Membrane Pore Spacing Can Modulate Endothelial Cell-Substrate and Cell-Cell Interactions

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SUPPORTING INFORMATION

Ultrathin Silicon Dioxide (SiO_2) Membrane Fabrication and Device Assembly

SiO_2 membranes were fabricated using conventional microfabrication techniques at the Rochester Institute of Technology Semiconductor Microfabrication Laboratory (SMFL).[1] Briefly, 300 nm of SiO_2 was deposited using plasma enhanced chemical vapor deposition (PECVD) on double-side polished 150 mm diameter wafers. Wafers were backside patterned with a mask that results in 5.4 x 5.4 mm square dies with 2 x 2 mm windows when the wafer was through-etched. The oxide membrane was front-side patterned with an ASML PAS 5500/200 i-line stepper to create 0.5 μm and 3.0 μm pores in a hexagonal tiling pattern with a center-to-center spacing of two diameters (~23% porosity). The pores were patterned and aligned such that there was an approximately 100 micron non-porous exclusion zone at the edge of the suspended membrane. The oxide film was etched with a Drytek 482 Quad Etcher using reactive ion etching. We stabilized the film stress in a slightly tensile state through a 600 °C anneal in nitrogen. This tensile film is substantially more robust than the native film, enabling its use in routine cell culture. The wafer was through-etched from the backside using ethylenediamine pyrocatechol (EDP) in a custom fabricated one-sided heated etch cell as reported previously.[2] After etching, the wafer was cleaved into individual chips. In order to culture cells on the membranes, the chips were bonded to silicone gaskets using a handheld corona surface treatment wand (Nbond, Littleton, CO) as described previously.[3] The silicone gaskets were produced from defined thickness sheets of restricted grade silicone (Silicone Specialty Fabricators, Paso Robles, CA) that was cut using a digital craft cutter.[4] The silicone gaskets were used to help retain cells during seeding.

All substrates were pre-treated for 30 minutes with 1:100 dilution of Geltrex® (0.15 mg/mL concentration) that contains laminin, vitronectin and collagen IV. Substrates were rinsed with PBS immediately prior to seeding cells and were not permitted to air dry. Immunofluorescence confirmed the absence of fibronectin, but a relatively uniform presence of collagen IV on the surface.

Cell Culture

All cell culture and immunofluorescence reagents were purchased from Thermo Fisher (Carlsbad, CA) unless specified otherwise. Pooled HUVECs were cultured in M200 with GIBCO Large Vessel Endothelial Supplement (LVES). Cells were detached and subcultured per manufacturer's instructions using TrypLE. HUVEC media was exchanged every 2-3 days and cells were passaged at 80% confluence. HUVECs were used between passages 3-5.

For focal adhesion and fibronectin experiments, cells were seeded at a density of 800 per membrane. The intent was to achieve 5-10% coverage with minimal cell-cell contact. Cells were cultured for 24 hours, fixed permeabilized and stained as described below. Due to migration of HUVECs over the 24-hour period, fibronectin matrix assembly can appear widespread and suggest a much denser population of cells.

For ZO-1 staining, cells were seeded at 1600 per membrane (10-20% coverage). Cells were fed at 2 days and then fixed and permeabilized at 4 days as they reached confluence. Cells were stained as described below.

Immunofluorescence

Cells were fixed with 3.7% formaldehyde for 15 minutes, washed three times with PBS and then permeabilized with 0.1% Triton X-100 for 3 minutes. Cells were blocked with 1% BSA for 15 minutes and again washed with PBS.

For visualization of stress fibers and focal adhesions, cells were stained with DAPI (300 nM), 1:400 AlexaFluor 488 conjugated phalloidin and 1:100 eFluor570 conjugated anti-vinculin, Clone 7F9 (Affymetrix eBioscience, San Diego, CA).

For visualization of fibronectin fibrillogenesis the cells and substrates were stained with 1:100 AlexaFluor488 conjugated anti-fibronectin, Clone FN-3 (Affymetrix eBioscience, San Diego, CA).

For visualization of tight junctional protein ZO-1 the cells were stained with 1:100 AlexaFluor488 conjugated anti-ZO-1/TJP1, Clone ZO1-1A12 (Affymetrix eBioscience, San Diego, CA).

For visualization of focal adhesions, cells were permeabilized with 0.1% Triton X-100 for 30 seconds, washed twice with PBS, and then fixed with 3.7% formaldehyde for 15 minutes. Cells were blocked with 1% BSA for 15 minutes and again washed with PBS. Cells were stained with DAPI (300nM), 1:400 AlexaFluor 488 conjugated phalloidin, and 1:100 eFluor570 conjugated anti-vinculin Clone 7F9 (Affymetrix eBioscience, San Diego, CA).

