APPENDIX LITHOGRAPHIC PATTERNING OF INTRINSICALLY PHOTOREACTIVE CELL-ADHESIVE PROTEINS

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A.1 Introduction

Control of the spatial arrangement of proteins on surfaces is essential in a number of emerging biotechnologies. Defining the location of specific proteins on the micro- or nanoscale improves the quality of protein microarrays, increases the sensitivity of biosensors,[1,2] and allows tissue engineering scaffolds to organize multiple cell types.[3] Patterning is also a powerful tool in cell biology, where cell arrays are used to elucidate key factors that mediate migration, proliferation, and cell-cell interactions.[4-6] Although photolithography holds a preeminent place as a patterning method in the microelectronics industry, optical lithography of proteins has been hampered by the need either to use traditional chemical photoresists or to modify proteins chemically by attachment of photoreactive functional groups; both methods can compromise protein function.[7]

Production of a protein "photoresist" without the need for post-translational chemical modification would require an intrinsically photoreactive

protein. Recently, the incorporation of photoreactive, non-canonical amino acids into proteins has been reported via both site-specific[8,9] and residue-specific techniques.[10] Here we describe the microbial expression of artificial proteins bearing the photosensitive non-canonical amino acid *para*-azidophenylalanine (*p*N_3Phe). The recombinant proteins, designated artificial extracellular matrix proteins with aryl azides (aE-N_3), belong to a family of engineered proteins designed to exhibit mechanical properties similar to those of native elastins[11] and to support adhesion of endothelial cells through cell-binding domains (CS5 or RGD) derived from fibronectin (Figure A.1A).[12] These proteins can be crosslinked efficiently upon irradiation at 365 nm. The physical properties of the crosslinked films can be tuned by changing the extent of *p*N_3Phe incorporation, which is accomplished simply by changing the concentration of the non-canonical amino acid in the expression medium. Thin films of aE-N_3 proteins can be patterned on surfaces via simple photolithographic techniques. We demonstrate the utility of the method by creating cell arrays through selective endothelial cell attachment to lithographically prepared protein patterns.

A.2 Results and discussion

Preparation of aE-N_3 proteins containing the CS5 cell binding domain was accomplished through residue-specific incorporation of *p*N_3Phe in *E. coli*. This method of incorporation relies on competitive activation of phenylalanine (Phe) and *p*N_3Phe by the phenylalanyl-tRNA synthetase (PheRS), the enzyme responsible for charging Phe to its cognate tRNA.[13] The PheRS used for this

study was a previously characterized mutant with relaxed substrate specificity.[13] Proteins were expressed in a Phe-auxotrophic *E. coli* strain grown in cultures supplemented with *p*N$_3$Phe and purified by taking advantage of the temperature-dependent phase behavior of proteins with elastin-like repeats.[14] Incorporation efficiency was determined by integration of the aromatic proton signals in the ^{1}H NMR spectra of the purified proteins (Figure A.4); the extent of Phe replacement varied from 13% to 53%, depending on the concentration of *p*N$_3$Phe in the expression medium (see Methods section below; Figure A.5).

