SUPPLEMENTARY INFORMATION

Nucleation and Growth of Ordered Arrays of Silver Nanoparticles on Peptide Nanofibers: Hybrid Nanostructures with Antimicrobial Properties

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EXPERIMENTAL

Chemicals

All peptide synthesis reagents and amino acid derivatives were purchased from *NovaBiochem (EMD Millipore)*; standard amino acids were purchased as protected Fmoc amino acids with the standard side chain protecting scheme: Fmoc-Ala-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Glu(OAll)-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Val-OH. C-terminal amide peptides were synthesized on a 0.25 mmol scale using a 0.36 mmol/g loading Fmoc-Rink Amide MBHA LL resin from *NovaBiochem (EMD Millipore)*. All other chemicals were purchased from *Aldrich* or *Fluka*. All solvents were dry and synthesis grade, unless specifically noted. Water was purified using a Milli-Q system (*Millipore*).

Instrumentation

Peptides were synthesized using a *CEM Liberty* microwave-assisted peptide synthesizer at the Peptide Synthesis Core at the Simpson Querrey Institute for Bionanotechnology (SQI).

Preparative and Semi-Preparative High-Performance Liquid Chromatography (HPLC) were performed with a *Varian ProStar 210*, using a *Phenomenex Gemini* column (C_{18} stationary phase, 5 µm, 110 Å pore size, 30 × 150 mm or C_{18} stationary phase, 5 µm, 110 Å pore size, 10 × 250 mm). Analytical HPLC was performed with an *Agilent 1260* Series, using an *Inspire* analytical column from *Dikma* (C_{18} stationary phase, 5 µm, 100 Å pore size, 4.6 × 250 mm). The standard gradient used for analytical and preparative HPLC was 5 → 95% 0.1% NH_4OH, CH_3CN/0.1% NH_4OH, H_2O over 30 min, and 20 → 65% 0.1% NH_4OH, CH_3CN/0.1% NH_4OH, H_2O over 30 min, respectively. Electrospray Ionization Mass Spectrometry (ESI/MS) was performed with an *Agilent 6520 Q-TOF* in positive scan mode using direct injection of the purified peptide solution.

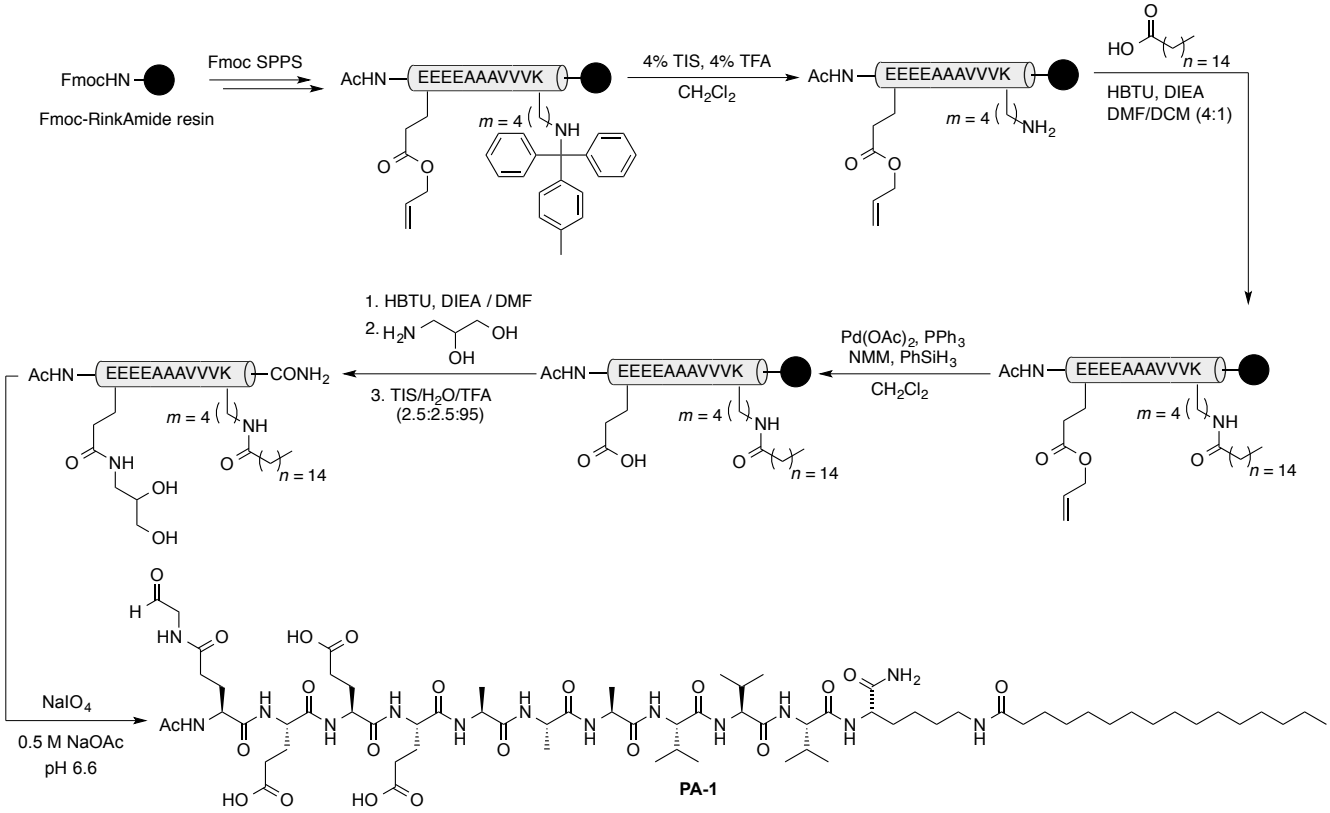
UV measurements were made in a *Perkin Elmer Lambda 1050* spectrophotometer using a standard *Hellma* Semi-Micro cuvette (114-QS) or Macro cuvette (110-QS).

