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SUPPORTING INFORMATION FOR

Coloured cornea replacements with anti-infective properties: expanding the safe use of silver nanoparticles in regenerative medicine

By

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Materials and methods

Chemicals and reagents

Silver nitrate ($AgNO_3$), trisodium citrate, 2-Hydroxy-1-[4-(2-hydroxyethoxy)phenyl]- 2-methyl-1-propanone (I-2959), sodium chloride, and phosphate buffer saline were purchased from Sigma-Aldrich. Otherwise indicated all aqueous solutions were prepared using Milli-Q water (18.2 MΩ·cm).

Synthesis of Thiol modified LL37 (LL37-SH)

A modified LL-37 peptide, CSG-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-NH_2, was synthesized as we previously described [1]. LL37-SH was prepared 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, using 0.1 mmol scale synthesis used Fmoc-PAL-PS resin and four-fold excess of amino acids for each coupling. Then, the peptide was cleaved from the resin by treatment with a mixture of trifluoroacetic acid, water and triisopropylsilane (95:2.5:2.5 v/v; 10 mL per gram of polymer) for 2 h at ambient temperature. Crude LL37-SH was filtered and TFA evaporated. LL37-SH was then precipitated with cold diethyl ether, centrifuged and lyophilized. The peptide was purified using a reversed-phase HPLC in a semi-preparative C-18 column (Grace Vydac, Helsingborg, Sweden), followed by MALDI-TOF analysis of the sample (M+1 4741).

Synthesis of citrate@AgNPs

Citrate@AgNPs were as described in the literature [2] [3-5]. A deoxygenated (30 min N_2) aqueous solution containing 0.2 mM $AgNO_3$, 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 ± 0.5°C) for 30 minutes. The reaction evolution was monitored at the plasmon absorption band (397 nm). Absorbance spectra were recorded in a Libra S50 UV–Vis spectrophotometer (Biochrom, Cambridge, UK) at room temperature, employing 1.0 cm pathlength cuvettes.

Synthesis and characterization of large spherical citrate@AgNPs (Green-1)

Green coloured AgNPs were prepared using a modification of the synthetic approach used for the synthesis of citrate@AgNPs as described above. Briefly, a deoxygenated (30 min N_2) aqueous solution containing 0.2 mM $AgNO_3$, 0.2 mM I-2959, and 0.33 mM sodium citrate was irradiated with UVA light (8 lamps, in a Luzchem LZC-4 photoreactor at 25.0 ± 0.5°C) for 40 minutes. The resulting particles were stable only in the presence of LL37-SH at > 5.0 μM, otherwise the colloid precipitates within hours after particle preparation. X-ray photoelectron spectroscopy (XPS) was also carried out in a Kratos analytical model Axis Ultra DLD, using monochromatic aluminum Kα X-rays at a power of 140 watts. XPS spectra shown the presence of Ag^0 with its peak centered at binding energies of 366.0 eV similar to the reported for us in the preparation of collagen capped AgNPs [3].

Synthesis of LED-reshaped AgNPs (Green-2 and Blue)

LED mediated reshaping of 4.0 nm spherical citrate@AgNPs was employed for preparing AgNPs with different colours green or blue (Green-2 and Blue) as previously reported in the literature [5]. Briefly, solutions (200 ml) of citrate@AgNPs were irradiated in a 250 nm Erlenmeyer using 740 nm (LedEngin, 2.10 lm, LZ440R308) and 590 nm (LedEngin, 2.10 lm, LZ420A100) LEDs for Green-2 and Blue (2.5 cm from the center of the Erlenmeyer), respectively. The irradiations were carried out at room temperature until the plasmon spectrum showed no further changes (up to 144 h for Blue AgNPs), overall changes in sample temperature were detected to be up to +2.0ºC during the irradiation.

