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 (C₁₈ stationary phase, 5 µm, 110 Å pore size, 30 x 150 mm or C₁₈ stationary phase, 5 µm, 110 Å pore size, 10 x 250 mm). Analytical HPLC was performed with an Agilent 1260 Series, using an Inspire analytical column from Dikma (C₁₈ stationary phase, 5 µm, 100 Å pore size, 4.6 x 250 mm). The standard gradient used for analytical and preparative HPLC was 5 → 95% 0.1% NH₄OH, CH₃CN/0.1% NH₄OH, H₂O over 30 min, and 20 → 65% 0.1% NH₄OH, CH₃CN/0.1% NH₄OH, H₂O 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 °C. 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(CH2CHO)EEEEEAAAVVVK(C16)-NH2 and PA-2: Ac-EAAAEEAVVVK(C16)-NH2) 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.

Mt+ 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 C16-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), PPh3 (1.5 equiv), N-Methylmorpholine (NMM) (10 equiv), and PhSiH3 (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), diethylthiocarbamate (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-(1H-7-aza-benzotriazol-1-yl)-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% H₂O, 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% NH₄OH, CH₃CN/0.1% NH₄OH, H₂O (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 C₇₁H₁₂₅N₁₄O₂₂ = 1525.90, found = 763.47 [MH₂]²⁺ (33 mg, 9% yield for 0.25 mmol scale).

![Graph 1](image1)

**PA-2:** ESI-MS [MH]+ calcd for C₆₃H₁₁₁N₁₂O₁₈ = 1323.81, found = 1345.82 [MNa]+; 662.45 [MH₂]²⁺ (9 mg, 6% yield for 0.125 mmol scale).

![Graph 2](image2)
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 NaIO₄ (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]⁺ calcld for C₇₀H₁₂₁N₁₄O₂₁ = 1493.88, found = 1515.83 [MNa]⁺; 1494.05 [MH]⁺; 780.39 [M-H+3Na]²⁺; 769.42 [MNa₂]²⁺; 758.39 [MNa]⁺; 747.43 [MH]²⁺ (6.1 mg, 42% yield).

Formation of silver nanoparticles over PA nanofibers

Tollens’ solution was prepared by mixing aqueous solutions of AgNO₃ (625 µL, 0.5 M) and NaOH (125 µL, 3 M), followed by the addition of NH₄OH 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.
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.

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, AgNO₃, 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 to 16 h in the presence of AgNO₃ in the following concentrations: 0, 100, 250, 500, 750 nM, 1, 1.5, and 2 µM.

![Graph showing bacterial growth inhibition profile for E. coli in the presence of AgNO₃](image)

**Fractional area**

- [AgNO₃] / M
- OD 600 nm
- Time / h

| Concentration | OD 600 nm |
|---------------|-----------|
| 0             | 0         |
| 100 nM        | 0.1       |
| 250 nM        | 0.2       |
| 500 nM        | 0.3       |
| 750 nM        | 0.4       |
| 1 µM          | 0.5       |
| 1.5 µM        | 0.6       |
| 2 µM          | 0.7       |

**Best-fit values**

- MIC: 1.216 ± 0.007
- MIC: 1.020 ± 0.002
- MIC: 7.322 ± 0.005

Figure S16. MIC and NIC fittings for AgNO₃.

![MIC and NIC fittings graph](image)

- MIC: 1.216 ± 0.007
- NIC: 7.322 ± 0.005
- Best-fit values:
  - MIC: 1.020 ± 0.002
  - NIC: 7.322 ± 0.005

Figure S17. Bacterial growth inhibition profile for *E. coli* 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.

![Graph showing bacterial growth inhibition profile for E. coli in the presence of PA-1](image)

- OD 600 nm
- Time / h
- Concentration
  - 0
  - 50 nM
  - 125 nM
  - 250 nM
  - 375 nM
  - 500 nM
  - 750 nM
  - 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 AgNO₃ 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.

![Graph](image)

**Figure S18.** Cell viability versus silver concentration when treated with AgNO₃, 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 CaCl₂ in Milli-Q water.

![Image](image)

**Figure S19.** Picture of a PA-1–AgNPs gel over a 40 mM CaCl₂ 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.