Electronic Supplementary Material (ESI) for Chemical Communications.

Electronic Supporting Information

Micro- and nano-patterned conductive graphene-PEG hybrid scaffolds for cardiac tissue engineering

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Experimental Procedures

Substrate preparation

Polyethylene glycol (PEG) was chosen as a base platform material for its biocompatibility and its demonstrated capacity to support the generation of micro- and nano-scale topographies with desired geometry and dimensions using capillary force lithographic techniques[1]. 800 nm groove and ridge widths (with a depth of 600 nm) were selected based on previous results demonstrating optimal dimensions for promoting structural development in cultured cardiac cells[1, 2].

PEG/graphene substrates were fabricated using a two-step process. First, PEG polymer substrates were patterned with nanoscale ridge-groove dimensions of 800 x 800 x 600 nm (ridge width x groove width x groove depth). Second, the graphene film was transferred in water onto the patterned PEG polymer substrates. Patterned PEG substrates were constructed through application of capillary force lithography (CFL) techniques, and using *perfluoropolyether* (PFPE) molds as negative patterns[3]. Briefly, UV-curable PFPE molds were fabricated by mixing Fluorolink MD-700 (Solvay) with a photoinitiator (2-hydroxy-2-methyl-propiophenone; Sigma-Aldrich; 2% by weight). The liquid mixture was drop-dispensed onto a prefabricated silicon master, and a flexible, transparent polyethylene terephthalate (PET) film was brought into contact with the liquid mixture. The construct was then exposed to UV light to promote free radical polymerization. Once cured, the PFPE mold was removed from the PET film and silicon master and stored at room temperature until use.

PEG nanostructures were fabricated on glass substrates. To increase the adhesion of the PEG, the glass was piranha cleaned and treated with 10 mM 3-(acryloxypropyl)-trichlorosilane. A small amount (~0.1 to 0.5 mL) of PEG-DA (M.W. 285) precursor was then drop-dispensed onto treated glass coverslips, and a PFPE mold was placed directly onto the surface. The PEG-DA precursor was spontaneously drawn into the cavities of the PFPE mold by capillary action, and was cured for 30 seconds under a UV lamp. After curing, the PFPE mold was peeled from the substrate to reveal the PEG nanostructures.

Graphene was grown on copper (Cu) foil by chemical vapor deposition (CVD) at 990°C using methane as a precursor[4]. Following deposition, a thin film of poly(methyl methacrylate) (PMMA) was spin-coated on the graphene. The Cu foil was then etched away in dilute HNO_3, and the graphene/PMMA construct was rinsed twice in deionized water. In order to prevent the graphene/PMMA film from folding up, graphene transfer took place at an air-water interface, where the surface tension of the water kept the graphene flat. The transfer of the graphene layer onto patterned PEG substrates was done while floating the graphene/PMMA construct at the air-water interface with the graphene side facing down. The PEG substrate was placed in the water, with the patterned PEG side facing up underneath the graphene/PMMA, and brought up through the air-water interface at an angle to facilitate confluent contact with the graphene layer. Once the

graphene/PMMA was transferred to the top of the PEG nanostructures, the Graphene-PEG/PMMA composite was left in the hood to dry overnight. Finally, the PMMA was removed using isopropyl alcohol, and the resulting Graphene-PEG nanostructure was rinsed twice in de-ionized water. Prior to use, Graphene-PEG substrates were either treated with oxygen plasma, or left pristine. Surfaces to be treated were placed in an O_2 plasma chamber (Femto Science), and subjected to 25W, 0.5 Torr O_2 plasma for 1 or 2 seconds depending on the specific experiment being conducted.

Substrate characterization

The surface topographies of graphene-functionalized PEG nanostructures were imaged using a Dimension 3100 atomic force microscope (AFM, Vecco Dimension 3100, Bruker, Inc.) equipped with a NanoScope IVa Controller and Si tip (resonant frequency = 75 kHz, force constant = 3 N/m, Bruker, Inc.). Topographic images were collected in tapping mode (scanning rate = 1 Hz). Raman spectra of prepared substrates were collected using a Renishaw inVia Raman microscope system with a 514 nm excitation wavelength (Renishaw, Inc.).

Conductive atomic force microscopy

The electro-conductivities of graphene-functionalized PEG nanopatterns were measured using conductive atomic force microscopy. Samples were imaged with an atomic force microscope (Asylum Research, MFP-3D). Platinum-coated, contact-mode AFM tips with diameters less than 25 nm were used (Budget Sensors, BS-ElectriCont). Voltages were applied between the edges of the graphene substrate and the conductive probe tips, and the current was recorded by the AFM's internal preamplifier (Asylum Research, ORCA head model 59). Current maps were collected using a DC bias voltage of 20 mV. The topography and current were measured simultaneously in contact mode.

