

***Supporting information for LL37 peptide@silver nanoparticles:
Combining the best of the two worlds for skin infection control***

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Materials and methods

Chemicals and reagents

Silver nitrate (AgNO_3), silver sulfadiazine (AgSD) and trisodium citrate were purchased from Sigma-Aldrich. 2-Hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (I-2959) was a generous gift from BASF.

Synthesis of LL37

A modified LL-37 peptide, CSG-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES-NH₂, was synthesized on a Symphony automated peptide synthesizer (Protein Technologies Inc., Tucson, AZ, U.S.A.) using standard fluorenylmethoxycarbonyl (Fmoc) chemistry. HCTU (ChemPep Inc., Wellington, FL, USA) was used as the activating reagent. The 0.1 mmol scale synthesis used Fmoc-PAL-PS resin (Applied Biosystems, Life Technologies Europe BV, Sweden) and four-fold excess of amino acids in each coupling. The resulting peptides were cleaved from the resin by treatment with a mixture of trifluoroacetic acid (TFA), water and triisopropylsilane (TIS) (95:2.5:2.5 v/v; 10 mL per gram of polymer) for 2 h at ambient temperature. They were then filtered and the TFA was evaporated. The LL37 was then precipitated by the addition of cold diethyl ether, centrifuged and lyophilized. Purification was done using reversed-phase HPLC on a semi-preparative C-18 column (Grace Vydac, Helsingborg, Sweden) and peptide identity was confirmed by their MALDI-TOF spectra (M+1 4741).

Synthesis of LL37@AgNP and citrate@AgNP

Citrate@AgNPs were prepared from deoxygenated (30 min) 0.2 mM AgNO_3 , 0.2 mM I-2959, and 1.0 mM sodium citrate irradiated with UVA light (8 lamps, in a Luzchem CCP-V4 photoreactor at $25.0 \pm 0.5^\circ\text{C}$). A similar procedure was used for preparing LL37-coated AgNPs (LL37@AgNPs) but in this case sodium citrate was replaced by a solution of 2.5 μM LL37. The methodology is based on the Norrish Type I photocleavage of I-2959 leading to ketyl radicals with a quantum efficiency of 0.29,¹ which acts as a reducing agent, reducing in this case, Ag^+ to Ag° .²⁻⁴ The reaction evolution was monitored at the plasmon absorption band. Absorbance spectra were

recorded using a Cary-100-Bio UV–Vis spectrophotometer (Varian) at a scan speed of 1515 nm/min at room temperature, employing 0.7 cm pathlength cuvettes.

TEM images, dynamic light scattering, and zeta potential measurements

Samples for electron microscopy were prepared by delivering ~10 µl of solution to carbon-coated copper grids (400 mesh) and dried in a vacuum system. Electron microscopy images were obtained in a JSM-7500F FESEM from JEOL Inc., operating in the transmission mode (TEM). Nanoparticle sizes were calculated from TEM imaging by using ImageJ software.⁵ The zeta potential and hydrodynamic sizes were measured in a Malvern Zetasizer Nano ZS, using 1.0 cm pathlength disposable cuvettes and disposable 0.5 ml folded capillary cells (from Malvern) for DLS and zeta potential, respectively.

Biocompatibility and cell proliferation experiments

The cytotoxicity of LL37@AgNPs and citrate@AgNPs was evaluated *in vitro* using low passage (<8) primary human dermal fibroblasts (ATCC, USA). The fibroblasts were grown in Dulbecco's modified eagle's medium (DMEM, Gibco Life Technologies, Burlington, Canada) containing 10% fetal calf serum and 1.0 % penicillin/streptomycin, and cultured at 37°C, 5.0 % CO₂, and 100 % humidity. Cell viability was evaluated using CellTiter 96® Aqueous MTS colorimetric viability assay (Promega) and employed as a measure of cell survival after 14 h of incubation. Briefly, from confluent fibroblasts cultured, 100 µl of a 5 x 10⁴ cells/ml solution were seeded in a 96-well plate followed by overnight incubation, prior to addition of nanoparticles, AgSD or AgNO₃. After 14 h of sample incubation, the well contents were replaced by 100 µl fresh cell culture medium and 20 µl of MTS solution, and wells were incubated for 2 h. The absorbance at 490 nm was measured in a 96-well plate in a SpectraMax 5 from Molecular Devices and viable cells were plotted as % of cell survival. Cell proliferation experiments were performed in 96-well plates, where 100 µl of a 1 x 10⁴ cells/ml solution were seeded in each well. After overnight incubation, solutions of nanoparticles, AgSD and AgNO₃ in DMEM were added, and replaced every 48 h. Pictures were taken every 24 h with a JuLI Microscope and cells were counted with ImageJ and referred to cells/cm².