Transmission electron microscopy (TEM) images

Samples for electron microscopy were prepared by delivering ~5.0 μl of 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., operating in the transmission mode (TEM) at 15 kV. Mean nanoparticle size for Green-1 was calculated from TEM images using ImageJ software [6] from measuring >400 particles selected from different areas in the grid. Additional, high resolution TEM imaging was carried out for

samples of Blue and Green-1 AgNPs. Samples were prepared as described above, but the imaging was carried out in a FEI Tecnai G2 F20 TEM system operating at 75 kV.

Stabilization protocol and stability tests

Post-stabilization of Green-1, Green-2, and Blue AgNPs was assessed using different concentrations of the LL37-SH peptide, see Scheme 1 main text. To this end, the peptide (1.0 mM stock in DMSO) was added to the colloidal solution of AgNPs and vigorously stirred. The plasmon spectrum was measured before, immediately and after 6 h addition of the peptide. The range of peptide concentration tested was between 0.5-10 μM, being for all three kind of nanoparticles 5.0 μM the optimal range in terms of stability and protection, see Figure 1 main text. Stability experiments were carried out using concentrated stock solutions of NaCl (1.0 M) and phosphate saline buffer (10XPBS) that were added to the colloidal solution of AgNPs with and without LL37-SH, see main text. The solutions (20 ml) were then flash frozen and kept at -80ºC overnight and lyophilized for 96 h in a Labconco Freezone 6.0 Lyophilizer for 96 h. Then, the resulting powder was resuspended in 5 ml ultrapure water (Sigma) prior preparation of the hydrogels and used within 24 h.

Preparation of hydrogels containing AgNPs

Hydrogels containing the LL37-SH protected AgNPs were prepared using type I porcine collagen crosslinked using the epoxy crosslinker BDDGE under basic conditions [7, 8]. Calculated volumes of aqueous solutions of BDDGE were then added, volumes depend on the total mass of collagen (a 2.7% Lysine content was assumed in the calculation). The crosslinking was carried out at pH 11, pH close to the isoelectric point of the lysine residues on the collagen molecules (pKa 10-11) [9], avoiding the use of catalyst for reaction. Thus, 0.4 ml aliquots of 10 w/w% collagen were weighed out and mixed in a T-piece system [7, 10, 11], and the mixture buffered with a 0.036 M Na_2CO_3 and 0.064 M $NaHCO_3$ buffer, and the pH further was adjusted to 11 by adding 10-20 μl of 2.0 N aqueous NaOH. For control gels the BDDGE crosslinker (15-25 μl) was immediately added, mixed 40 times and dispensed into either flat glass or cornea moulds separated by 500 μm thick

spacers, cured at 100% humidity at room temperature for 24 h, post-cured at 37˚C for 1 day, then washed extensively in PBS to remove any non-crosslinked substrate. For the samples containing AgNPs, following pH adjustment and thorough mixing, 100 µl of the working solutions (4X stock) were injected to the system and thorough mixed. The total amount of Ag^+ added roughly corresponded to the total silver content found in the 0.20 µM spherical citrate@AgNPs hydrogels (≈500 µM total silver).

Mechanical properties and stability of hybrid hydrogels

The tensile strength, Young's modulus and elongation at break for the materials were measured on an Instron mechanical universal tester (Model 3342, Instron, Norwood, MA) equipped with Series IX/S software, using a crosshead speed of 10 mm min^{-1}. To this end, the materials were equilibrated in PBS (100 mM, pH 7.4) for 1 h and cut into 10 × 5 mm rectangular pieces. Prior clamping in the system, the rectangular pieces were gently blotted with paper immediately. Water content of the materials was evaluated by weighing the "wet weight" (W_0) of the sample, equilibrated in PBS for 96 h at 4.0ºC. The material was then dried under vacuum at room temperature for 96 h to obtain the dry mass (W). The total water content of the hydrogels (W_t) was then calculated according to the equation:

$$W_t = \frac{(W - W_0)}{W} \times 100$$

Differential scanning calorimetry measurements for the materials were carried out in a Q2000 differential scanning calorimeter (TA Instruments, New Castle, DE) in the range of 8 to 80˚C using a scan rate of 5.0 ˚C min^{-1}. Collagen samples with masses between 5.0-10 mg were surface-dried with filter paper and hermetically sealed in an aluminium pan. The denaturation temperature (Td) was measured at the onset of the endothermic peak. Finally, material stability against biodegradation by collagenase enzyme was assessed by incubating 50 mg samples of hydrogel in vials containing collagenase enzyme in a PBS solution at 37ºC (5.0 ml of a 10 U/ml solution of type I collagenase).

Proliferation assays

The biocompatibility of the composites was determined by measuring the ability to support cell proliferation. $2.5x10^3$ cells/cm^2 of immortalized human umbilical vein endothelial cells (HUVEC) or green fluorescence protein (GFP) transfected human corneal epithelial cells (HCECs, [12]) were seeded onto 6 mm circular pieces of the hydrogels. HUVECs were maintained in supplemented M200 medium (Life Technologies) containing 1.0% penicillin/streptomycin and 0.1% gentamicin. GFP-HCEC were cultured in keratinocyte-serum free medium (KSFM; Life Technologies, Invitrogen) containing 50 µg/mL bovine pituitary extract and 5 ng/ml epidermal growth factor) and 1.0% penicillin/streptomycin and 0.1% gentamicin. Both cell lines were cultured in a humidified 37˚C incubator with 5% CO_2 in all cases cells were used at low passage number ($p<4$). Cell proliferation for HUVECs was assessed using bright field pictures of the cells at 1, 3 and 5 days after initial seeding in an inverted AxioVert A1 (Carl Zeiss). For GFP-HCEC, cell proliferation was measured following the fluorescence emission upon 488 nm excitation of the samples AxioVert A1 (Carl Zeiss), equipped with an epifluorescence cube for GFP fluorescence emission. All experiments were carried out by quadruplicate and cell counts were made using image-J software [6] in blind fashion. Additional set of cell proliferation assays was carried out for gels impregnated with ionic silver, at the same total silver concentration than the ones containing AgNPs, for HECC.

Antimicrobial activity

The antimicrobial properties of the materials were assessed against Gram (-) *P. aeruginosa,* PA01 strain, which has been well-characterised and used as a reference strain for profiling clinical strains. Briefly, an overnight culture from a single colony of the bacterium was diluted to a cell density of ~10^6 cfu/ml in Luiria Bertani (LB) broth and 100 µl of that suspension seeded onto 6 mm circular pieces of the materials contained in 96 well plates, followed for 24 h incubation at 37ºC and 50 rpm orbital shaking. After this time, the absorbance of the samples (n=4) was read and 10 µl of each well plated onto agar plates and incubated for 18 h at 37°C. The ensuing colonies were counted manually in a blinded manner.

In-vivo assays

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, each weighing 20-25 g, were chosen to assess the activation of IL-6 by the implanted hydrogels. Hydrogels were prepared under sterile conditions and circular pieces, 6 mm in diameter, were cut 12 h prior surgery and kept in sterile PBS at 4ºC. During surgery, mice were anesthetized with 3% isofluorane through a nose cone inhaler and their backs were shaved and washed with betadine and 70% ethanol. Paravertebral incisions were made 1.0 cm away from the vertebral column and subcutaneous pockets were created by blunt dissection using hemostats. The circular implants were placed one per pocket, and the incisions were closed using 5.0 silk suture (n=8 samplesper group). An addition sham group underwent the surgical procedure but without the implant insertion (n=3). All animals were observed for signs of inflammation and pain was managed by buprenorphine administered post-surgery. Mice were euthanized after 24 and 72. Implants along with surrounding skin and connective tissue were collected for cytokine analysis.

