Electronic supplementary information for

Electroconductive nanoengineered biomimetic hybrid fibers for cardiac tissue engineering

By

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Materials and Methods

Chemical and reagents

Silver nitrate ($AgNO_3$), 2-Hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (I-2959), *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-Hydroxysuccinimide (NHS) and Trifluoroethanol (TFE) from Sigma-Aldrich were used as receive. Type I medical grade porcine collagen (TheraCol®) was purchased from Sewon Cellontech Co. Ltd. (Seoul, South Korea). Unless otherwise indicated all solutions were prepared using Milli-Q water.

Synthesis of collagen-capped AgNPs

Collagen protected silver nanoparticles (AgNP@collagen) were prepared according to a previously described protocol [1]. Briefly, an aqueous solution (Milli-Q) containing 0.2 mM $AgNO_3$ and 0.2 mM I-2959 was prepared and purged with N_2 for 60 min to deoxygenate the solution. Type I medical grade porcine collagen (1.0 μM) was then added and the mixture was purged with nitrogen for additional 90 min. This was followed by exposure to UVA irradiation at 25˚C in a temperature controlled Luzchem CCP-4V photoreactor. The reaction evolution was monitored at the plasmon absorption band (SPB). Spectra were acquired with a 1515 nm/min scan rate at room temperature in a Cary-100-Bio UV-Visible spectrophotometer by using 1.0 cm path-length cuvettes. The solution was then divided into 32.5 mL aliquots, put into the -80°C freezer for 30 minutes, and then freeze-dried for 4 days.

Preparation of electro-aligned fibers

A solution containing 14 mg of AgNP@collagen and 21 mg of pure collagen in 400 μL of TFE was prepared and sequentially sonicated and vortexed until fully combined. Separate 2 M solutions of EDC and NHS were prepared in 100 μL of TFE. Both solutions were sequentially sonicated and vortexed until fully dissolved. 72 μL of the EDC solution and 72 μL of the NHS solution were then added to the collagen solution and subsequently sonicated and vortexed until fully dissolved. The distance between the nozzle of the infusion pump and the rotor of the electrospinner was 20 cm.

The rotor was set to spin at 400 rpm and the parameters of the voltage were set at 1.55 mAmps and 15.0 kV. The AgNP@collagen solution was then loaded into a 1 mL syringe and connected to the infusion pump of the electrospinner. The system was primed with the solution and then the infusion rate was set at 3.33 μL/min.

Characterization of fibrous materials

Fiber diameters was calculated from Scanning Electron Microscope (SEM) images. The samples were prepared by collecting the fibers from the rotor directly onto glass slides. The samples were then covered with a 5.0 nm carbon coating prior to SEM imaging using a low vacuum coater Leica EM-ACE200. The samples were imaged by using the secondary emission (SEI) detector in a JSM-7500F FESEM from JEOL Inc., operating at 5.0 and 10 kV for collagen fibers and AgNPs, respectively. ImageJ® software was used to measure the fiber diameter. Over 100 individual fibers were measured from randomly selected areas of the sample. A similar protocol was followed for quantifying nanoparticle size on the collagen fibers.

Differential scanning calorimetry (DSC) was performed for the AgNP@collagen and pure collagen fibers, which were collected and dry for 3 days. The glass transition temperature (T_g) of the samples was measured using a Q2000 DSC (TA Instruments). 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 fibers with and without the addition of nanosilver. The samples used were dried for 3 days, and 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. A WITec Alpha 300 confocal microscope (WITec, Germany) was used to carry out Atomic Force Microscope (AFM) measurements using Digital Pulsed Force Mode (DPFM). Collagen fibers were prepared as described above. AFM images were obtained with a cantilever with a tetrahedral tip (radius ~10 nm), a resonance frequency of 75 kHz and a nominal spring constant of 2.8 N/m (ArrowTM FM Nanoworld, Switzerland). Set point and

amplitude ranged between 1-2 V, with a frequency of 1.0 kHz. DPFM curves were converted from deflection [V]-phase [°] into force [N]-displacement [nm] according to a previously published procedure [2]. From these curves, the stiffness was calculated (μN/m). At least 50 independent points were randomly selected to calculate the overall stiffness values.

Conductivity measurements were recorded on a Princeton Applied Research Parstat 2273 analyzer controlled by Power Suite software (version 2.58). 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 set-up.