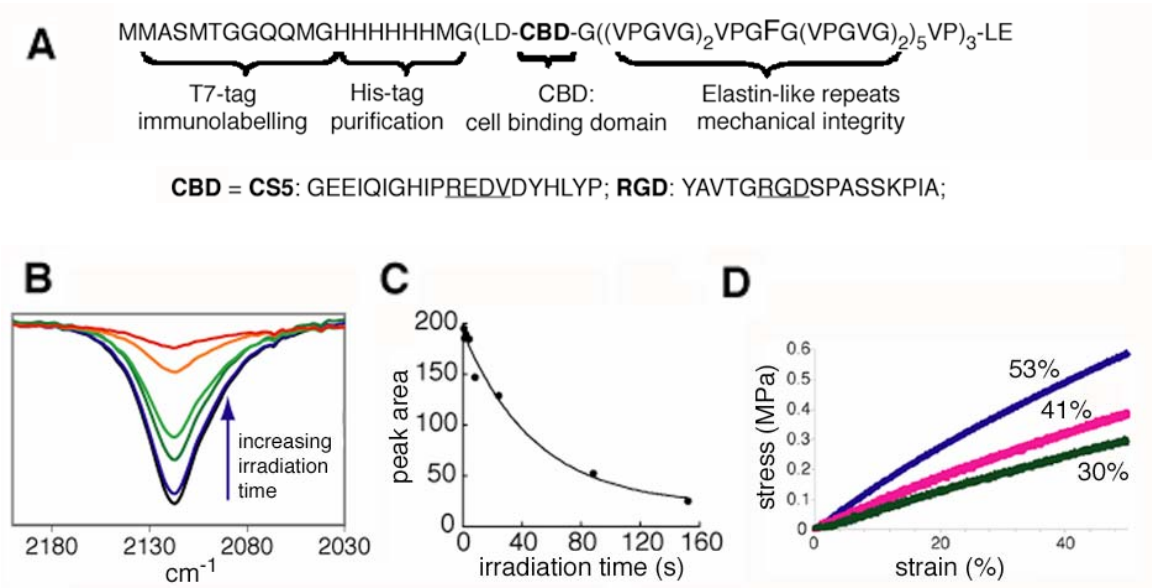

Figure A.1 Response of aE-N$_3$ to irradiation. (A) Primary sequence of aE-N$_3$; the CS5 variant was used in all studies described herein. (B) FTIR demonstrates loss of the characteristic azide asymmetric stretch as a function of time of irradiation of aE-N$_3$ films. (C) Peak area vs. irradiation time yields a first order decay with $t_{1/2}$=34 sec. (D) Uniaxial tensile testing of irradiated mold-cast films of aE-N$_3$ with differing *p*N$_3$Phe contents; modulus increases with increasing concentration of photoreactive side chains.

Understanding the response of the photoreactive protein to irradiation is crucial for high-resolution pattern formation. We measured the rate of azide decomposition under irradiation by monitoring loss of the characteristic infrared (IR) asymmetric stretch at 2130 cm^{-1} (Figure A.1B).[15] Measurements were performed on thin films of aE-N_3 spin coated directly onto zinc selenide wafers and irradiated using a Karl Suss contact aligner filtered to 365 nm in constant intensity (7 mW/cm^2) mode, with a quartz wafer in place of the mask. Azide loss under these conditions was rapid, following first-order kinetics with a half-life of 34 seconds (Figure A.1C). It is noteworthy that none of the other infrared bands were noticeably altered, indicating that irradiation under the conditions used here activates the aryl azide without substantial modification of any of the canonical amino acids. This is as expected given that none of the natural 20 amino acids absorb above 310 nm.[16]

Elastic moduli of irradiated aE-N_3 films were determined by uniaxial tensile testing under simulated physiological conditions (Figure A.1D). As expected, the elastic modulus correlated with the extent of *p*N_3Phe incorporation. Irradiated aE-N_3 films in which 30, 41, or 53% of the encoded Phe residues were replaced by *p*N_3Phe yielded elastic moduli of 0.53 ± 0.10, 0.94 ± 0.09, and 1.39 ± 0.09 MPa, respectively, which are in the range of native elastin (0.3 – 0.6 MPa). Replacement of less than 20% of the encoded Phe residues gave films that were too weak to test, and films made without *p*N_3Phe yielded no evidence of crosslinking. The fact that modulus can be controlled simply by changing the *p*N_3Phe concentration in the expression medium is an attractive feature of the

method, as recent work has highlighted the role of mechanical transduction mechanisms in mediating the physiology of adherent cells.[17,18]

To investigate the potential of photoreactive proteins as substrates for studies of cell adhesion and growth, we created patterns of adherent endothelial cells on proteins patterned by photolithography. Protein films created by spin coating 10% solutions of protein in dimethylsulfoxide directly on poly(ethylene glycol) (PEG)-coated glass coverslips were clear and homogeneous by optical microscopy. Protein films were dried at 50°C for 5 minutes and subsequently irradiated for 30 seconds at 365 nm through a chrome-on-quartz mask using a Karl Suss contact aligner. Efficient stripping of the masked areas was accomplished by washing in mild aqueous detergent (0.05% aqueous sodium dodecylsulfate).

