C h a p t e r I V

LITHOGRAPHIC PATTERNING OF AN INTRINSICALLY PHOTOREACTIVE CELL-ADHESIVE PROTEIN*

IV.1 Abstract

This chapter describes a novel, simple method for the photolithographic patterning of cell-adhesive proteins. Intrinsically photoreactive proteins are synthesized in *Escherichia coli (E. coli)* through incorporation of the noncanonical photoactive amino acid *para*-azidophenylalanine. Upon ultraviolet irradiation at 365 nm, proteins form crosslinked films with elastic moduli that can be tuned by varying the concentration of photoreactive amino acid in the expression medium. Films of these proteins can be directly patterned using standard photolithographic techniques. Processing of irradiated films is performed under mild aqueous conditions, allowing these proteins to retain biological activity. We demonstrate the utility of this method of protein patterning by creating stable arrays of endothelial cells on an engineered protein "photoresist."

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IV.2 Introduction

Control of the spatial arrangement of proteins on surfaces is an essential factor 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 interact with 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, growth, and cell-cell interactions [4-6].

Although photolithography holds a preeminent place as a method to create patterns 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 photoactive functional groups; both methods can compromise protein function [7]. To circumvent these issues, new techniques such as microcontact printing and dip-pen nanolithography have emerged that allow direct placement of adhesive proteins or peptides on a surface [8, 9]. Despite these advances, the hallmarks of photolithography — high registry, throughput, and fidelity — are not yet matched by current methodologies.

Production of a protein "photoresist" without the need for post-translational chemical modification would require an intrinsically photoreactive protein. Recently, the incorporation of photoreactive noncanonical amino acids into proteins has been reported using both site-specific [10, 11] and residue-specific techniques [12]. Here we describe the microbial expression of artificial protein bearing the photosensitive noncanonical amino acid *para*-azidophenylalanine (*p*N_3Phe). Aryl azides have been previously shown

to mediate crosslinking efficiently in sensitive biological systems [13]. The recombinant proteins, designated artificial extracellular matrix proteins with aryl azides (aECM-N_3), belong to a family of engineered proteins designed to exhibit mechanical properties similar to those of native elastins [14], and to support adhesion of endothelial cells through a cell-binding domain derived from the CS5 region of fibronectin (Figure IV-1a) [15]. We demonstrate that these proteins can be efficiently crosslinked 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 noncanonical amino acid in the expression medium. Furthermore, thin films of such proteins can be patterned on surfaces using simple photolithographic techniques. We demonstrate the utility of the method by creating cell arrays through endothelial cell attachment to lithographically prepared protein patterns.

IV.3 Methods

IV.3.1 Protein expression

Samples of aECM-N_3 were expressed using a phenylalanine auxotrophic derivative of *E. coli* strain BL21(DE3), designated AF-IQ [16] and harboring the plasmid pNS-CS5-ELF [17], which encodes both the desired aECM sequence and the phes* gene for the A294G mutant *E. coli* PheRS. 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).

IV.3.2 Protein purification

The aECM-N_3 protein was purified using a modified temperature cycling procedure previously described for elastin-containing aECM constructs [18, 19]. Expression cultures were pelleted at room temperature (10000*g*, 10 min, 25°C), resuspended in 20 mL of TEN buffer (10 mM Tris, 1 mM EDTA, 0.1 M NaCl) by sonication with a probe sonicator, and frozen at –20°C. To frozen lysate, 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 then centrifuged at room temperature (22000*g*, 60 min, 25°C), which is above the expected lower critical solution temperature of aECM-N_3. The target protein was extracted from the pellet by stirring overnight in 4 M urea at 4°C. This suspension was centrifuged (22000*g*, 60 min, 2°C), and the supernatant was dialyzed in 12-14 kD MWCO dialysis tubing against cold (4°C) distilled water for three days with six water changes. Precipitate formed during dialysis was removed by centrifugation (22000*g*, 60 min, 2°C). The supernatant, which contained aECM-N_3, was lyophillized.