Live/Dead® experiments

The Live/Dead® kit for mammalian cells (Invitrogen L-3244, Carlsbad, CA) was employed to further gather information regarding the effect of the different silver sources on cultures of human skin fibroblasts. The cells were seeded in 8 well chambers Lab-Tek (Thermo Scientific, Nunc) borosilicate sterile wells at a density of ≈ 3500 cells/cm², same density used for the MTS and cell proliferation counting experiments described above. After an overnight incubation, solutions of nanoparticles, AgSD and AgNO₃ in DMEM were added to the chambers and the cells incubated for different time intervals as shown in Figure 3B. Prior to staining and cell counting, the cells were washed out with PBS and incubated in 4 μ M ethidium, 2 μ M calcein solution for 3.0 min in dark. Fluorescence images were obtained in a Zeiss Observer A1 Fluorescence Inverted Microscope using a 10X planar objective. The experiments were carried out by duplicated. The cells were counted under the microscope and the numbers shown in Figure 3B, correspond to the average of at least 4 different regions in a well.

Flow cytometry experiments

Flow cytometry experiments were also carried out to further gather information regarding the operative cell death mechanism of human skin fibroblasts incubated with the different silver sources. Briefly, human skin fibroblasts were culture in 150 cm² cell culture bottles (at ≈ 3500 cells/cm², $\approx 5 \times 10^5$ cells/bottle) for 14h and the corresponding silver sources in DMEM, or pure DMEM for the control, added. After 12h of incubation the cells were trypsinated, counted and suspensions of 1×10^6 cells/ml prepared. Those suspensions were stained with Alexa Fluor®488 annexin V/Dead cell apoptosis kit (V13241, Molecular Probes) following the recommendations of the provider (<http://www.lifetechnologies.com/order/catalog/product/V13241>). The cells were then analyzed in a Beckman-Coulter FC500 flow cytometer using 488 nm laser excitation and 525 \pm 25 and 575 \pm 25 nm detection channels for apoptosis and necrosis, respectively.

Antimicrobial activity

The antimicrobial properties of the LL37@AgNPs, citrate@AgNPs, AgSD and AgNO₃ were assessed against the gram (-) *E. coli* (CF073) and *P. aeruginosa* (PA14) as well as the gram (+) *S. epidermidis* (SE19) and *S. aureus* (ATTC 25923). The Minimal inhibitory concentration (MIC) was determined by the broth microdilution method,

slightly modified from the standard Clinical and Laboratory Standards Institute (CLSI) protocol.⁶ An overnight culture from a single colony of each bacterium was diluted to a cell density of $\sim 10^5$ cfu/ml in Luria Bertani (LB) broth that contained a two-fold dilution of the test compound. The absorbance was estimated at 630 nm measured at time 0 and after 18 h of incubation at 37°C. The lowest concentration of test-substance at which the absorbance of the bacterial inoculum did not increase substantially ($\Delta OD_{630} < 0.03$ OD units/18 h) was taken as the MIC. All measurements were carried out in triplicate, on two independent days and median results were reported. For the time kill assay, exponentially growing cultures of each of the four bacterial strains were exposed for 3 to 12 h in 96-well plates, to 2X MIC concentrations of each test substance while constantly stirred at 37°C. Estimates of the density of surviving bacteria were obtained by plating saline-diluted samples on LB agar and allowing for growth at 37°C for 18-24 h. The ensuing colonies were counted manually.