Circular dichroism experiments were made with a *Jasco J-815*, using a *Precision Cells*, Type 20 Demountable O-Shaped Circular Dichroism Cuvette (0.1 mm light pass) at 20 ºC. Samples contained 500 µM of peptide-amphiphiles and 1 mM or 3.36 mM of silver, when present. The reported spectra are the average of 10 scans, and are processed using the "smooth" macro implemented in the program *KaleidaGraph* (v 4.1.3 by *Synergy Software*).

Conventional TEM was performed using a *Hitachi HT-7700* Biological TEM with a S-type tungsten filament at 20 kV and an accelerating voltage of 100 kV. The sample (7 µL) was deposited on 300 mesh copper grid with carbon film support (*Electron Microscopy Sciences*, *EMS*). Images were acquired using an *Orius SC 1000A* CCD camera.

Peptide synthesis and purification procedures

Peptide amphiphiles (**PA-1**: Ac-E(CH_2CHO)EEEAAAVVVK(C_{16})-NH_2 and **PA-2**: Ac-EEEAAAVVVK(C_{16})-NH_2) were synthesized following standard microwave Fmoc-solid phase peptide protocols. Amino acid couplings were performed using 4 equiv of protected amino acid, 4 equiv of *O*-(benzotriazole-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU), and 8 equiv of *N*,*N*-diisopropylethylamine (DIEA) in dimethylformamide (DMF) at 75 °C for 5-10 minutes. Fmoc deprotection was performed using 20% 4-methylpiperidine with 0.1 M hydroxybenzotriazole (HOBt) in DMF at 75 °C for 3-4 minutes. Capping following the addition of each amino acid was performed using a mixture of 0.5 M acetic anhydride, 0.125 M DIEA, and 0.015 M HOBt in DMF at 65 °C for 2 minutes.



Scheme S1. PA-1 synthetic scheme.

Mtt deprotection of **PA-1** and **PA-2** was done treating the peptide attached to the solid support with a solution of 4% triisopropylsilane (TIS) and 4% trifluoroacetic acid (TFA) in dichloromethane (DCM) (2 × 5 min). Then, the C_{16}-tail was attached to the Lys-deprotected side chain, using 4 equiv palmitic acid, 3.95 equiv of HBTU and 6 equiv of DIEA in DMF/DCM (4:1 v/v) overnight at rt.

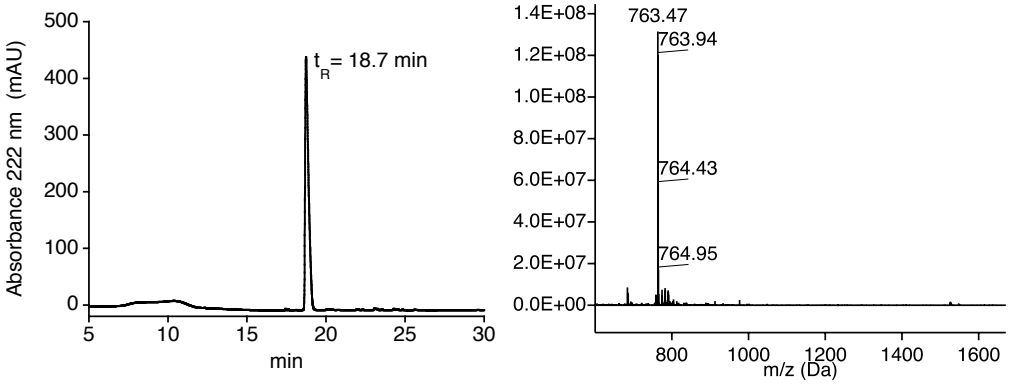
Once the **PA-1** was fully assembled in solid phase, the side chain of the Glu(OAll) residue was selectively deprotected, for specific attachment of 3-amino-1,2-propanediol, following this procedure: 0.25 mmol of peptide attached to the solid support was treated at room temperature for 12 h with a mixture of $Pd(OAc)_2$ (0.3 equiv), PPh_3 (1.5 equiv), *N*-Methylmorpholine (NMM) (10 equiv), and $PhSiH_3$ (10 equiv) in DCM (10 mL). The resin was

then filtered and washed with THF (1 × 15 mL × 2 min), DMF (2 × 15 mL × 2 min), diethyldithiocarbamate (DEDTC, 75 mg in 15 mL of DMF, 2 × 5 min), DMF (2 × 15 mL × 2 min), and DCM (2 × 15 mL × 2 min).

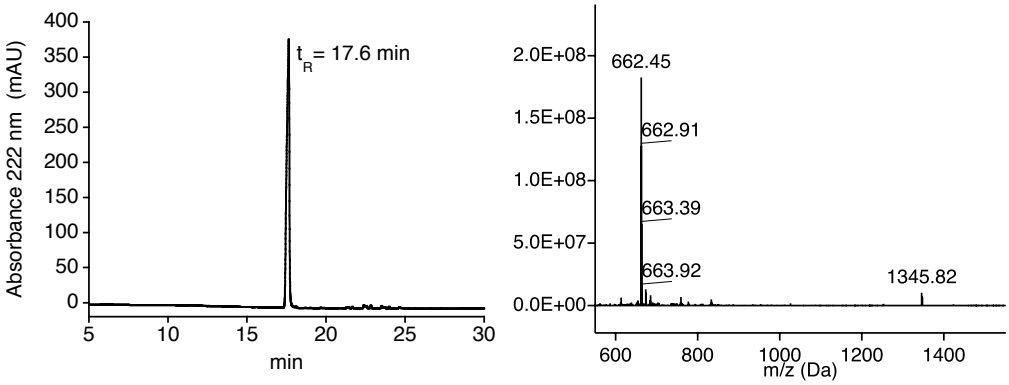
The allyl-deprotected peptide attached to the resin (0.25 mmol) was suspended in dry DMF (10 mL). 2-(1*H*-7-*aza*-benzotriazol-1-*yl*)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 1 equiv) and DIEA (1.5 equiv) were added to the solution. After five minutes, 3-amino-1,2-propanediol (4 equiv) was added to the mixture and the resin suspension was shaken for 2 h. After filtration, the resin was washed with DMF (3 × 10 mL × 3 min) and DCM (3 × 10 mL × 3 min), and dried under nitrogen.