Fluorescence immunolabeling with anti-T7-tag antibody showed that the aE-N_3 protein was localized only within the irradiated areas of the pattern (Figure A.6). Films prepared from the protein lacking *p*N_3Phe formed no detectable patterns even after prolonged exposure times. Non-contact atomic force microscopy (AFM) of dried aE-N_3 patterns demonstrated uniform features (Figure A.7), which varied in height depending on the conditions used for spin coating. Films spun at 1400 and 2000 rpm were 84 and 4 nm thick. Protein patterns stored either dry or in aqueous solutions were stable for weeks.

To create cell arrays, human umbilical vein endothelial cells (HUVEC) were plated on aE-N_3 patterns in the absence of serum. After six hours of incubation, the medium was supplemented with 5% serum. After 24 hours of incubation,

HUVEC exhibited well-spread morphologies and had proliferated to confluence exclusively within the patterned areas (Figure A.2). HUVEC monolayers in the interior of the patterned regions were indistinguishable from monolayers grown on homogenous fibronectin coatings; however, cells positioned along the aE-N_3 pattern edges were elongated and oriented parallel to the pattern border, consistent with previous studies.[19]

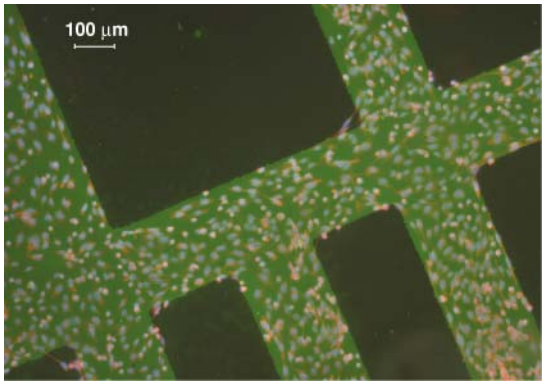

Figure A.2 Fluorescence microscopy of HUVEC attached to photopatterned aE-N_3. Immunostaining with anti-T7 (green) demonstrates colocalization of aE-N_3 protein and cells stained with nuclear stain (blue) and cytoskeletal stain (red).

HUVEC patterns were stable in serum for 48 hours after reaching confluence, consistent with known behavior of PEG coatings as cell-resistant backgrounds.[20] At longer times, cells began growing beyond the protein pattern at sites where the protein pattern formed right angles, presumably in concert with cellular synthesis and secretion of extracellular matrix proteins.

To establish the specificity of the observed adhesion, aE-N_3 proteins with sequence-scrambled CS5 and RGD cell binding domains were created, in which

the positions of just two adjacent amino acids were swapped. HUVEC spread well on uniformly photocrosslinked aE-N_3 films with RGD (RGD-N_3) but not on the scrambled version (RDG-N_3), seen in Figure A.3 below.

