Electronic supplementary information for

Multi-functional thermo-crosslinkable collagen-metal nanoparticle composites for tissue regeneration: Nanosilver vs. Nanogold

by

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Materials and Methods

Chemical reagents

Silver nitrate ($AgNO_3$), gold (III) chloride trihydrate, 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (I-2959), 25% glutaraldehyde solution, glycine, trisodium 2-hydroxypropane-1,2,3-tricarboxylate (sodium citrate), sodium hydroxide (NaOH), chondroitin sulphate, phosphate buffered saline (PBS) and hydrochloric acid (HCl) were purchased from Sigma-Aldrich (St, Louis, MO). Type I rat tail collagen (0.375%) from Corning® (Corning, NY) were used as received. Unless otherwise indicated, all solutions were prepared with Milli-Q water. Thiol modified LL37-SH (CSG-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-NH_2) was synthesized as previously described,[1] by using a Symphony automated peptide synthesizer (Protein Technologies Inc., Tucson, AZ, USA) 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 LL37 protected AgNP solution

Citrate capped AgNPs (AgNP@citrate) were prepared according to a previously described protocol.[2] Briefly, an aqueous solution containing 0.2 mM $AgNO_3$, 0.2 mM I-2959 and 1mM of citrate was prepared and purged with N_2 for 30 min to deoxygenate the solution. Once purged, the solution was irradiated with UVA light (8 lamps) at 25 °C in a temperature controlled Luzchem LZC-4 photoreactor (Luzchem Research Inc., Ottawa, Canada) for 30 min. Yellow translucent solutions were obtained in all cases and were kept at room temperature protected from light. LL37 (10 μM) was added to AgNP@citrate solution and incubated overnight on ice. Then, the solution was centrifuged and re-suspended into 10x concentration.

Synthesis of LL37 protected AuNP solution

An aqueous solution containing 0.33 mM $HAuCL_4$ and 0.2 mM I-2959 was prepared and exposed to UVA irradiation at 25°C in a temperature controlled Luzchem LZC-4 photoreactor for 20 min. LL37 (10 μM) was added to the solution and incubated overnight on ice. The solution was centrifuged and re-suspended into 100x concentration.

Synthesis of metal NP-conjugated hydrogels

The collagen hydrogels were prepared by using collagen media (10x DMEM supplemented by 10x 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin). Rat tail collagen type I (6ml) was added to 1ml of collagen media mixed with chondroitin at 20% this 10x medium with 520 mL of 20% chondroitin sulfate and kept on ice. The final pH was adjusted to 7.5 using NaOH (2N). After adding 100 μl of 1.5% glutaraldehyde, 22 μl/ml of AuNP@LL37 or 25 μl/ml of AgNP@LL37 solution was added to the collagen solution respectively, and incubated for 1 hr on ice. The glutaraldehyde crosslinking reaction was quenched by adding 500 μl of glycine (20%) and incubated for 1 hr on ice. Hydrogels were incubated for 30 min at 37in for solidification.

Characterization of materials

The surface plasmon band (SPB) was measured by UV-visible spectroscopy with a Synergy Mx multi-mode microplate reader (BioTEK, Winooski, USA). Stability tests were performed by following the changes in the SPB of the AgNPs or AuNPs contained in the collagen matrices, in PBS at 37ne up to 24hr.

Microstructure analysis of the collagen matrices was assessed using low temperature cryo-Scanning Electron Microscope (c-SEM) images. Samples were prepared as previously described.[3] Cross-sections of the samples were coated with a 5.0 nm thick carbon film prior to SEM imaging using a low vacuum coater Leica EM-ACE200 (Wetzlar, Germany), and imaged by using the secondary emission detector in a JSM-7500F FESEM from JEOL Inc.(Peabody, MA). ImageJ (National Institute of

Health, Bethesda, MD) software was used to measure the pore sizes. Over 100 individual pores were measured from randomly selected areas of the sample.

Differential scanning calorimetry (DSC) was performed for the collagen matrices. The glass transition temperature (T_g) of the samples was measured using a Q2000 DSC (TA Instruments, New Castle, DE). Heating scans were recorded in the range of 8 to 210°C at a scan rate of 10°C/min. T_g was measured on the onset of the endothermic peak.