The resin-bound peptide (0.25 mmol) was placed in a 50 mL falcon tube to which 15 mL of the cleavage cocktail (2.5% TIS, 2.5% H_2O, and 95% TFA) were added. The resulting mixture was shaken for 3 h. The resin was then filtered, and the TFA filtrate was concentrated under reduce pressure to a volume of approximately 2 mL. The residue was added to ice-cold diethyl ether (20 mL). After 10 min, the precipitate was centrifuged and washed again with 10 mL of ice-cold ether and centrifuged. The solid residue was dried under nitrogen and redissolved in 0.1% NH_4OH, CH_3CN/0.1% NH_4OH, H_2O (1:9) and purified by preparative reversed-phase HPLC. The collected fractions were identified by ESI-MS, lyophilized and stored at –20 °C.

PA-1-diol: ESI-MS $[MH]^+$ calcd for $C_{71}H_{125}N_{14}O_{22}$ = 1525.90, found = 763.47 $[MH_2]^{2+}$ (33 mg, 9% yield for 0.25 mmol scale).



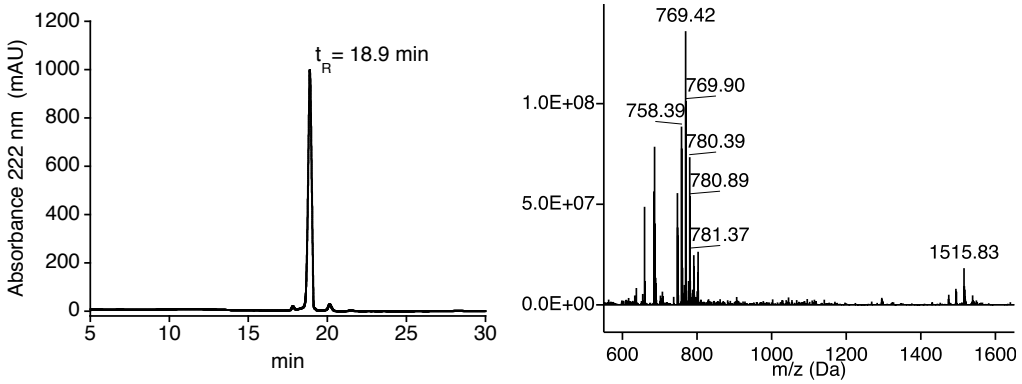
PA-2: ESI-MS $[MH]^+$ calcd for $C_{63}H_{111}N_{12}O_{18}$ = 1323.81, found = 1345.82 $[MNa]^+$; 662.45 $[MH_2]^{2+}$ (9 mg, 6% yield for 0.125 mmol scale).



PA-1-diol oxidation

PA-1-diol (15 mg, 0.0098 mmol) was dissolved in NaOAc (1.78 mL, 0.5 M, pH 6.6-6.8) and $NaIO_4$ (220 µL, 70 mM) was added. The mixture was shaken at room temperature for 45 min, and the crude was then purified by semi-preparative reversed-phase HPLC. The collected fractions were identified by ESI-MS, lyophilized, and stored at –20 °C.

PA-1: ESI-MS $[MH]^+$ calcd for $C_{70}H_{121}N_{14}O_{21}$ = 1493.88, found = 1515.83 $[MNa]^+$; 1494.05 $[MH]^+$; 780.39 $[M\text{-}H+3Na]^{2+}$; 769.42 $[MNa_2]^{2+}$; 758.39 $[MHNa]^{2+}$; 747.43 $[MH_2]^{2+}$ (6.1 mg, 42% yield).



Formation of silver nanoparticles over PA nanofibers

Tollens' solution was prepared by mixing aqueous solutions of $AgNO_3$ (625 µL, 0.5 M) and NaOH (125 µL, 3 M), followed by the addition of NH_4OH solution (180 µL, 14%, 7.4 M) until the black precipitate is completely dissolved.

Centrifuged Tollens' solution was added to a 500 µM solution of **PA-1** in Milli-Q water (pH 6.6-6.8, and aged overnight) to give a final silver concentration of 1 mM or 3.36 mM. The formation of silver nanoparticles was monitored measuring the UV-vis spectrum every 5-10 min. The cuvette was kept closed during this time to prevent evaporation of ammonia.

UV-vis spectroscopy

To 249.2 µL of a 500 µM solution of **PA-1** in Milli-Q water (pH 6.6-6.8), 0.75 µL of the Tollens' solution were added at rt. UV-vis spectra were recorded 30 s after the addition of Tollens' solution, and then every 10 min using a 1 mm path length cuvette.