Focal adhesions were imaged with 40x and 63x long working distance objectives. Fibronectin and ZO-1 were imaged with a 20x air objective. All imaged were collected on a Leica DMI6000 microscope (Leica Microsystems, Buffalo Grove, IL) using a Rolera em-c^2 camera (QImaging, Surrey, BC Canada).

Distinct focal adhesions were identified as punctate fluorescent (anti-vinculin) features that could be readily identified at 40x using the same exposure time and illumination on all substrates. Focal adhesions were typically elliptical and 2-5 microns in the major axis. Only isolated cells or cells with less than 10% cell-cell contact (as defined by perimeter path) were analyzed for focal adhesions. Total sample size was greater than 20 for each substrate type collected from 3-4 independent membranes or substrates.

Statistical Comparisons

Comparisons between spread area were made using one-way ANOVA with a Tukey post hoc analysis (Figure S1). Comparisons between focal adhesions at 24 hours were made using one-way ANOVA with a Tukey post hoc analysis in Minitab (Figure 2). Percent of cells with distinct focal adhesions is from the sum total across the 3-4 independent membranes or substrates of each type ($n > 20$). Normalized radial histograms of stress fiber alignment (Figure 5) show the distribution of aligned cells (n = 32-37 for each substrate from 3-4 independent substrates for each type). Student's unpaired t-test comparisons were made between 1-day and 6-day focal adhesion measurements on non-porous, 0.5 μm and 3.0 μm pore glass membranes (Figure S2). Statistical comparisons of fibronectin intensity (Figure 3 B) and fibril length (Figure 3 C and D) from the different substrate types were each performed *via* one-way ANOVA with a Tukey post hoc analysis ($\alpha = 0.05$).

Cell Spread Area

For cell spreading experiments, HUVEC were seeded at a density of 500 cells/membrane, which was found to minimize the degree of cell-cell contact after 24 hours. After 1, 4, or 24 hours, cells were fixed, permabilized, and stained for nuclei and F-actin cytoskeleton. Four 10x tiled images covering each 2 x 2 mm membrane were analyzed, overlapping regions were excluded. The borders of the cells were identified by thresholding the F-actin cytoskeleton images and then converting to a binary image. The spread-area of each cell was found using the measure tool on each binary object. The DAPI channel was used to confirm that each binary object had a single nucleus and that the cell was not undergoing mitosis. More than 50 cells were analyzed for each substrate type. Box and Whisker plots were created in Microsoft Excel (Seattle, WA) using a custom script. The box represents Q1-Q3 with the median identified as a line. The Whiskers represents Q3 + 1.5 IQR and Q1 – 1.5 IQR. No significant differences were found using one-way ANOVA with a Tukey post hoc analysis.

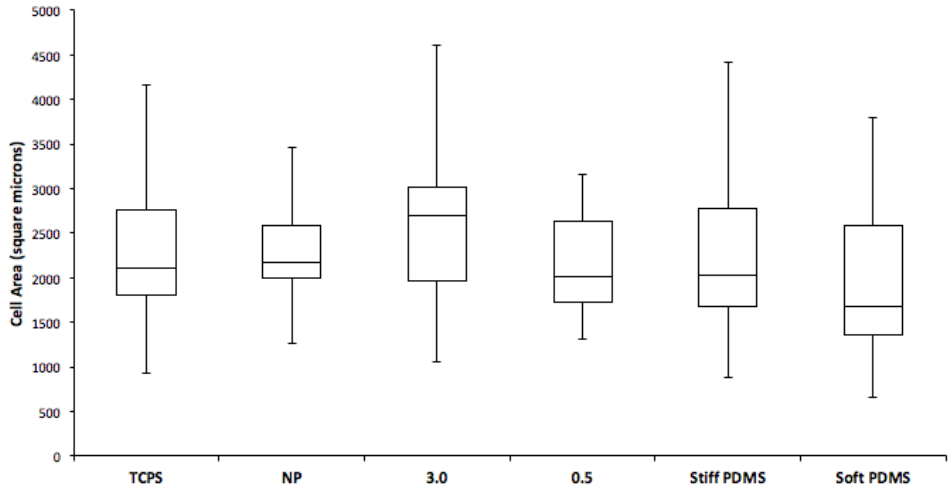

Figure S1. Cell-spread area tissue culture treated polystyrene (TCPS), non-porous, 3.0 μm and 0.5 μm pore diameter SiO_2 membranes after 24 hours. The box plots represent median and IQR of spread area (whiskers are the highest and lowest datum within +/- 1.5 IQR).