Macroscopic resistivity measurements

Substrate resistance over centimeter length scales on Graphene-PEG nanostructures was measured with a custom-built conductivity meter and a custom-written Labview program. The I-V curve for each examined surface was collected by sweeping the bias voltage from –10 to 10 V.

Cardiac tissue culture

Neonatal rat ventricular myocytes (NRVMs) were isolated from 2 day-old newborn Fischer 344 rats, as described elsewhere[5], using methods approved by the University of Washington's Animal Care Committee, and in accordance with federal guidelines.

Enzymatically dissociated cardiac cells were re-suspended in feeding medium at a density of 750 cells/mL and plated onto sterilized substrates at a concentration of 1.5 million cells per substrate. The medium consisted of a 4:1 ratio of Dulbecco's modified Eagle's medium (DMEM; HyClone, Inc) and M199 (10% horse serum, 5% fetal bovine serum, 20 mM HEPES, 100 U/mL penicillin G, 100 mg/mL streptomycin, and 4 mM glutamine). Culture medium was changed daily for 7 days, and was supplemented with 10 mM arabinosylcytosine starting at day 3 to remove proliferating fibroblast cells from the population.

Immunocytochemistry

Cells on nanopatterned coverslips were fixed in 4% paraformaldehyde for 15 minutes at room temperature following 7 days *in vitro*. They were then permeabilized with 0.1% Triton-X-100 diluted in phosphate buffered saline (PBS) for 10 minutes before being incubated in a blocking solution for 1-hour at room temperature. The blocking solution consisted of 0.5% bovine serum albumin and 5% serum in PBS. After blocking, cells were incubated overnight at 4^{o}C in a primary antibody solution consisting of the antibodies diluted in blocking solution. The antibodies used in this study were mouse anti-α-actinin (diluted 1 in 1000; Sigma) and rabbit anti-connexin43 (Cx43; diluted 1 in 1000; Life Technologies).

Following primary incubation, cells were washed 3-times in PBS before being incubated in a secondary antibody solution consisting of donkey-anti-mouse IgG conjugated to Alexafluor 488 (1 in 200; Life Technologies) and donkey-anti-rabbit IgG conjugated to Alexafluor 568 (1 in 200; Life Technologies) diluted in blocking solution. A DAPI (4',6-diamidino-2-phenylindole; Sigma Aldrich) counterstain was added to the secondary antibody incubation to label nuclei. After 2 hours in the secondary antibody solution, cells were washed 3-times in PBS before the coverslips were mounted onto glass microscope slides using a drop of mounting medium (VectaShield). Immunostained cells were visualized using a Nikon Ti300 confocal microscope and associated software.

Western blot

NRVMs were lysed on day 7 of the culture using a RIPA buffer that contained 1 mM polymethylsulfonyl fluoride and a 1x complete protease inhibitor cocktail. Lysates were boiled for 5 min, resolved on a 10% SDS-PAGE gel, and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk powder in Tris-buffered saline (containing 0.1% (v/v) Tween 20) for 60 minutes at room temperature. Antibodies targeting connexin 43 (Cx43), GAPDH, and sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA2) were applied to the membranes at a dilution of 1:1000, and incubated overnight at 4°C. The immunoblotted membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour, and the resulting immunoreactive bands were detected using enhanced digitalized chemiluminescence.

Calcium imaging

Fluo-4/AM was obtained from Molecular Probes (Life Technologies). Measurement of $[Ca^{2+}]_i$ transients, and the preparation of the HEPES buffered physiological saline solution (HBSS) were performed as previously described[1]. All NRVMs were analyzed following 7 days *in vitro*. Experiments were performed at room temperature (22–24°C), using a Nikon confocal microscope Ti300.

Action potential waveform analysis

Analysis of action potential waveforms was performed visually using a high-speed (5 ms resolution), CMOS-based fluorescence imaging system (MiCAM Ultima; SciMedia Ltd.). To visualize electrical activity, 7 day old confluent NRVM monolayers cultured on Graphene-PEG nanostructures were first stained with a voltage-sensitive dye, di-4-ANEPPS (10 μM), and then placed in the recording chamber, which was continuously superfused with warm (37^{o}C) Tyrode's solution. The excitation light source, emission filter, instrumentation, and staining procedure have all been previously described[1, 6].