IV.3.3 ^{1}H-NMR of aECM-N_3

Purified aECM-N_3 proteins were lyophilized completely and dissolved in DMSO-d_6 at 40 mg/mL. Spectra were collected on a Varian Inova NMR spectrometer with proton acquisition at 599.69 MHz

IV.3.4 FTIR spectroscopy

Infrared spectra were taken using a Perkin Elmer 1600 series FT-IR. Protein samples were drop-cast onto zinc selenide wafers from DMSO. To measure azide decomposition kinetics, a 10% aECM-N_3 solution in DMSO was spun onto a ZnSe crystal at 2000 rpm for 100 seconds. The resulting film was dried for 2 minutes at 50°C. Films were irradiated at 365 nm through at transparent mask in a Karl Suss mask aligner to mimic the protein lithography conditions. Infrared spectra were taken of the irradiated film at various time points until the azide peak was no longer changing.

IV.3.5 Mechanical testing of bulk films

Flims were prepared by drying aECM-N_3 solutions (10 wt % in DMSO) at 50°C overnight in Teflon molds followed by irradiation for 30 s approximately 8 inches from an unfiltered Oriel 100W medium pressure mercury lamp. The crosslinked samples were removed from the mold and immersed in 4°C water overnight to fully hydrate. Swollen flims were cut into testing strips of approximately 3 mm x 10 mm.

Tensile testing was performed using an Instron device with a constant strain rate of 10 % per minute on films equilibrated in a bath of PBS buffer at 37°C. Elastic

modulus (E) was obtained from the slope of the steepest part of the initial plot. Each protein sample was tested 3-6 times.

IV.3.6 Preparation of aminated glass surfaces

Glass coverslips 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 ethanol, dried briefly with canned air, and then dried at 50°C for 30 min. 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 filtered water (2 % DETA in 5% acetic acid). Aminated coverslips were rinsed under a stream of water, rinsed under a stream of ethanol, dried with canned air, and cured for 4 h at 50°C.

IV.3.7 Preparation of PEG-modified slides

Aminated coverslips were placed in a covered dish containing a reservoir of pyridine. The coverslips were covered dropwise with a 100 mM solution of PEG-SPA-5000 (Nektar Therapeutics) in pyridine. After 12 h, the PEGylated coverslips were rinsed under a stream of water, rinsed under a stream of ethanol, dried with canned air, and used immediately.

IV.3.8 Spin coating of protein films

A 12.5 mg/mL solution of aECM-N_3 in DMSO was centrifuged for 1 min at 14000 rpm. This solution was added dropwise to cover the top of a PEGylated 12 mm circle glass coverslip (~8 μL). The coverslip was 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.

IV.3.9 Protein lithography

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 at the California Institute of Technology by chrome deposition and stripping from a 3000 dpi transparency [20]. Irradiated slides were washed overnight in 0.05 % sodium dodecyl sulfate (SDS) to remove uncrosslinked protein from the masked regions and then rinsed for 6 h in doubly distilled, filtered water.

IV.3.10Cell 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 3 and 8 were used for all experiments.

All coverslips were sterilized by immersion in a 75% aqueous ethanol solution for at least 2 min and then dried using canned air. Substrates were placed in sterile 6-well polystyrene culture plates and blocked with a solution of 2% heat-denatured BSA in PBS for 30 min. For cell patterning studies, freshly harvested HUVEC cells were plated on the prepared substrates at a density of 4.2×10^4 cells/cm^2. Cell viability was measured

using a standard Wst-1 assay and cell adhesion was quantified using the buoyant centrifugation method described in Liu and coworkers [21].

