Supporting Information

Cell instructive microporous scaffolds through interface engineering

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Experimental Details

Preparation of polyHIPE matrices.

The monomer divinylbenzene (DVB) (80% mixed with m- and *p*-ethyl styrene, Sigma Aldrich) was passed through a basic alumina column (Fluka, Brockmann activity I) to remove the inhibitor; *p*-tert butlycatechol. Potassium persulfate (Sigma Aldrich), polystyrene-b-poly(ethylene oxide) (PS-PEO, Mw= 22500-27500 g/mol, polydispersity index, PDI = 1.1-1.3, Sigma Aldrich), Poly(Styrene)-b-poly(acrylic acid) (PS-PAA, Mw= 8100-8500 g/mol, PDI=1.1-1.3, Sigma Aldrich)) and Span 80 (Sorbitan monooleate, Sigma Aldrich) were all used as received. The monomer: surfactant ratio was maintained at $2.5x10^4$:1 for PS-PAA and PS-PEO. PS-PAA was first dissolved in tetrahydrofuran (10 µl/mg) before solubilizing in the monomer. Emulsions with copolymer mixtures at the indicated molar ratios were prepared (Table 1). The aqueous phase, consisting of 0.1 wt/wt % potassium persulfate initiator adjusted to pH 10 with 1M NaOH, was added drop wise to the oil phase (DVB and surfactant) using a peristaltic pump at a rate of 10 ml/min. All HIPEs were prepared with a porosity of 80% based on aqueous phase volume. Emulsions with higher porosities were found to be unstable at the polymerization temperature resulting in rapid droplet coalescence and foams with broad polydispersity of void diameters. Once the aqueous phase was added, the resulting emulsion was stirred for 5 minutes to homogenize. The emulsion was polymerized at 60 °C for 24 hours. The resulting foam was then extracted in a soxhlet for 48 hours using a 50/50 v/v% of deionized water / isopropanol.

Chemical Force Spectroscopy Mapping

CFSM provides adhesive force maps generated by measuring the rupture force between bonds that form between a functionalized tip and the surface. As shown in Supplemental Fig. 2a, tips were functionalized by poly-L-lysine (PLL, M_w 1000-5000, Sigma-Aldrich) based on a technique described elsewhere (4). Briefly, AFM tips were cleaned by chloroform and immersed in 5 M ethanolamine-hydrocholide in dimethyl sulfoxide (Sigma Aldrich) overnight. After washing with phosphate-buffered saline (PBS), cantilevers were immersed in 25 mM BS3 (bis[sulfosuccinimidyl] suberate; Pierce) for 30 min. After washing again with PBS, cantilevers were then immerged in 1 mg/ml poly-L-lysine for 30 min. All steps were done at room temperature. Functionalized cantilevers were air-dried and kept in 4°C until use. CFSM was performed on the MFP3D-BIO atomic force microscope (AFM, Asylum

Research) to measure the adhesive interactions with films maintained at pH 9 overnight and immerged in pH 7 immediately before tested.

Functionalized cantilevers were calibrated before indenting samples to account for batch-to-batch variability in tip functionalization. Using PEO100 (large adhesion forces) and PAA100 (minimal adhesion forces), force maps of 16 × 16 resolution over 20×20 μm were performed so that adhesive forces could be internally calibrated to each cantilever. Maximum adhesion forces for PEO100 and PAA100 were 10 and 2 nN, respectively. After calibration, the adhesive forces lower than one standard deviation from the average adhesion of the PEO100 map were set as non-adhesive regions. Those low tip interaction forces also overlapped with the maximum forces measured from the PAA100 map, and thus we determined this threshold for to be an acceptable definition of adhesion versus non-specific adhesion (see Supplemental Fig. 3). Using this definition, a distribution of the normalized adhesion forces was generated from all images (Fig. 3, top right) and the ratio of adhesive to total area was determined for each image, i.e. the PEO area fraction (Fig. 3, bottom left). A nano-domain, calculated by ImageJ software, was defined as at least 4 adjacent data points higher or lower than the threshold in the case of PEO or PAA domains, respectively (Fig. 3, bottom right). Force curves were generated with a 2 μm/s approach velocity, 1 nN surface trigger force, 3 second dwell time between tip indentation and retraction, and a 32 × 32 scan resolution over 2 × 2 and 20 × 20 μm scan areas (resolutions of 62.5 and 625 nm, respectively). Typical force curves for interactions without (top) and with adhesion (bottom) are shown in Supplemental Fig. 2b. Area fraction and domain surface areas were determined using ImageJ software from a thresholding scheme.

