ADVANCED BIOSYSTEMS

Supporting Information

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Electron Microscopy for 3D Scaffolds–Cell Biointerface Characterization

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Electron microscopy for 3D scaffolds-cell biointerface characterization

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SUPPLEMENTARY INFORMATION

EXPERIMENTAL PROCEDURE

Material and methods

Fabrication of ordered scaffolds. DLW-2PP was performed on a Nanoscribe Photonic Professional GT system (Nanoscribe GmbH). This system uses a 780 nm Ti−sapphire laser emitting ∼100 fs pulses at 80 MHz with a maximum power of 150 mW and is equipped with a 63X, 1.4 NA oil immersion objective. The substrate was placed in a holder that fits into a piezoelectric x/y/z stage. A galvo scanner determines the laser trajectories. A droplet of a negative tone photoresist, known as Ormocomp (Micro Resist Technology GmbH), was poured on a square glass coverslip previously washed with 2-propanol and dried with nitrogen. Ormocomp was fabricated at room temperature with an output power of 60 mW and a writing speed of 1000 μm/s. Structures were written in a "bottom-up" sequence where the first layer was attached on the substrate surface. To minimize the optical aberrations related to the immersion-oil configuration and then get the best results in terms of resolution, the photoresist-immersion configuration (Dip-in Laser Lithography, DiLL) was used. After exposure, the material was developed for 20 minutes in the mr-DEV600 (Micro Resist Technology GmbH) developer and washed with 2-propanol.

Fabrication of non-ordered scaffolds. Macroporous scaffolds were prepared following a slightly modified version of the procedure reported in previous studies [25,31]. Scaffolds were fabricated by the ice-templating technique, using an aqueous dispersion of PEDOT:PSS (PH1000, Heraeus LTD) to which 3% (v/v) (3-glycidyloxypropyl) trimethoxysilane (GOPS, Sigma Aldrich) was added as a crosslinker to improve scaffolds water stability, and 0.25% (v/v) dodecyl benzenesulfonic acid (DBSA, Sigma Aldrich) was introduced to increase scaffolds electrical conductivity. 400 μl of dispersion were poured into a 11 mm cylindrical mold that was then placed in a freeze-dryer (Pilote de Paillasse, Cryotec). Samples were frozen down to −50 °C at a defined rate (−0.35 °C min^{-1}). Ice crystals were then sublimed from the scaffolds ramping the temperature from −50°C to 20°C at 0.12°C min^{-1} (in 10 hours).
The obtained scaffolds were then cured at 60°C for 3hours. 400 μm thick slices were then prepared using a LEICA VT1200 Vibrating Blade Microtome to be then used for subsequent cell seeding.

Cell culture on scaffolds. Ordered scaffolds were sterilized with ultraviolet radiation (UV) for 1 hour and washed with distilled water. In order to improve cells adhesion 20 μl of fibronectin (Sigma Aldrich) were diluted in 1 mL of DPBS (Sigma Aldrich). 200 μl of obtained solution were added on the samples and incubated at 37 °C. After two hours, the coating solution was removed and samples were washed twice with DPBS. Human glioblastoma astrocytoma (U87) were grown in minimum essential medium (EMEM,

Sigma Aldrich) supplemented with fetal bovine serum (FBS, Sigma Aldrich), 2 mM L-glutamine (Gibco) 100 IU/ml streptomycin/penicillin (Gibco) and 1% of non-essential aminoacids (NEAA, Sigma Aldrich) at 37°C with 5% CO_2. Cells were detached from culture flask *via* trypsination and suspended in EMEM with a density 25,000 cells per milliliter. 200 µl of cells suspension were pipetted on each sample.
Non-ordered scaffolds were baked at 50°C for two hours in order to induce Gops crosslinking. Scaffolds were then sterilized by immersion in 70% ethanol for one hour, followed by extensive rinsing to remove any traces of ethanol. For these scaffolds, human adipose derived stem cells (hADSC by Lonza, p9) were adopted. hADSC were expanded in T75 flasks using high glucose Dulbecco's modified Eagle medium (DMEM glucose 4.5 g/l) supplemented with 20% FBS (Thermofisher Scientific), 1% Glutamax (Thermofisher Scientific), 5 µg/ml gentamicin (Thermofisher Scientific). Cells were cultured in an incubator with a humidified atmosphere with 5% CO_2 at 37°C. Scaffolds were seeded with 20 µl of cell suspension using about 12000 cells/scaffold (1500 cells/mm^3). Attention was paid as to obtain an as homogenous as possible distribution of the cell suspension on the scaffold surface. Cells were let adhere to the scaffolds for about 30 minutes in the incubator and then a volume of 0.5 ml of complete medium was added to each well. Cells were cultured for 5 days before being prepared for further analysis.