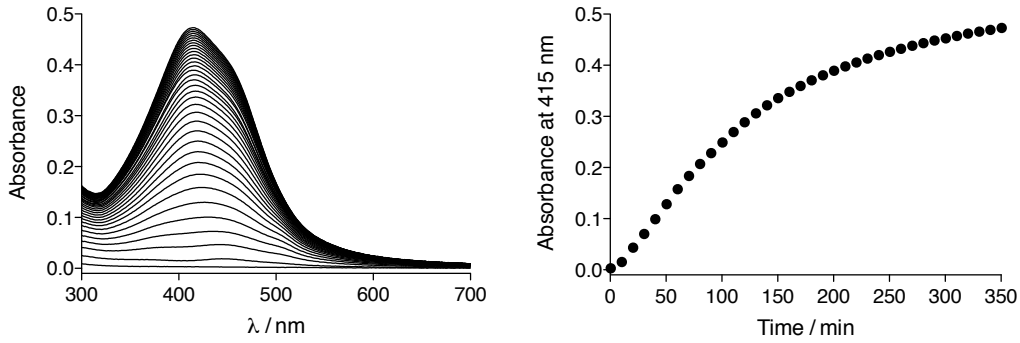


Figure S1. Left. UV-vis spectra of a 500 μM **PA-1**, 1 mM Ag^+ solution over time. **Right.** Absorbance intensity at 415 nm of the same solution over time.

To 247.5 μL of a 500 μM solution of **PA-2** in Milli-Q water (pH 6.8), 2.5 μL of the Tollens' solution were added at rt. The UV-vis spectra were recorded 30 s after the addition of Tollens' solution, and then every 10 min using a 1 mm path length cuvette.

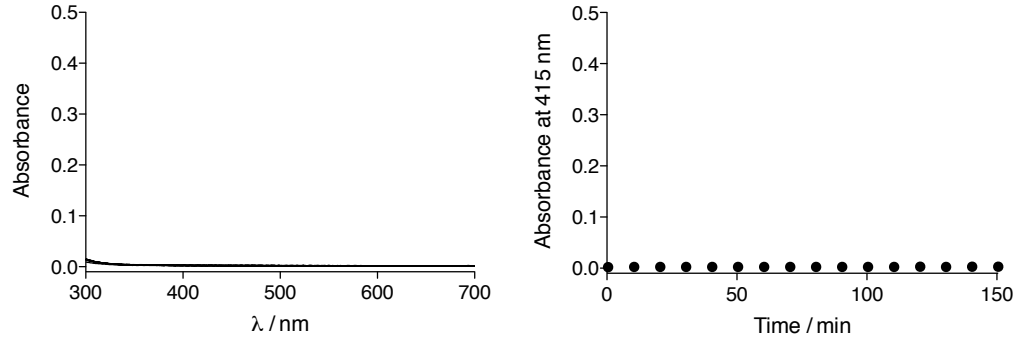


Figure S2. Left. UV-vis spectra of a 500 μM **PA-2**, 3.36 mM Ag^+ solution over time. **Right.** Absorbance intensity at 415 nm of the same solution over time.

To 247.5 μL of Milli-Q water, 2.5 μL of the Tollens' solution were added at rt. The UV-vis spectra were recorded 30 s after the addition of Tollens' solution, and then every 10 min using a 1 mm path length cuvette.

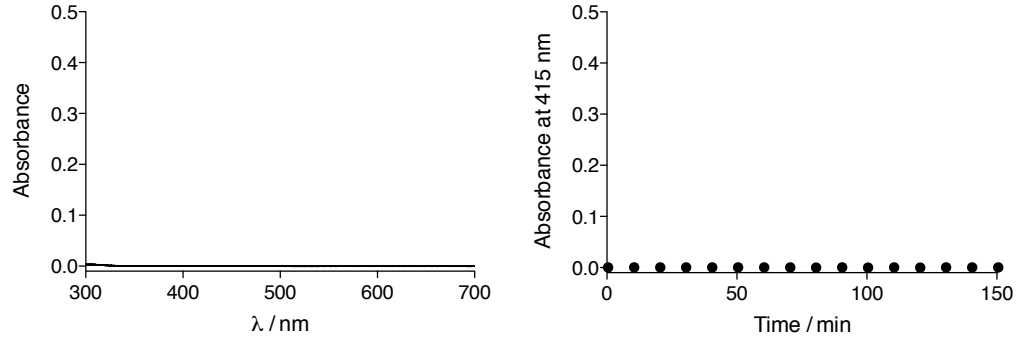


Figure S3. Left. UV-vis spectra of a 3.36 mM Ag^+ solution over time. **Right.** Absorbance intensity at 415 nm of the same solution over time.

Transmission Electron Microscopy

7 µL of 500 µM solutions of peptide amphiphiles (**PA-1** and **PA-2**) were deposited on carbon coated copper grids. The samples were stained with a 2% (w/v) uranyl acetate solution and dried for at least 2 h before the TEM analysis.

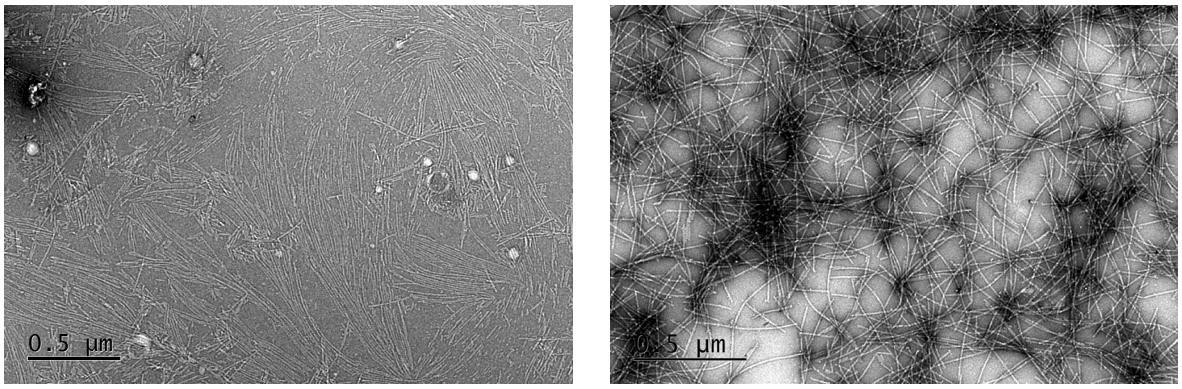


Figure S4. Left. TEM micrograph of **PA-1. Right.** TEM micrograph of **PA-2**.

