remained at 70°C for another 4 minutes. At the end of the stage the resin was washed by DMF three times. Fmoc deprotection step was conducted by 20% piperidine in DMF. The resin was heated to 80°C for 2 minutes and the temperature remained at 80°C for another 3 minutes. For 20-mer peptides 20 coupling cycles were carried out, and for 10-mer peptides 10 coupling steps were carried out. After synthesis procedure, lysine Boc protecting groups were removed by shaking the beads in 4 mL 50% TFA : 50% DCM suspension for 10 minutes. Then, the beads were washed by DMF and DCM to remove TFA leftovers.

Synthesis of free RPMs

Procedure was identical to synthesis in microwave described above, but was carried out on Fmoc-RAM resin, as described previously[1]. At the end of the synthesis, peptides were cleaved from the resin and Boc protecting groups were de-protected by shaking in 95% TFA, 2.5% DDW and 2.5% TIPS solution for 3 hours at room temperature. Peptides were centrifuged twice with 40 mL cold Diethyl Ether at 8228g for 5 minutes. The pellet was dried by air, suspended in 2 mL DDW, frozen by liquid nitrogen and lyophilized.

Amino acid analysis

LK 20-mer beads were placed in unique sterilized glass tube for amino acid analysis. The peptides were hydrolyzed by HCl 6N for 22 hours at 110°C under vacuum and nitrogen flushes. Then, the sample was dried under vacuum at ambient temperature for 2 hours. The sample separation was conducted using HPLC at 37°C, and detection conducted using PDA and Fluorescence SF detectors. The amino acid composition was determined by AccQ.

Bacterial strains

For the mutants experiment WT strain of *E. coli* K12 BW25113, and single-gene knockout mutants of this strain (obtained from the *E. coli* Keio collection[2] and kindly received from Shimshon Belkin's lab) were used (Table 1). For survival assays, confocal microscopy analysis and antimicrobial column assay *E. coli* K12 MG1655 (rp) was used. Survival assays were also carried out on Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* PAO1. The WT strains were grown in lysogeny broth (LB) or LB agar plates at 37°C. The mutants were grown at the same conditions, with the addition of 50 μg mL^{-1} kanamycin. Bacteria were stored at -80°C in 25% glycerol and at 4°C on LB agar plate.

bactericidal activity of the beads

To examine the bactericidal activity of the beads, survival assays have been carried out. *E. coli* rp, Methicillin-resistant *Staphylococcus aureus* (MRSA) or *Pseudomonas aeruginosa* PAO1 cultures were grown overnight in LB, and were then centrifuged and washed in phosphate

buffered saline (PBS) (pH 7.4) for three times. The suspension was diluted to OD_{600nm} of 0.1, and then diluted 1000-fold more, to obtain ~10^4 CFU mL^{-1}. Incubation of 1 mL bacteria suspension was performed in round bottom tubes in agitation (200 RPM) with bioactive beads in PBS buffer at 37°C. For the peptides properties comparison assays, incubation was performed with 10 μmol mL^{-1} of beads for 1/2 hour and an hour. To examine the effect of initial bacterial load, incubation was performed with LK 20-mer beads in a concentration of 4 μmol mL^{-1}, and samples were taken after 1/2 hour of incubation. For the kinetics curve of bacterial elimination by LK 20-mer beads, the beads' concentration was 10 μmol mL^{-1} and samples were taken after 0, 1/4, 1/2 and 1 hour of incubation. To assess eradication of MRSA and *P. aeruginosa* the beads' concentration was 10 μmol mL^{-1}, and samples were taken after 1 hour of incubation. Tubes containing only bacteria were used as control. After incubation, the beads submerged, samples were taken from the supernatant and the concentration of bacteria remained in the suspension was numerated by CFU method on LB agar.

Antimicrobial activity of the beads in growth media

E. coli rp cultures were grown overnight in LB. The suspension was diluted in LB to OD_{600nm} of 0.1, and then diluted 1000-fold more, to obtain ~10^4 CFU mL^{-1}. Incubation of 1 mL bacteria suspension was performed in round bottom tubes in agitation (200 RPM) with 10 μmol mL^{-1} bioactive beads in LB at 37°C for 1, 3 and 6 hours. Tubes containing only bacteria were used as control. After incubation, the beads submerged, samples were taken from the supernatant and the concentration of bacteria remained in the suspension was numerated by CFU method on LB agar.

Antimicrobial activity of the beads against *E. coli* mutants

Survival assays were carried out as described above, with some changes. *E. coli* K12 BW25113 WT strain culture was grown overnight in LB, and the mutant strains were grown overnight in LB with 50 μg mL^{-1} kanamycin. The bacteria were then centrifuged and washed in PBS buffer for three times. The suspensions were diluted to OD_{600nm} of 0.1, and then diluted again in 10 fold, to obtain final bacterial concentration of ~10^6 CFU mL^{-1}. Incubation was carried out as described above, in the presence of 10 μmol mL^{-1} LK 20-mer beads. Samples were taken after 1/2 hour of incubation and the microbial load of the suspension was numerated by CFU method on LB agar. Samples from the mutants' tubes were spread after serial dilutions in PBS on LB agar plates with 50 μg mL^{-1} kanamycin to generate CFU\mL values.

