A short amphipathic peptide-based injectable hydrogel with antibacterial properties

Dorien Aerssens[a], Atiruj Theppawong[a], Hue Vu[a], Petra Rigole[b], Marleen Van Troys[c], Tom Coenye[b], Richard Hoogenboom[d], Steven Ballet[e] Charlotte Martin[e] and Annemieke Madder[a]

[a.] *Organic and Biomimetic Chemistry Research Group, Department of Organic and Macromolecular Chemistry, Ghent University, 9000 Ghent, Belgium.*
[b.] *Department of Pharmaceutical analysis, Ghent University, 9000 Ghent, Belgium.*
[c.] *Department of Biomolecular Medicine, Ghent University, 9000 Ghent, Belgium.*
[d.] *Supramolecular Chemistry Group, Centre of Macromolecular Chemistry (CMaC), Department of Organic and Macromolecular Chemistry, Ghent University, 9000 Ghent, Belgium.*
[e.] *Research Group of Organic Chemistry, Vrije Universiteit Brussel, B-1050 Brussels, Belgium*

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1. Materials and methods

1.1 Materials

Rink Amide AM resin (0.68 mmol/g) was obtained from Novabiochem. L-amino acids were used throughout the synthesis. Fmoc-β-(2-furyl)-L-alanine was purchased from Chem-Impex International. Dimethylformamide (DMF) peptide synthesis grade and N-methylpyrrolidone (NMP) were purchased from Biosolve. Dichloromethane (DCM) and N,N-diisopropylethylamine (DIPEA) were obtained from Aldrich. Trifluoroacetic acid (TFA), Hexafluoroisopropanol (HFIP), Fmoc-protected amino acids, and coupling reagents were obtained from Iris Biotech GmbH.

1.2 HPLC analysis and purification

RP-HPLC analyses were performed on an Agilent 1100 Series HPLC system equipped with Phenomenex Kinetex C18 100 Å (150 x 4.6 mm, 5 µm, at 35 °C). A two solvent system was used: 0.1% TFA in H_2O (A) and 0.1% TFA in CH_3CN (B). Samples were eluted using a gradient from 0 % to 100 % B over 6 minutes at a flow rate of 1.5 mL/min at 40°C.

RP-HPLC-MS analyses were performed on an Agilent 1100 Series instrument with diode array detector, equipped with a Phenomenex Kinetex C18 100 Å (150 x 4.6 mm, 5 µm, at 35 °C), hyphenated to an Agilent ESI-single quadrupole MS detector type VL. Mass detection operated in the positive mode. A two solvent system was used: 0.1 % HCOOH in H_2O (A) and CH_3CN (B). Samples were eluted using a gradient from 0 % to 100 % B over 15 minutes at a flow rate of 1.5 mL/min at 35 °C.

Purification was performed on an Agilent instrument with Prostar 410 injector, Prepstart pumps detector (UV-VIS dual wavelength) and 440-LC fraction collector. A Phenomenex AXIA packed Luna C18 column (100 A, 250 x21.2 mm, 5 micron at 35 °C) using a gradient of 0-100% B in 25 min unless mentioned otherwise. The following solvents were used in a flow rate of 17.5 mL/min: 0.1% TFA in water (A) and ACN (B)

1.3 Peptide synthesis

To synthesize the peptides, 100 mg of the Rink amide resin (0.69 mmol/g) was used. Automated peptide synthesis was done with a SYRO Multiple Peptide Synthesizer (Multisyntech) or with a MultiPep RSi (Intavis), making use of the Fmoc/tBu strategy. Usually, synthesis with double couplings was performed as following: the resin is swollen in DMF for 20 minutes. A mixture of 5 equivalents (eq.) amino acid in DMF (0.5 M), 5 eq. N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) in DMF (0.5 M), and 10 eq. DIPEA in NMP (2 M) is added to the resin, with subsequent reaction for 40 minutes at room temperature. The reaction mixture is removed and the resin is washed with DMF (4 x 30 sec). The Fmoc group is removed by reaction with 20% piperidine in DMF for 12 minutes.

1.4 Gel preparation

Stock solutions of the cargoes were made first in phosphate-buffered saline solution (PBS) at the desired concentration. Gelation was achieved by adding 100 µL of this stock solution to x mg of peptide hydrogelator, in an Eppendorf tube (1.5 mL). All gels were then repetitively vortexed (1 min) and sonicated (5 min) after which they were left to rest overnight.

2. Peptide characterization

2.1 Table of all synthesized peptides

Table SI 1: Overview of the peptide sequences: their corresponding codes, the calculated molecular weight (MW), the obtained MW via HRMS analysis, the residence time in LCMS analysis (t_r), their calculated isoelectric point (calcd pI), and minimum gelation concentration (MGC)*.

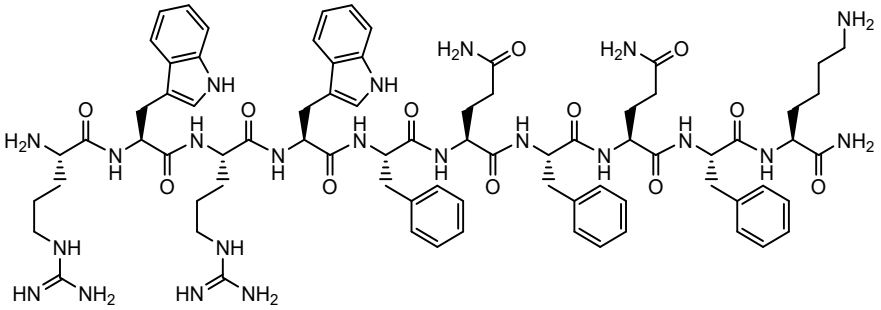
Name code	***Peptide sequence***	***Calcd MW (Da)***	***HRMS (Da)***	***t_r (min)***	***Calcd pI***	***MGC (w/v%)***
P1	H-RWRWFQFQFK-NH_2	1527.80	1527.60	3.84	11.84	ND**
P2	H-WRWRFQFQFK-NH_2	1527.80	1528.50	3.86	11.84	ND
P3	H-RRWWFQFQFK-NH_2	1527.80	1528.50	3.85	11.84	ND
P4	H-RWRWQFQFK-NH_2	1380.62	1380.40	3.69	11.84	1
P5	H-RRWWQFQFK-NH_2	1380.62	1381.50	3.67	11.84	ND
H-RWRW-NH_2	H-RWRW-NH_2	701.83	703.30	3.40	11.84	ND
H-FQFQFK-NH_2	H-FQFQFK-NH_2	843.00	844.50	3.30	9.07	1

***ND = Not determined*

** The MGC was determined via the inverted tube test. 100 µL hydrogels were prepared in a 1.5 mL Eppendorf tube and let rest overnight (see section 1.4). The MGC was concluded as the minimum concentration at which no flowy liquid upon inverting the tube was observed.*

2.2 *LC-MS characterization*

A)



Exact Mass: 1526,80

B)