Stability tests were performed following the changes in the surface plasmon band (SPB) of the AgNPs contained in the collagen fibers, in phosphate buffered saline solution (PBS) at 37°C. AgNP containing fibers were collected on the surface of a plastic cuvette (10 mm path-length), and kept in a dry place protected from light for 1 day prior to performing the test. Absorption spectra were recorded and absorbance at 406 nm corrected for scattering before and after PBS incubation (up to 24h) in a Libra S50 UV-Vis spectrophotometer (Biochrom, Cambridge, UK).

Degradability of the fibers in the presence of type I collagenase was measured for fibers with and without AgNPs, that were collected on plastic cuvettes and incubated in Tris-HCl buffer (pH=7.4) for 30 min at 37°C. The cuvettes were removed from the solution, carefully blotted, and then the absorbance was registered for both samples following the SPB and the light dispersion, for the AgNP and pure collagen fibers samples, respectively. Measurements were repeated in time intervals up to 5h in Tris-HCl solutions containing type I collagenase (1 unit/mL) at 37°C.

Cardiomyocyte isolation and culture conditions

Neonatal rat ventricular myocytes (NRVMs) were freshly isolated as we described (Liang et al. 2015) [3]. First, trypsin (Amersham Biosciences, Piscataway, NJ,

USA) and collagenase (type II; Worthington Biochemical, Freehold, NJ, USA) were used for digestion of heart ventricle tissues collected from 2-day-old rats (Sprague–Dawley, Harlan, Indianapolis, IN, USA). Isolated NRVMs were resuspended in M-199 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 19.4 mM glucose, 2 mM l-glutamine, 2 unit/m penicillin, 0.8 μg/ml vitamin B12, 10 mM Hepes, and 1× MEM non-essential amino acids. 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 22x22 mm square glass slides coated with the collagen fibers. The samples were then placed in 6-well plates (BD Biosciences, San Jose, CA, USA) and cultured for 12h without electrical stimulation. Then, cells were submitted to electrical stimulation for 24h (1V, 5ms pulse duration and 5Hz frequency) using a C-PACE® system.

Cell fluorescence microscopy and flow cytometry

After the 24h of pacing, cells were rinsed twice with Hank's buffer saline solution and 1.0 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. Following blocking/permeabilizing, the cells were incubated overnight at 4.0 ^{0}C in alpha-actinin (sarcomeric, α-SA) and anti-connexin 43 antibodies (Cx43) both from Sigma-Aldrich. Ki67 primary antibody was purchased from Santa Cruz. Secondary antibodies conjugated to Alexa Flour® 488 and Alexa Flour® 546 (Life Technologies) and a DAPI counterstain (Vector Labs) to the cell nucleus were used for fluorescent visualization. Cells were imaged with Zeiss Axiovert 200M Fluorescence microscope equipped with an AxioCam MR camera using 63X oil immersion objective. Excitation and emission wavelengths were; DAPI (365±12 and 445±25 nm, DAPI), Alexa Fluor® 488 (470±20 and 525±25 nm, Cx43 and Ki67), and Alexa Flour® 546 (550±12.5 and 605±35 nm, α-SA). Integration time for images in all cases were maintained < 100 ms to minimize background

contribution. Confocal pictures in all cases were taken within the first two hours after secondary staining was completed.

NRVMs were cultured onto the fibers that were electrically paced using the C-PACE system (24 h total), were then collected and prepared for flow cytometric analysis. A small sample of cells was removed from a sample to provide autoflourescent-gating criteria. Cells were incubated with connexin 43 antibody (Sigma) and DAPI (Sigma) for 30 min on ice. Following a wash step with PBS, cells were then incubated with Alexa Flour 488 secondary antibody (Life Technologies) for 30 min on ice. Cells were then washed with PBS and analyzed via flow cytometry. Appropriate compensation and internal controls were applied to the experiment.

Macrophage adhesion and polarization

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; tibia bones were collected and flushed with media to isolate the bone marrow. The freshly isolated cells were cultured for 1 week in DMEM supplemented with 10% FBS, 20% L929 conditioned media and penicillin-streptomycin. Following 7 days of culture cells were lifted using 5mM EDTA/HBSS (without Ca^{2+}/ Mg^{2+}) and plated on either collagen fibers or collagen fibers containing AgNp for 4 days. Cells were collected from the fibers by digesting the collagen using a 3mM $CaCl_2$ HBSS solution containing 250 units of collagenase I (Gibco). Macrophage polarization was assessed by flow cytometry (FACS Aria III; Becton Dickinson) using CD86 (Biolegend) to identify macrophages with a M1 phenotype and CD206 (Biolegend) for those with a M2 phenotype.