7 µL of a 500 µM **PA-1,** 1 mM silver solution aged for 6 h, 7 µL of the same solution aged for a week with additional 2.5 mM silver added, and then aged one extra day, and 7 µL of a 10-fold diluted solution, diluted after aging for 6 h, (50 µM **PA-1,** 100 µM silver) were deposited on carbon coated copper grids, and the samples were dried for at least 2 h before the TEM analysis. Particle diameter of the AgNPs in each sample was measured using the *ImageJ*® software by averaging 3 conventional TEM micrographs per sample at the same magnification. Data was tabulated using *Prism 5* (*GraphPad* software) and fitted into a Gaussian model to calculate the mean particle size.

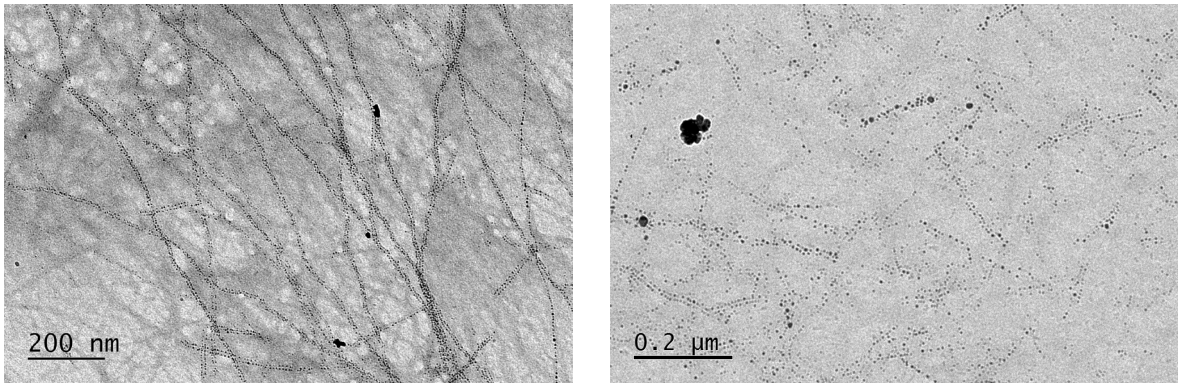


Figure S5. Left. TEM micrograph of 500 µM **PA-1**, 1 mM silver, aged for 6 h. **Right.** TEM micrograph of 500 µM **PA-1**, 3.5 mM silver, aged for a week.

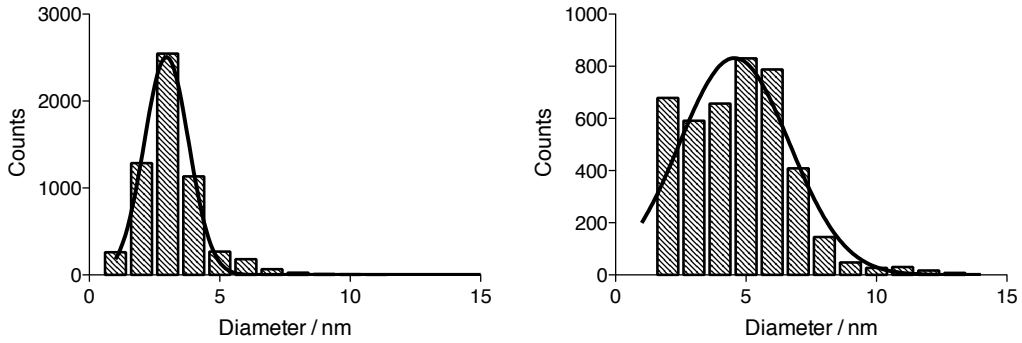


Figure S6. Left. Size distribution of AgNPs for 500 μM **PA-1**, 1 mM silver, aged for 6 h; d = 2.96 ± 0.85 nm. **Right.** Size distribution of AgNPs for 500 μM **PA-1**, 3.5 mM silver, aged for a week; d = 4.56 ± 2.11 nm.

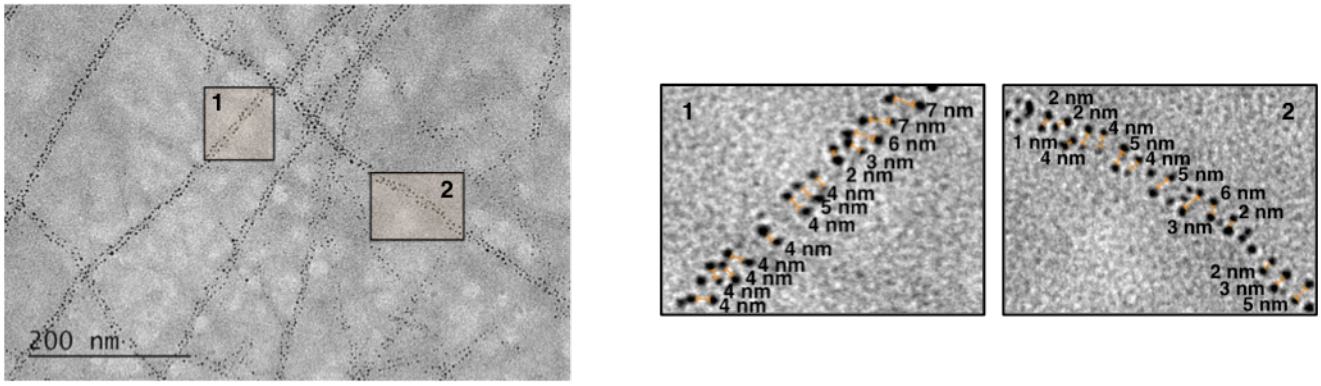


Figure S7. Left. TEM micrograph of 500 μM **PA-1**, 1 mM silver, aged for 6 h. **Right.** Interparticle distances measured for the two sections highlighted in the TEM micrograph on the left.