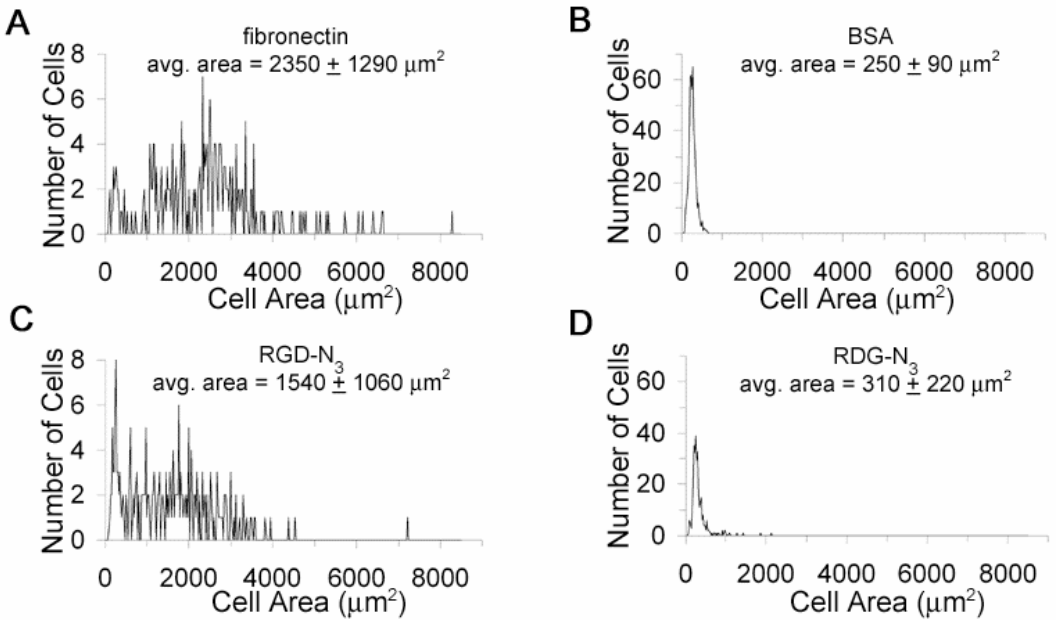

Figure A.3 HUVEC spread areas on (A) fibronectin (positive control), (B) BSA (negative control), (C) RGD-N_3, and (D) RDG-N_3 demonstrate the sequence-specificity of cell spreading.

The availability of intrinsically photoreactive proteins enables a facile new method for patterning of proteins. The technical simplicity of the method allows rapid production of samples with a wide variety of feature shapes and sizes, while permitting straightforward engineering of the elastic modulus of the crosslinked protein. The method is a promising approach to the study of adherent cells, providing control over mechanical properties, ligand-receptor inter-actions, and geometric shape. Applications in medical devices, tissue engineering, and array technologies are readily imagined.

A.3 Methods and additional figures

A.3.1 Cloning of aE protein constructs.

Synthetic oligonucleotides encoding the CS5 and RGD cell-binding domains, and their sequence-scrambled analogs "SC5" and "RDG", were annealed, phosphorylated, and ligated into pEC2[21] to produce pEC2-CS5, pEC2-SC5, pEC2-RGD, and pEC2-RDG. An oligonucleotide encoding the elastin-like repeat $(VPGVG)_2VPGFG(VPGVG)_2$ was similarly ligated into pUC19 (New England Biolabs) between *Eco*R1 and *Bam*H1. The elastin-like insert was cut out using *Ban*1 and self-ligated to form multimers. The multimerization mixture was ligated with *Ban1*-linearized pEC2-CS5, pEC2-SC5, pEC2-RGD, or pEC2-RDG. Transformants with the pentamer insert were selected, digested at *Xho*1 and *Sal*1, and ligated into a modified pET28a (Novagen). This step was repeated twice to obtain the final $[CBD(ELF)_5]_3$ construct under control of the T7 promoter. Finally, the *pheS** gene encoding the alpha subunit of the A294G mutant of *E. coli* phenylalanyl-tRNA synthetase was subcloned into the *Sph*I site from the pKSS vector kindly provided by Dr. Peter Kast[22] to produce pNS-CS5-ELF[23], pSM-SC5-ELF, pSM-RGD-ELF, and pSM-RDG-ELF.

A.3.2 Protein expression and purification.

The target protein was expressed using a phenylalanine auxotrophic derivative of *E. coli* strain designated AF-IQ[24] harboring either pNS-CS5-ELF, pSM-SC5-ELF, pSM-RGD-ELF, or pSM-RDG-ELF. To express proteins from

these strains, a culture was grown overnight in 2xYT medium and used to inoculate 1 L of M9AA medium supplemented with the antibiotics chloramphenicol and kanamycin. At an OD_{600} of 1.0, expression of target protein and T7 RNA polymerase was induced by adding 1 mM IPTG. After 10 additional minutes of growth, the cells were washed twice with 0.9% NaCl and resuspended in M9 medium containing 19 amino acids (excluding phenylalanine) to a final volume of 1 L. The cultures were supplemented with either 25 mg/L phenylalanine (positive control) or up to 250 mg solid *p*N_3Phe and grown for 4 h. Protein expression was monitored by SDS-PAGE and Western blotting with anti-T7 tag-horseradish peroxidase conjugate antibody (Amersham). Cell pellets, produced by spinning down (10,000×g, 10 min, 25°C) 1L of expression culture, were resuspended in 20 mL of TEN buffer (10 mM Tris, 1 mM EDTA, 0.1 M NaCl) by sonication and frozen. Frozen lysate was treated with 1 mM PMSF, and 10 μg/mL each of DNase and RNase was added. This mixture was agitated for 4 h at 37°C and centrifuged at a temperature above the expected LCST of the protein (22000×g, 60 min, 25°C). The target protein was extracted from the pellet into 4 M urea at 4°C. This suspension was clarified by centrifugation below the LCST (22000×g, 60 min, 2°C). The resulting supernatant was exhaustively dialyzed in cold (4°C) distilled water and subsequently lyophilized. Typical experiments yielded 40 mg of protein per liter of culture.