Fibronectin Matrix Preparation and Imaging

NIH3T3 fibroblasts were cultured at 100% confluence on 25 mm coverslips for 7 days in growth media (89% Dulbecco's Minimum Eagle's Media, 1% Penicillin-Streptomycin, and 10% fetal bovine serum). The serum used to culture the fibroblasts was fibronectin-depleted by passing the serum first over a gelatin column prior to use in the media. All serum was confirmed to be fibronectin free by silver staining and western blotting methods. Growth media was then spiked with human fibronectin to a concentration equal to what was depleted from the serum originally.

Once the fibroblasts had been grown for 7 days, the cells were lysed and removed by a previously described method that maintains the fibronectin network intact (1). The

rabbit polyclonal R457 antibody was then used to stain all fibronectin fibrils (2) while cell adhesion sites were detected specifically on the human fibronectin that had been incorporated into the network during fibroblast-mediated assembly by the hFN7.1 antibody (Developmental Studies Hybridoma Bank; Iowa City, IA). Images were acquired on a BD CARV II spinning disc confocal fluorescent microscope using 20x objective. Image analysis was performed with NIH ImageJ software.

Preparation of films

As indicated, 2D films were characterized in place of 3D foams. 2D film preparation employs the same compositions and oil:aqueous phase volume fractions as the 3D foams (Table 1). Briefly, the aqueous phase was added to a glass vial using a syringe. The oil phase (monomer + block copolymer surfactant) was then carefully layered on top of the aqueous phase to maintain phase separation. The copolymer mixtures were then allowed to adsorb at the oil-water interface for 24 hours without agitation and then polymerized at 60 °C for 24 hours. The resulting films were washed extensively with deionized water, left in ethanol for 1 week to remove unreacted monomers, and dried before analysis.

SEM and Image analysis

Scaffold morphologies were characterized by scanning electron microscopy. Fractured segments from various parts of the foam were mounted on an aluminium stub with a sticky carbon pad. Samples were gold coated (approx 15-20 nm) using an Emscope SC 500 A sputter coater unit and viewed with an FEI Inspect F field emission gun scanning electron microscope. Samples were viewed at an accelerating voltage of 5 kV and a spot size of 3.0.

Porosities of the scaffolds were measured from SEM micrographs using the image analysis software Image J (NIH Image). A random selection of 100 voids were measured from several micrographs of the same foam to obtain a more representative measurements. The assumption that the fractures of the segments exactly bisect the voids is made, which means that the measured values are all underestimates of the true value. Therefore a statistical correction is introduced (3). This was done by evaluating the average of the ratio R/r, where R is the equatorial void diameter and r

is the measured diameter on the micrograph (see figure). The statistical factor is calculated using the following formula:

$$h^2 = R^2 - r^2 \quad (1)$$

Where the probability of the sectioning takes place at a distance given by h, from the centre of the void is the same for all values of h. This means that the average probability h is R/2. By substituting this in the above equation we get $R/r = 2^{(1/3)}$, which is the statistical correction. By multiplying this number to the measured diameters, a more representative value is obtained. Surface roughness of 3D foams was measured by image analysis using Image J.

Micro-Computational Tomography

To evaluate the porosity of the scaffolds micro CT analysis was performed using SkyScan 1172 high-resolution scanner. Scaffolds with a diameter of 1.4 cm and height of 2-3 mm were used. The applied X-Ray voltage was 35 kV and no filter was used. The pixel size (resolution) was 1.7 μm. A total of 1400 scans were achieved and reconstructed using the SkyScan micro-CT analysis software package. Circular regions of interest (diameter = 1.25 mm, height = 0.6 mm) were chosen and 3D models were generated using the adaptive rendering algorithm available in the SkyScan software which also calculated scaffold open porosity, closed porosity, volume and pore strut thickness.

X-ray Photoelectron Spectroscopy (XPS)

Surface analysis was carried out with a Kratos Ultra DLD X-ray photoelectron spectrometer. A monochromated Al Kα X-ray source at a power of 150 W was used. The spot size was 300 μm by 700 μm. The pressure in the main ultra-high vacuum chamber was maintained below 1×10^{-8} mbar for all samples. As the polymers analyzed here are electrical insulators, charge neutralization was required to prevent positive charge build up on the substrate's surface; an electron flood gun was focused onto the sample to compensate for the positive charging effect. High-resolution spectra of the elemental core level C 1s were also completed to assess surface carbon. The parameters used were a binding energy range of 275 to 300 *eV,* a pass energy of 20 *eV* and a step interval of 0.1 *eV.* All data collected was then analyzed using CasaXPS software peaks were again fitted by removing unwanted background using

CasaXPS software. Asymmetry of the peaks was fixed at zero and the position of each peak was fixed relative to the hydrocarbon peak. After initial rough automatic peak fitting, the carbonyl peak was then moved slightly to obtain a good fit of the C 1s linescan. Subsequent carbon functional group peaks were then calculated from the total of the C 1s peak to give the carbon environment composition.