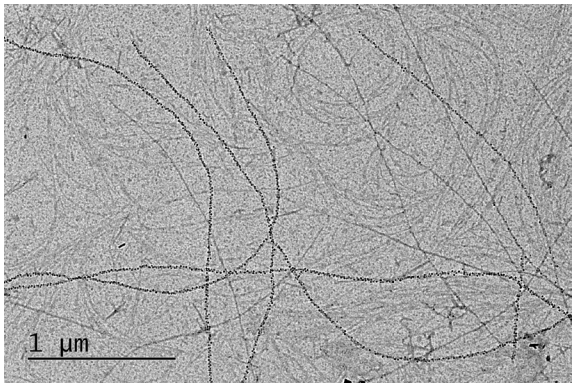


Figure S8. TEM micrograph of 50 μM **PA-1**, 100 μM silver.

7 μL of a 33.6 mM Ag^+ solution (10-fold diluted Tollens' solution), and 7 μL of a 500 μM **PA-2**, 33.6 mM Ag^+ solution aged for 6 h, were deposited on carbon coated copper grids, and the samples were dried for at least 2 h before the TEM analysis.

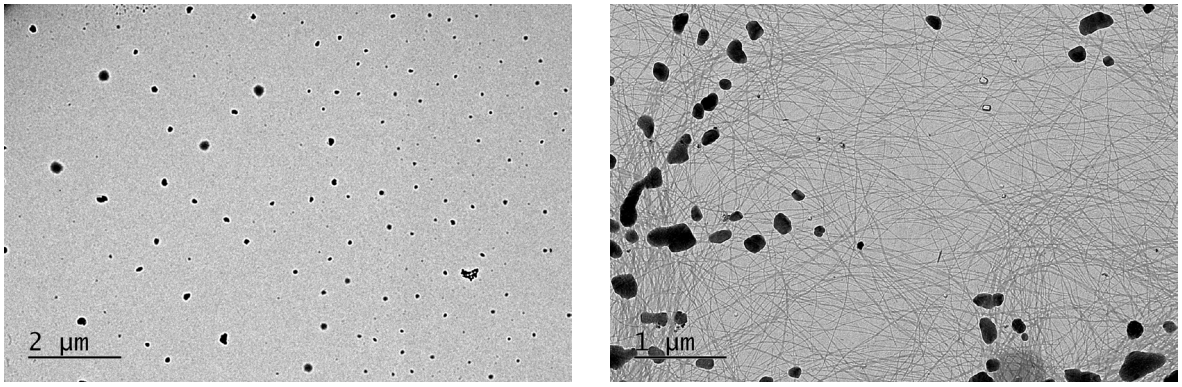


Figure S9. Left. TEM micrograph of a 10-fold diluted Tollens' solution (33.6 mM Ag^+). **Right.** TEM micrograph of 500 µM **PA-2**, 33.6 mM Ag^+.

Nanofiber effect on AgNPs formation

To 249.2 µL of a 500 µM **PA-1** solution in Milli-Q water (pH 6.6-6.8, and aged overnight), 0.75 µL of Tollens' solution were added and the mixture was incubated for 6 h at rt. This solution was then diluted in Milli-Q water (2 µL of PA-silver solution in 998 µL of Milli-Q water) to a final concentration of 1 µM **PA-1,** 2 µM silver, and the UV-vis spectrum was recorded using a 1 cm path length cuvette. (Figure S10 black line).

2 µL of a fresh 500 µM **PA-1** solution in Milli-Q water (pH 6.6-6.8) were diluted with 998 µL of Milli-Q water to a final concentration of 1 µM **PA-1**, and this solution was aged overnight. Then, 0.6 µL of a 100-fold diluted Tollens' solution (3.36 mM Ag^+) were added at rt, and after 6h the UV-vis spectrum was recorded using a 1 cm path length cuvette. (Figure S10 gray line).

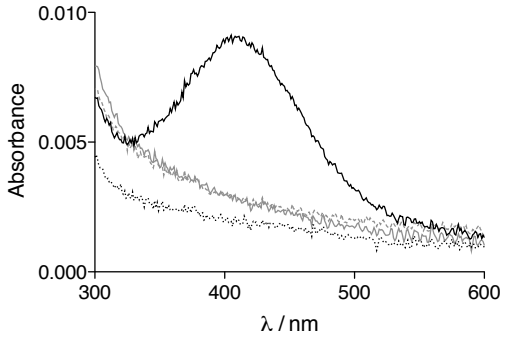


Figure S10. UV-vis spectra of: 1 µM **PA-1** solution, aged then diluted (black dashed line); 1 µM **PA-1**, 2 µM silver solution, aged then diluted (black solid line); 1 µM **PA-1** solution, diluted then aged (gray dashed line); 1 µM **PA-1**, 2 µM silver solution, diluted then aged (gray solid line).

7 µL of both solutions (1 µM **PA-1,** 2 µM silver) were deposited on carbon coated copper grids, and the samples were dried for at least 2 h before the TEM analysis. Fibers and AgNPs were only found for the **PA-1** sample diluted after being aged with silver for 6 h.