Biofilm formation

2.0 mL of LL37@AgNP, LL37 0.156 μ M or water were added into 6 well tissue culture plates (Costar) under sterile conditions. The plates were then incubated at 37°C, 90% humidity for 24h and employed to assess biofilm formation using a modified air liquid interface (ALI) assay.⁷ Saturated overnight cultures were diluted 1/100 into a minimal medium (M63-arginine) made up of M63 salts (1% potassium phosphate [monobasic] and 0.2% ammonium sulfate) supplemented with arginine (0.4%) and MgSO₄ (1 mM).⁸ The diluted cultures were added to the wells of angled (30°) 6-well plates, followed by the incubation at 37°C for 16 h. After removing the culture from the wells and extensively washing with 0.9% NaCl, the biofilms were analyzed on an inverted microscope by phase-contrast microscopy.

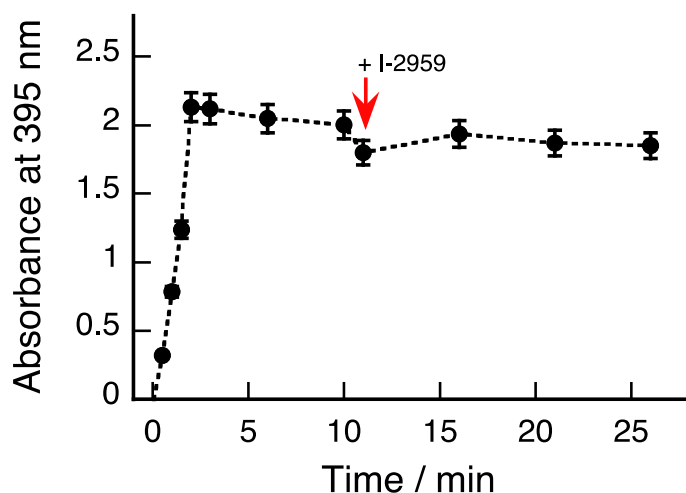


Figure S1. Changes on AgNP-SPB absorption at 395 nm measured as function of the irradiation time for a sample containing 0.2 mM AgNO₃, 0.2 mM I-2959 and 2.5 μ M LL37 peptide. The red arrow indicates the addition of another extra 0.2 mM I-2959. Note that there is no further increment in the absorbance which suggests that all the available Ag⁺ has already been reduced. All the measurements were carried out in free oxygen solutions at room temperature under UVA irradiation, see experimental.

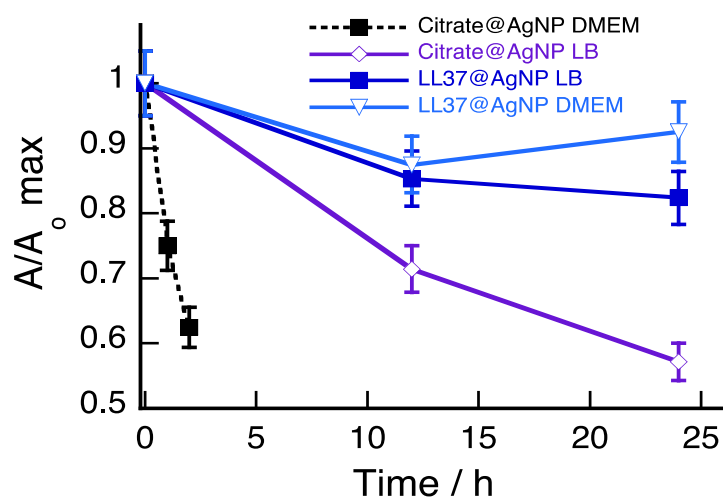


Figure S2. Changes on AgNP-SPB represented as A/A_0 at their maxima absorption measured at different time intervals after addition into LB or DMEM media. The samples were kept at 37°C. All measurements were carried out at room temperature. 10% arbitrary error bars have been included for comparison purposes.