IV.3.11 Phase contrast and fluorescence microscopy

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 a 6-well plate, and each well was washed 3 times with 2 mL PBS before 1 mL ice-cold acetone was applied for exactly 1 min. The wells were again washed 3 times with 2 mL PBS before 2 mL of a 10% BSA solution was applied for 30 min at room temperature. After blocking, 0.2 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 2 mL PBS for 5 min without agitation. A secondary antibody/phalloidin solution composed of 862 μL PBS, 100 μL secondary antibody (Cy2-labeled anti-mouse, 0.5 mg/mL, Chemicon) and 38 μL rhodamine-phalloidin (Molecular Probes) was incubated with the samples in the dark for 1 h. Labeled samples were washed with 2 mL PBS for 10 minutes with agitation followed by 2 mL PBS 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 2 mL PBS and mounted to a glass slide using filtered mounting solution of 1:1 PBS:glycerol and clear fingernail polish as sealant.

IV.3.12 Atomic force microscopy

Atomic force microscopy was conducted on dried, patterned aECM-N_3 protein films on PEGylated coverslips in constant-force, non-contact mode with an autoprobe M5 atomic force microscope (Parker Scientific).

IV.4 Results and Discussion

Large-scale production of the aECM-N_3 protein was accomplished through residue-specific incorporation of *p*N_3Phe in *E. coli*. This method of incorporation relies on competitive activation of phenylalanine and *p*N_3Phe by the phenylalanyl-tRNA synthetase, the enzyme responsible for charging phenylalanine to its cognate tRNA. The phenylalanyl-tRNA synthetase used for this study was a previously characterized mutant with relaxed substrate specificity [12, 22]. Proteins were expressed in a phenylalanine-auxotrophic *E. coli* strain grown in cultures supplemented with *p*N_3Phe and purified by taking advantage of the inverse temperature phase behavior of proteins with elastin-like repeats [23]. Incorporation efficiency was determined by integration of the aromatic proton signals in the ^{1}H NMR spectra of the purified proteins (Figure IV-1b); the extent of phenylalanine replacement varied from 13% to 53% depending on the concentration of *p*N_3Phe in the expression medium (Figure IV-2).

a.

MMASMTGGQQMGRKTHHHHHHHMG{LDGEEIQIGHIPREDVDYHLYPG[(VPGVG)$_2$(VPGFG)(VPGVG)$_2$]$_5$LP}$_3$LE

CS5 domain Elastin-like sequence

b.