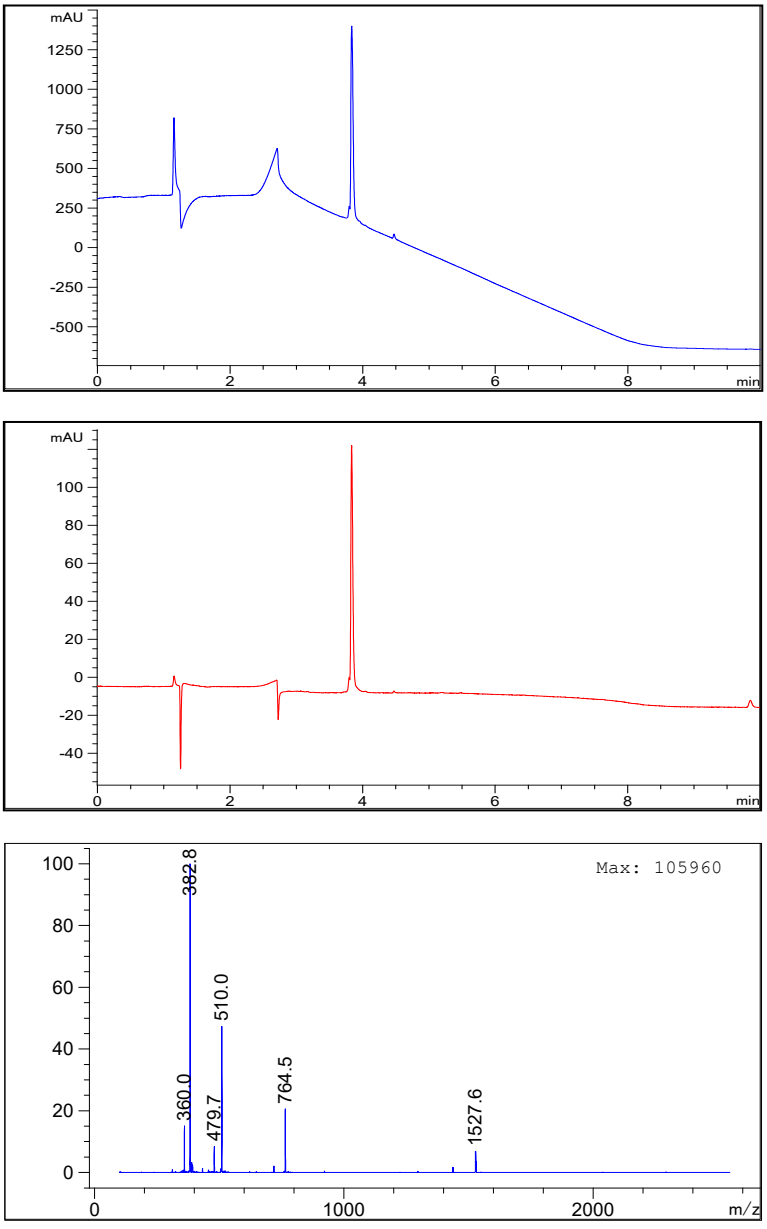
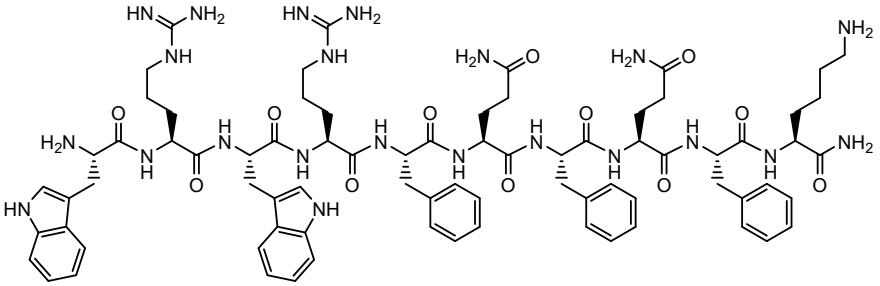


Figure SI 1: A) Chemical structure of the **P1** peptide. B) HPLC-UV trace at 214 nm of **P1**, C) HPLC-UV trace at 254 nm of **P1,** and D) mass spectrum of **P1.**

A)



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B)

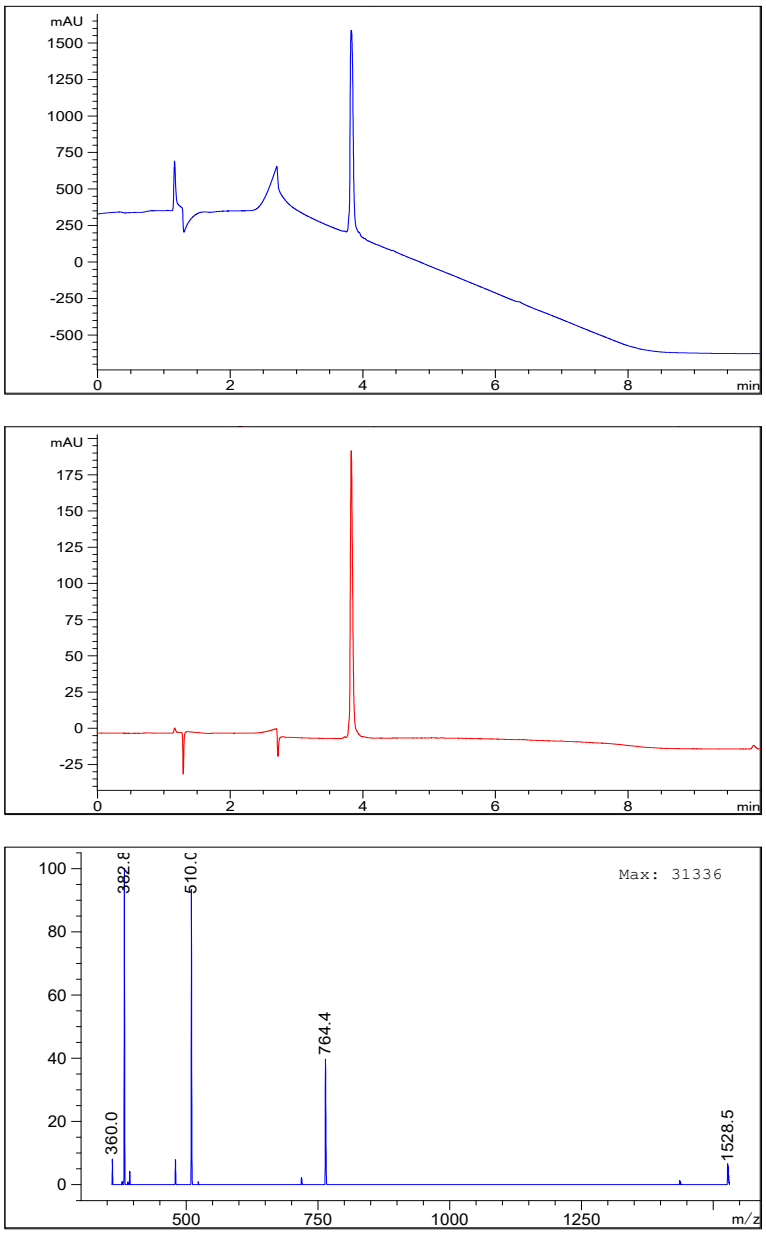
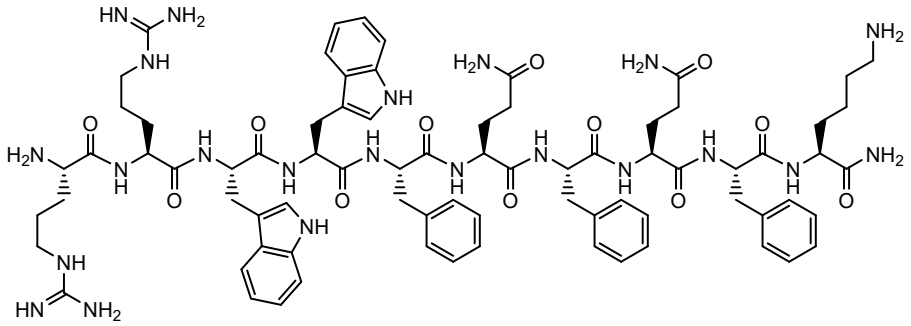


Figure SI 2: A) Chemical structure of the **P2** peptide. B) HPLC-UV trace at 214 nm of **P2**, C) HPLC-UV trace at 254 nm of **P2,** and D) mass spectrum of **P2.**

A)



Exact Mass: 1526,80

B)