Staining of cells for confocal imaging. **Staining of cells for confocal imaging**. For cells on ordered scaffolds, nuclei were stained with DAPI (Sigma Aldrich) opportunely diluted in PBS 1:10000 for fifteen minutes at RT. For Actin labeling phalloidin 647 was used and diluted 1:200 in PBS and incubated for one hour at RT. Before images acquisition all the samples were washed gently with PBS (Sigma Aldrich). hADSCs after 5 days of proliferation on the macroporous scaffolds were fixed with paraformaldehyde 4% for twenty minutes at RT. They were then either used for fluorescence staining or for Immunocytochemical staining. For Actin labeling, cells were first permabilized by incubating them with a solution of Triton X-100 0.1% in PBS for 5 min, and then rinsed 3 times with PBS. Phalloidin-iFluor 594 (Abcam) was used and diluted 1:1000 in PBS and incubated for one hour at RT (according to the manufacturer instructions). Nuclei were then stained with Hoechst 3342 (Sigma Aldrich) 1µg/ml in PBS for fifteen minutes at RT. Samples were rinsed with PBS before images acquisition 3 times with PBS. Immunocytochemical staining was instead performed for vimentin labelling. Cells were fixed with paraformaldehyde 4% for twenty minutes at RT. Samples were rinsed with PBST for 10 minutes, they were then incubated with the blocking solution containing 1% BSA and 2% horse serum for 45 minutes and were gently rocked. Primary antibody incubation in 1/10 blocking solution containing 10ug/ml Vimentin ab (Vimentin monoclonal antibody, sp20, Thermofisher Scientific) was performed over night at 4 ºC. Samples were then rinsed with PBST and incubated with Alexa Fluor 568 donkey anti-rabbit IgG (Invitrogen) diluted 1:100 in 1/10 blocking solution for one hour, gently rocked. They were then rinsed with PBST and counterstained with bisBezamide (1ug/ml) in PBS for 15 minutes at RT under gently

shaking and then rinsed with PBS. Images were acquired for samples layered with mounting medium on a glass slide, using microscope Axio Observer Z1 (Carl Zeiss MicroImaging GmbH, Oberkochen, Germany).

Sample preparation for SEM/FIB. Samples with cells were prepared using a thin plasticization protocol as previously presented [27,28]. Cells were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer (pH 7.3, Electron Microscopy Sciences) at 4°C overnight, washed 3 times in the same buffer and quenched in 20 mM glycine (Sigma Aldrich) for 20 minutes at 4°C. After rinsing with buffer (3 x 5 minutes at 4°C), specimens were post-fixed with equal volumes of 4% osmium tetroxide (Electron Microscopy Sciences) and 2% potassium ferrocyanide (Electron Microscopy Sciences) for 1 hour on ice, then washed (3 x 5 minutes) with chilled buffer. Samples were incubated with 1% thiocarbohydrazide (Electron Microscopy Sciences) at room temperature for 20 minutes and washed (3 x 5 minutes) in distilled water. The thiocarbohydrazide solution was freshly prepared. Substrate with cells were post fixed with 2% osmium tetroxide (aqueous solution) for 1 hour at room temperature, washed 3 times in distilled water and *en-bloc* stained with 4% aqueous uranyl acetate (Sigma Aldrich) overnight at 4°C. After rinsing with chilled deionized water, only ordered scaffold specimens were treated in 0.15% tannic acid buffer for 3 minutes and again rinsed (3 x 5 minutes) with water. All samples were dehydrated in increasing concentration of ethanol (10%, 30%, 50%, 70%, 90%, 100%, Sigma-Aldrich). Each step was performed for 10 minutes on ice, except for the last step in 100% ethanol solution that was performed at room temperature. Ordered scaffold samples with cells were infiltrated with increasing concentration of Spurr's low viscosity embedding resin (Electron Microscopy Sciences) in absolute ethanol, at room temperature in a sealed container, using these ratios: 1:3 (2 x 3 hours), 1:2 (2 x 3 hours), 1:1 (overnight), 1:2 (2 x 3 hours), 2:1 (2 x 3 hours), 3:1 (2 x 3 hours). Non-ordered scaffolds with cells were infiltrated with Epon resin (Sigma Aldrich) with same concentrations and incubation times. Resin-ethanol mixtures were replaced with freshly 100% resin and samples were infiltrated at room temperature overnight. To remove the excess of resin, each specimen was mounted vertically for 2 hours and then quickly rinsed with absolute ethanol prior to polymerization in oven at 70°C overnight. Samples were mounted with colloidal silver paste (Ted Pella Inc.) to a pin stub and a 10 nm layer of platinum-palladium alloy was deposited before imaging.