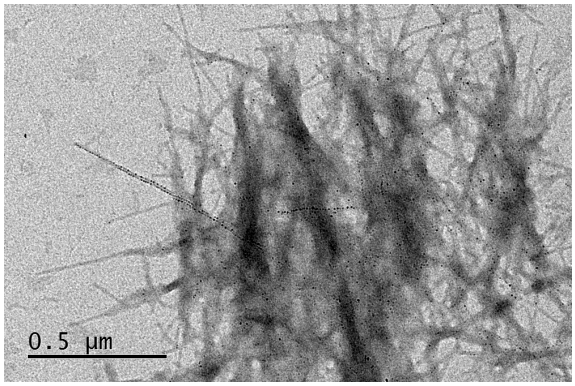


Figure S11. TEM micrograph of 1 µM **PA-1**, 2 µM silver.

Circular Dichroism spectroscopy

To 145.6 or 135 µL of 500 µM solutions of peptide amphiphiles (**PA-1** and **PA-2**) in Milli-Q water (pH 6.6-6.8, and aged overnight), 4.46 or 15 µL of a 10-fold diluted Tollens' solution ([Ag^+] = 33.6 mM) were respectively added at rt. After 6 h of incubation in the dark, the circular dichroism spectra were recorded at 20 ºC.

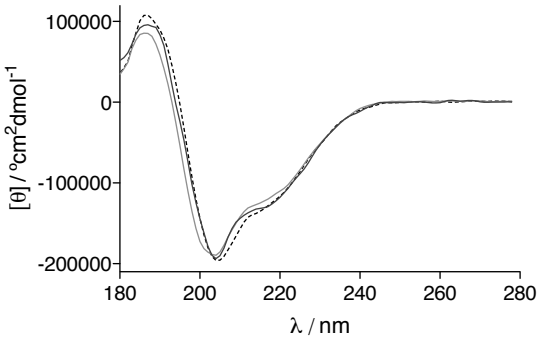


Figure S12. CD spectra of: 500 µM **PA-1** solution (black dashed line); 500 µM **PA-1**, 1 mM silver solution (light gray solid line); 500 µM **PA-1**, 3.36 mM silver solution (dark gray solid line).

Bacteriostatic assay

A saturated culture of *E. coli* (*Bioline*, BIO-85027) was used to inoculate Lurie Broth (LB) medium at a 1:500 dilution in all assays. This inoculum was then aliquoted into several tubes, to which 1/10 of their volume of serial dilutions of 10x solutions of **PA-1–AgNPs**, $AgNO_3$, or **PA-1** in sterile Milli-Q water were added. Then, 400µl of these solutions were put into single or duplicate wells of a 24 well plate, and bacterial growth (or lack of) was recorded by measuring their optical density at 600 nm every hour for a period of 16 h in a *Cytation 3* instrument (*BioTek*), with continuous orbital shaking at 37 ºC. Each condition was tested in 2-3 independent experiments. Data was plotted using *Prism 5* (*GraphPad* software) and shown are the mean and the SEM together with a

fitted line obtained from a sigmoidal dose-response equation with variable slope. MIC and NIC values were calculated using *Prism 5* (*GraphPad* software), and following published methods[1] based on a modified Gompertz function to fit the fractional area under the curve versus log[Ag].

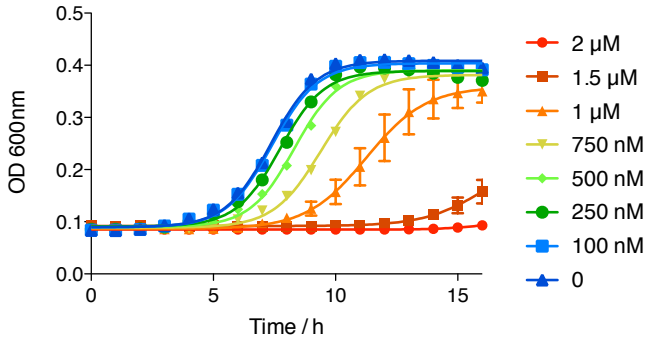


Figure S13. Bacterial growth inhibition profile for *E. coli* up to 16 h in the presence of **PA-1–AgNPs** in the following concentrations: 0, 100, 250, 500, 750 nM, 1, 1.5, and 2 µM silver content, and 0, 50, 125, 250, 375, 500, 750 nM, and 1 µM PA content, respectively.

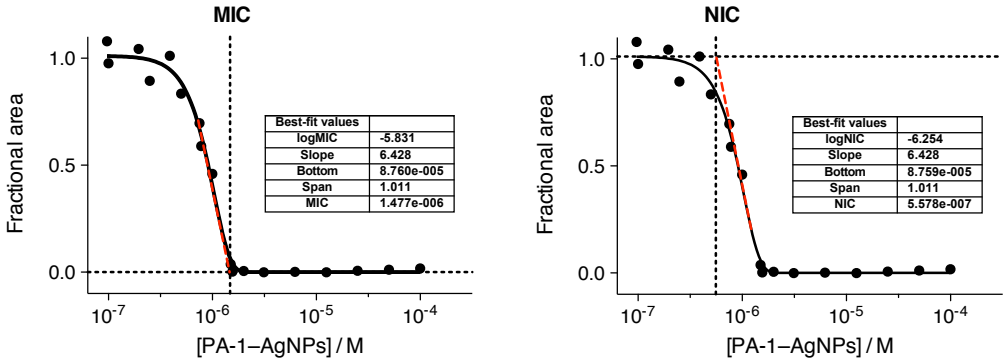


Figure S14. MIC and NIC fittings for **PA-1–AgNPs**.

1 *J. Appl. Microbiol*. **2000**, *88*, 784-790.