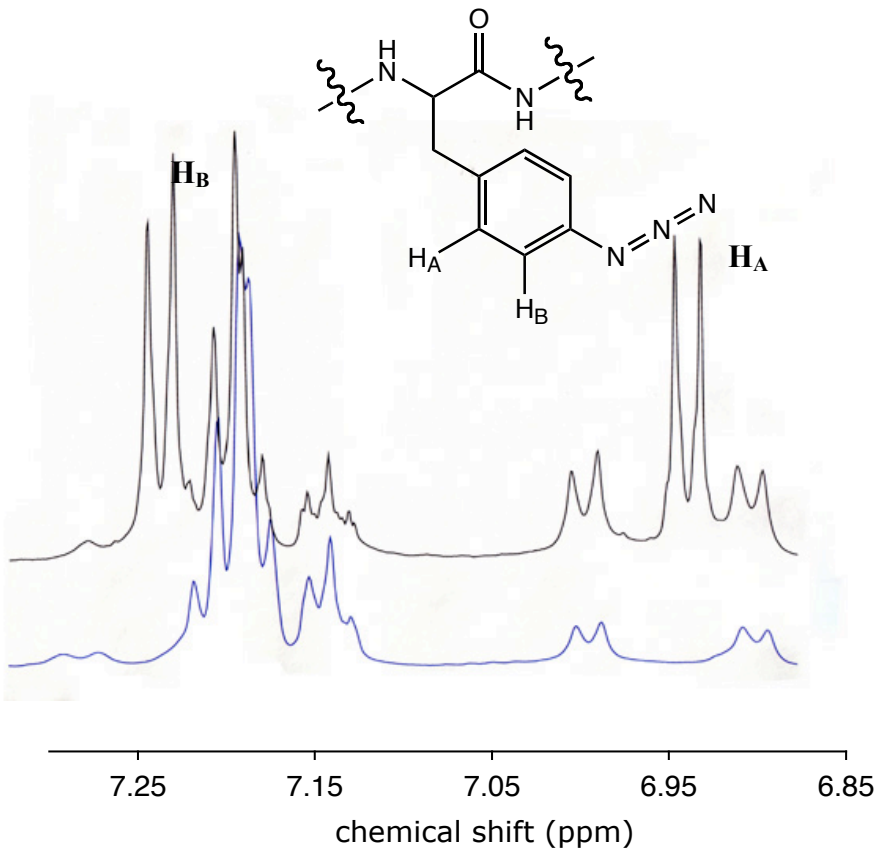


Figure IV-1. Design and production of aECM-N_3. (**a**) The aECM-N_3 primary sequence encodes a cell-binding domain (the CS5 region of fibronectin) and a structural domain (the pentapeptide VPGVG elastin-like repeat with periodic phenylalanine sites for incorporation of *p*N_3Phe). (**b**) ^{1}H NMR (600 MHz, DMSO-d_6) of the artificial protein expressed in the presence of phenylalanine (bottom spectrum) or in the presence of 250 mg/L *p*N_3Phe (top spectrum). Integration indicates 53% *p*N_3Phe incorporation.

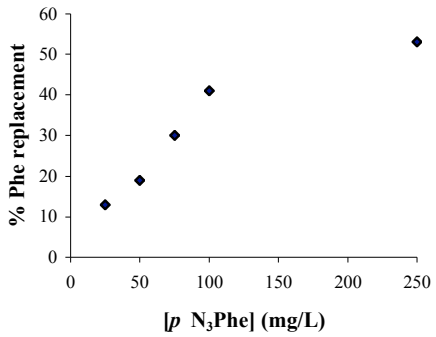


Figure IV-2. Incorporation of *p*N_3Phe into aECM-N_3 as a function of concentration in the expression medium.

Understanding the response of the designed 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 IV-3a) [24]. Measurements were performed on thin films of aECM-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 IV-3b). It is noteworthy that none of the other infrared bands were noticeably altered, implying that irradiation under the conditions used here specifically affects the aryl azide without substantial modification of any of the canonical amino acids. This is expected given that none of the canonical amino acids absorb above 310 nm [25].