Fourier transform infrared (FTIR) analysis was carried out on collagen matrices. The samples were dried for 3 days, manually powdered and mixed. Infrared measurements were carried out in a Nicolet 6700 FT-IR, equipped with a Smart iTR Attenuated Total Reflectance (ATR) sampling accessory. Sixty-four individual spectra were collected for each sample.

Degradability of nanometal-conjugated collagen hydrogels in the presence of type I collagenase was assessed. The samples were incubated in Tris-HCl buffer solution containing type I collagenase (5 units/mL) at 37°C and weigh evaluated up to 4 h.

Viscosity measurements were performed in a BROOKFIELD RS-CPS+ Rheometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA). The measurements were carried out in triplicate for each hydrogel at 37°C.

For the electrical conductivity measurements, each sample of hydrogels was plated onto a glass slide between 2 strips of Copper electrode, and incubated overnight at 37°C. Electrical conductivity measurements were recorded on a Princeton Applied Research Parstat 2273 analyzer controlled by Power Suite software (AMETEK Inc., Berwyn, PA). Electrochemical impedance spectra, based on ± 10 mV potential modulation, were recorded over a frequency range of 2 MHz to 10 KHz using a standard 4 electrode setup.

Macrophage polarization and migration

Bone marrow-derived macrophages were generated from C57BL/6J mice aged 8-12 weeks as previously described.[4] Briefly, mice were euthanized by CO_2 inhalation and cervical dislocation; femur and tibia bones were harvested and flushed with media to

isolate the bone marrow. The freshly isolated cells were cultured for 1 week in DMEM (life technologies, Carlsbad, CA) supplemented with 10% FBS, 20% L929 conditioned media and 1% penicillin-streptomycin (Sigma-Aldrich).

For the polarization assay, cells were incubated in the supplemented DMEM containing 20% of homogenized hydrogels using Bullet Blendert Blendersing Bullet. Following 4 days of culture, cells were collected using 5mM ethylenediaminetetraacetic acid (EDTA)/Hank's balanced salt solution (HBSS) (without Ca^{2+}/Mg^{2+}) and subjected to flow cytometry (FACS Aria III; Becton Dickinson, Franklin Lakes, NJ) using CD86 (Biolegend, San Diego, CA) to identify macrophages with a M1 phenotype and CD206 (Biolegend) for those with a M2 phenotype.

For the migration assay, macrophages were activated with 20 ng/ml interferon-γ (R&D System, Inc., Minneapolis, MN) and 100 ng/ml lipopolysaccharide (Sigma-Aldrich) to derive M1 macrophages or 20 ng/ml interleukin-4 (R&D System, Inc.) to derive M2 macrophages for 72hr. Cells were lifted using 5 mM EDTA/HBSS and stained with CellTracker™ Dye (Thermo Fisher, Waltham, MA). Either 50 μl of collagen hydrogel or collagen hydrogel containing AuNP or collagen hydrogel containing AgNP was applied to the upper wells of 24-well Transwell® (Corning, NY), and solidified by 30 min incubation at 37°C. Then, either 20,000 cells of M0 or M1+M2 (M1:M2 = 1:1) were plated on the upper wells with 150 μl of DMEM containing 10% FBS. In the lower well, 500 μl of DMEM containing 10% FBS and 20% L929 were put, which contains granulocyte macrophage colony-stimulating factor. After culture for 3 days, invaded cells located at the lowest layer of the hydrogel in the upper wells were observed under the microscope 200x magnification. Invaded cells were counted on randomly selected 12 fields in each sample and the averages of number were calculated to compare.

Cardiac fibroblasts isolation and culture conditions

Cardiac fibroblasts were isolated from 9-weeks old C57BL/6 and cultured in DMEM/high glucose media (containing 10% FBS, 1% Penicillin and Streptomycin, and 1% Ampicillin). Cells were maintained at low passage number ($p<3$) to minimize fibroblast activation.