Contact Angle Measurements

Scaffold hydrophobicity was ascertained by determining the water contact angle with the substrate using a Ramé-Hart contact angle goniometer. 2 - 4 μL droplets of neutral (pH 7) and acidic (pH 2.2) deionized water (pH 7) were used. Measurements were made in triplicate for each sample.

Topographical imaging by AFM

Tapping mode atomic force microscopy (TM-AFM) was applied to determine height images of 2D polymer surfaces. All samples were examined in distilled water at pH 7 at room temperature on MFP3D-BIO atomic force microscope (Asylum Research; Santa Barbara, CA) with acoustic and vibration isolation. A gold-coated, pyramidal AFM tip (TR400PB; Olympus; Center Valley, PA) with a nominal radius of 40 nm, half-angle of 35°, and spring constant of 20pN/nm was then used in TF-AFM to image the surfaces. TM-AFM images were made with a set point to resting amplitude ratio (A/A_0) of 0.5, $A_0 \sim 1$ V, drive frequency of ~22 kHz for the tapping, scan rate at 0.5 Hz, and a 512×512 image resolution for scan sizes of 2×2 and 20×20 μm^2. Surface roughness was determined by Igor-pro software (Wavemetrics; Portland, OR) following the formula of height root mean squared:

$$RMS = \sqrt{\Sigma y^2 / n} \qquad (2).$$

Chemical Force Spectroscopy Mapping (CFSM)

described below.

Preparation of 3D scaffolds for cell culture

For 3D experiments, circular scaffolds off all compositions (1.2 cm in diameter and 0.3-0.5 cm in height) were first sterilised in 70% ethanol overnight and then washed with PBS three times. Scaffolds were then pre-wet prior to seeding at a density of 10^5

cells per scaffold in total volume of 50 µl to allow maximum cell attachment. Cells were then incubated at 37 ℃ for 1 hour before media (1 ml/well) was added. Media was replenished every 2 days. Note that no soluble induction factors were used in any experiments.

Protein adsorption and detection on films

Cylindrical foams of ~12 mm in diameter and ~4 mm thick were placed in a 24-well tissue culture plate and submerged in the culture media. After 2 hrs in culture media, foams were rinsed with PBS 3 times. Absorbed protein was dissociated with mRIPA buffer (1% Triton-X, 1% sodium deoxycholate, 0.1% SDS, 10% glycerol, 0.5 mM $MgCl_2$, 150 mM NaCl, 50 mM HEPES pH 7.5) plus the protease inhibitor phenylmethanesulfonylfluoride overnight. Protein concentration within the lysates was measured by the bicinchoninic acid (BCA) assay (Pierce).

Micro- and nano-scale protein clustering was visualized by immunofluorescence and CFSM. 100 µg/ml rat plasma fibronectin in PBS was incubated with film samples for 2 hours at 37°C and washed with PBS. For those samples observed by confocal microscopy, films were incubated with the R457 fibronectin antibody (5, 6) in 2% BSA in PBS (1:500) for an hour at 37°C. After rinsing with PBS, samples were incubated with an Alexa Fluor® 647-conjugated secondary antibody in 2% BSA in PBS (1:1000) for an hour at 37°C. A spinning disc confocal fluorescent microscope (BD CARV II, BD Bioscience) and 60X water-immersion objective was used for image acquisition. For those samples observed by CFSM, the R457 fibronectin antibody was bound to an AFM tip using the same chemistry as PLL. All CFSM parameters were described as the same as the previous paragraph. The scan area was set to 2 × 2 µm with 62.5 nm resolution. The specific interaction between the antibody and fibronectin was identified from the rupture force-loading rate graph, providing the threshold as 300 pN, which we have previously established for fibronectin (4). Again, area fraction and domain surface areas were determined using ImageJ software.

Cell Culture

Human embryonic stem-mesoderm progenitor (hES-MP) cells (Cellartis, UK) were cultured in basal alpha modified-modified eagle's medium (Gibco, UK) supplemented

with 10% FBS, 1% penicillin/streptomycin and 0.25% fungizone and maintained at 37 C and 5% CO_2. Media was replenished every 2-3 days and passaged at 70-80% confluency. Human bone marrow derived- mesenchymal stem cells (hBMSC) (Lonza) were cultured in growth media (20% fetal bovine serum, 79% Dulbecco's Minimum Eagle's Media, and 1% Penicillin-Streptomycin). Passages between 5-10 were used for all experiments and all cell sources.