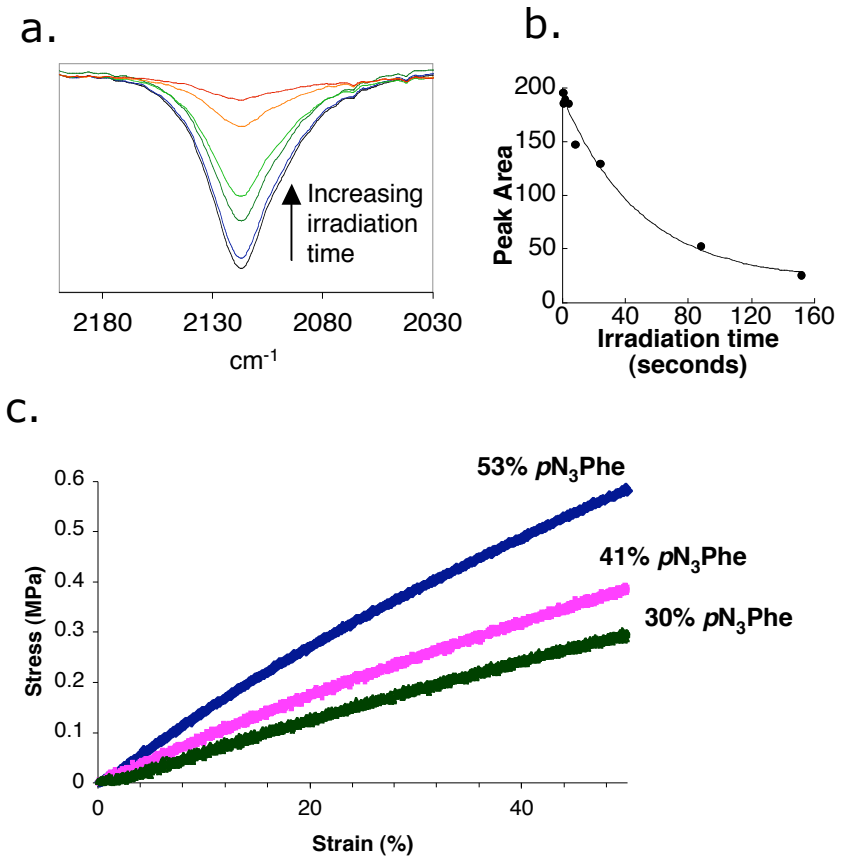


Figure IV-3. Characterization of azide decomposition by infrared spectroscopy and mechanical photocrosslinked films. **(a)** FT-IR spectrum of aECM-N_3 films as a function of irradiation time in the region of the aryl azide asymmetric stretch. Irradiation times are 0 (bottom), 0.5, 8, 24, 88, and 124 seconds (top). **(b)** Peak area versus irradiation time yields a first order exponential decay. **(c)** Uniaxial tensile testing of aECM-N_3 films with variable *p*N_3Phe incorporation.

Elastic moduli of irradiated aECM-N_3 bulk films were determined through uniaxial tensile testing under physiological conditions (Figure IV-3c). As expected, the elastic modulus correlated with the extent of *p*N_3Phe incorporation. Irradiated aECM-N_3 films in which 30, 41, and 53% of the encoded phenylalanine residues were replaced with *p*N_3Phe yielded elastic moduli of 0.53 ± 0.10, 0.94 ± 0.09, and 1.39 ± 0.09 MPa, which

are in the range of native elastin (0.3 – 0.6 MPa) [26]. Replacement of less than 20% of the encoded phenylalanine residues gave films that were too weak to test, and films made without *p*N_3Phe yielded no evidence of crosslinking. The fact that the 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 [27, 28].

To investigate the potential of our 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 12.5% solutions of protein in dimethylsulfoxide directly onto prepared poly(ethylene glycol) (PEG)-coated glass coverslips were clear and homogeneous by optical microscopy. These protein films irradiated for 30 seconds at 365 nm through a chrome-on-quartz mask using a Karl Suss contact aligner. Stripping of the masked areas was accomplished by washing in mild aqueous detergent (0.05% aqueous sodium dodecasulfate).

Fluorescence immunolabeling with anti-T7-tag IgG antibody revealed that the aECM protein was localized only within the irradiated areas of the pattern (Figure IV-4a). Films prepared from protein lacking *p*N_3Phe formed no detectable patterns even after prolonged exposure times.