Data was linearly detrended and low-pass filtered with a cutoff frequency of 32 Hz and convolved with a 5x5 spatial Gaussian filter. A Fourier transform for each channel was calculated

and used to exclude channels that had a different dominant frequency than the pacing frequency. A 5-point derivative was calculated, and channels with more than 6 signal direction changes (i.e. derivative zero-crossings) per pace were excluded to eliminate noisy channels. Activation maps were constructed using the time of maximum upstroke rate (derivative) of the action potential.

Statistical analysis

Differences in measurements of electrical resistance in parallel with the nanopatterns and traversing them were assessed for significance using unpaired t tests. Structural and electrophysiological measurements recorded from cells grown on PEG, Graphene-PEG, and PEG/oxidized graphene surfaces were compared using one-way analysis of variance (ANOVA) on ranks, with *post hoc* tests for multiple comparisons. For all statistical tests, analysis was performed using SigmaPlot, and a p value of < 0.05 was considered significant. All data is presented as the mean ± the standard error of the mean.

References

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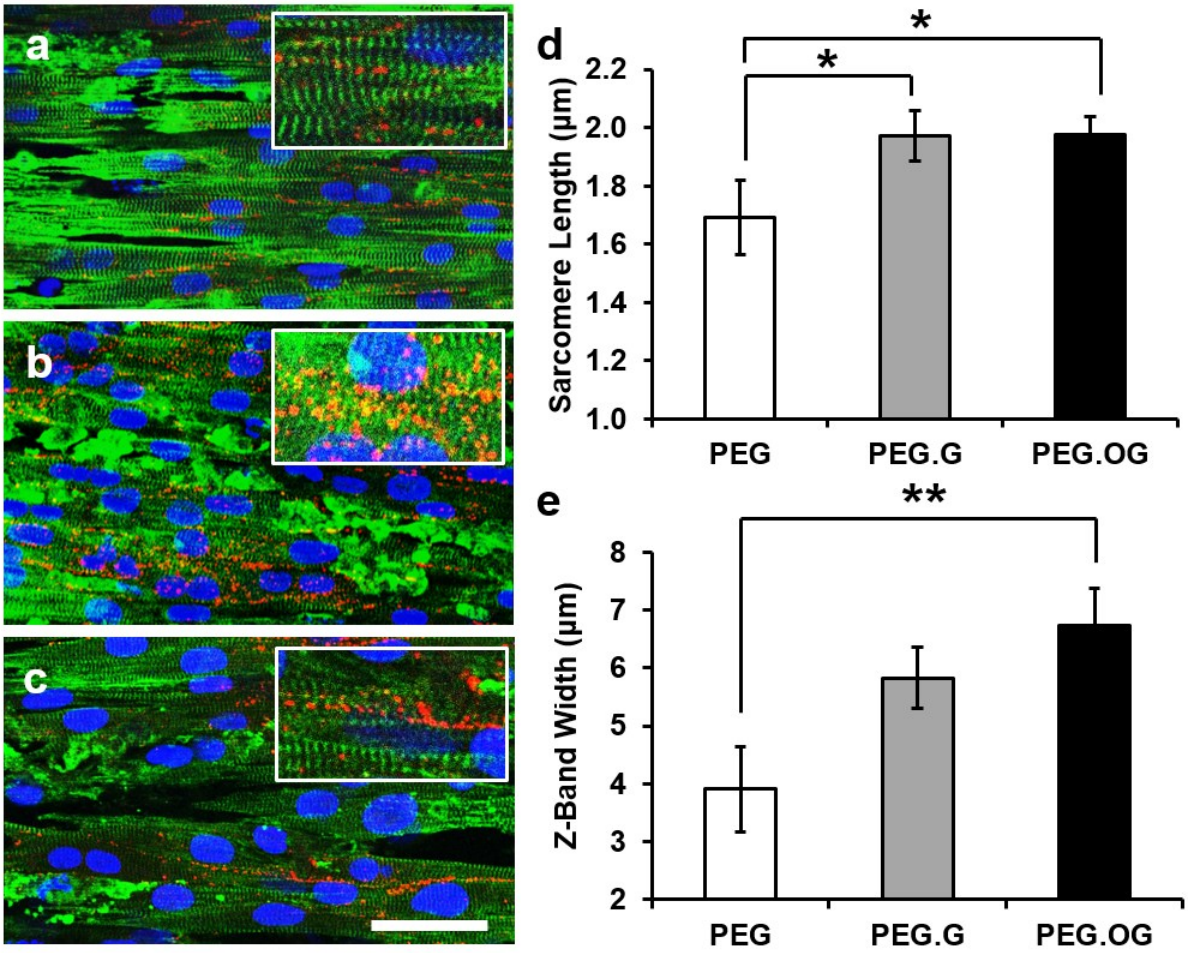
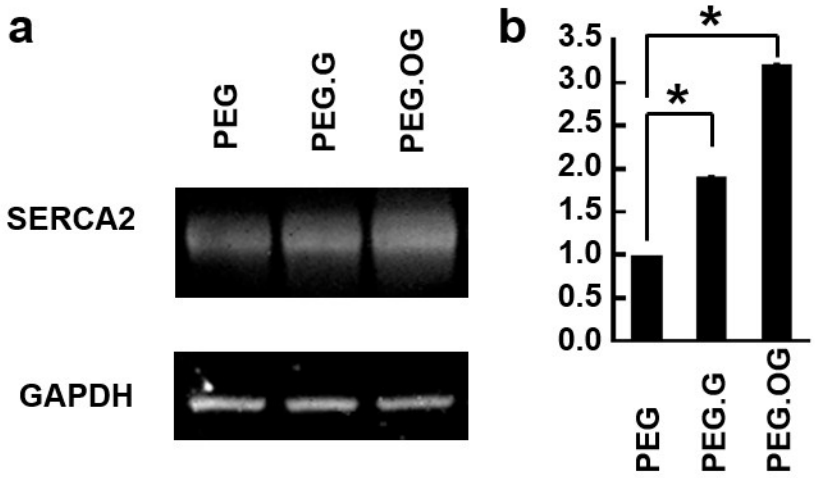
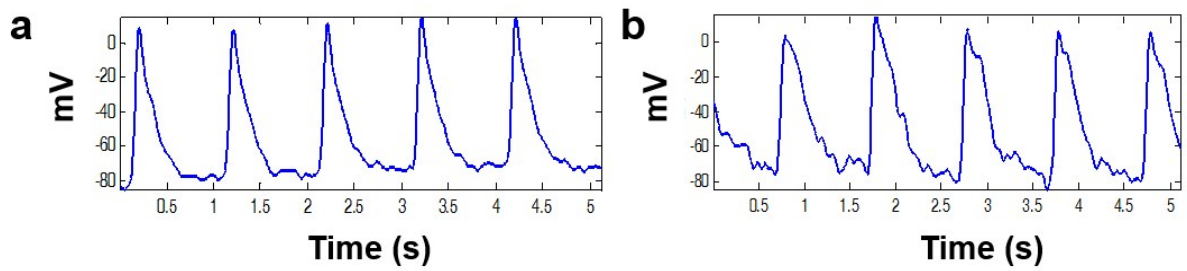
Supplementary Figures