Cardiomyocytes isolation and culture conditions

Neonatal rat ventricular myocytes (NRVMs) were freshly isolated as we described in previous work[5]. First, trypsin (Amersham Biosciences, Piscataway, NJ) and collagenase type II (Worthington Biochemical, Freehold, NJ) were used for digestion of heart ventricle tissues collected from 2-day-old rats (Sprague–Dawley, Harlan, Indianapolis, IN). Isolated NRVMs were resuspended in M-199 medium (Life Technologies) supplemented with 10% FBS, 19.4 mM glucose, 2 mM l-glutamine, 2 unit/mL penicillin, 0.8 μg/mL vitamin B12, 10 mM HEPES, and 1x MEM non-essential amino acids (Sigma-Aldrich). Cardiac fibroblasts were removed by two rounds of 60-min preplating, which allow the fibroblasts to attach to the dish bottom and removed from the cell suspension. After the preplatings, cells were plated at 40,000 cells/cm^2 onto 12 mm diameter circle glass slides coated with the hydrogels. The samples were placed in 24-well plates and cultured for 12 hr without electrical stimulation. Successively, cells were submitted to electrical stimulation for 24h (0.4V, 5ms pulse duration and 5Hz frequency) using a C-PACE® system (Ion Optix LLC., Westwood, MA).

After the 24h of pacing, cells were rinsed twice with HBSS and 0.25 ml of pre-warmed 4.0% PFA added and incubated for 15 min at room temperature. Then, 2-rinsing steps using PBS containing 5.0 mM NH_4Cl, followed by twice rinsing with cold PBS. Those samples were blocked and permeabilized using a BSA/PBS/Triton-x solution for 90 min at room temperature. Mouse anti-alpha sarcomeric actinin antibody (α-SA; 1:400, Sigma-Aldrich) and rabbit anti-connexin 43 antibody (Cx43; 1:200, Sigma-Aldrich) were used for primary antibodies, and incubated overnight at 4.0 °C. Secondary antibodies conjugated to Alexa Flour® 488 and Alexa Flour® 546 (Life Technologies, Carlsbad, CA) and a DAPI counterstain (Vector Laboratories, Burlingame, CA) to the cell nucleus were used for fluorescent visualization by 2-hr incubation at room temperature. The samples were imaged with Zeiss Axiovert 200M Fluorescence microscope equipped with an AxioCam MR camera (Carl Zeiss, Oberkochen, Germany). For the quantitative analysis, the number of Cx43 positive cells was counted at randomly selected 4 areas. Each samples was assessed by n=3.

Statistical analyses

Student's t-test (unpaired data with unequal variance) using a confidence interval of $p<0.05$ was considered to identify statistically significant differences. Analyses were carried out in Kaleida Graph 4.5®.

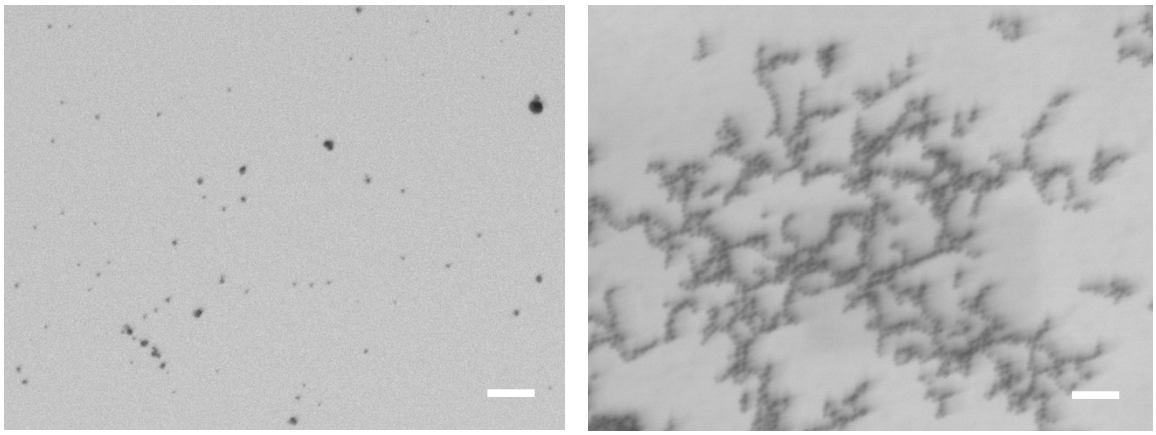

Figure S1. Representative SEM images of AgNP (left) and AuNP (right) solution. Scale bar = 100nm.

