

Electronic supplementary information for

## **Sprayable peptide-modified silver nanoparticles as barrier against bacterial colonization**

By

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## **Materials and Methods**

### **Chemical and reagents**

Silver nitrate ( $\text{AgNO}_3$ ), trisodium citrate, 2-Hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (I-2959), glycine and 25% glutaraldehyde solution were purchased from Sigma-Aldrich. Rat tail collagen (Discovery Labware, USA) was used as received. All solutions were prepared using Milli-Q water. All cell culture media and reagents were purchased from thermofisher scientific Gibco brand unless otherwise specified. Thiol modified LL37-SH (CSG-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES-NH<sub>2</sub>) was synthesized as described [1], using a Symphony automated peptide synthesizer (Protein Technologies Inc., Tucson, AZ, U.S.A.) employing standard fluorenylmethoxycarbonyl (Fmoc) chemistry (ChemPep Inc., Wellington, FL, USA). LL37-SH was purified using a reverse-phase HPLC, followed by MALDI-TOF analysis of the sample (M+1 4741).

### **Synthesis of citrate-capped AgNPs**

Citrate-capped AgNPs were prepared similar to as described in the literature [2-4]. Briefly, a deoxygenated (30 min N<sub>2</sub>) aqueous solution containing 0.2 mM  $\text{AgNO}_3$ , 0.2 mM I-2959, and 1.0 mM sodium citrate was irradiated with UVA light (8 lamps, in a Luzchem LZC-4 photoreactor at  $25.0 \pm 0.5^\circ\text{C}$ ) for 30 min. Yellow translucent solutions were obtained in all cases and the solutions were kept at room temperature protected from light.

### **Hydrodynamic sizes and zeta potential measurements**

Changes in hydrodynamic sizes and zeta potential for citrate@AgNPs upon addition of the LL37-SH peptide were carried out in a Malvern Zetasizer Nano ZS at 20°C in 1.0 cm pathlength disposable plastic cuvettes. Reported values correspond to the average of three independent batches, each measured in triplicate.

### **Preparation of spray-on AgNPs formulation**

Formulations were prepared in a laminar hood under sterile conditions. Microliter quantities of a 1.0 mM LL37-SH solution (50 nM final concentration) were added to a citrate@AgNPs solution and incubated for 10 min at 4.0°C. To this mixture, rat tail collagen Type I (3.2 µg/ml final concentration) was added and vigorously mixed. The pH of the solution was adjusted to 7.4 using 5X PBS; total silver concentration was  $\approx$  150 µM. The cross-linking was performed by adding 1.5 % glutaraldehyde to the AgNP@LL37 solution, and allowing the reaction to proceed for 30 min. Excess glutaraldehyde was quenched with a 20% glycine solution and incubated again for another 30 min. The final resulting solution was then diluted to a final total silver concentration of 100 µM in PBS. The same protocol was followed to prepare the AgNO<sub>3</sub> sample solution. A sterile solution of 1x PBS was used as control.

### **Transmission electron microscopy (TEM) and X-ray photoelectron spectroscopy**

Briefly, to fresh samples of citrate@AgNPs, 10 µM LL37-SH was added and incubated for 30 minutes. Then, samples for electron microscopy were prepared by delivering  $\sim$ 5.0 µl of that solution to carbon-coated copper grids (400 mesh) and dried in a vacuum system for three days. Electron microscopy images were obtained in a JSM-7500F FESEM from JEOL Inc., working in the transmission mode (TEM) at 15 kV. X-ray photoelectron spectroscopy (XPS) for samples of LL37@AgNPs was also carried out in a Kratos analytical model Axis Ultra DLD, using monochromatic aluminum K $\alpha$  X-rays at a power of 140 watts. Samples were prepared by depositing 500 µL of the samples in 18 mm square cover slides, which were dried under vacuum for 4 days. XPS spectra shown the presence of Ag<sup>0</sup> with its peak centered  $\approx$  366.0 eV similar to the reported for us in the preparation of multishaped LL37 capped AgNPs [5], see Figure S2.