MTS assay

Cell viability was examined by the MTS assay on both the hES-MP cells and hBMSC cultured on scaffolds for 7 days. Experiments were carried out in triplicate. Growth media was removed and treated with 600 µl of MTS/PMS solution (Promega, UK) in culture medium at the final concentration of 333 µg/ml and 25 µM respectively were added to each well. Cells were then incubated for 3 hours at 37^{o}C to let the substrate react with the dehydrogenase enzyme present in mitochondria. 100 µl of the resulting solution was transferred to flat-bottomed 96-well plates in duplicate and the absorbance was measured at 490 nm. Number of viable cells on the scaffolds was calculated from a calibration curve using cell densities ranging from 0 to $2.5x10^4$ cells. Student's t-test was performed to check for statistical differences in viability across the different surface chemistries.

Immunofluorescent staining

hES-MP and hBMSCs cells were cultured on the foams and films for 48 hours and 7 days. The materials were washed with PBS, fixed in 3.7% paraformaldehyde for 30 min, washed with PBS twice before 0.2% Triton-X100 was added to each combination for 15 minutes. The materials were washed with the solution A (250mM $MgCl_2$ in PBS) twice and then incubated in Texas Red- or Rhodamine-labeled phalloidin and 4',6-diamidino-2-phenylindole (DAPI) in PBS (Invitrogen) at room temperature for 1 hour before washing again in PBS. For adipogenic staining, PPAR-γ2 antibody (1:100 dilution, Santa Cruz Biotech) was incubated overnight in 4°C. Materials were mounted and visualized under either an inverted Zeiss LSM 510 META confocal laser-scanning or BD CARV II spinning disc confocal fluorescent microscope using 10x and 20x objective lenses, respectively. Microscopy was performed in a sequential multiple channel fluorescence scanning mode. The

polystyrene-based foams were auto-fluorescent at 488 nm in the green channel. Image analysis was performed with NIH ImageJ software.

Supplemental Figure Legends

Supplemental Fig. 1: Heterogeneous adhesion in a Fibronectin Matrix. (a) A cell-derived fibronectin matrix (red) was dual labeled with 1 μm diameter beads (green) bound to an antibody that recognizes the fibronectin region containing the cell binding site. Beads were used to directly probe the region as they may approximate filopodia and identify cell accessible regions. Closed arrowheads indicate beads that were on top of fibrils (yellow coloured) and open arrowheads indicate beads that were adjacent and bound to matrix fibrils (green coloured). The representative image here is a confocal section through the matrix just below its surface. Scale bar is 20 μm. (b) Quantification of the minimum bead-to-bead distance for fibronectin matrix.

Supplemental Fig. 2: **Surface Characterization.** RMS surface roughness of foams a) determined from SEM images in Fig. 1a and b) films from 2 x 2 μm scans from CFSM. Porosity and interconnectivity (c) values obtained from micro-CT scans. DVB matrix volume represents the total volume occupied by the HIPE matrix in the selected region of interest. Atomic percentages from XPS plots at the indicated binding energies for d) PEO100 and e) PAA100 foams.

Supplemental Fig. 3: Chemical Force Spectroscopy Mapping (CFSM). a) Probe for CFSM which is covalently bond to bis[sulfosuccinimidyl] substrate (BS3) and poly-l-lysine (PLL). b) Force curves with (bottom) and without (top) interaction between probe and surface. Note the adhesion force indicated during retraction (black line) versus indentation (grey line).

Supplemental Fig. 4: Force Histograms. An adhesive force threshold was estimated using the difference between PAA and PEO films by normalizing the distribution curves for PAA100 (light grey bars) and PEO100 (dark grey bars). Virtually all events observed from PAA100 were below one standard deviation below the PEO100 film average; the grey box illustrates this threshold.

Supplemental Fig. 5: Adhesion Maps. Examples of CFSM images from 20 × 20 μm scans corresponding to the indicated formulations.

Supplemental Fig. 6: Domain Spacing. a) The distribution of inter-domain spacing for PAA domains in a sample PEO75 film. b) Minimum distance between PAA domains as a function of bulk PEO composition. [a] and [b] indicate $p < 0.05$ and 0.01 versus all data not in the group, respectively. c) Schematic relationship between inter-domain spacing and domain size. The dark grey arrow indicates the observed behaviour of PEO: with increasing PEO content, PEO domain size increases while inter-domain spacing of PAA domains decreases.

Supplemental Fig. 7: Foam Toxicity. Toxicity testing of scaffolds with the indicated copolymer mixtures compared to tissue culture plastic and polystyrene controls, i.e. Span 80. Cell viability was determined by MTS assay for cells seeded in plastic wells and treated with foam conditioned media.

Supplemental Fig. 8: 3D cell culture. a) SEM micrographs of hES-MPs cultured on a) PEO75 and PEO50 foams at day 7 showing integration with the scaffold and cells beginning to secrete its own matrix. b) Confocal Z-projection of hES-MPs at day 7 on PEO75 and PEO50 foams.

Supplemental References

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