IL-6 ELISA

The concentration of pro-inflammatory cytokine IL-6 was assessed in homogenized skin and connective tissue harvested from the implant area after 24 h and 72 h using a mouse IL-6 enzyme-linked immunosorbent assay kit (ELISA, Life Technologies). Samples were stored in sterile PBS at -80ºC, and then thawed on ice prior to the assay. The sample concentrations were all normalized to 0.01 g/mL; previously determined by bicinchoninic acid assay (BCA, ThermoFisher scientific). A serial dilution of freshly prepared IL-6 standard (10,000 pg/mL) was completed to create a working range of 0 – 500 pg/mL, following instructions from the manufacturer (see https://tools.thermofisher.com/content/sfs/manuals/KMC0061_KMC0062_Ms_IL6.pdf).
For this solid-phase sandwich ELISA, 100 µL of samples and standards were added to wells pre-layered with designated 1º antibodies and incubated for 2 h at room temperature. Afterwards, 100 µL of mouse IL-6 biotin conjugate solution was added to the wells and

incubated for 30 min. Next, 100 µL of streptavidin-HRP was added to the wells and incubated for 30 min. In preparation for the reading at 450 nm (SynergyMx, Biotek), 100 µL of chromogen was added to the wells, incubated for 30 min in the dark, and halted with the addition of 100 µL stop solution. All wells were aspirated and washed four times with washing buffer before adding the next reagent (except for chromogen and stop solution). Controls were prepared with PBS, while detection controls were prepared with PBS without biotin conjugate and streptavidin-HRP. The concentration of IL-6 in samples was determined using a standard curve generated after the reading. Additional experiments were carried out by spiking 40 pg/mL of IL-6 into homogenized tissue samples and pure PBS rendered 38 and 39.0 pg/mL, after discounting background, respectively, which demonstrates that tissue samples were not masking any IL-6 readings.

Silver release from hydrogels and silver content in tissues

Initial silver release into the lysogeny broth (LB) from the materials after 24 h was determined using ICP-MS. Materials without any NPs were also tested as controls. Briefly, circular, 6 mm diameter, hydrogels were placed in 100 µl 25% LB in 96 well plates and incubated at 37ºC with constant shaking. Then, 50 µl of the liquid removed and digested in a *Digi*Prep MS system (SCP Science) prior analysis in an inductively coupled plasma-mass spectroscopy system (ICP-MS; Agilent 7700x and silver content determined monitoring the 107 m/z signal (100 ms integration). Argon was used as carrier gas (0.85 ml/min, Ar plasma gas flow: 15 L/min). Limit of detection (LOD) for silver was determined as 2.0×10^{-6} µg/ml. For the analysis of silver content in the tissues, we followed a protocol that we recently described for determining the silver concentration in different murine organs.[8] Briefly, implant and adjacent surrounding tissue were excised from euthanized animals, flash-frozen and subsequently freeze-dried for 48 h. The solid material was digested in a *Digi*PREP MS system (SCP Science), n=4 per group, and sham n=3.

Freeze-dried samples were placed in *Digi*TUBES and 1.0 mL of deionized, distilled water and 2.5 ml of concentrated nitric acid added and the tubes placed into the *Digi*PREP. Tubes were hermetically sealed to avoid sample evaporation. Samples were heated up to 100ºC, in increments of 1.0 ºC/min and then held for 3.5 h. Then they were cooled for 20 minutes

and 1.5 mL of hydrogen peroxide added to each tube and incubated at 95ºC for another 60 min. Finally; the samples were diluted with deionized water to a final volume of 10 mL, vortexed and injected into the ICP-MS system. The data reported here corresponded to total silver determined from interpolation of a calibration curve run the same day of the experiment. The resulting error is the standard deviation. The limit of detection (LOD) was 0.0092 μg/kg (0.0092 ppm) of homogenized tissue.