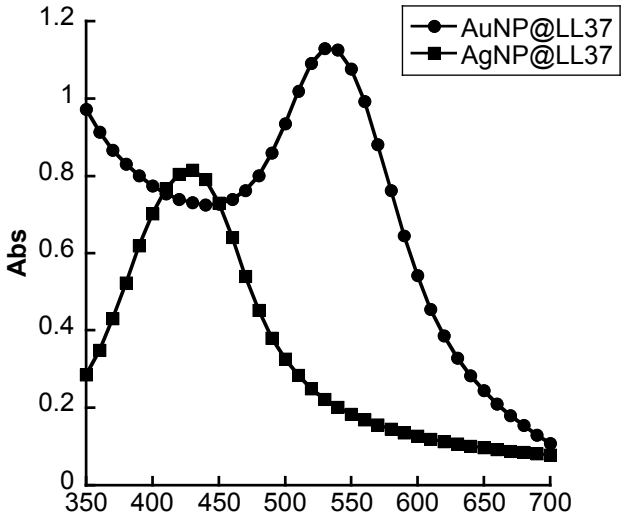

Figure S2. SPB for AgNP@LL37 and AuNP@LL37 stock solution. All measurements were carried out at room temperature.

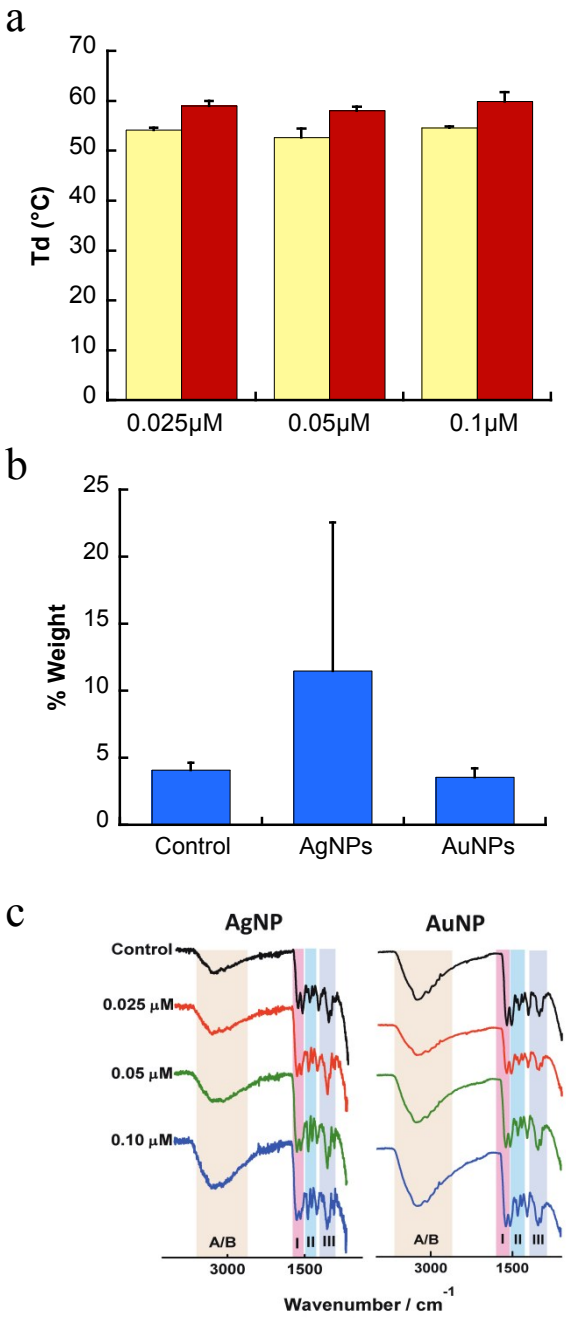

Figure S3. (a) Results of DSC for the collagen hydrogels containing AgNPs (yellow bar) and AuNPs (red bar), with different concentrations. (b) Result of water content measurement of the collagen hydrogels. The concentration of AgNPs and AuNPs were both 0.025μM. The samples were collected and dry for 3 days. (c) Result of FTIR. Error bars correspond to standard errors calculated from triplicated samples.