Antibiofilm assays

Biofilms of *Pseudomona aeruginosa* PA14 were grown on glass cover slips with/out fibers in 6-well plates. Briefly, an overnight culture of PA14 was diluted to 1/100 in M63 buffer. Then, 1 ml 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. 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 were imaged in a JuLI Fl fluorescence live cell movie analyzer (Nanoentek).

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®.

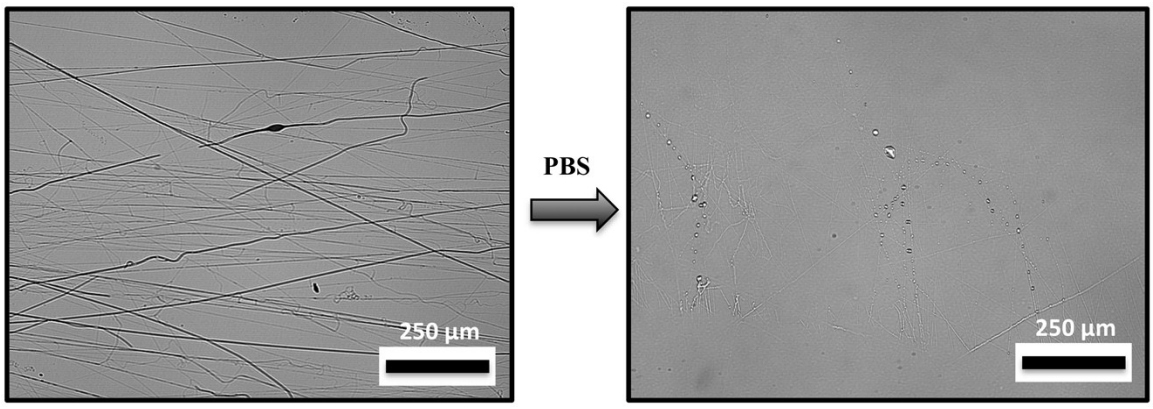

Figure S1. Representative bright field images for collagen fibers containing AgNPs produced without the addition of the crosslinkers EDC/NHS. Left shows an image for the fibers freshly prepared and collected onto the glass slides. The arrow in the middle corresponds to a 1X wash with PBS. Right shows the field seen after the PBS wash. Note how the fibers are not longer visible under the microscope.

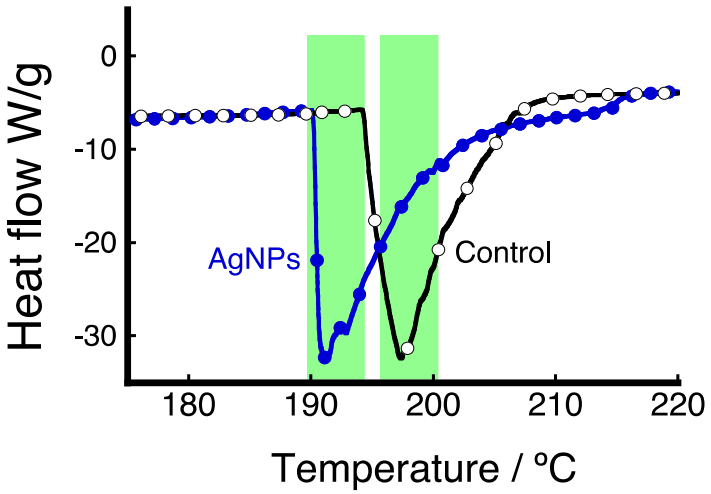

Figure S2. Representative DSC plots for AgNPs containing collagen fibers and control sample without AgNPs. Heating scans were recorded in the range of 8 to 210°C at a scan rate of 10°C/min. However, in the figure we have zoomed in the area where the most intense transition takes place. T_g values were measured on the onset of the endothermic peak.