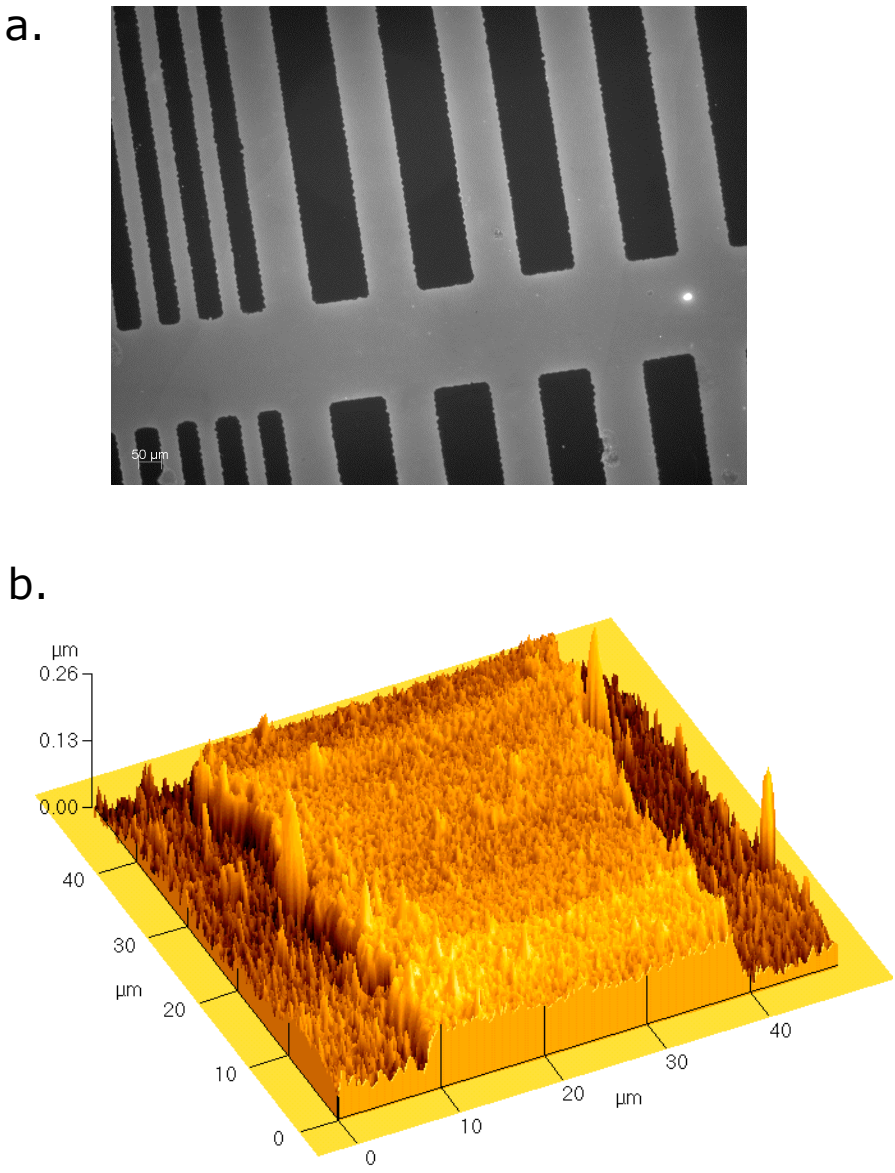


Figure IV-4. Characterization of photopatterned aECM-N_3 features by fluorescence and atomic force microscopy. **(a)** Fluorescence microscopy of photopatterned aECM-N_3. PEGylated glass slides were spin coated with aECM-N_3, irradiated for 30 seconds, and washed overnight in 0.05% SDS to produce well-defined protein features on a non-adhesive background. Protein patterns were immunolabeled with an anti-T7-tag primary antibody and an anti-mouse Cy2-conjugated secondary antibody. **(b)** AFM image of patterned aECM-N_3. The image was taken of a dried aECM-N_3 patterned film in constant-force, non-contact mode with an autoprobe M5 atomic force microscope.

The edge roughness of protein pattern features is a consequence of the roughness of the mask (created from a 3000 dpi print) and not the inherent resolution of the films (Figure IV-5). Non-contact AFM of dried aECM-N_3 patterns demonstrated uniform features, which varied in height depending upon the conditions used for spin coating. Films spun at 1400 rpm were 84 nm thick (Figure IV-4b), whereas those spun at 2000 rpm were approximately 4 nm thick. Protein patterns stored either dry or in aqueous solutions were stable for weeks.