### **Cell lines and culture conditions**

A human dermal fibroblast cell line (ATTC) transfected with green fluorescence protein (GFP) was cultured in DMEM media supplemented with 10% fetal bovine serum (FBS), 1% penicillin, 1% streptomycin and 0.1% gentamicin. Cells were grown at 37°C with 5% CO<sub>2</sub>. The media was changed every 3 days and cells were passaged at 90%

confluency using 0.5% trypsin EDTA solution. In all cases cells were used at passage numbers lower than ten.

### **Preparation of 3D skin model**

A 3D skin model was prepared by encapsulating human dermal fibroblasts within a chemically crosslinked collagen-chondroitin hydrogel. The hydrogel was prepared on ice by mixing a 3.1– 3.5 mg/ml rat tail type I collagen solution with a collagen medium containing 36% (v/v) FBS, 32% (v/v) 10X DMEM M199 (pH = 7.2) and 0.5% (v/v) gentamicin in 10X HEPES buffer. The ratio of collagen:collagen media was 6:1. The acidic solution (pH~2) was then neutralized to pH 7.5 by titration with 1M NaOH. A 20% (w/v) chondroitin sulfate solution, prepared in 1X PBS buffer, was then added such that the final mixture contained 7% (v/v) chondroitin. The collagen-chondroitin mixture was crosslinked with 1.3% (v/v) glutaraldehyde. The crosslinking reaction was allowed to incubate on ice, protected from light for 1 hour. To quench any unreacted glutaraldehyde in the hydrogel solution, 25% (w/v) glycine prepared in 1X PBS, was added to a final concentration of 1.2% (v/v). The hydrogel solution was allowed to quench on ice for 1 hour, protected from light. Human dermal fibroblasts expressing GFP (GFP-fibroblasts) were seeded within the hydrogel solution at a density of  $1.3 \times 10^5$  cells/ml. A 6-well cell culture plate was then coated with 400  $\mu$ l of GFP-fibroblast hydrogel solution and the gels were incubated at 37°C for 30 min in order to solidify. The average thickness of the 3D skin models was 420  $\mu$ m with an initial seeding density of  $5 \times 10^4$  cells/well. The 3D skin models were supplemented with DMEM media containing 10% FBS (v/v) and the media was changed every 3 days.

### **Spray treatment of *in vitro* 3D skin model**

After 5 days in culture, the 3D skin models were treated with either AgNP or AgNO<sub>3</sub> spray treatment, while others were not-treated and served as controls. The AgNP spray treatment was prepared as described above, while the AgNO<sub>3</sub> spray consisted of 100  $\mu$ M of AgNO<sub>3</sub> in water. Prior to the spray treatment, the media was removed and all gels except the one receiving the spray treatment were covered with filter paper. The spray treatments were delivered using an Airbrush Makeup System (Belloccio Inc.), held

~10-11 cm from the surface of the 3D skin model. The treatment duration was 20 sec at high-speed setting, delivering approximately 1.5 ml of treatment per sample. The treatment of two 20 sec sprays was administered twice daily for two days with 4 hours between treatments. The spray treatments were removed 1 hour after application to allow time for the AgNP@LL37 collagen film to form and DMEM with 10% FBS media was added to each well. Fluorescence microscopy images were taken at different time points up to 48h hour after the first treatment to calculate the density of GFP-fibroblasts within the 3D skin models.

### **Bacterial inoculation for 3D model and spray on treatment**

In all cases, bacteria suspensions were freshly prepared from overnight cultures. *Pseudomona aeruginosa* (PAO1) bacteria were inoculated into each 3D collagen hydrogels one hour after the fourth spray treatment as described above. Excess spray treatment was removed prior to adding 1 ml of a  $10^6$  cfu/ml solution of PAO1 in 25% (w/v) LB broth. The inoculated samples were then allowed to incubate overnight at 37°C. Subsequently, the solution from each 3D skin model was plated and counted (4 times per 3D skin model performed).