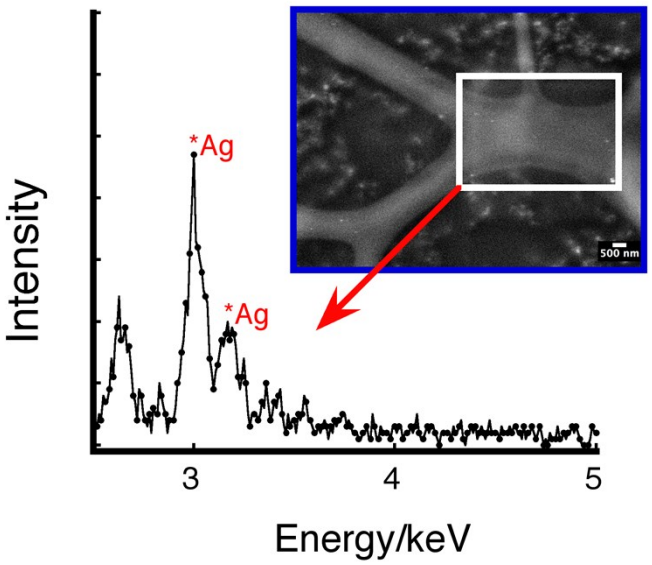

Figure S3. Representative EDS spectrum for a particular region, white rectangle, of collagen fibers containing spherical nanosilver The inset in the Figure corresponds to a scanning electron microscopy image for the fibers. Characteristics energy emissions for silver are indicated in the plot with red asterisks.

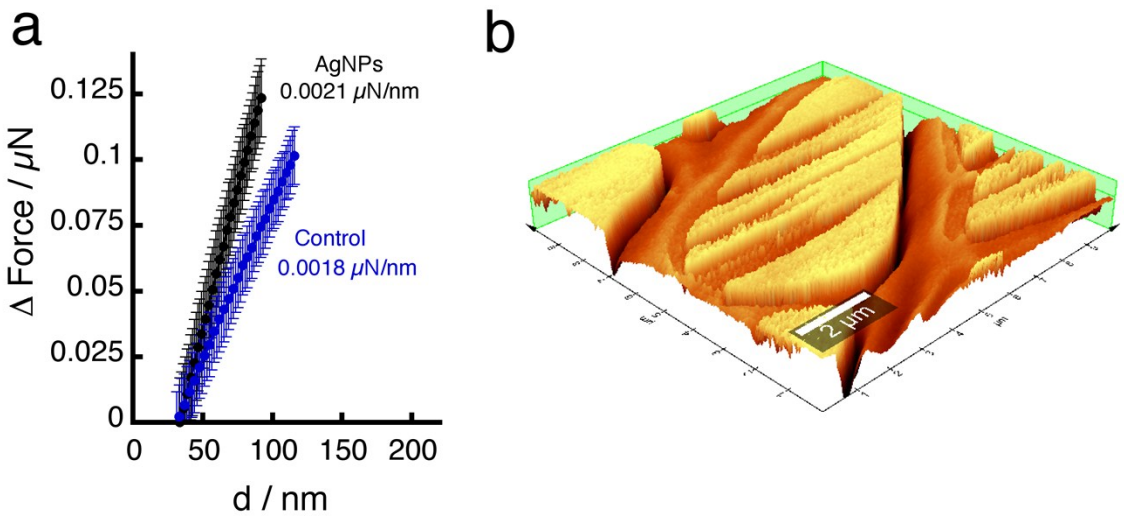

Figure S4. **(a)** Stiffness vs. displacement curves for collagen fibers (black) with and without nanosilver (blue) measured by atomic force microscopy (AFM). Error bars correspond to the standard deviation calculated from measuring 45 individual points from different random areas in the plot. **(b)** Representative image for collagen fibers prepared without nanosilver. False color scale represents differences in maximum force, being higher for areas shown with darkest color intensities.