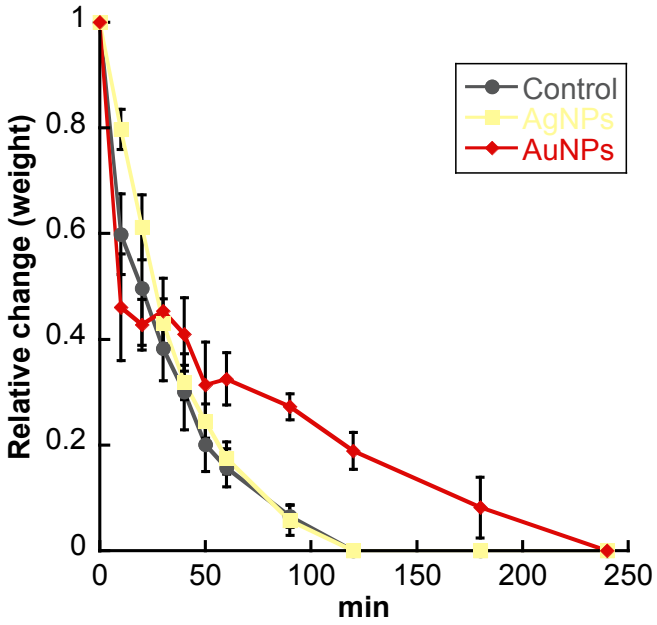

Figure S4. Degradability assessment of collagen hydrogels in the presence of type I collagenase. The concentration of AgNPs and AuNPs were both 0.025μM. Results are shown as relative-change in hydrogels weight compared to the initial weight. Error bars correspond to standard errors calculated from triplicated samples.

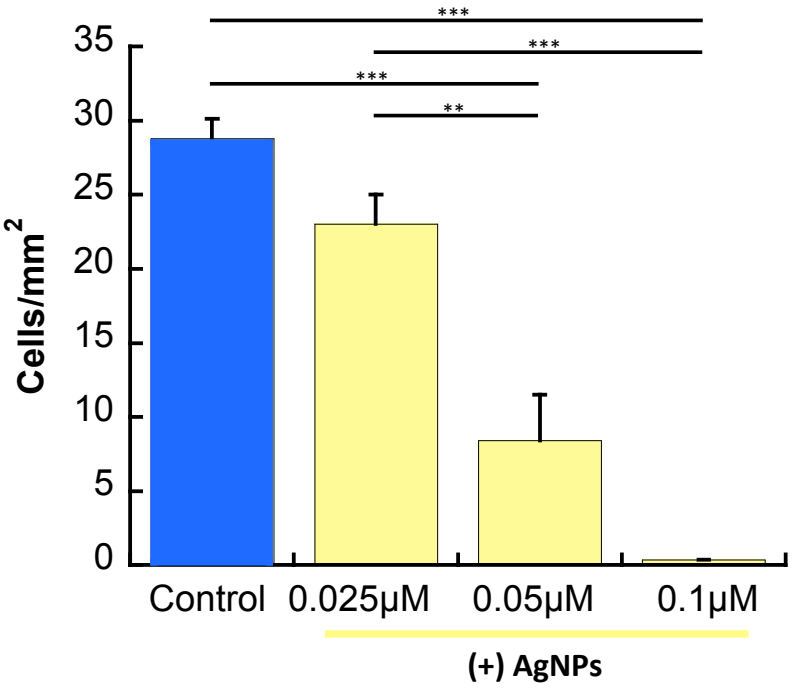

Figure S5. Result of cardiac fibroblasts culture. Cells were incubated with or without AgNPs for 3 days. The numbers were counted from different areas and from at least three slides, from different experiments of the same group. Error bars correspond to standard errors. *P*-values were calculated from Tukey-Kramer test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

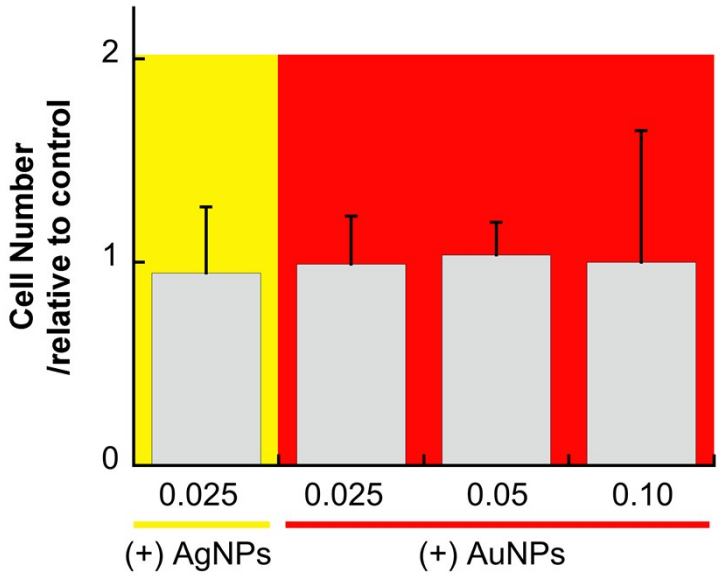

Figure S6. Cell toxicity for cardiomyocytes cultured onto the matrices with and without the nanomaterials at different concentrations. The number of cells expressing DAPI signals were counted after 36h incubation. In all cases >50 cells were counted from different areas and from at least three slides, from different experiments of the same group. Error bars correspond to standard errors. Control groups were cells seeded onto collagen hydrogels containing no nanoparticles.