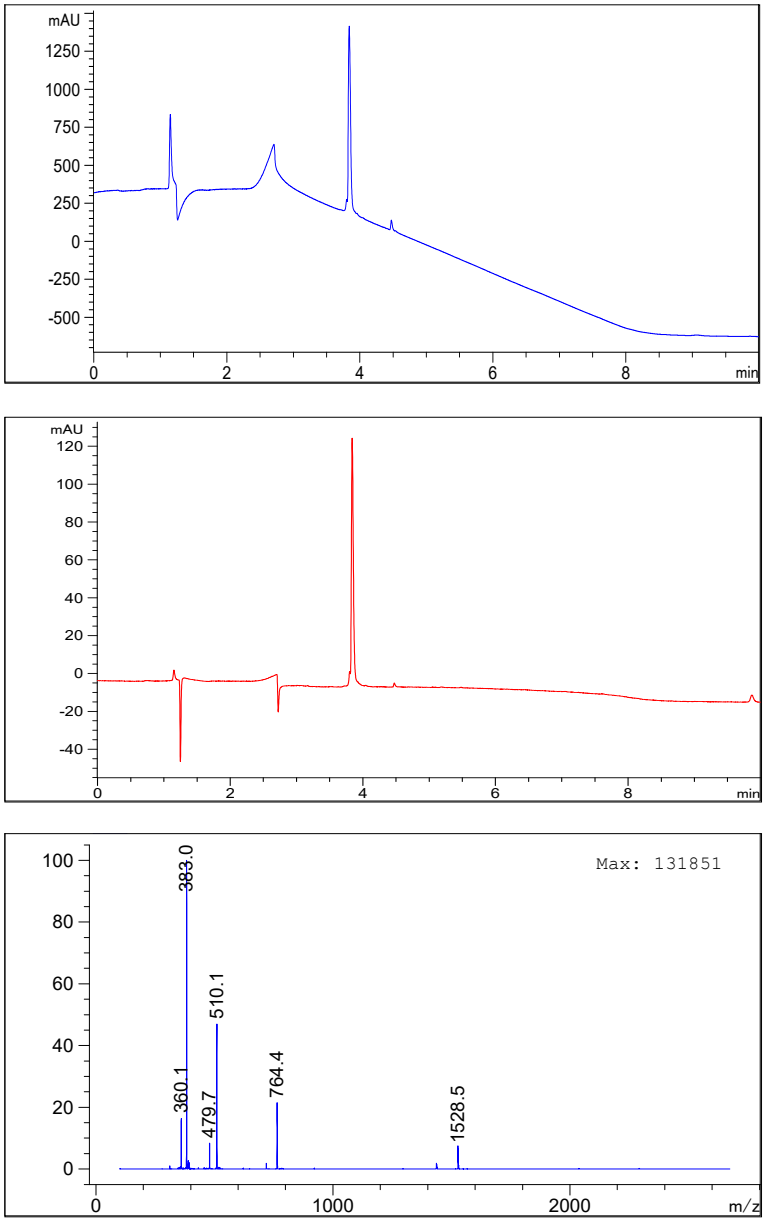
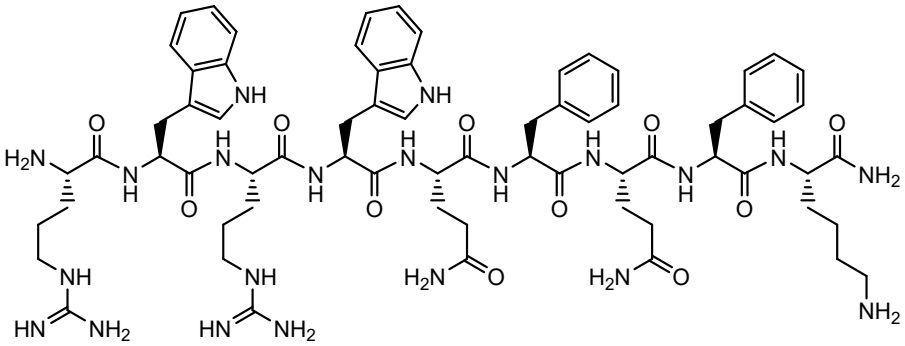


Figure SI 3: A) Chemical structure of the **P3** peptide. B) HPLC-UV trace at 214 nm of **P3**, C) HPLC-UV trace at 254 nm of **P3,** and D) mass spectrum of **P3.**

A)



Exact Mass: 1379,74

B)

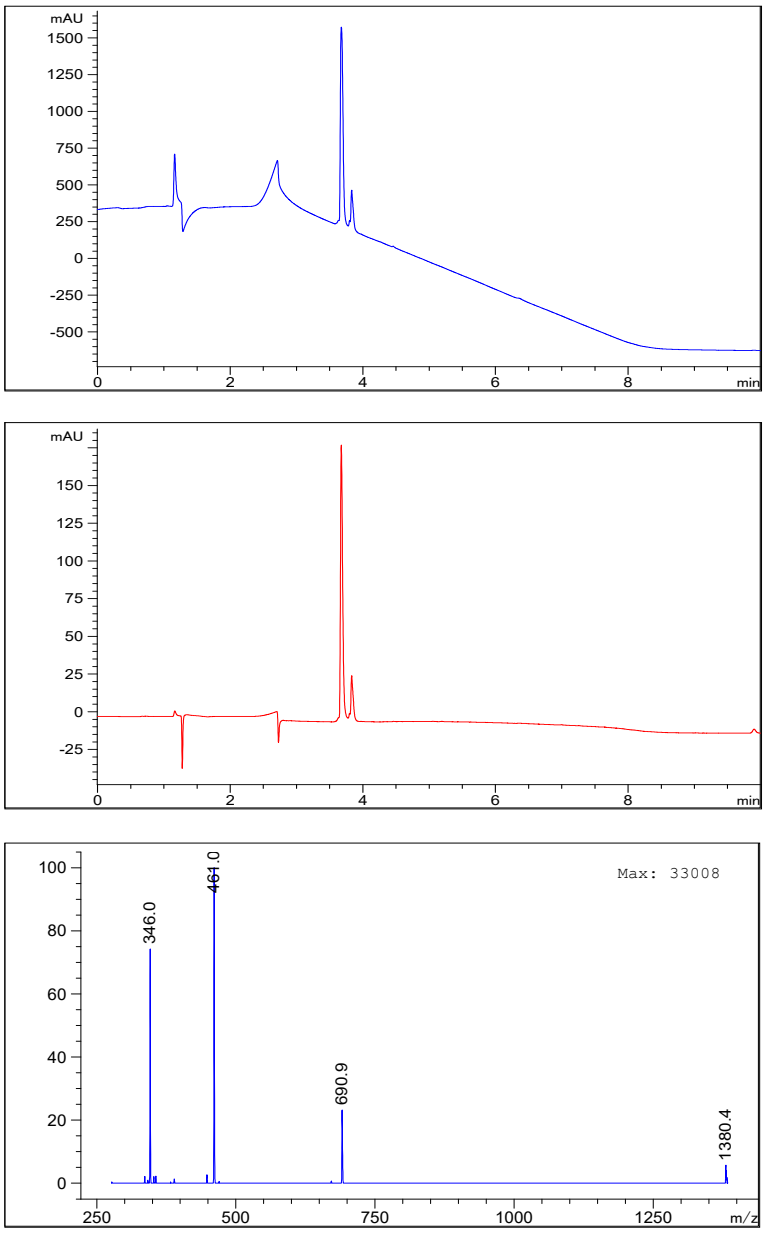
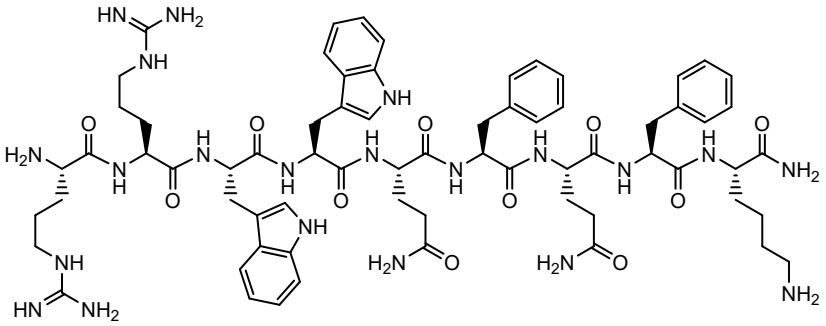