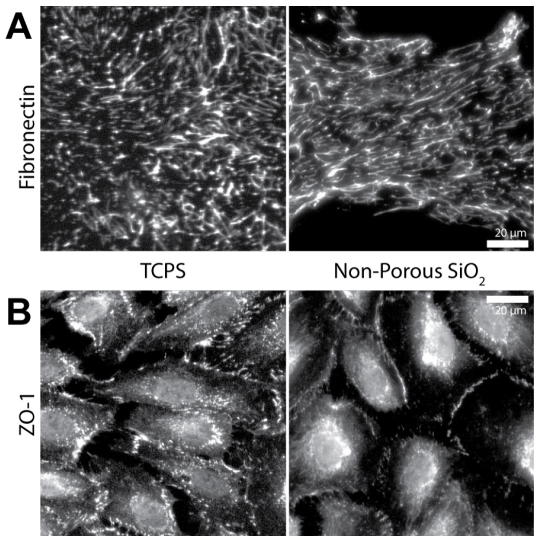

Figure S2. Fluorescent images of Fibronectin and ZO-1 on TCPS and non-porous SiO_2 membranes are similar in morphology and intensity.

Fibronectin Intensity Analysis

For each 10x image of fibronectin fibrils, five 100 µm x 100 µm regions were randomly selected from cellular regions and a 50 µm x 50 µm region was arbitrarily chosen from the non-cellular regions as background. The average intensity of each selected region was background subtracted and pooled together to produce a sample size of at least fifteen for each substrate type collected from multiple (3 or 4) independent substrates. Statistical comparisons of the fibronectin intensities from the different substrate types were performed *via* one-way ANOVA with a Tukey post hoc analysis ($\alpha = 0.05$).

Fibronectin Fibril Length Analysis

For each 40x image of fibronectin fibrils, the edges of the fibronectin fibers were detected using the Laplacian of Gaussian method in MATLAB. Described briefly, the 2nd spatial derivative of image intensity was calculated and the edge was defined at wherever there is a zero-crossing. The half perimeter of the edge was used as the approximation of fiber length. We quantified the total number of long fibers (≥ 5 µm and ≥ 10 µm) across multiple samples (n = 3-4) for each substrate. The numbers of long fibers from the different substrate types were compared *via* one-way ANOVA with a Tukey post hoc analysis ($\alpha = 0.05$).

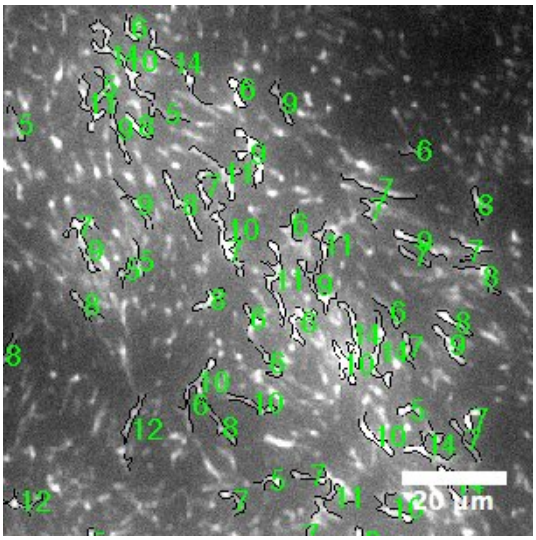

Figure S3. Example of fibronectin fibril length analysis using the Laplacian of Gaussian method on a non-porous SiO_2 membrane. Numbers are the estimated lengths of each detected fibril ≥ 5 µm.

Cell Alignment Analysis

We measured the orientation of a cell's primary actin stress fibers in ImageJ (Figure S5) and categorized the cell as aligned in either ***a*** (-7.5° to 7.5°), between ***a*** and ***b*** (7.5° to 22.5°), ***b*** (22.5° to 37.5°) or between ***b*** and ***a*** (37.5° to 52.5°). All porous membrane images were oriented with the ***a*** direction as shown in Figure S5. Non-porous images were not rotated and should represent a random distribution result. Due to the three axes of symmetry, all angle data was mapped to the primary directions listed above. The sum total within each direction was used to create the radial histograms in Figure 5.