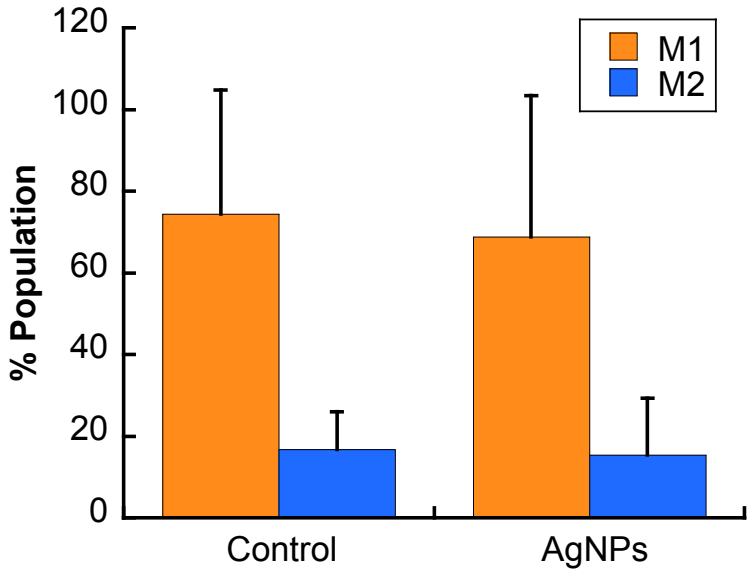

Figure S7. (a) Results of flow cytometry for the assessment of macrophage polarization. Cells were cultured with collagen hydrogels with or without AgNPs (0.025μM) for 4 days. M1 and M2 correspond to inflammatory and pro-healing macrophage populations, respectively. Error bars correspond to standard errors calculated from quadruplicated samples.

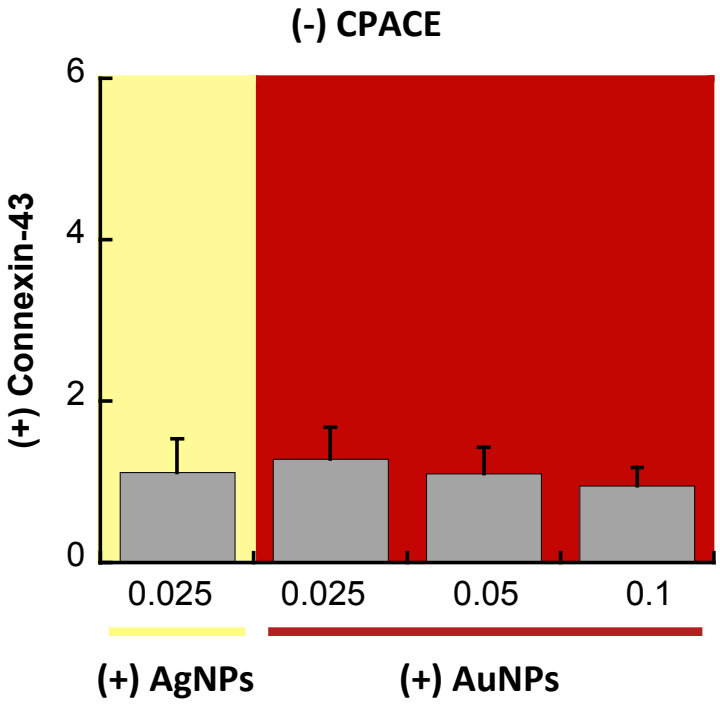

Figure S8. Relative number to control without nanoparticles of $Cx43^{+}$ foci per cell for cells without electrical stimulation. In all cases >50 cells were counted from different areas and from at least three slides, from different experiments of the same group. Error bars correspond to standard errors. Control groups were cells seeded onto collagen hydrogels containing no nanoparticles.

References

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