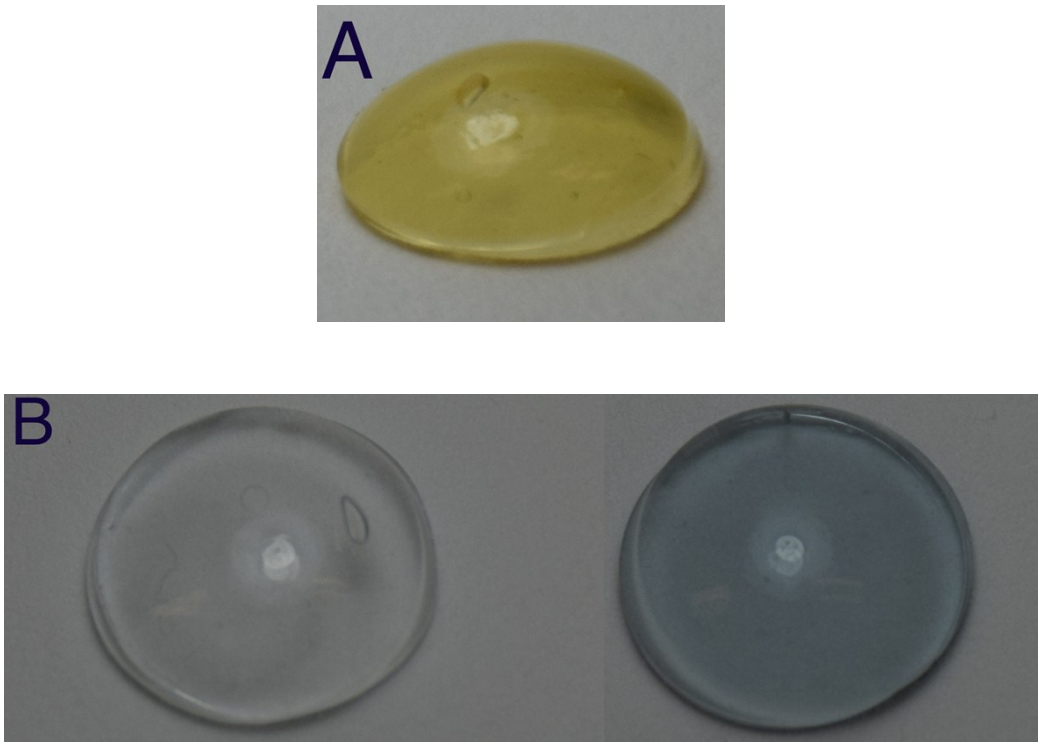


Figure S1. **(A)** Collagen hydrogel moulded as a corneal implant, prepared containing collagen-protected spherical AgNPs.**(B)** Representative images for moulded collagen hydrogels, containing Blue AgNPs either unprotected (left) or protected with LL37-SH (right).

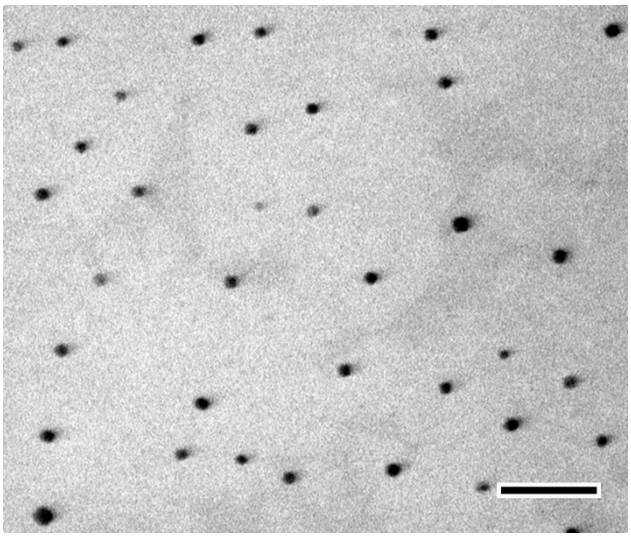


Figure S2. Representative TEM image for Green-1 AgNPs. Scale bar, 100 nm.