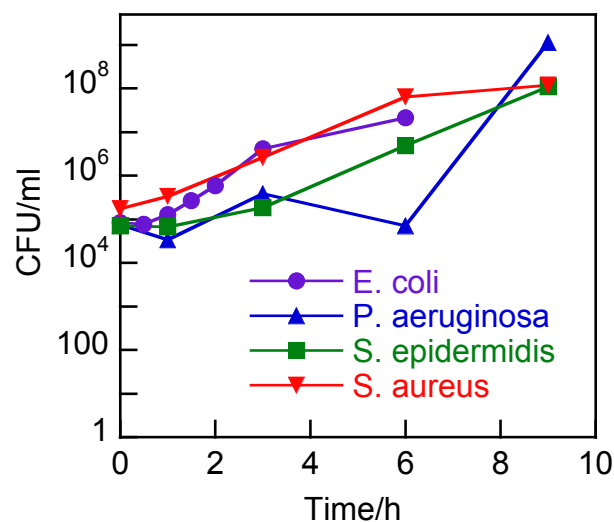


Figure S3. Number of survival bacteria colonies counted in the presence of 0.156 μ M LL-37 peptide which corresponds to \approx 12.5 μ M Ag measured for the different bacteria strains as *E. coli* (⊗), *P. aeruginosa* (Ⓢ), *S. epidermidis* (Ⓢ), and *S. aureus* (Ⓢ). All experiments were carried out in 25% LB and colonies counted after 18 h of incubation at 37°C on LB agar plates.

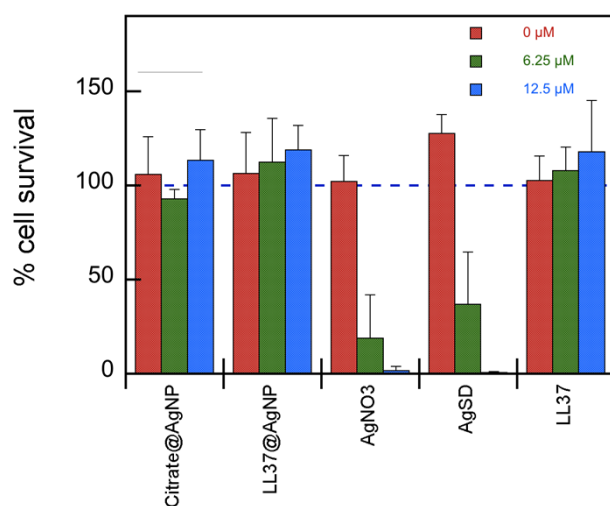


Figure S4. Human skin fibroblasts cell toxicity in the presence of different silver sources measured after 14 h incubation using MTS colorimetric assay. Error bars correspond to SD obtained from three samples.

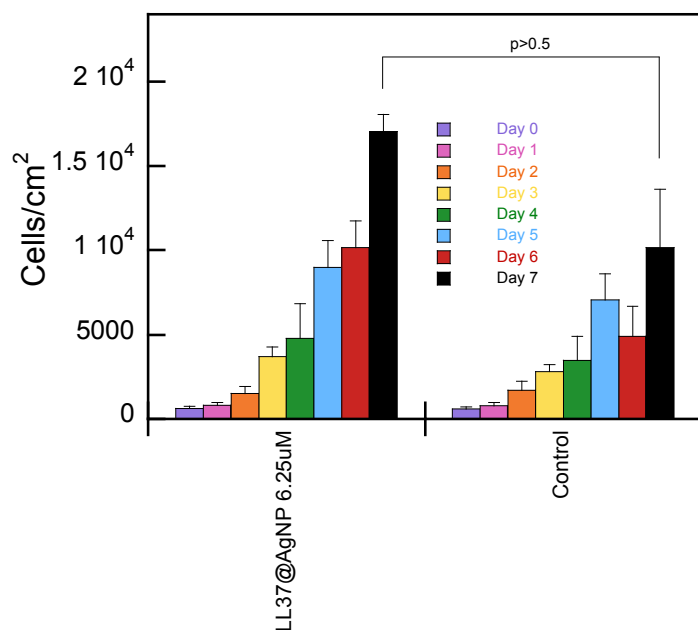


Figure S5. Effect of LL37@AgNPs at 6.25 μ M silver concentration on the proliferation profile of human skin fibroblasts. The LL37@AgNP solution was changed every 48 h. Error bars correspond to SD from six different samples obtained from triplicate experiments. Two tail t-Student test, $p>0.5$.