Figure SI 4: A) Chemical structure of the **P4** peptide. B) HPLC-UV trace at 214 nm of **P4**, C) HPLC-UV trace at 254 nm of **P4,** and D) mass spectrum of **P4.**

A)



Exact Mass: 1379,74

B)

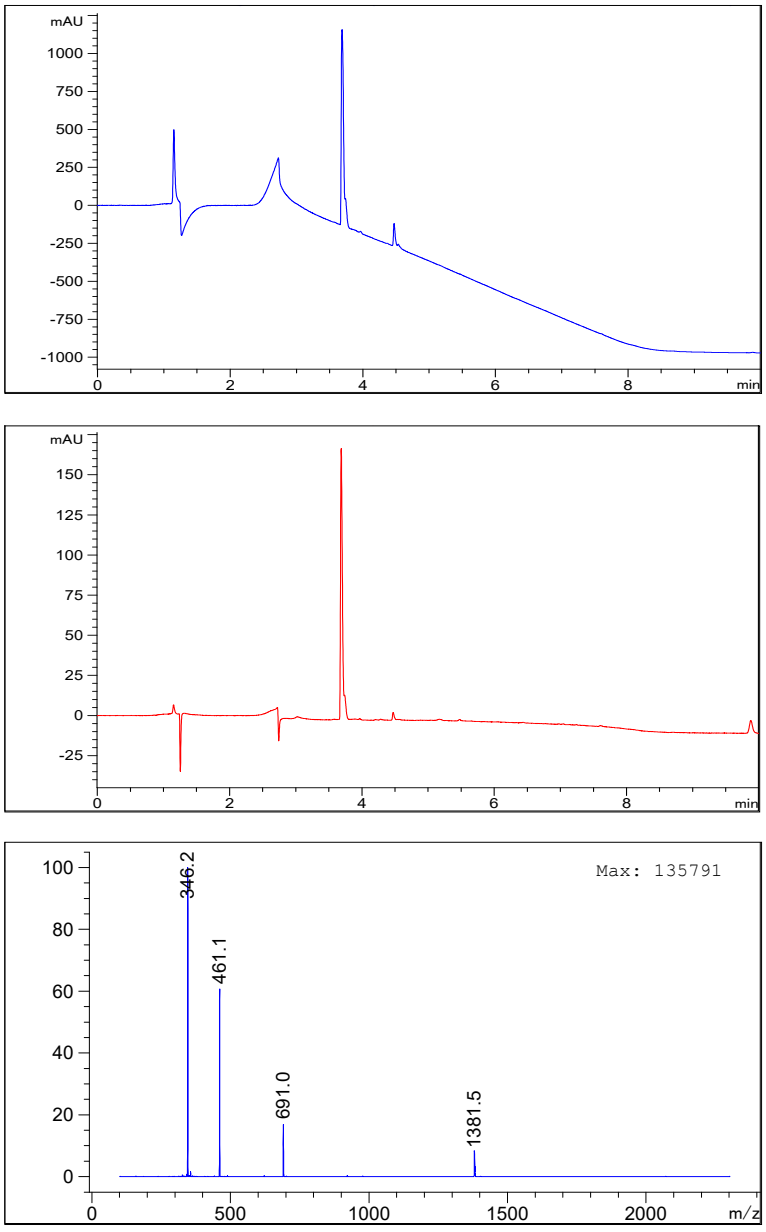
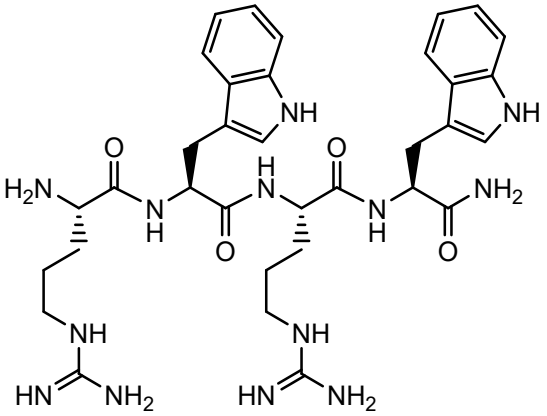


Figure SI 5: A) Chemical structure of the **P5** peptide. B) HPLC-UV trace at 214 nm of **P5**, C) HPLC-UV trace at 254 nm of **P5,** and D) mass spectrum of **P5.**

A)



Exact Mass: 701,39

B)

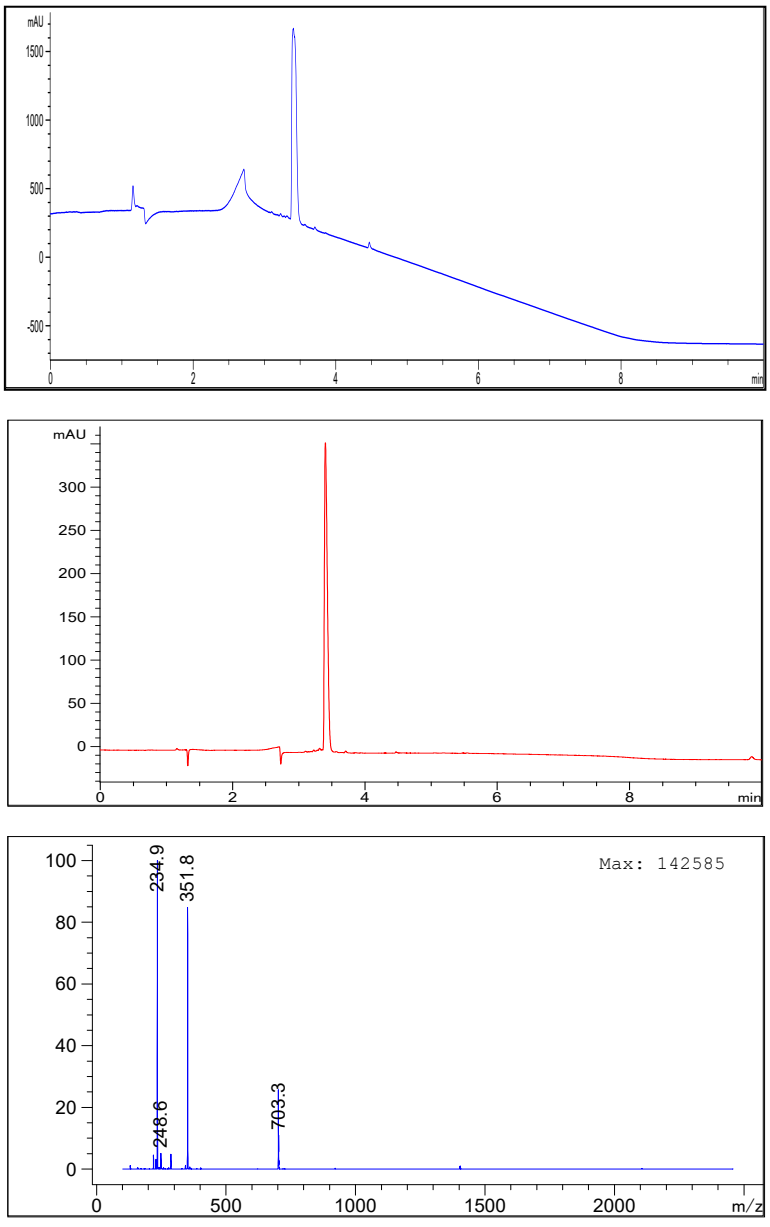
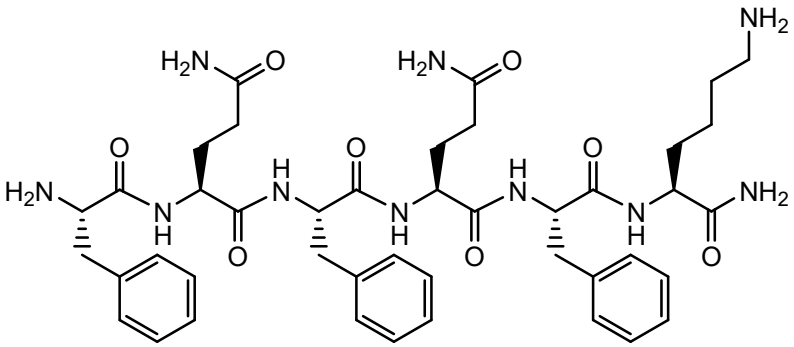