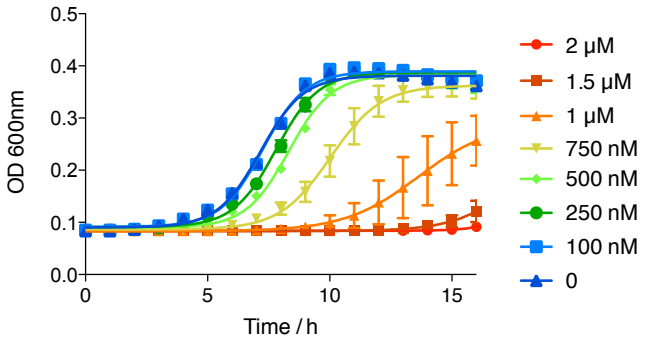


Figure S15. Bacterial growth inhibition profile for *E. coli* up up to 16 h in the presence of $AgNO_3$ in the following concentrations: 0, 100, 250, 500, 750 nM, 1, 1.5, and 2 µM.

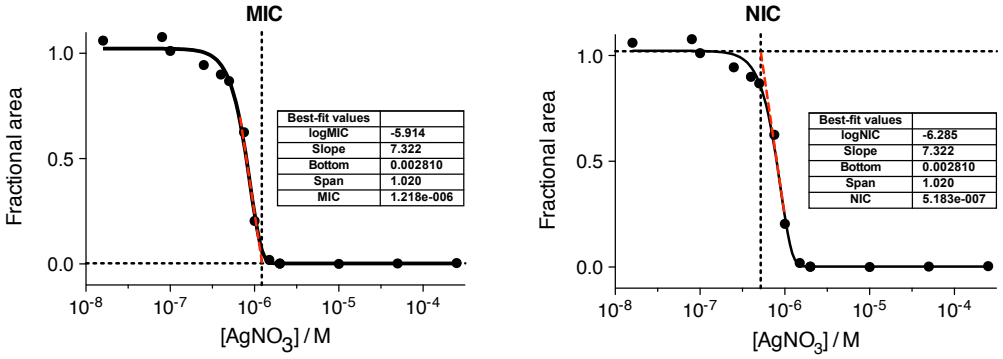


Figure S16. MIC and NIC fittings for $AgNO_3$.

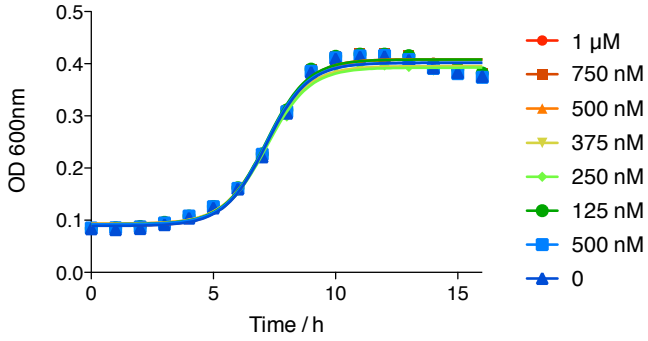


Figure S17. Bacterial growth inhibition profile for *E. coli* up up to 16 h in the presence of **PA-1** in the following concentrations: 0, 50, 125, 250, 375, 500, 750 nM, and 1 µM.

C2C12 cell cytotoxicity

C2C12 cells were seeded at 10,000 cells in 180 µL of 10% FBS media (48-well plate). After 3 h incubation, 20 µL of a 10X solution of **PA-1**, **PA-1–AgNPs**, or $AgNO_3$ in sterile Milli-Q water were added to each well. Following, cells were incubated for 5 h, and then stained with calcein (live cells) and propidium iodide (dead cells). *ImageJ* software analysis tool was used to quantify the number of live and dead cells.

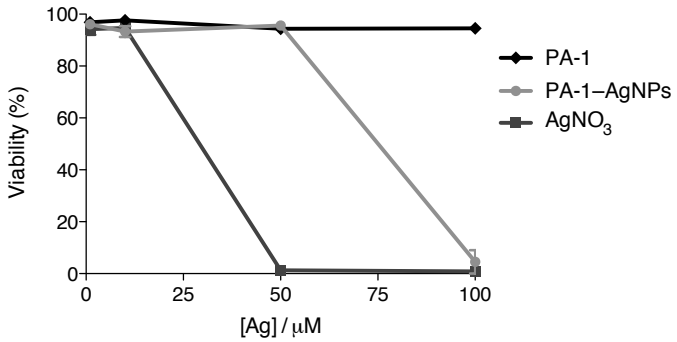


Figure S18. Cell viability versus silver concentration when treated with $AgNO_3$, **PA-1–AgNPs** ([**PA-1**] = [Ag]/2), and **PA-1** (same PA concentrations than **PA-1–AgNPs**).

PA Gelation

A **PA-1–AgNPs** solution (13 mM **PA-1**, 26 mM silver, previously annealed at 80 ºC for 30 min and cooled down overnight), was gelled by pipetting 5 µL out into a "gelling solution" made of 40 mM $CaCl_2$ in Milli-Q water.

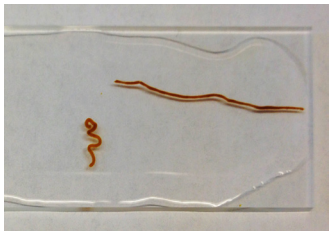


Figure S19. Picture of a **PA-1–AgNPs** gel over a 40 mM $CaCl_2$ solution.

Antibacterial properties of metallized nanofiber gels

In order to test the bacteriostatic effect of the metallized nanofiber gels, 200 µL of a saturated culture of *E. coli* (*Bioline*, BIO-85027) were homogeneously spread onto 10 cm LB-agar plates so that a confluent layer of bacteria would be formed upon growth. After the adsorption of the 200 µL of bacteria was complete, **PA-1–AgNPs** gels were placed and incubated at 37 ºC for 16 h. After the incubation period, the area surrounding the gel was analyzed and used as readout for bacterial growth inhibition.