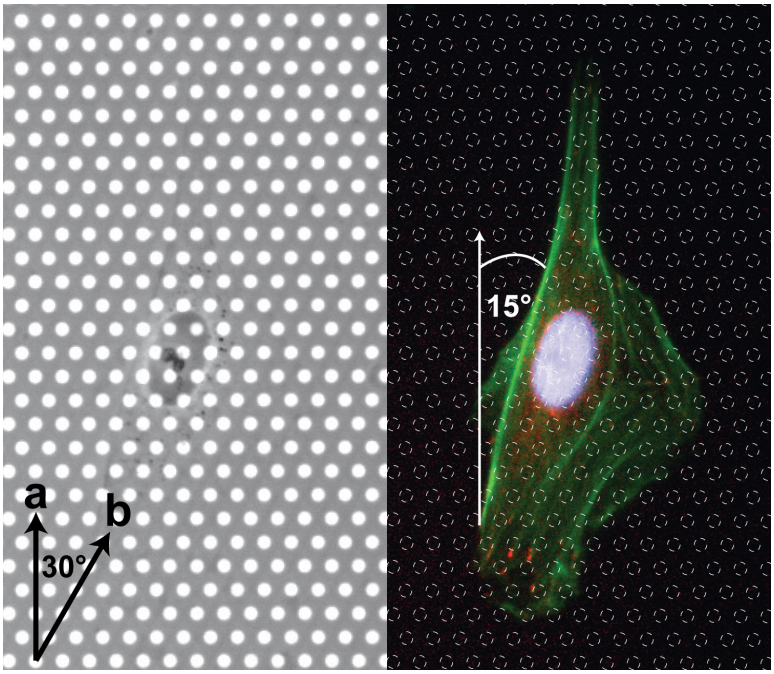

Figure S4. Phase contrast and fluorescence microscopy images of a cell on a SiO_2 show how alignment of F-actin was measured. This representative image also shows that punctate focal adhesions do not significantly overlap pores at 24 hours.

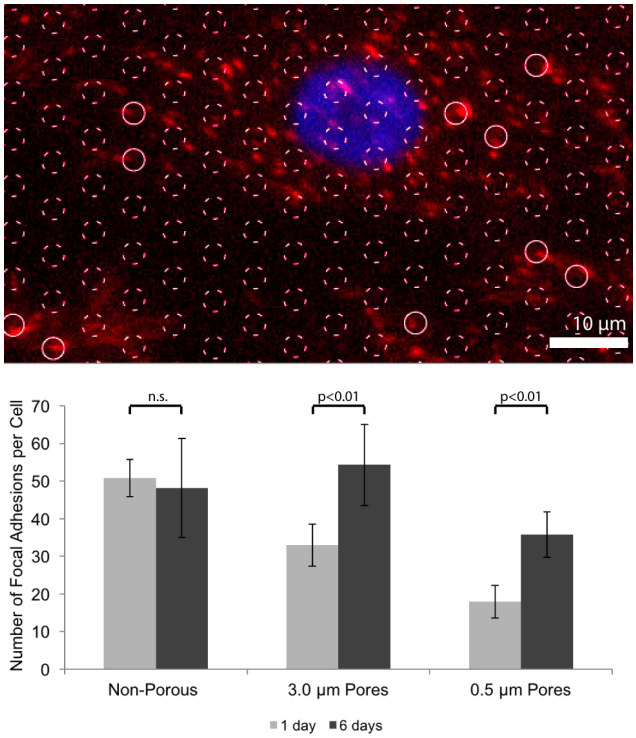

Figure S5. Fluorescent image of a cell on a 3.0 µm pore diameter SiO_2 membrane after 6 days stained for focal adhesions (anti-vinculin, red) and the nucleus (DAPI, blue). White outlines identify locations of a pore. Solid circles indicate where focal adhesions overlap pores. Bottom: Quantification of the number of focal adhesions after 1 day and 6 days ($n > 20$ for each substrate; mean ± standard deviation; Student's unpaired t-test comparisons).

References

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(2) Striemer, C. C.; Gaborski, T. R.; McGrath, J. L.; Fauchet, P. M. Charge- and size-based separation of macromolecules using ultrathin silicon membranes. Nature 2007, 445 (7129), 749–753 DOI: 10.1038/nature05532.

(3) Yang, C.; Wang, W.; Li, Z. Optimization of corona-triggered PDMS-PDMS bonding method; 2009; pp 319–322.

(4) Yuen, P. K.; Goral, V. N. Low-cost rapid prototyping of flexible microfluidic devices using a desktop digital craft cutter. Lab Chip 2010, 10 (3), 384–387 DOI: 10.1039/b918089c.