Live/Dead staining and confocal microscopy analysis

E. coli rp culture was grown overnight in LB, and was then centrifuged and washed in PBS buffer for three times. The suspension was diluted to OD_{600nm} of 0.1, and then diluted 10-fold more, to get ~10^6 CFU mL^{-1}. To watch the beads after incubation with bacteria, incubation of 1 mL bacteria suspension was performed in agitation (200 RPM) with 4 μmol mL^{-1} LK 20-mer beads in PBS buffer at 37°C for 1/2 hour. Incubation of the bacteria with PAM resin without peptides was used as control. To explore the viability of bacteria in the supernatant over time incubation was performs for 1/2, 1 and 3 hours and bacteria only was used as control. After incubation, the supernatant was transferred and the remaining beads were stained using LIVE/DEAD BacLight Bacterial Viability Kit (ThermoFisher). Each sample was stained with 2.5 μL propidium iodide (red) and 2.5 μL SYTO9 (green) and incubated for 15 minutes in the dark. Afterwards fluorescent images were taken by 63* water lens (PMT emission 490-530 for SYTO9 and 600-644 for propidium iodide) using a Leica SP8 Confocal Laser Scanning Microscope.

Scanning electron microscopy (SEM) analysis

E. coli rp culture was grown overnight in LB, and was then centrifuged and washed in PBS buffer for three times. The suspension was diluted to OD_{600nm} of 0.1, to get ~10^7 CFU mL^{-1}. Incubation of 1 mL bacteria suspension was performed in agitation (200 RPM) with 10 µmol mL^{-1} LK 20-mer beads in PBS buffer at 37°C for 1 hour. Incubation of the bacteria with PAM resin without peptides, bacteria in PBS, LK 20-mer beads in PBS and PAM resin in PBS were used as control. After incubation, the beads submerged, the PBS was taken out and 100 µL 4% glutaraldehyde in PBS was added to the samples for an hour at room temperature. The bacteria control was centrifuged (4000 RPM for 5 minutes), the supernatant was taken out and the bacteria were washed by 100 µL 4% glutaraldehyde in PBS followed by 1 hour of incubation at room temperature. Then the beads were washed 3 times in PBS, and the bacteria control were centrifuged (4000 RPM for 5 minutes) and washed in PBS for 3 times. The drying of the beads and PAM resin without bacteria samples was performed by washing 3 time in Dichloromethane. Bacterial control was then attached to a glass disk, 50 µL at each side of the disk. Dehydration of the samples with bacteria was done in increasing ethanol concentrations (25, 50, 75, 95, 100% ethanol in deionized water), 5 minutes twice for each concentration. Drying was performed with K850 critical point dryer (CPD BAL-TEC CPD-030) and iridium coating was performed with Q150T ES Spatter Coater. The microphotographs were recorded using imaging Jeol JSM 7800 SEM.

Beads hemolysis assay

One mL of human blood was suspended in 50 mL 20 mM Tris 150 mM NaCl pH 7.2 buffer. Then the suspension was centrifuged (3500 RPM for 8 minutes) and washed by the buffer for three times to extract the red blood cells (RBCs). LK 20-mer beads were suspended in 0.5 mL buffer and then 0.5 mL RBCs suspension was added, so that the beads' final concentration was 10 µmol mL^{-1} and the final concentration of the RBCs was 1%. The treatments were: 1). 50 µg mL^{-1} free LK 20-mer peptide. 2). 10 µmol mL^{-1} bioactive beads. 3). PAM resin not loaded with peptides. 4). RBCs only as negative control. 5). Treatment of 1% tween20 as positive control. Incubation of 1.25 hours was conducted in round bottom tubes in agitation (200 RPM) at 37°C. Tubes were centrifuged (3500 RPM for 8 minutes), and 100 µL of the supernatant was transferred into 96-wells plate (ThermoFisher). The hemolytic effect of the treatments was determined by measuring the absorption of the samples in 405 nm wavelength in a Tecan Infinite Pro Plate reader. The hemolysis percentage was normalized with respect to the tween20 control, which was determined as 100% hemolysis.

Hemolysis effect of released peptides

Ten µmol mL^{-1} LK 20-mer beads were incubated in 1 mL DDW in agitation for 1, 2, 3, 4, 5 and 14 days. The supernatant was filtered, lyophilized and was stored in -20°C. One mL human red blood cells was suspended in 50 mL 20 mM Tris 150 mM NaCl pH 7.2 buffer. Then the suspension was centrifuged (3500 RPM for 8 minutes) and washed by the buffer for three times. The lyophilized supernatant was suspended in 0.5 mL buffer and then 0.5 mL RBCs suspension was added. Fifty µg mL^{-1} free LK 20-mer peptide was also used and 1% tween20 was used as positive control. Incubation of 1.25 hours was conducted in agitation (200 RPM) at 37°C. Tubes were centrifuged (3500 RPM for 8 minutes), and 100 µL of the supernatant was transferred into 96-wells plate. The hemolytic effect of the treatments was determined by measuring the absorption of the samples in 405 nm wavelength in a Tecan Infinite Pro Plate

reader. The hemolysis percentage was normalized with respect to the tween20 control, which was determined as 100% hemolysis.