Figure SI 6: A) Chemical structure of the **H-RWRW-NH$_2$** peptide. B) HPLC-UV trace at 214 nm of **H-RWRW-NH$_2$**, C) HPLC-UV trace at 254 nm of **H-RWRW-NH$_2$,** and D) mass spectrum of **H-RWRW-NH$_2$**.

A)



Exact Mass: 842,44

B)

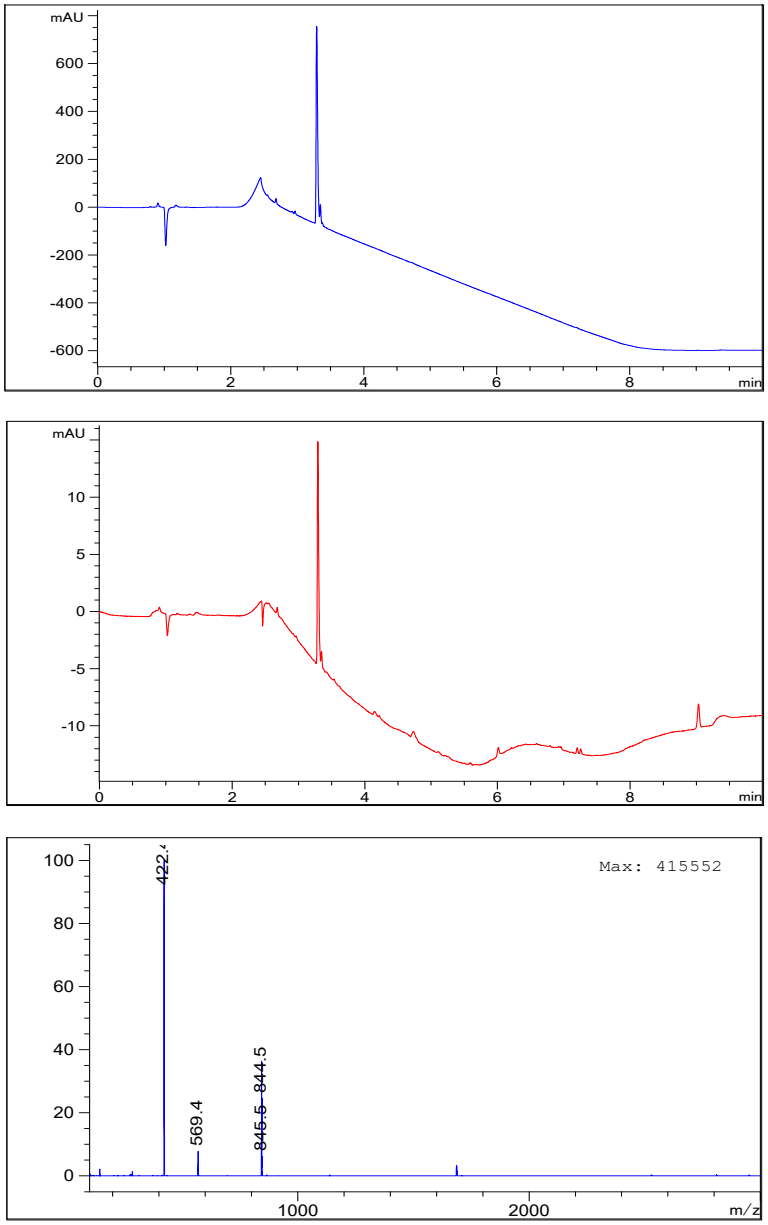


Figure SI 7: A) Chemical structure of the **H-FQFQFK-NH$_2$** peptide. B) HPLC-UV trace at 214 nm of **H-FQFQFK-NH$_2$**, C) HPLC-UV trace at 254 nm of **H-FQFQFK-NH$_2$,** and D) mass spectrum of **H-FQFQFK-NH$_2$**

3. MIC and MBC values

MIC screening was based on the EUCAST procedure. The optical density (OD) was plotted as a function of concentration. For this set of experiments a stock solution of 4 mg/mL (in MQ water) was prepared for each peptide. The concentration at which the OD reaches zero, corresponds to the MIC value. This screening was performed with **H-FQFQFK-NH$_2$** (grey), **P4** (pink), **P2** (blue), **P3** (green), **P5** (lilac) and on *S. aureus*, *B. subtillis*, *P. aeruginosa*, *A. baumannii* and *E. coli*. The MIC values corresponding to the graphs in Figure SI 10 are summarised in Table 3 and Figure SI 11.

