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A cell-assembled, spatially aligned extracellular matrix to promote directed tissue development

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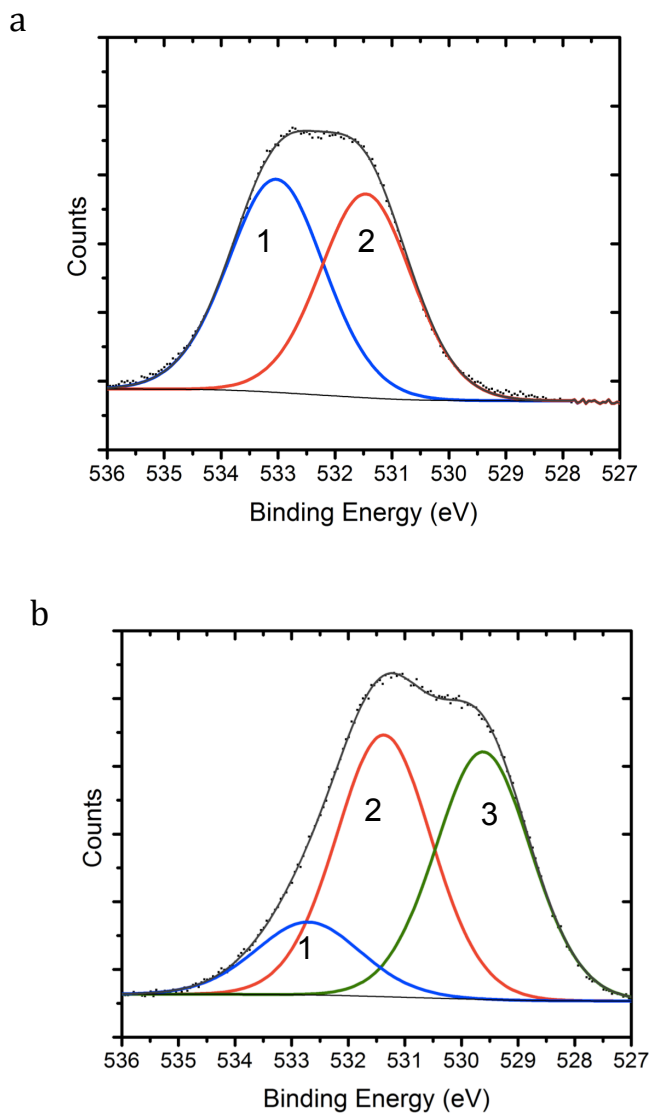

Supplementary Figure 1. (a) The O(1s) region of PET measured at 60° from the surface normal to emphasize surface features. Two peaks (**1** and **2**) are observed, corresponding to the oxygen atoms of the ether and carbonyl moieties of the ester group, respectively;[1] (b) the same region measured at 60° following synthesis of the ZrO_2 interface, showing diminution of one of these peaks (peak **1**; BE = 533.0 eV) and the appearance of a new peak (peak **3**; BE = 529.61 eV) assigned to oxygen bonded to Zr. The peak at BE 531.4 eV (peak **2**) remains constant; the black line in both sets of spectra is the smoothed data trace.

Experimental Procedures

Chemical patterning on polyethylene terephthalate (PET). Micro-patterning on PET was achieved by combining photolithography with the chemical vapor deposition as described by Donnelly et al.[2] A 0.05 mm thick PET sheet (Goodfellow Corporation, Oakdale, PA) was cut into ~0.5 cm x 0.5 cm square pieces, cleaned by sonication in isopropanol for 15 minutes, dried in a stream of nitrogen gas, and baked at 95º C for 10 minutes. The surface was spin-coated at 3000 rpm with hexamethyldisilazane (Electron Microscopy Sciences, PA) followed by diazonaphthoquinone sulfonic ester positive photoresist AZ5214-E (Clariant Corp.). The resist was cured for 45 seconds at 95º C, and exposed to UV light through a photolithographic mask. The film was developed in tetramethyl-ammonium hydroxide solution AZ312 MIF to dissolve away the photoresist from the UV-exposed regions.