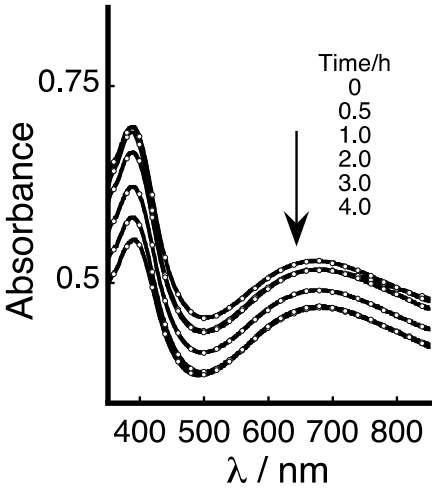


Figure S3. Changes in the surface plasmon band after synthesis for Green-2 AgNPs without LL37-SH. Measurements were carried out at room temperature in a 1.0 cm optical pathlength plastic cuvette. Note how the plasmon band progressively decreases over time, which indicates reduced stability of AgNPs.

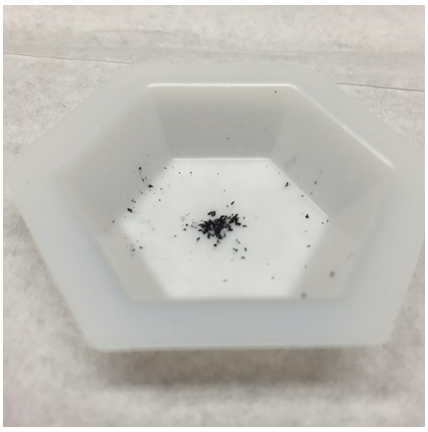
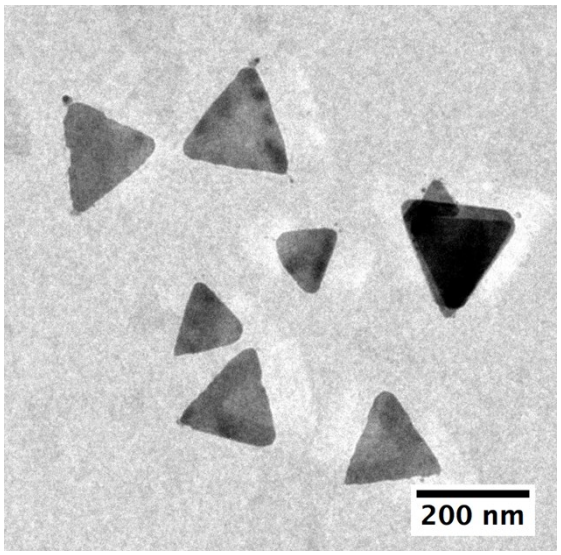


Figure S4. Representative picture of the powder obtained for Green-1 AgNPs capped with LL37-SH after 72 h lyophilization, see main text.