Supplementary Fig. 1: Structural analysis of cardiac cells maintained on graphene-PEG patterns. Immunostaining images of cardiac cells grown on engineered substrates: (a) PEG topographies, (b) graphene-PEG topographies, and (c) graphene-PEG graphene topographies. Cardiac cells were stained for expression of α-actinin (green), Cx43 (red), and nuclei (blue). For each image, the inset panel highlights the sarcomeric structure observed in each culture condition. Scale bars: 30 µm. (d) Sarcomere length analysis from cardiac cells maintained on Graphene-PEG topographies. *p = 0.05. (e) Z-band width analysis from cardiac cells maintained on Graphene-PEG topographies. **p = 0.006.



Supplementary Fig. 2: Analysis of SERCA2 protein expression in cardiac cells. (a) Representative chemiluminescence results from western blot analysis of SERCA2 expression normalized to GAPDH. (b) Quantitative analysis of blot data represented in (a), *$p < 0.05$.



Supplementary Fig. 3: Analysis of action potential waveform in cardiac cells grown on PEG and graphene–PEG topographies. (a) Representative temporal analysis of cardiac cell action potential waveforms derived from optical mapping data of a PEG patterned culture. (b) Representative temporal analysis of cardiac cell action potential waveforms derived from optical mapping data of a graphene–PEG patterned culture.

Supplementary video legends

Supplementary video 1. Optical mapping analysis of action potential propagation on PEG patterns. Video constitutes a 5-second recording across a representative 1 cm^2 field of view.

Supplementary video 2. Optical mapping analysis of action potential propagation on graphene-PEG patterns. Video constitutes a 5-second recording across a representative 1 cm^2 field of view.