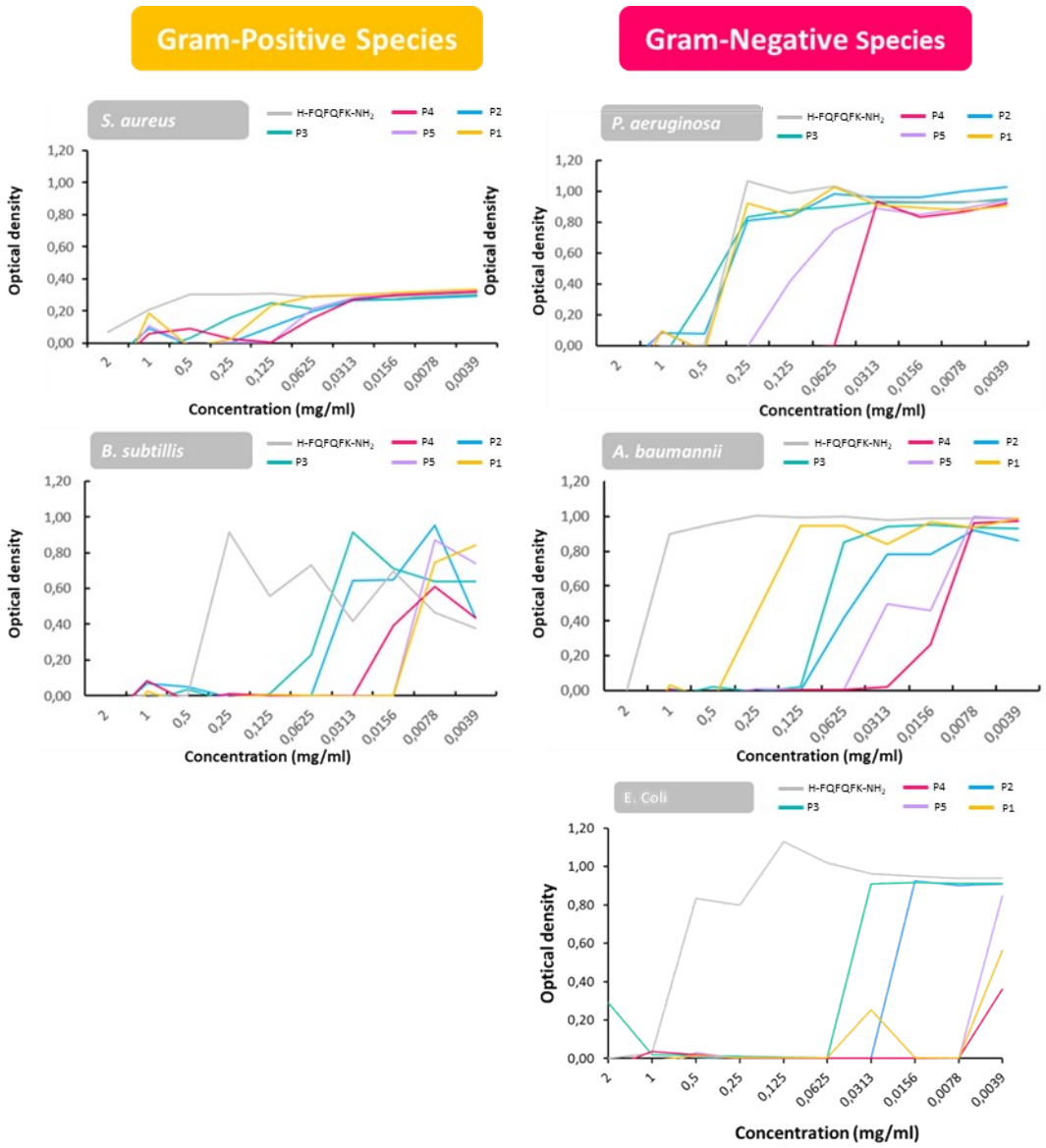


Figure SI 8: Optical density of gram-positive (*S. aureus* and *B. subtillis*) and gram-negative (*P. aeruginosa*, *A. baumannii* and *E. coli*) bacteria as a function of the concentration of the candidate antibacterial biogels: **H-FQFQFK-NH$_2$** (grey), **P1** (yellow), **P2** (blue), **P3** (green), **P4** (pink) and **P5** (purple).

Table SI 3: The obtained MIC values (µmol/mL) obtained for gram-positive (*S. aureus* and *B. subtillis*) and gram-negative (*P. aeruginosa*, *A. baumannii* and *E. coli*) bacteria for all candidate biogelators

Sequence	MIC values (µmol/mL)				
	S. aureus	*B. subtillis*	*P. aeruginosa*	*A. baumannii*	*E. coli*
H-FQFQFK-NH$_2$	>2	0.52	0.52	2.08	1
P1	0.071	0.0088	0.28	0.28	0.0044
P2	0.14	0.035	0.28	0.14	0.018
P3	0.57	0.071	0.57	0.14	0.035
P4	0.077	0.019	0.039	0.039	0.0048
P5	0.077	0.0096	0.155	0.040	0.0048

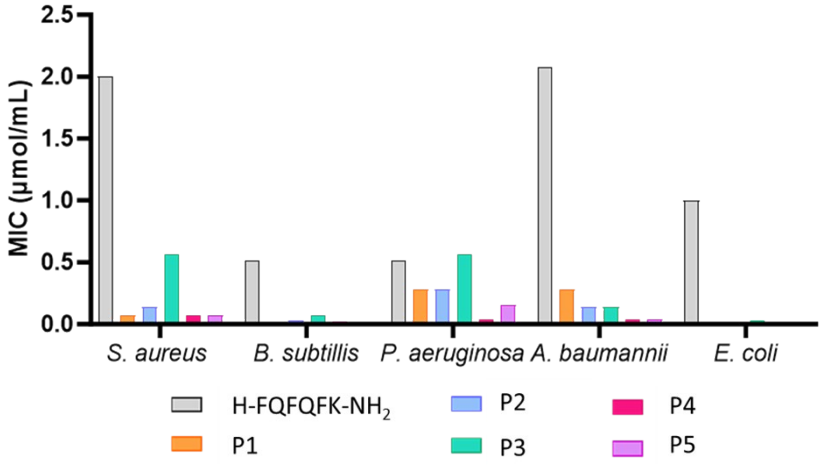


Figure SI 9: The obtained MIC values (µmol/mL) obtained for gram-positive (S. aureus and B. subtillis) and gram-negative (P. aeruginosa, A. baumannii and E. coli) bacteria for all candidate biogelators **H-FQFQFK-NH$_2$** (grey), **P1** (yellow), **P2** (blue), **P3** (green), **P4** (pink) and **P5**(purple).

Table SI 4: The obtained MBC values (µmol/mL) obtained for gram-positive (S. aureus and B. subtillis) and gram-negative (*P. aeruginosa*, *A. baumannii* and *E. coli*) bacteria for all candidate biogelators

Sequence	MBC values (µmol/mL)			
	S. aureus	*B. subtillis*	*P. aeruginosa*	*A. baumannii*
H-FQFQFK-NH$_2$	2.08	0.52	2.08	2.08
P1	0.28	0.0088	0.57	0.57
P2	0.28	0.035	1.13	0.14
P3	1.13	0.14	1.13	1.13
P4	0.15	0.019	0.62	0.077
P5	0.15	0.0097	0.62	0.039

4. Biogel characterization

4.1 Circular Dichroism

Experiments were performed on a 410-model CD spectrometer (Aviv Biomedical Inc., Lakewood, NJ) using 300 µL quartz cuvettes with a 1 mm path length. Spectra were recorded from 260 to 195 nm at 25°C. The peptide concentration was 500 µg/mL in phosphate buffer (75 mM Na_2HPO_4, 25 mM NaH_2PO_4, pH 7.11).

CD spectra are reported as the molar ellipticity ([θ], deg cm^2 $dmol^{-1}$), calculated by the equation:

$[\theta] = (\theta_{obs} \times MW) / (10*l*c)$

where θ_{obs} is the ellipticity in millidegrees, MW is the molecular weight, l is the path length of the cuvette in centimetres, and c is the peptide concentration in milligrams per millilitre.

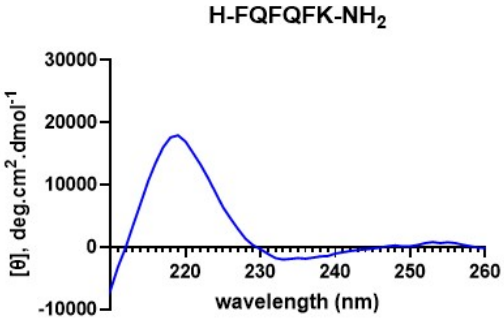


Figure SI 10. CD spectrum of H-FQFQFK-NH2 at 500 µg/mL in phosphate buffer (100 mM). The typical cruve of a highly-twisted β-sheet structure with a red shift of approximately 20 nm corresponding to a maximum around 218 nm and a minimum around 233 nm was observed.

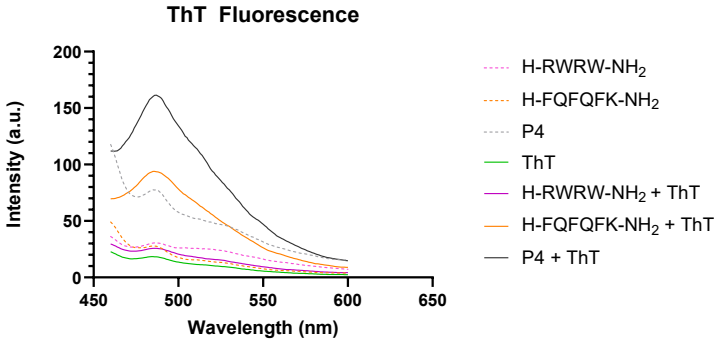


Figure SI 11. Fluorescent spectra of ThT upon incubation with **P4** at 1 w/v% (black), **H-FQFQFK-NH_2** at 1 w/v% (orange), and **H-RWRW-NH_2** at 0.55 w/v% (purple). The corresponding dash lines are background fluorescence of the peptides (without ThT). All peptides were prepared in PBS (pH 7.4).