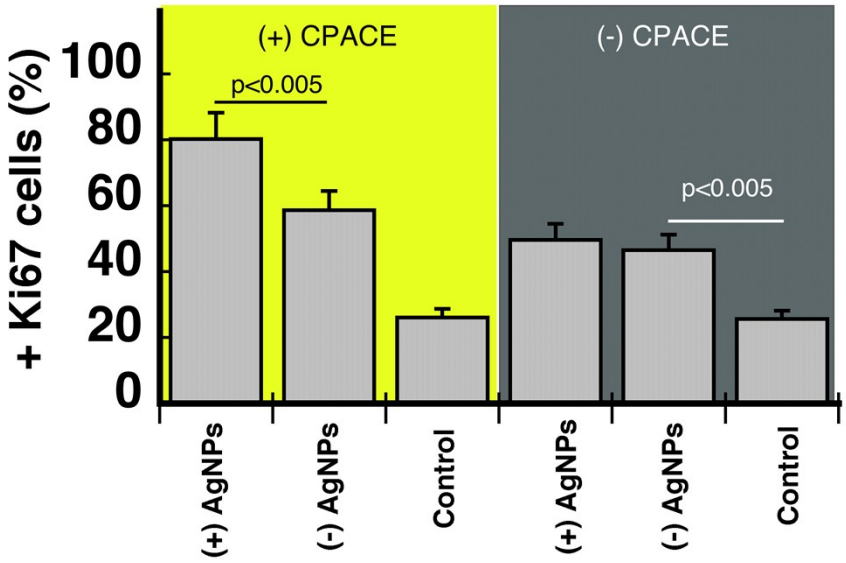

Figure S5. Percentage of neonatal rat cardiomyocytes cells for Ki67 measured for cells seeded onto collagen fibers with and without nanosilver (+ or - AgNPs). Control group for samples without fibers are also included. Cells were either submitted or not to CPACE, see experimental for further details. In all >100 individual cells were counted from at three independent experiments.

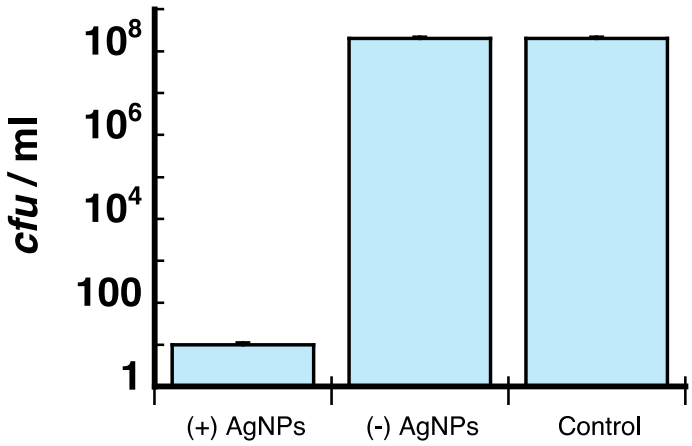

Figure S6. Number of *Pseudomona aeruginosa* counted after 6 h incubation at 37ºC on glass slides with the collagen fibers and/or control without any fibers.

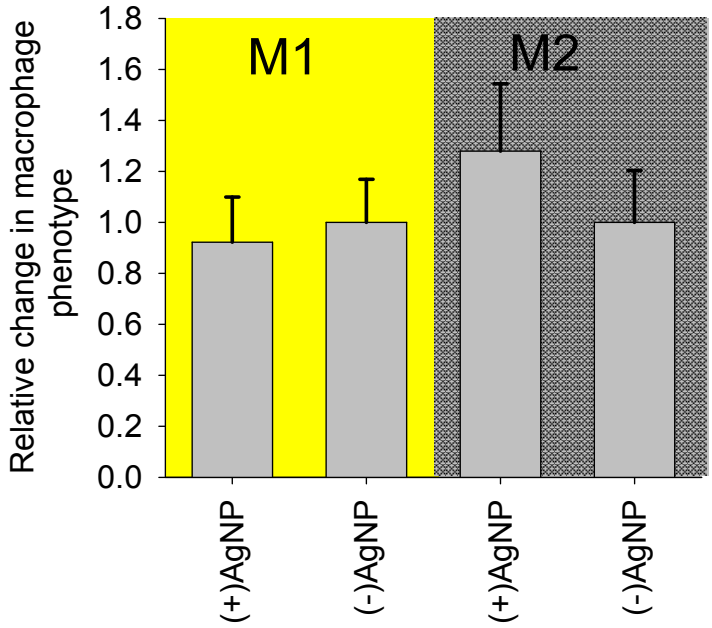

Figure S7. Relative change in bone marrow derived macrophage polarization (M1-CD86; M2-CD206) after 4 days of culture on collagen fibers with and without nanosilver (+ or - AgNPs).

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