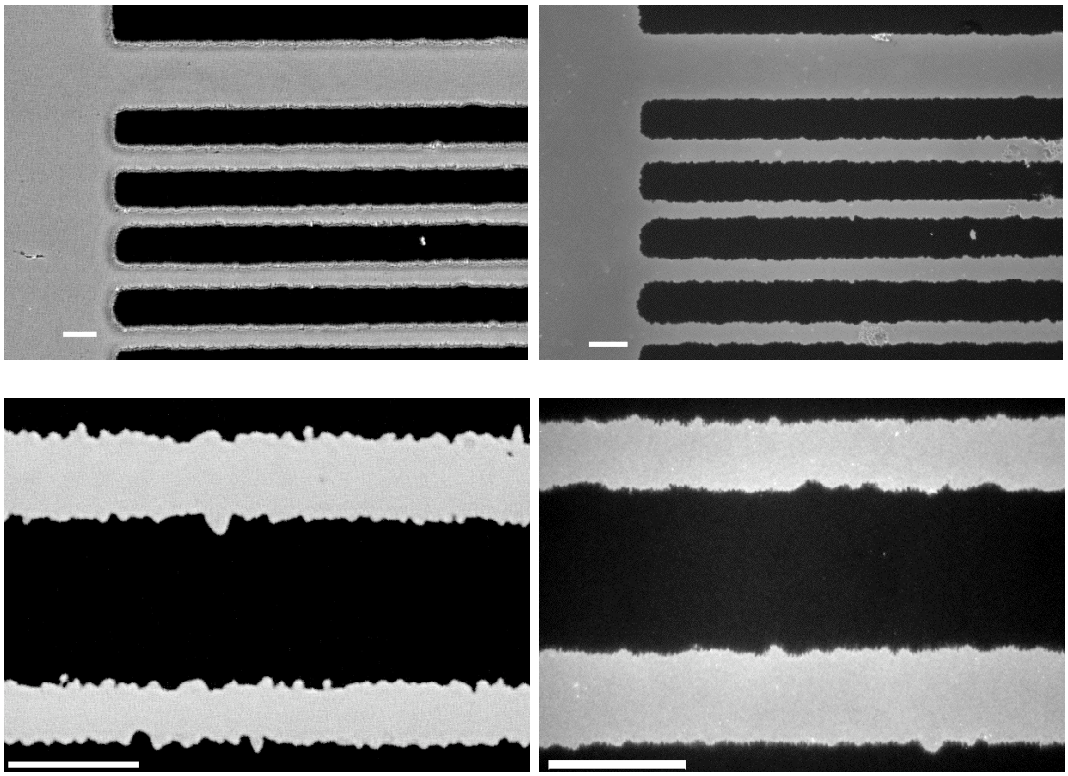
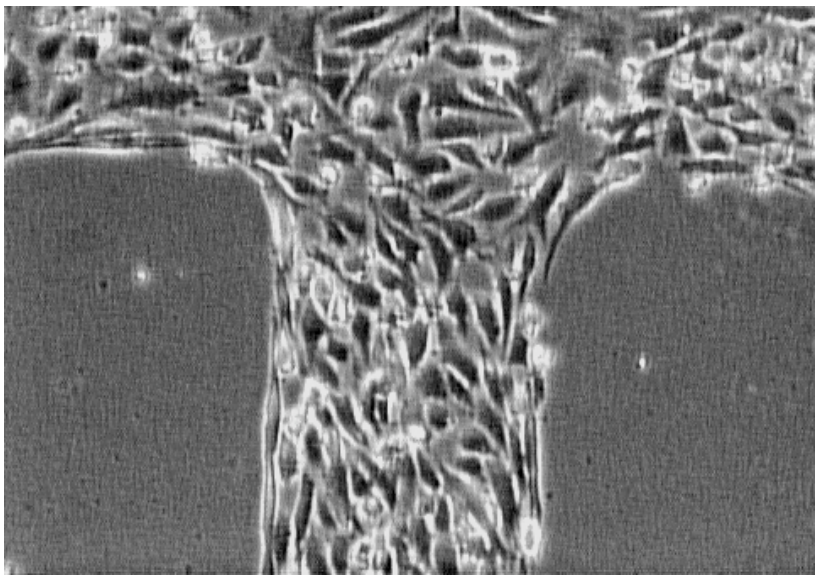


Figure IV-5. Phase contrast images of the chrome mask used in photopatterning (left panels) compared with fluorescence microscopy images of the protein patterns (right panels). Scale bars represent 50 microns. In the top panels (lower magnification), the protein pattern could be visually matched to precisely the region on the mask that created it. The bottom panels (higher magnification) show two separate regions with features of similar sizes.