4.2 Rheology

For the rheology measurements, an Anton Paar Physica MCR 301 rheometer was used. The results were analysed with Rheoplus software. The shear recovery experiments were performed on 350 µL of

hydrogel. The hydrogel was ejected on a 25 mm plate. After injection of the sample, the sample was allowed to rest for 5 minutes, prior to starting the measurement.

For the shear recovery experiment, the G' and G" were first measured for 60 seconds at a frequency of 1 Hz and a shear strain (γ) of 1%. After these 60 seconds, the γ was increased up to 50 % for 30 seconds. This cycle was repeated 4 times in each experiment. The measurements were performed in threefold with a normal force of 1N onto the sample at a temperature of 25◦C.

For the amplitude sweep experiment, the shear strain (γ) was increased from 0 to 500% while the frequency was kept constant at 0.15 Hz and temperature at 25◦C. The hydrogel sample was priorly prepared and let rested for 4 hours before applying on the plate (25 mm in diameter) via a syringe. 300 µL of sample was used for each measurement.

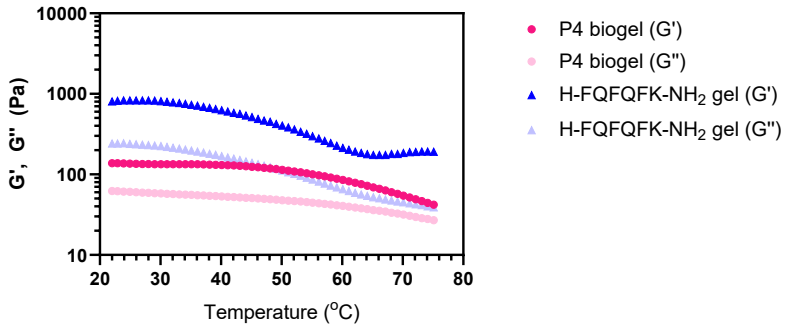


Figure SI 12. Data from temperature ramps done on 2 w/v% gels of H-FQFQFK-NH2 (blue triangle) peptide and P4 peptide (pink circle). A heating rate of 3°C/min was applied under constant shear value of 1% and frequency of 1 Hz. No cross-over point was observed for the investigated temperature range in both cases.

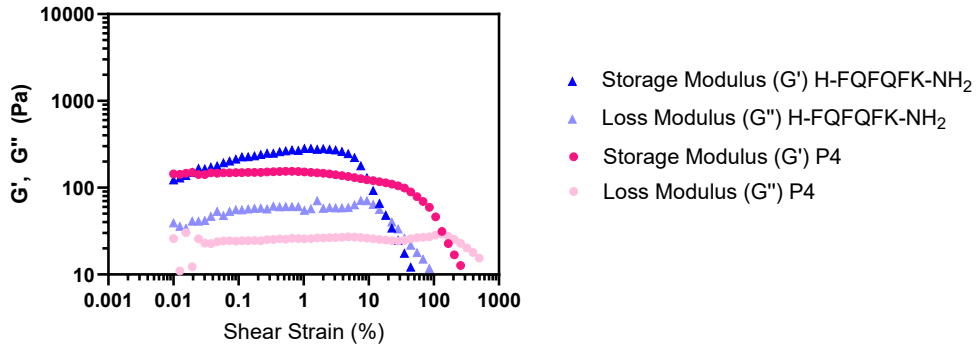


Figure SI 13. A rheological amplitude sweep (0.01 to 500% shear value). The frequency was kept constant at 0.15 Hz, no normal force was applied. The measurement was done at 25°C. P4 hydrogel (pink) started to showed liquid-like behavior at the shear strain (γ) of 166% while H-FQFQFK-NH_2 hydrogel (blue) showed crossed-over point at approximatelyshear strain (γ) of 20%. Hydrogels were priorly prepared in PBS 1X (pH 7.4) and let rested for 4 hours before mearement.

5. Cytotoxicity

5.1 XTT-assay

The cytotoxicity of the peptides in solution were determined by performing an XTT assay (Roche, Sigma, kit II). For this purpose, murine NIH 3T3 fibroblasts, collected from a sub-confluent culture using 0.25 w/v% trypsin/1mM EDTA were seeded into a 96-well plate (Corning® 96-well Flat Clear Bottom). The

optimal cell density in this standardized procedure is 4000 cells/well. Next, this inoculated 96-well plate is incubated for 24 hours at 37°C and 5% CO_2.

After 24h incubation at 37°C and 5% CO_2 in which the cells were able to attach, the NIH3T3 fibroblasts were exposed to peptides at various concentrations.

The peptides (H-FQFQFK-NH_2, H-RWRW-NH_2) were initially prepared as a 20 mM stock solution in MQ water and subsequently adjusted to final concentrations ranging from 250 to 6.25 µM using a cell growth medium. Next, 100 µL of this peptide solution was added to each well in the 96-well plate. Per condition 3 replicate wells were used. The blank samples contained no cells and no peptide, whereas the control samples contained cells but no peptide. Moreover, the cells were incubated with the peptides for 48 hours at 37°C, 5% CO_2.

After 48 hours, the medium was replaced in each well with the XTT reagents to quantify the metabolic activity of the cells. In brief and following the instructions of the manufacturer, 50 µL XTT-solution (containing the electron coupling reagent and the labelling reagent in a 1:50 ratio) and 100 µL cell culture medium was added per well. During the subsequent 3-hour reaction at 37°C, the metabolically active viable cells in the well, converted the XTT reagent into a colored soluble formazan. The absorbance of the formazan was measured at 450 nm using an iMark microplate reader.

The obtained absorption was corrected for background measurement at 595 nm and for the mean blank value (from replicate wells without cells). In this experiment, a t_0-plate similarly seeded with 4000 cells per well was prepared alongside the main set-up and used to quantify the number of viable cells at the start of the experiment (24 h after seeding) and before the addition of the peptides. This is similarly done using the XTT-assay as described above. The absorbance measurements in the t_0 plate and in the main set-up were processed as follows.

First, the absorbance at 595 nm was subtracted from absorbance value obtained at 450 nm to correct for background ($A_{450} - A_{595}$). Secondly, the mean of blanks was subtracted from this background corrected absorbance value to obtain the net absorbance (A450 net). This was done for the samples on the accompanying t_0-plate and for the main setup. Next, the net absorbance values at t_{48} were divided by the net mean absorbance value at t_0 ($\mathbf{A_{450}\ net\_t_{48}\ /\ A_{450}\ net\_t_0}$) for each condition. Finally, data was imported in GraphPad for plotting and performing nonparametric statistical analysis using Kruskal-Wallis followed by a Dunn multiple comparison tests to correct for multiple testing. The significance level was set to 0.05. The experiment was performed in duplicate-independent (n = 6).

5.2 Cytotoxicity hydrogel

To see whether the hydrogels as such would have an effect on the NIH3T3 fibroblasts, 100 µL of hydrogels (4 w/v% of P4, 2.46 w/v% of H-FQFQFK-NH_2 and 2.46 w/v% of H-FQFQFK-NH_2 + 1.13 w/v% of H-RWRW-NH_2) were ejected in the middle of a 24 well plate. First the gels were incubated with cell medium for two hours. Afterwards the cells were seeded in the well and the total volume in each well equalled 750 µL. After 24 hours of incubation, Sytox Green (200 nM final concentration) was added to each well, so that visualization of dead cells was possible see Figure SI 10.