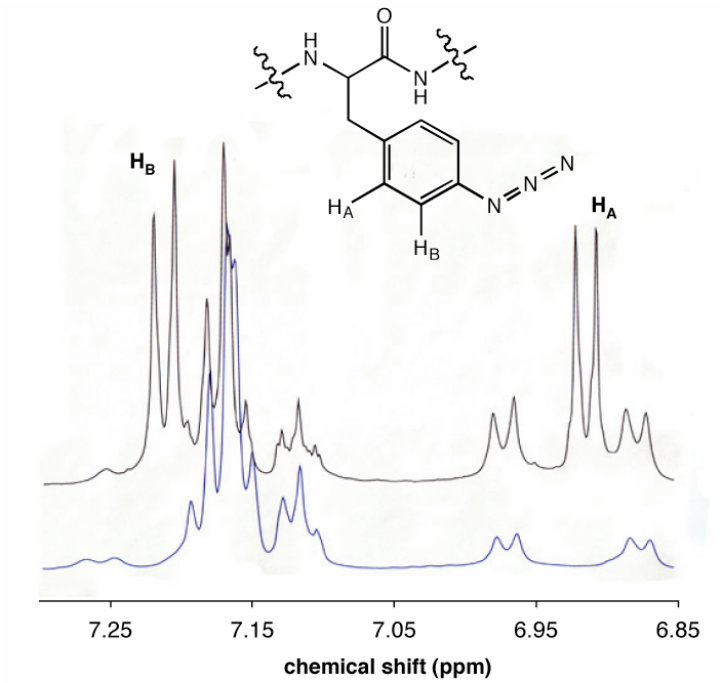

Figure A.4 ^{1}H NMR spectrum of aE-N$_3$ expressed in media supplemented with phenylalanine (bottom spectrum) or with 250 mg/L *p*N$_3$Phe (top spectrum). Spectra are identical except for two additional doublets in the top spectrum resulting from the aromatic protons of *p*N$_3$Phe; integration indicates 53% *p*N$_3$Phe incorporation. 600 MHz spectra were taken on 1 mM samples in DMSO-d$_6$ at 23°C.

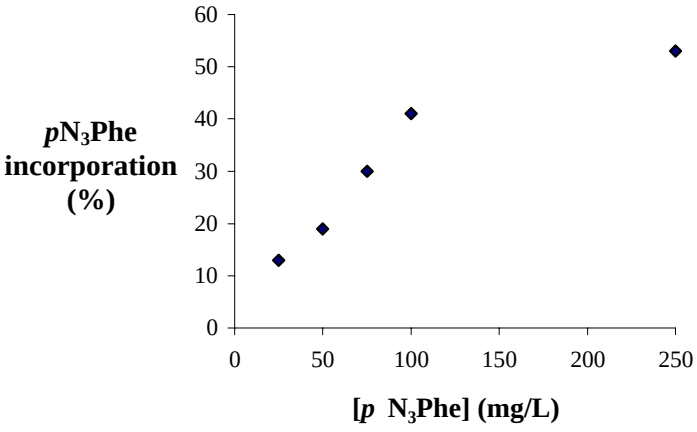

Figure A.5 Incorporation of *p*N$_3$Phe into aE-N$_3$ as a function of concentration in the expression medium.

A.3.4 Mechanical testing.