### **qPCR**

Gene expression analysis of GFP-fibroblasts treated with AgNPs sprays was performed using the SYBR green system (Roche). Primers utilized were for the apoptotic Bax and anti-apoptotic Bcl2 markers while 18S gene was used as a control. Primer sequences were: Bax Forward 5'-AAC ATG GAG CTG CAG AGG AT-3', Bax Reverse 5'-CAG TTG AAG TTG CCG TCA CA-3', Bcl2 Forward 5'-TGG GAT GCC TTT GTG GAA CT-3', Bcl2 Reverse 5'-GAG ACA GCC AGG AGA AAT CAA AC-3', 18S Forward 5'-CGG CTA CCA CAT CCA ACG-3', and 18S Reverse 5'-CTG GAA TTA CCG CT-3'. The thermocycler program used consisted of an initial denaturation at 95°C for 5 min, and 45 cycles of 95°C for 15 sec, 60°C for 20 sec and 72°C for 20 sec. Data was analyzed using the  $\Delta\Delta C_t$  method, where each experimental sample was corrected to both 18S housekeeping gene and the untreated control samples.

## **Antibiofilm assays**

Biofilms of PAO1 were grown on glass cover slips in 12-well plates. Briefly, an overnight culture of PAO1 was diluted to 1/100 in M63 buffer. Then, 750  $\mu$ l of this solution was added to each plate with a cover slip. The plate was placed in a humidity chamber at an angle of approximately 40 degrees. The chamber was incubated at 37 °C for 6 h to form a thin biofilm. Non-adherent cells were removed from the biofilms by washing the surface 4 times with saline solution. Then, spray treatments were applied following the same protocol described above. Biofilms received the spray treatments and were washed three times with 1X PBS, followed by 1 h incubation. One set of cover slips was processed in order to quantify the number of bacteria in the biofilms. The cover slips were scraped, and the bacteria were plated and counted. The second group of cover slips was processed so that the biofilms could be visualized with crystal violet. The biofilms on the cover slips were stained with crystal violet (0.1%), incubated for 15 min at room temperature, rinsed with water 4 times and dried for 1 h and imaged by a Celestron Handheld Digital microscope.

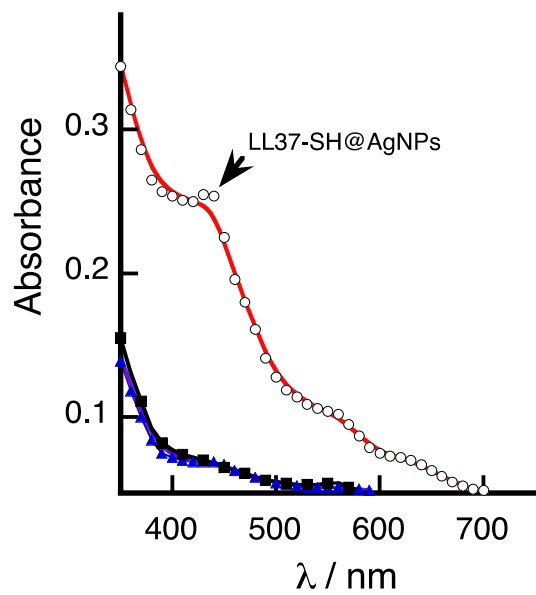
## **In vivo mouse skin wound model and spray-on treatment**

All *in vivo* studies were conducted with ethical approval from the University of Ottawa Animal Care Committee and in compliance with the National Institutes of Health Guide for the Use of Laboratory Animals. Eight week old female C57 mice were anesthetized with 2.5% isoflurane and a dorsal full thickness skin wound was made with a circular punch, 6 mm in diameter. The skin surrounding the wound was covered with sterile filter paper and the mice received four 5 sec spray treatments with 2 min between treatments. Mice were randomly assigned to four groups, one control group and three treatment groups as follows: PBS spray, AgNP@LL37 collagen spray and AgNP collagen spray (n=4/group). AgNP@LL37 collagen spray treatment was prepared as described above, while the AgNP collagen spray was prepared with the same protocol but with water substituted for the LL37 peptide capping agent. Controls did not receive any treatment. The mice were sacrificed four days following the spray treatments using CO<sub>2</sub> and the heart, lung, spleen, kidney, axial lymph nodes, as well as the wound area and a

non-treated section of dorsal skin were harvested for each mouse. The organs and skin wounds were freeze dried and silver content analyzed by ICP-MS (see below).