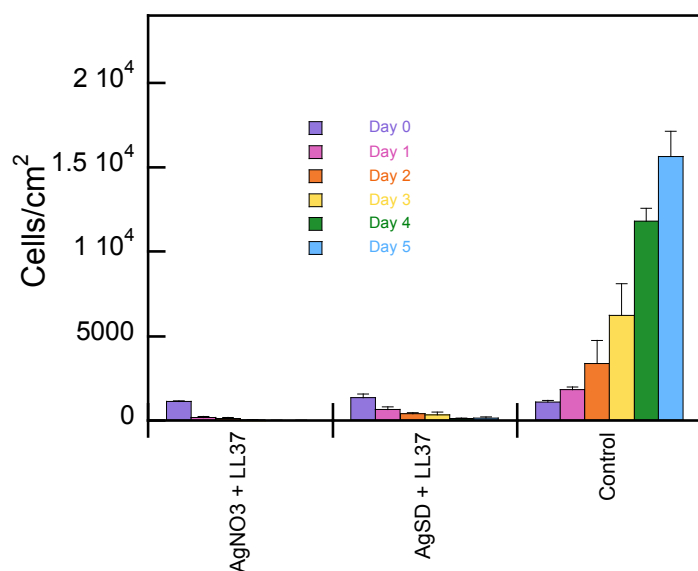


Figure S6. Effect of AgSD and AgNO₃ at 6.25 μ M silver concentration on the proliferation profile of human skin fibroblasts in the presence of 0.078 μ M LL37 peptide. The solutions of Ag and LL37 were changed every 48 h. Error bars correspond to SD from six different samples obtained from triplicate experiments.

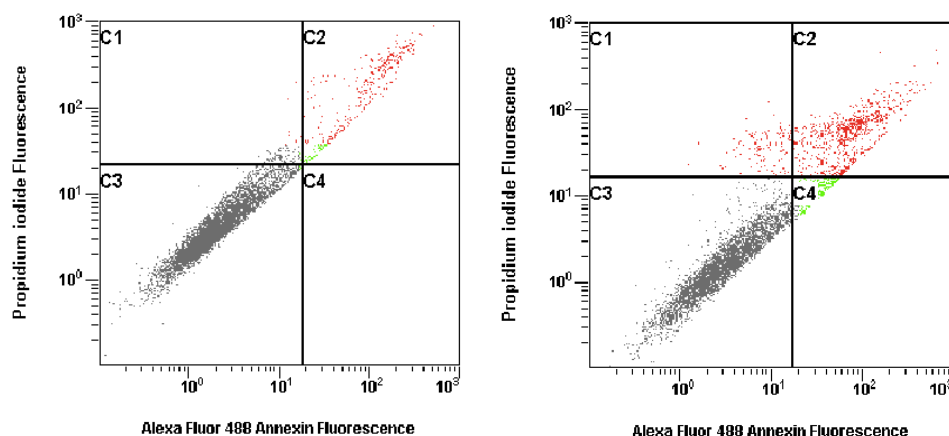


Figure S7. Representative flow cytometry profiles for human skin fibroblasts ($\approx 1 \times 10^6$ cells/ml) stained with Alexa Fluor®488 annexin V/Dead cell apoptosis kit for control samples (left) or incubated with AgSD (right). C1 and C2 correspond to the regions where propidium iodide fluoresces and can be interpreted as necrotic cells while C4 is the green channel where apoptotic cells would appear. Unstained cells are contained in C3 and correspond to viable human skin cells.

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