Samples were created in polytetrafluoroethylene molds by drying 10% aE-N_3 solutions in DMSO overnight at 50°C and were irradiated with a 100 W mercury lamp for 30 minutes. Samples were removed from the mold, swollen in 4°C water overnight to fully hydrate, cut into testing strips, and finally equilibrated in PBS at 37°C. Films were approximately 3 mm x 10 mm. Uniaxial tensile testing of equilibrated films was performed at 37°C in phosphate buffered saline at pH = 7.4 on an Instron Universal Testing Machine model 5542 with a 5 N load cell. Films were extended at a rate of 10% length/minute. Each protein sample was tested 3 to 6 times.

A.3.5 Photolithographic patterning of aE-N_3.

Glass coverslips (12 mm circles) were sonicated for 15 min in a saturated solution of potassium hydroxide in ethanol. Clean coverslips were rinsed under a stream of filtered (0.2 μm) doubly distilled water followed by a stream of filtered ethanol and dried briefly with canned air. Dried coverslips were immersed for 30 min in a freshly prepared solution of 1 mL 3-(trimethoxysilylpropyl)-diethylenetriamine (DETA), 2.5 mL acetic acid, and 46.5 mL methanol (2 % DETA). Aminated coverslips were rinsed under streams of water and then ethanol, dried with canned air, and cured for 4 h at 50°C. Cured coverslips were placed in a covered dish containing a reservoir of pyridine and were covered dropwise with a 100 mM solution in pyridine of mPEG-SPA-2000 (Nektar Therapeutics, MW = 2000 Da). After 12 h, the PEGylated coverslips were

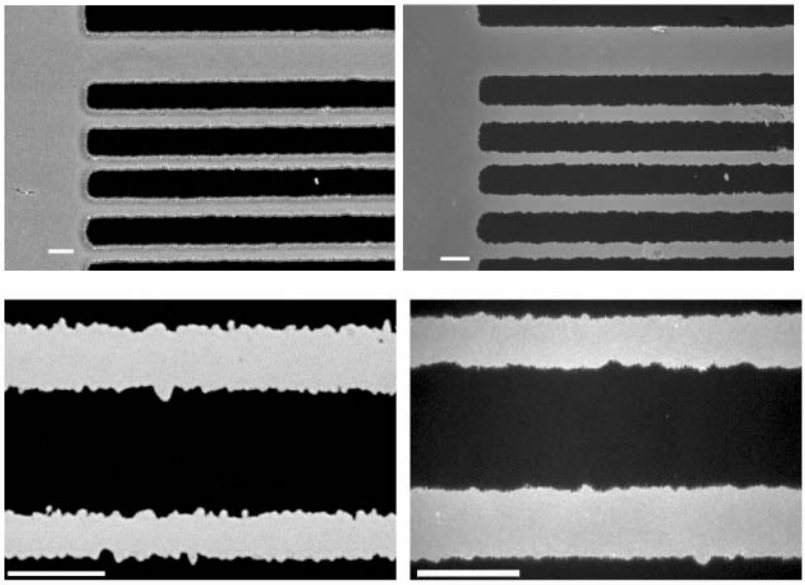

Figure A.6 Phase contrast microscopy images of the chrome mask used in photopatterning (left panels) compared with fluorescence microscopy images of aE-N_3 patterns (right panels). Scale bars represent 50 μm. In the top panels (lower magnification), the protein pattern could be visually matched to precisely the region on the mask that created it. The bottom panels (higher magnification) show two separate regions with features of similar sizes.

rinsed under streams of water and then ethanol, dried with canned air, and used immediately. PEGylated coverslips were covered dropwise with 8 μL of a 12.5 mg/mL solution of aE-N_3 in DMSO that had been centrifuged for 1 min at 14000 rpm to remove particulates. Coverslips were spun for 100 s at 1400 rpm on a Specialty Coating Systems model P-6000 spin coater. Protein-coated slides were dried at 50°C for 30 min. Exposure of protein to sunlight was avoided until protein photolithography was complete. Protein-coated slides were exposed for 30 s in a Karl Suss mask aligner (365 nm) under a chrome-on-quartz mask prepared by Dr. Michael Diehl by chrome deposition and stripping from a 3000 dpi transparency. Irradiated coverslips were washed overnight in 0.05% sodium

dodecyl sulfate to remove soluble protein from the masked regions and then rinsed for 6 h in filtered water.