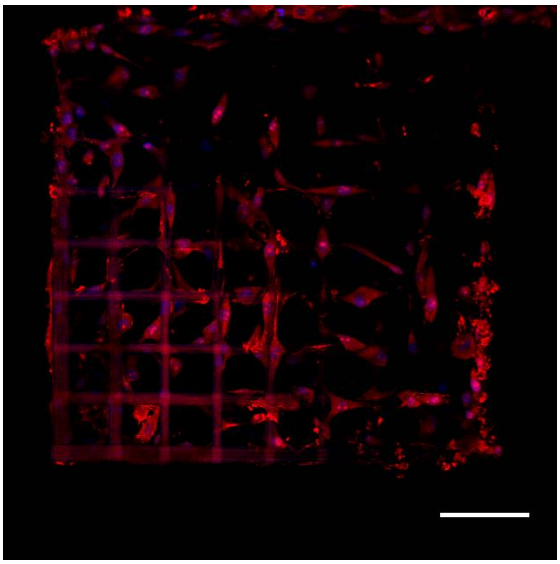
SEM imaging and FIB cross sectioning. Samples were loaded in to a dual beam machine (Helios 650 and Helios 600i, FEI Company) and secondary imaging has been performed with an 'in lens' detector with a voltage in the range 3-10 kV. Prior to cross sectioning, the region of interest located on the sample has been covered with a 1 µm thick layer of platinum deposited *via* electron beam-assisted deposition first and then *via* ion beam-assisted deposition. After trenching out and polishing the cross section (operational parameters are described in Santoro et al.),[28] imaging of the cross sectional area has been performed with an 'in lens' detector with a backscattered acquisition with a voltage between 3-10 kV and a current between 0.6 - 40 nA. Images were then post-processed with Fiji (ImageJ, NIH).

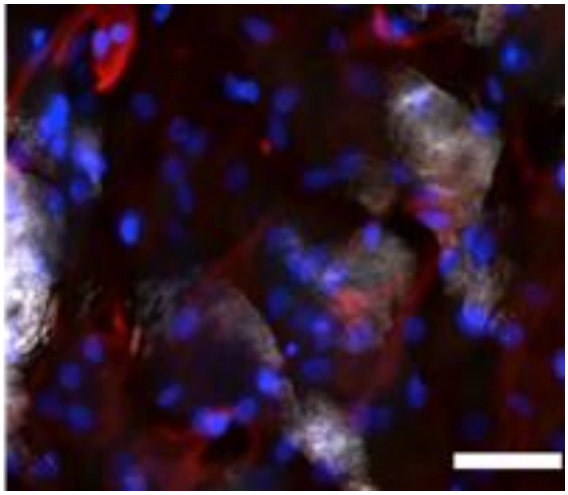
STATISTICAL ANALYSIS

For the ordered scaffolds, 12 samples have been prepared for UTP procedure, on each sample 2-6 scaffolds have been prepared. Overall, about 30 cells have been sectioned (2-10 sections per cell) via SEM/FIB. For non ordered scaffold, 8 samples have been prepared, overall about 12 cells have been sectioned. SEM images have been post-processed with ImageJ (NIH, USA).