To create cell arrays, HUVEC were plated on aECM-N_3 patterns in the absence of serum. After six hours of incubation, the medium was supplemented with 2% serum. After 24 hours of incubation, HUVEC exhibited a well-spread morphology (Figure IV-6a) and had proliferated to confluence exclusively within the patterned areas (Figure IV-6b). HUVEC monolayers in the interior of the patterned regions displayed morphology indistinguishable from monolayers grown on homogenous fibronectin coatings; however, HUVEC positioned along the aECM-N_3 pattern edges were elongated and oriented parallel to the pattern border (Figure IV-6a), consistent with previous studies. As expected, actin stress fibers within these elongated cells were aligned with the pattern edges (Figure IV-6b). Similar to other endothelial cell patterning techniques, this parallel cell alignment was generally observed for the first two to three cell layers adjacent to the pattern edges; therefore, cell alignment was enhanced on patterns with smaller feature sizes [29].

a.



b.

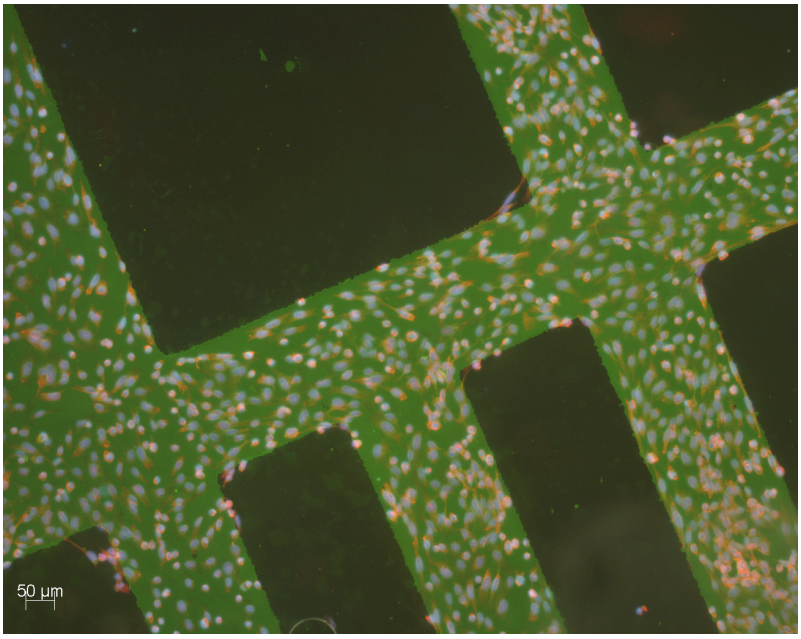


Figure IV-6. **(a)** Phase contrast and **(b)** fluorescence microscopy of HUVEC attached to photopatterned aECM-N_3. Cells were plated onto the prepared surfaces in serum free media and allowed to incubate 6 hours prior to supplementation with 2% serum. After 24 hours, the cells and substrates were fixed with acetone, immunolabeled with an anti-T7-tag primary antibody and anti-mouse Cy2-conjugated secondary antibody, and stained with phalloidin (specific to the actin cytoskeleton) and DAPI (specific to the nucleus).

In serum, HUVEC patterns were stable for 48 hours after reaching confluence, consistent with known behavior of PEG coatings as cell-resistant backgrounds [30]. At longer times, cells began growing beyond the protein pattern at the corners, probably in concert with synthesis and secretion extracellular matrix proteins.

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

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