A.3.6 Atomic force microscopy.

Topographical scans of aE-N_3 protein patterns on PEGylated coverslips were obtained with an AutoProbe M5 atomic force microscope (Park Scientific Instruments) in constant-force contact mode, using pyramidal tips (0.58 N/m, Veeco DNP-S). Imaging was performed in water: a glass slide was affixed to the back of the cantilever mount in the path of the laser, and the space between the sample and the slide was filled with water to provide a smooth and constant optical interface.

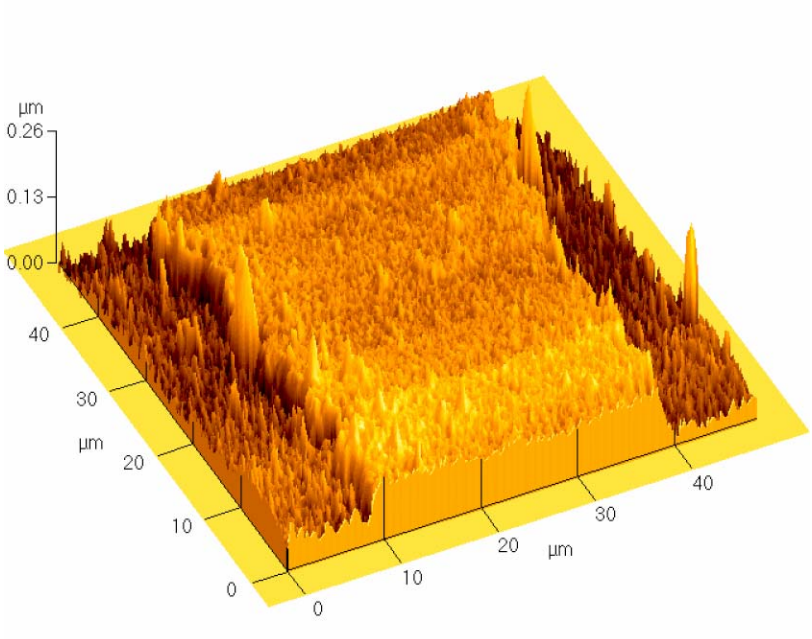

Figure A.7 AFM image of patterned aE-N_3. Full width of pattern feature (30 µm) is visible.

A.3.7 Cell culture.

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics and maintained in endothelial growth medium-2 (EGM-2, 2% serum, Clonetics, Walkersville, MD). Cells were kept in a humidified, 5% CO_2 environment at 37°C and passaged non-enzymatically using a 0.61 mM EDTA solution (Gibco, Grand Island, NY). Cells between passages 2 and 9 were used for all experiments.

A.3.8 Cell patterning.

Patterned coverslips were placed in a 24-well plate, and HUVEC were added at density of 2.0×10^5 cells/cm^2 in a total volume of 1 mL of endothelial cell serum-free defined medium (Cell Applications, San Diego, CA). Phase contrast pictures were taken on a Nikon Eclipse TE 300 microscope. Fluorescence pictures were taken on a Zeiss Axioplan II fluorescence microscope equipped with a monochrome Axiocam. To fix and fluorescently label cell patterns, the coverslips were placed in the wells of a 24-well plate, and each well was washed 3 times with 1 mL phosphate-buffered saline (PBS) before 0.5 mL ice-cold acetone was applied for exactly 1 min. The wells were again washed 3 times with 1 mL PBS before 0.5 mL of a 10% BSA solution was applied for 30 min at room temperature. After blocking, 0.25 μL of anti-T7 primary antibody (Novagen) was added and allowed to incubate at room temperature for at least 6 h. The wells were then washed three times with 1 mL of water for 5 min without agitation. A secondary antibody/phalloidin solution composed of 425 μL PBS, 50 μL

secondary antibody (Cy2-labeled anti-mouse, 0.5 mg/mL, Chemicon), and 25 μL Alexa Fluor 546 phalloidin (Molecular Probes) was incubated with the samples in the dark for 1 h. Labeled samples were washed with 1 mL of water for 10 minutes with agitation followed by 1 mL of water for 5 minutes without agitation. The samples were then incubated with 1 mL of DAPI solution (0.3 μM in PBS) for 5 minutes at room temperature. Samples were rinsed 3 times with 1 mL of water and mounted to a glass slide using filtered mounting solution of 1:1 PBS:glycerol and clear fingernail polish as sealant.