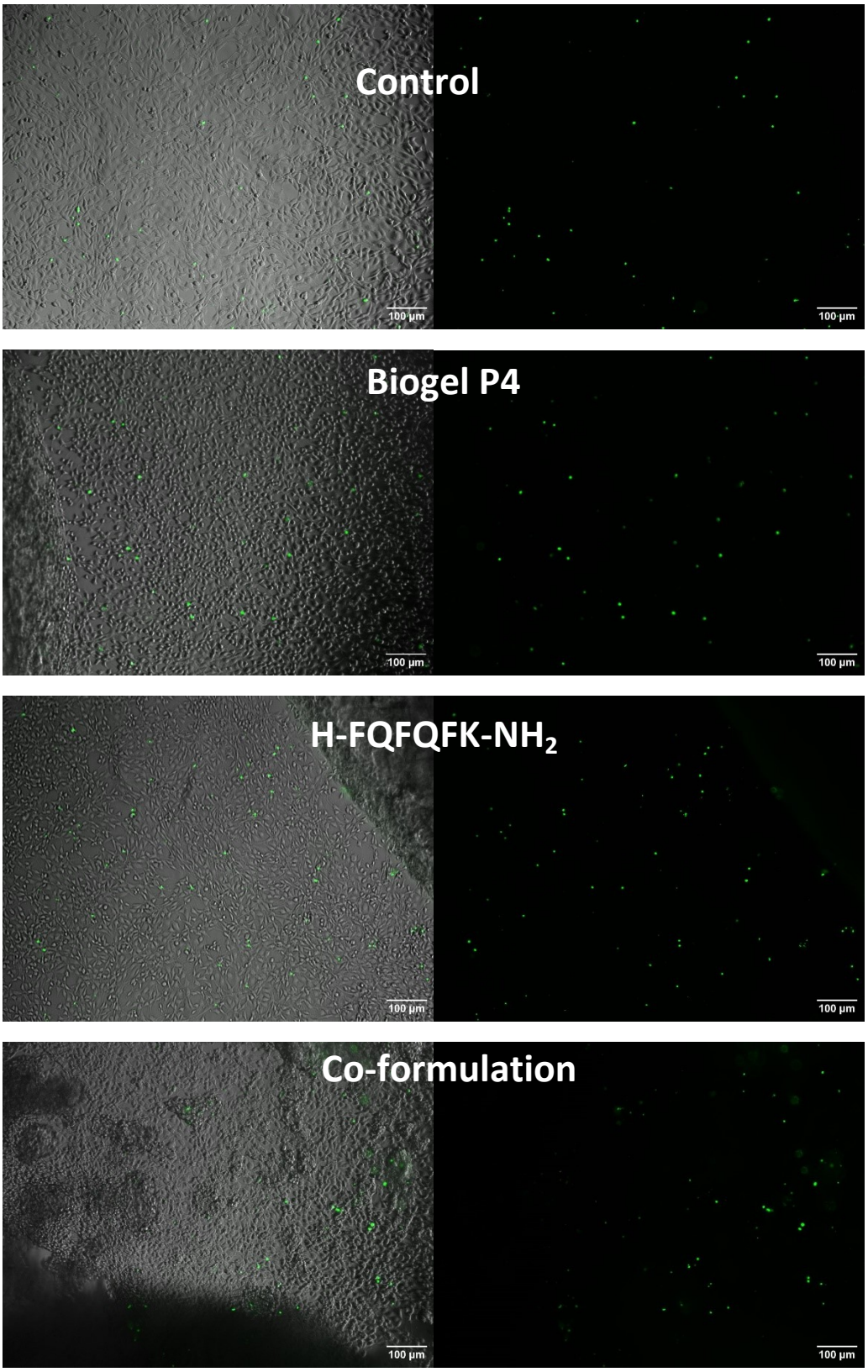


Figure SI 14: Phase contrast and fluorescent images of NIH3T3 cells incubated for 24 hours with only cell medium (control), the P4 biogel, the H-FQFQFK-NH_2 hydrogel and the hydrogel of H-FQFQFK-NH_2 co-formulated with H-RWRW-NH_2.

Phase contrast and fluorescent images of the cells were acquired using an Olympus IX81 microscope equipped with a Zeiss Axiocam 105 camera and ZEN Zeiss acquisition software. The images were processed in FIJI (https://fiji.sc)

6. In vitro wound model

6.1 Artificial dermis synthesis

Preparation of 1.5 % high-molecular-weight hyaluronic acid (first layer)

To 150 mg of sodium hyaluronate powder, 10 mL of sterile MQ water is added. This mixture is mixed and vortexed. Subsequently, the pH (6.8-7.2) is brought to pH 3.5 by adding drops of a 0.1 M HCl. Next, 30 mg of ethylene glycol diglycidyl ether (ρ = 1.18 g/cm^3) is added. Of this mixture, 1 mL is added to a freeze-drying container. This sample was then frozen at -80°C and freeze dried overnight.

Preparation of the second layer

First, a 1% hyaluronic acid solution is prepared by adding 3.4 mL of sterile MQ water to 34 mg of of sodium hyaluronate powder. This solution is vortexed and mixed and will be referred to as solution A. Next, a 1% hydrolyzed low molecular weight hyaluronic acid solution is obtained by adding 10 mL of sterile MQ water to 100 mg of sodium hyaluronate powder. This mixture is placed in the autoclave for 1h. Once the solution is cooled down, the volume is adjusted with sterile MQ water until it reaches a volume of 10 mL. In the following step, this solution will be referred to as solution B. Moreover, a 0.1 % collagen containing solution was prepared by weighing 3.3 mg of collagen and mixing it with 3.3 mL of sterile MQ water. This solution was subsequently placed at 50°C, and will be referred to as solution C. 3.4 mL of solution A was mixed with 3.3 mL of solution B and 3.3 mL of solution C. By adding a few drops of a 0.1 M NaOH solution, the pH was adjusted to 7.5. Of this mixture, 1 mL was added to the freeze-drying container in which the first freeze-dryed layer sheet is immersed. This container was first stored for several hours at 4°C after which it was frozen at -80°C.

Linking the first and the second layer

Both sides of the sheets were irradiated with the UV lamp for 20 minutes. These sheets were then placed in sterile glass containers and sterilized at 110°C for 1h to obtain a sterile artificial dermis.

6.2 Set-up of the infection protocol

The medium for this essay was prepared by dissolving 10 mL of plasma into 19 mL of sterile Bolton Broth. To this mixture 1 mL of horse blood and 10 U of herparine (20 µL of a 100 kU solution) was added. Next, an artificial dermis was placed into each well of a 24 well plate. On top of the dermis, 300 mL medium was added. In addition, 500 µL of the medium was placed around the dermis in order to keep the dermis moist.

The infection solution was prepared by selecting the bacterial culture of choice, centrifuging it and resuspending it in physiological saline. This solution was diluted to an optical density that corresponds to 1x10^8 CFU/mL. This suspension was further diluted to 1x10^6 CFU/mL. Of that suspension, 10 µL (corresponds to 10^4 CFU) was added on top of the dermis, after which the plate was incubated at 37°C for 4 hours. After the 4 hours of incubation, 200 µL of the gel or peptide solution was added on top of the dermis. This plate was then placed at 37 °C for another 20 hours.

6.3 Quantification

To quantify the CFU/dermis, the AD were placed into tubes containing 10 mL PS, the sessile cells were removed from the AD by three cycles of vortexing (30 s) and sonication (30 s; Branson 3510; Branson Ultrasonics Corp., Danbury, CT) and the number of CFU/dermis was determined by plating the resulting suspensions.

The *S. aureus* was plated on mannitol salt agar, the *P. aeruginosa* on pseudomonas isolation agar and the *A. baumanni* on MacConkey agar. A minimum of three samples of each type of hydrogel was analyzed.