### **Silver content quantification**

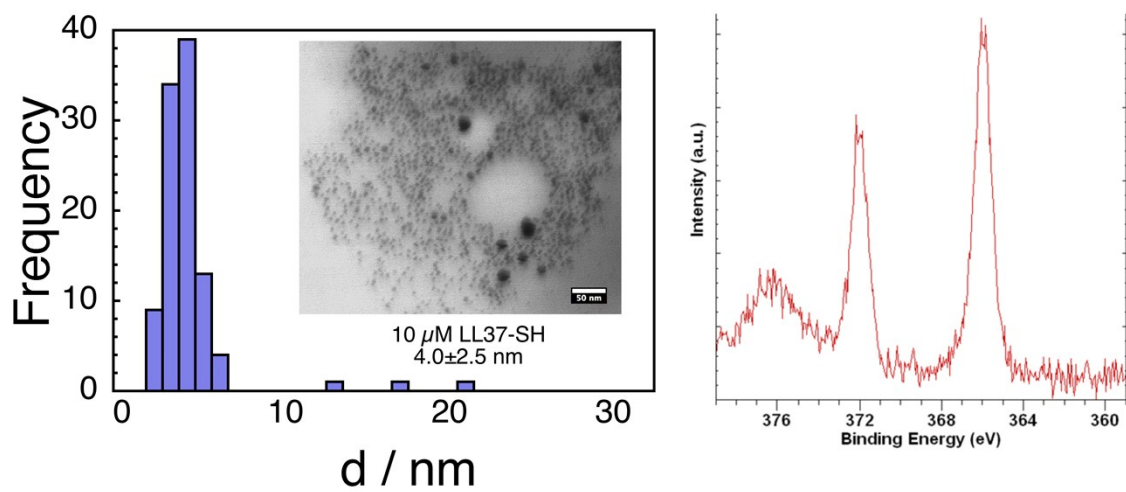
For measurement of silver content in the tissues, we followed a protocol similar to that recently described [5, 6]. Briefly, tissues were harvested from freshly euthanized animals, flash-frozen and subsequently freeze-dried for two days. The resulting solid material was digested in a *DigiPREP* MS system (SCP Science; n=4/group). Numbers reported correspond to total silver determined from interpolation in a calibration curve. Limit of detection (LOD) was 0.0091 µg/kg of homogenized tissue. Total silver concentration was then determined using an inductively coupled plasma-mass spectroscopy system (ICP-MS; Agilent 7700x and silver content determined monitoring the 107 m/z signal (100 ms integration), using Argon as a carrier gas (0.85 ml/min, Ar plasma gas flow: 15 L/min).



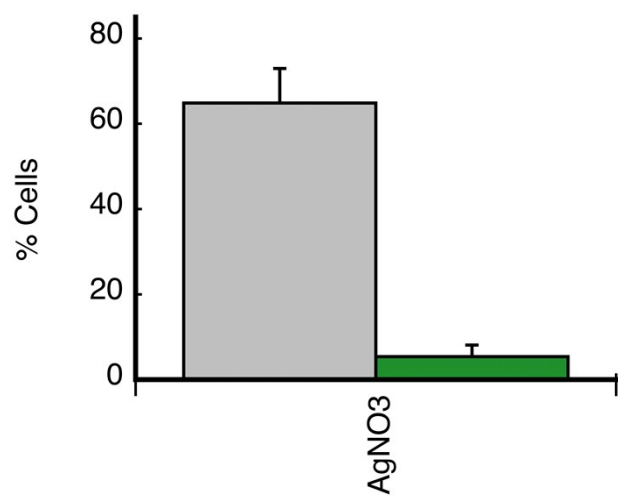
**Figure S1.** Representative absorption spectra for hydrogel matrices sprayed with LL37-SH@AgNPs formulations (red line, open circles) or with formulations containing AgNO<sub>3</sub> (blue triangles). Control non-sprayed sample is also included (black squares).

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**Figure S2.** Left: LL37@AgNPs size histogram obtained from measuring individual AgNPs (>100) from TEM images. Insert shows a representative TEM image for the sample, scale bar 50 nm. Average diameter calculated was  $4.0 \pm 2.5$  nm. Right: Representative XPS spectrum for the LL37@AgNPs.



**Figure S3.** Percentage of cells per hydrogel counted in fluorescence microscopy after 45 min (gray bar) or 48h (green bar) post-spray. Non-sprayed hydrogels were used as baseline numbers (n=4). Numbers were calculated by using non-sprayed cells as controls.

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## References

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