Antimicrobial beads column

E. coli rp culture was grown overnight in LB, and was then centrifuged and washed in PBS buffer for three times. The suspension was diluted to OD_{600nm} of 0.1, and then diluted 1000-fold more, to obtain 1L PBS inoculated with ~10^4 CFU mL^{-1}. A mixture of 5% LK 20-mer beads in sterile sand (250 mg beads with 4.750 g sand) was included in an antimicrobial column, 10 mm diameter and 40 mm length of active layer. Two sterile geotextile filters were placed in either end of the bead-sand mixture. The column was saturated with sterile DDW in a constant flow rate of 2.5 mL min^{-1} upwards to move captured air. The inoculated PBS was transported from downwards at a constant flow rate of 2.5 mL min^{-1}. The samples were taken after 1/2 hour and then every hour for 10 hours. The concentration of vaible bacteria in the emerging water after filtration was determined by CFU method on LB agar.

Kinetics analysis of bacterial filtration and killing in a column

Filtration results were simulated with a model which added a degradation term to Eq. (1) below, which considers convection and adsorption/desorption in a system including several solutes.[3] The presentation in Eq. (1) is for a solution which includes just one pollutant, e.g., a bacterium whose molar concentration is C(x,t), where x is a coordinate along the filter length and t is the time. The model considers a cylindrical column with a cross section A, and length L. The column is filled with a sorbent (beads in this case) whose molar concentration of adsorption sites is R_0. The top and bottom of the filter are at the coordinates X=0 and X=L, respectively. The bacteria concentration at the inlet, Co, is constant, i.e., C(X, t) = Co, X ≤ 0, where t denotes time.

$$dC(X,t)/dt = -v\bullet \partial C(X,t)/\partial X - C_1\bullet C(X,t)\bullet R(X,t) + D_1\bullet RL(X,t) \quad (1)$$

in which RL(X,t) is the concentration of occupied sites.

In Eq. (1), v denotes the flow velocity in the filter, which is given by:

$$v = Q_v/(A\bullet f) \quad (2)$$

in which Q_v is the flow rate (volume/time) and f is the fraction of pore volume out of the total volume of the filter. R(X,t) denotes the molar concentration of free adsorbing sites, i.e.,

$$R(X,t)= R_0 - RL(X,t) \quad (3)$$

C_1 is the forward rate constant of adsorption (M^{-1} min^{-1}) and D_1 (min^{-1}) is the rate constant of dissociation.

Degradation, or inactivation, of bacteria was assumed for adsorbed bacteria according to Eq. (4):

$$dRL(X,t)/dt = C_1\bullet C(X,t)\bullet R(X,t) - (D_1 + k)\bullet RL(X,t) \quad (4)$$

in which k (min^{-1}) is the rate constant of bacteria inactivation. The concentration of inactivated bacteria is given by DB (x,t), which is obtained by:

$$dDB(X,t)/dt = k \bullet RL(X,t) \quad (5)$$

The numerical solution of the above equations was essentially as described in Nir et al.[3,4] Mass conservation was tested both for bacteria and surface sites.

It turns out that the solution predicts a steady state phenomenon, i.e., after a certain time the numbers of bacteria emerging from the filter, i.e., C/C_0 and the concentration of adsorbed bacteria remain constant. This is illustrated in Table S2 above. The calculations required the knowledge of the quantities C_0 and R_0, initial concentrations of bacteria (known experimentally) and molar concentration of surface sites, respectively. The molar concentration of surface sites was fixed at the largest possible occupation of the beads. A division of the total of external surfaces of the beads by the total external areas of the bacteria yields the largest total number of bacteria (or number of moles of bacteria) which can hypothetically be adsorbed in the absence of their inactivation and release. A division of this number by the volume of the water filled pores in the filter (in Liters) gives the molar concentration, R_0. The value of $R_0 = 2.5 \bullet 10^{-11}$ M was determined for the case where 250 mg of beads were mixed with 4.75 g of sand, where water filled pores comprise 30% of the volume of the active layer in the filter (thus, the volume of pores in the filter is $\pi r^2 \bullet 4 \bullet 0.3$= 1 mL). It was preferred to fix the parameters $C_1 = 10^{12}$ M^{-1} min^{-1}, and $D_1 = 0.001$ min^{-1}, according to values deduced for *E. Coli* in filtration by means of other positively charged particles, such as micelle-clay composites.[5] Thus, in practice, just one parameter k (min^{-1}), the rate constant of bacteria inactivation, (Eq.(5)) had to be determined. It is of interest to note that the concentrations of inactivated bacteria at a steady state are given by:

$$DB(L,t2) - DB(L,t1) = k \bullet (t2-t1) \bullet (\text{total number of moles of adsorbed bacteria}) \quad (6)$$

References:

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