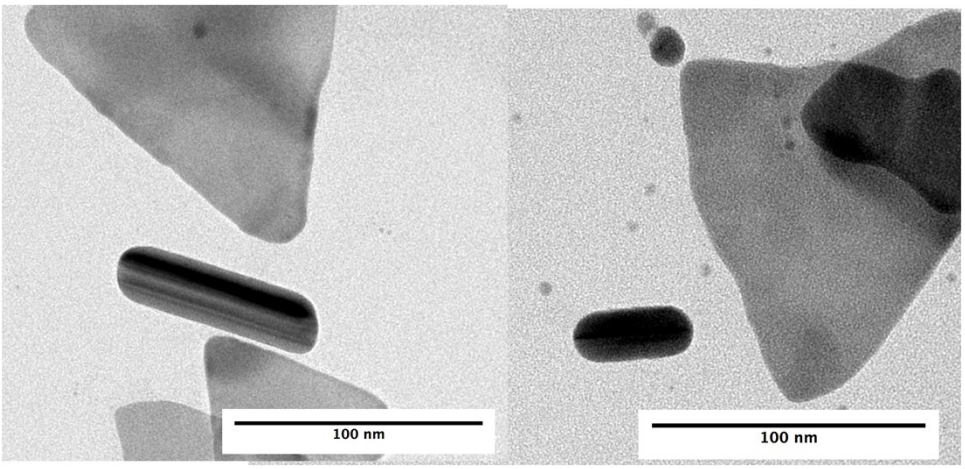


Figure S5. Representative TEM images for (Top) Blue and (Bottom) Green-1 AgNPs prepared in this work. Note that for Green-2, we observed the presence of rod-like structures as shown in the bottom image. Scale bar included in the figure corresponds to 200 nm, top, or 100 nm, bottom.

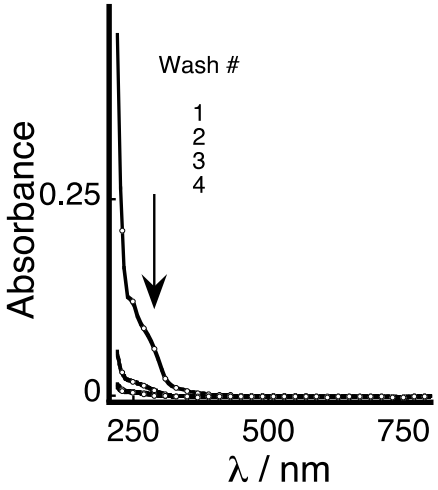


Figure S6. Absorption spectra for the supernatants for collagen hydrogels containing Blue AgNPs. Hydrogels were washed with 10 ml of sterile PBS for 3 h under constant shaking 100 rpm at room temperature. Spectra were measured at room temperature in 1.0 cm optical pathlength quartz cuvettes.

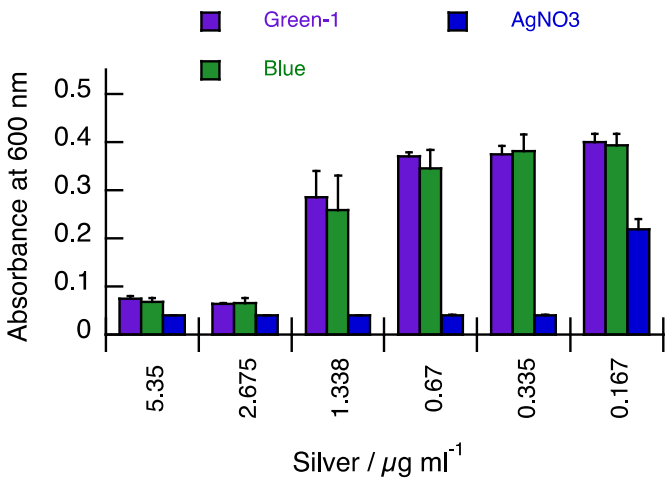


Figure S7. Absorbance at 600 nm of PAO1 cultures prepared in 25% LB media incubated for 14 h at 37ºC in the presence of different concentrations of AgNPs, Green-1 or Blue, or silver nitrate. Silver concentration is expressed as total silver concentration in all cases. Experiments were carried out by quadruplicated and initial bacteria density was 5.0 x10^5 cfu/ml. Silver concentration corresponds to total silver concentration in each well. Concentrations can be transformed to ppm by multiplying those numbers by 1,000.