Micro-patterned PET films were placed in a glass chamber with inlets for vacuum and vapors of zirconium tetra(tert-butoxide), $Zr(t\text{-}OBu)_4$ (Sigma-Aldrich). The chamber was evacuated to 10^{-3} torr, and samples were exposed to vapor for 5 minutes under vacuum followed by 5 minutes without external evacuation. The samples were heated to 47º C, allowed to cool to room temperature, and then immersed promptly in 1 mM 1, 4-butane diphosphonic acid (Acros Organic) in ethanol for about 15 hours. The films were sonicated 10 minutes in ethanol and 2 minutes in tetrahydrofuran to remove any residual photoresist. After rinsing with isopropanol, the substrates were dried in a stream of nitrogen gas. The patterns were characterized by AFM, SEM/EDX, and contact angle measurements, which gave equivalent results to those previously described for these ZrO_2/SAMP patterns.[2]

NIH3T3 cell culture and decellularized matrix preparation. SAMP-patterned (10 μm x 10 μm) and unpatterned control PET substrates were disinfected with 70% ethanol for 20 min,

washed three times with sterile phosphate buffered saline (PBS), and immersed in 1 mL DMEM in a 24-well plate before cell seeding. NIH3T3 (ATCC) cells were cultured in DMEM supplemented with 10% bovine calf serum (BCS; Hyclone). Cells were plated at 50,000 cells per well in DMEM. After 4 hr, unattached cells were removed and 1 mL of DMEM with 10% BCS was added. The medium was supplemented with 50 μg/mL ascorbic acid (Sigma) on day 6 and again on day 8 to augment synthesis of type I collagen. Phase contrast images of the cells on PET were captured using a Nikon Eclipse TS100 microscope equipped with a Cooke SensiCam QE High Performance camera. NIH3T3 cell cultures were fixed in formaldehyde and immunostained with R457 rabbit anti-fibronectin antiserum (1:100 in 2.5% BSA-PBS)[3] or rabbit anti-collagen type I antibodies (1:40 in 2.5% BSA-PBS) (Millipore) followed by Alexa Fluor 488-conjugated goat anti-rabbit secondary antibodies (1:200 in 2.5% BSA-PBS) (Invitrogen). Alignment of fibronectin and collagen fibrils was quantified[4, 5] using 8 cropped (20x magnification; 1024 x 1024 pixels) images; images were normalized and rotated to produce Fast Fourier Transform (FFT) maxima at 90° and 270°, and data were smoothed. 2D FFT results were nearly symmetrical, and radial intensity data were plotted from 0° to 180°, then normalized to 0 baseline value. The full width at half maximum (FWHM) for each peak was calculated and used for comparison. The Student's t-test was used to evaluate the difference between the groups. The data were considered statistically significant when $p < 0.05$.

NIH3T3 cells were seeded and allowed to assemble a dense fibronectin matrix for 10 days; cultures were then decellularized following the procedure of Mao and Schwarzbauer [6] except the second incubation in lysis buffer was extended to 75 min at 37˚ C. Samples were fixed and stained, and fibronectin and collagen were quantified as described above. Nanostructural morphology was examined by electron microscopy. Here, samples were fixed in 2.5%

glutaraldehyde, dehydrated in ethanol, immersed in hexamethyldisilazane, and air-dried. They were mounted for analysis using an environmental SEM (FEI Quanta 200) at 5 kV in low vacuum mode.

Neurite outgrowth on decellularized matrix. Decellularized matrices on patterned or unpatterned PET in wells of a 24-well dish were used as substrates for rat adrenal pheochromocytoma PC12 cells (kindly provided by Prof. Lynn Enquist, Princeton University). Cells were primed with nerve growth factor (NGF; Invitrogen) and seeded at 30,000 cells per well in 1 mL of differentiation medium (RPMI, 1% horse serum, 100 ng /mL NGF). After 72 hr of culture, cells were then fixed, permeabilized with 0.1% Triton-X100 in PBS, and stained with anti-collagen type I antibodies as above and 0.16 μM rhodamine-phalloidin in 1% BSA-PBS. The cells and fibrils were visualized with a Nikon TE2000U microscope equipped with QImaging Retiga 1300 camera.

Images were used to measure angles of neurites relative to matrix fibrils. Straight lines were drawn through the F-actin stained neurites and the cell bodies and angles were measured relative to a reference axis defined as orthogonal to the SAMP-patterned stripes. Orientations of about 200 neurites taken from 8 random images were quantified using ImageJ software and were plotted from 0 to 180º; a calculated value of 90° represents neurite alignment parallel to the SAMP pattern, and increased deviation from 90° indicates poorer alignment. A Gaussian was then fit to the distribution of measured angles using MATLAB (The MathWorks, Natick, MA) to obtain the FWHM. A lower FWHM indicates better alignment with the pattern and matrix fibrils.

References

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