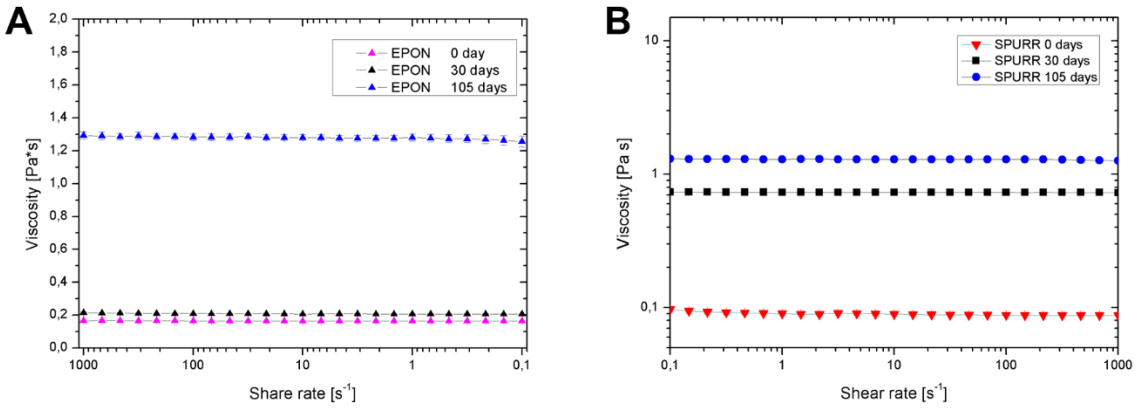
S1 – CONFOCAL IMAGES OF CELLS ON SCAFFOLDS

Cells on ordered scaffold labelled with phalloidin ad DAP for actin (red) and nuclei (blue) visualization, respectively (scale bar 200µm). Cells on macroporous scaffold were also characterized with immunocytochemical labelling of vimentin (red) and nuclei (blue) (scale bar 50 µm).



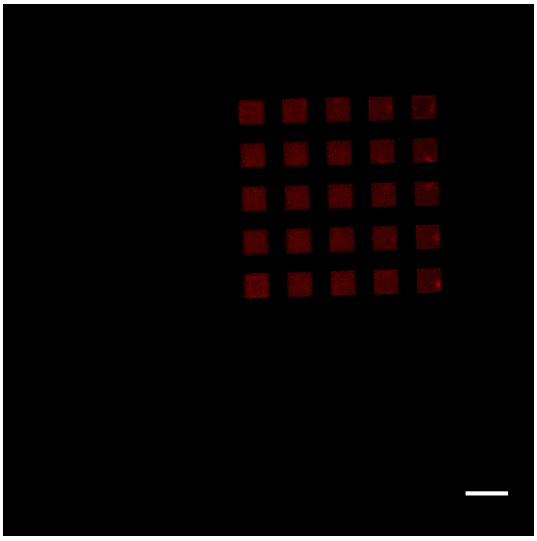


S2- RESIN VISCOSITY MEASUREMENTS. Rheological properties of EPON and SPURR resins have been investigated with a rotational rheometer, Anton Paar, model MCR302, using a cone-plate geometry, 50 mm diameter and 1° cone angle. Both types of resin (EPON and SPURR) have been tested at three different times to evaluate possible aging effects (day 0; day 30 and day 105), by measuring their viscosity. After calibrating the instrument and calculating inertia of the measurement system, 1mL of resin has been laid on the plate for each test, and then cone has been brought to the appropriate measurement distance. Air bubbles have been carefully removed to avoid measurement interferences. Viscosity values of resins (η) have been measured applying a logarithmic ramp of shear stresses (from 1*10^3 to 1*10-1 s-1), and each test has been repeated three times to assure the appropriate experimental reproducibility. All measurements have been performed at room temperature.

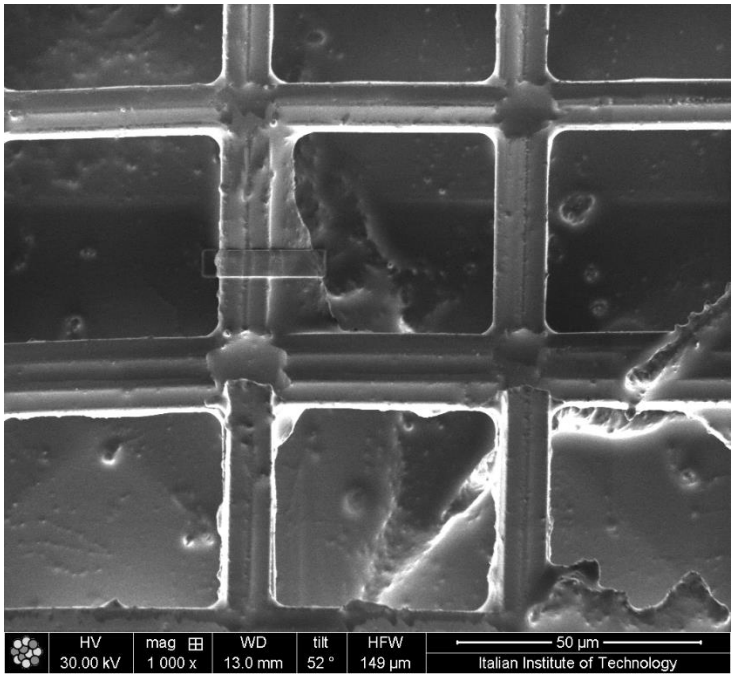


S3- EVALUATION OF THE PHOTORESIST AUTOFLUORESCENCE:

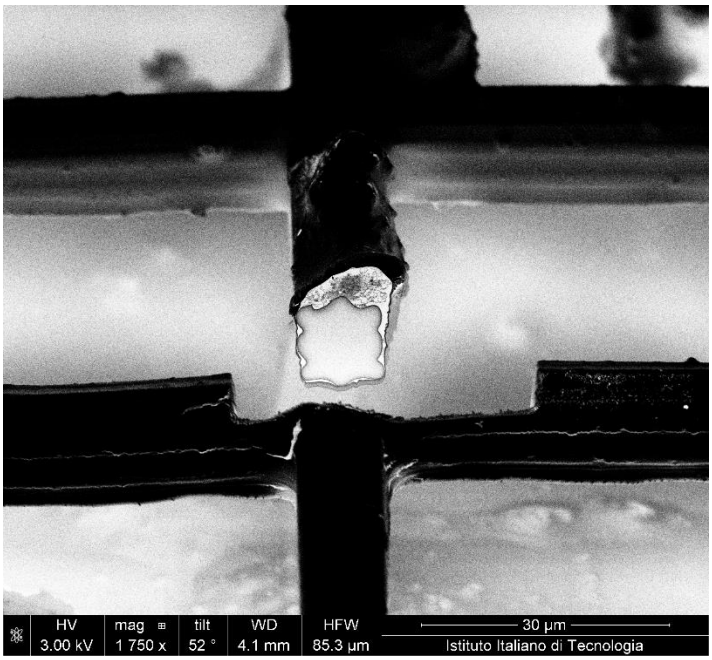
The autofluorescence of the material was evaluated with a multiphoton (MP) confocal microscope (TCS SP5 MP, Leica Microsystems, Germany). In particular, we observed the autofluorescence of the photoresist, fabricated as an array of parallelepipeds of 30X30X10 µm, in the far-red region. To this end, we illuminated the structures with a 633 nm wavelength tuned laser and observed their fluorescence emission in a 653-703 nm spectrum range. In the image, the scale bar is of 50µm.



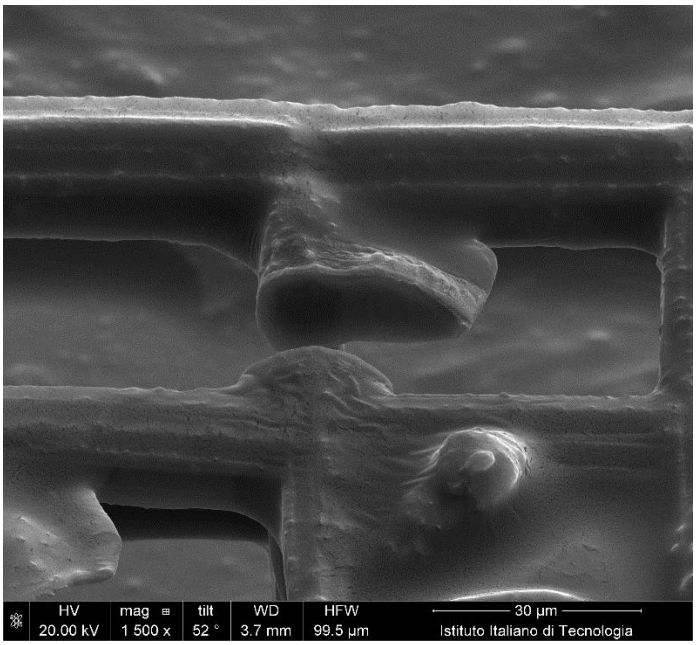
S4- PLATINUM DEPOSITION AT REGION OF INTEREST



S5- TRENCHING OUT AT REGION OF INTEREST



S6- REDEPOSITION


HV
20.00 kV
mag
1 500 x
tilt
52 °
WD
3.7 mm
HFW
99.5 µm
30 µm
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