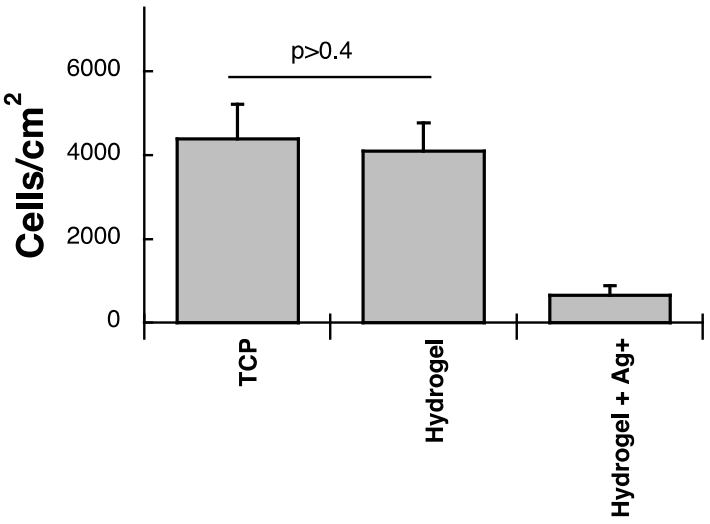


Figure S8. HECC cell density (cells/cm2) measured on gels with and without silver nitrate after 1 day of cell incubation. An additional set of experiments was carried out for the plate without any hydrogel (TCP). Cell proliferation was quantified from cell counting at different time points, see experimental for further details. All experiments were carried out by quadruplicated and error bars correspond to the standard deviation. P value shown in the figure was calculated from t-test assays. Silver loading employed in the preparation of the hydrogel corresponded to the same used for the preparation of the hydrogels containing AgNPs ≈ 500 μM.

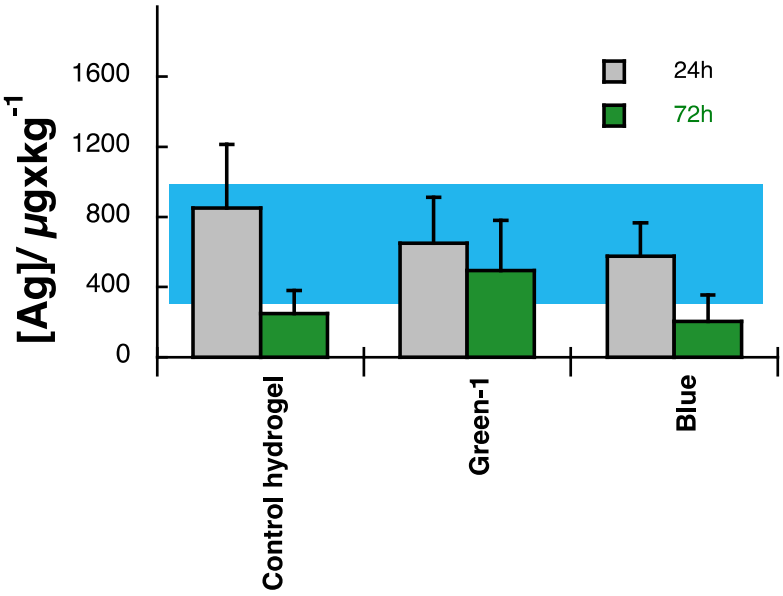


Figure S9. Total silver concentration in tissue sourronding the implant area, see experimental, measured at 24 and 72 h pots surgery. Highligthed area in the background corresponds to the average values for sham specimes that were subjected to surgery without implantation of the materials. Error bars correspond to the standard deviation.

Table S1. Concentration of mouse IL-6 in homogenized skin tissue extracted from implant region after 24 h and 72 h. A mouse IL-6 ELISA kit was used to determine the concentrations, with absorbance measured at 450nm. Values include standard deviation. In all cases p>0.1 when compared to control hydrogels, and/or sham group.

Condition	Concentration (pg/mL) 24 hrs	Concentration (pg/mL) 72 hrs
Sham	14.8 ± 2.6	17.8 ± 3.7
Control	11.8 ± 5.2	
Control hydrogel	19.3 ± 7.6	14.2 ± 1.4
Blue	13 ± 3.2	16.1 ± 4.5
Green-1	16.5 ± 5.3	15.3 ± 1.9

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