A.3.9 Cell spreading.

mPEG-SPA-5000 (100 mg) was dissolved in an excess of propargylamine (1 mL) and stirred overnight. The reaction mixture was poured into 200 mL ether, and the precipitate, alkynyl-mPEG-5000, was collected by centrifugation. ^{1}H-NMR ($CDCl_3$, 300 MHz): 2.21 (t, J = 2.54 Hz, $\underline{H}$-≡), 2.50 (t, J = 5.60, -O-CH_2-$C\underline{H}_2$-C(O)-), 3.36 (s, $C\underline{H}_3$-O-), 3.73 (t, J = 5.60, -O-$C\underline{H}_2$-CH_2-C(O)-), 4.03 (dd, J = 2.54, 2.85, -$C\underline{H}_2$-≡) yield: 50%, conversion: quantitative. RGD-N_3 and RDG-N_3 films were sub-quantitatively photocrosslinked for 60 s using an unfiltered Oriel 100W medium pressure mercury lamp. These films were immediately reacted with alkynyl-mPEG-SPA-5000 in a Cu(I)-catalyzed azide-alkyne [3+2] cycloaddition reaction in PBS buffer at pH 7.5 (200 μM $CuSO_4$, 400 μM tris(2-carboxyethyl)phosphine hydrochloride, 200 μM tris-triazole ligand, 200 μM alkynyl-mPEG-5000)[25]. PEGylated films were rinsed for 1 h in 1 mM EDTA, overnight in 0.05 % SDS, and for 2 h in distilled water. XPS data indicate the

average addition of 1 – 2 PEG molecules per protein chain (by comparing the C/N ratio before and after reaction).

For the fibronectin positive control, 1 mL of a 10 μg/mL fibronectin solution in PBS was adsorbed overnight in a 6-well plate at 4 °C. The wells were rinsed three times, blocked with a 0.2% BSA solution for 30 minutes at room temperature, and again rinsed three times. Coverslips with PEGylated aE-N_3 were adhered to the bottom of a BSA-blocked well by applying sterilized vacuum grease around the edge of the coverslip. Cells were resuspended in endothelial basal medium-2 (EBM-2, Clonetics) and seeded in a total volume of 3 mL per well at a density of 5.0 x 10^3 cells/cm^2. For quantification of spread area, pictures were obtained on a Nikon Eclipse TE 300 microscope. Cell areas were manually traced using ImageJ v. 1.33q (National Institutes of Health, Bethesda, MD). For each substrate, at least 200 cells total were examined in at least 4 independent experiments.

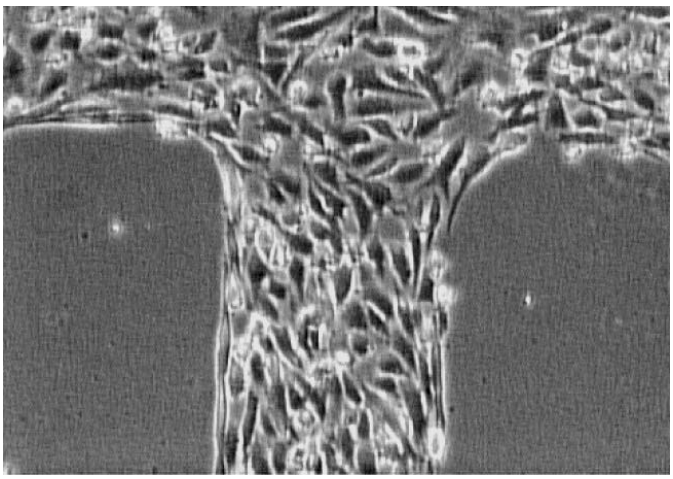

Figure A.8 Phase contrast microscopy of HUVEC attached to photo-patterned aE-N_3 (RGD variant).

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