Chapter 6

LITHOGRAPHIC PATTERNING OF INTRINSICALLY PHOTOREACTIVE CELL-ADHESIVE PROTEINS

6.1 Abstract

Cell-adhesive protein patterns were created through simple photolithographic techniques. The non-canonical amino acid *para*-azidophenylalanine (*p*N_3Phe) was introduced in a residue-specific manner into artificial extracellular matrix proteins containing the RGD and CS5 cell-binding domains (RGD-N_3 and CS5-N_3, respectively). The azide moiety allowed for efficient protein crosslinking at 365 nm. By varying the *p*N_3Phe concentration in the growth medium, the extent of *p*N_3Phe incorporation in proteins and the moduli of crosslinked films could be tuned. CS5-N_3 photopatterned on a non-adhesive background served as a template for cell adhesion. Furthermore, it was demonstrated that cells recognized the cell-binding domain of crosslinked RGD-N_3 in a sequence-specific fashion.

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6.2 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 (aECM-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 6.1a).[12,13] A control protein, RDG-N_3, was designed to be identical to RGD-N_3 except for a sequence swap of two amino acids within the cell-binding domain. 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 achieved simply by changing the concentration of the non-canonical amino acid in the expression medium. Thin films of aECM-N_3 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.

6.3 Materials and Methods

6.3.1 Cloning of aECM Constructs

Synthetic oligonucleotides encoding the CS5 cell-binding domains were annealed, phosphorylated, and ligated into pEC2[14] to produce pEC2-CS5.[15] An oligonucleotide encoding the elastin-like repeat $(VPGVG)_2VPGFG(VPGVG)_2$ was similarly ligated into pUC19 (New England Biolabs, Ipswich, MA) between *Eco*RI and *Bam*HI. The elastin-like insert was cut out using *Ban*I and self-ligated to form multimers. The multimerization mixture was ligated with *Ban*I-linearized pEC2-CS5. Transformants with the pentamer insert were selected, digested at *Xho*I and *Sal*I, and ligated into a modified pET28a (Novagen, San Diego, CA). This step was repeated twice to obtain the final $[CBD(ELF)_5]_3$ construct under control of the T7 promotor. A similar cloning strategy was used for the RGD and RDG constructs.[16] 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[17] to produce pNS-CS5-ELF,[15] pSM-RGD-ELF, and pSM-RDG-ELF.

6.3.2 Protein Expression and Purification

The target protein was expressed using a phenylalanine auxotrophic derivative of an *E. coli* strain designated AF-IQ[18] harboring either pNS-CS5-ELF, pSM-RGD-ELF, or pSM-RDG-ELF. To express proteins from these strains, a culture was grown overnight in 2×YT 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 min 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 (Novagen). 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 phenylmethylsulfonylfluoride and 10 μg/mL each of DNase and RNase. This mixture was agitated for 4 h at 37 °C and centrifuged at a temperature above the expected LCST of the protein (22,000*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 (22,000*g*, 60 min, 2 °C). The resulting supernatant was dialyzed exhaustively in

cold (4 °C) distilled water and subsequently lyophilized. Typical experiments yielded 40 mg of protein per liter of culture.

6.3.3 Mechanical Testing

Samples were created in teflon molds by drying 10% aECM-N_3 solutions in DMSO overnight at 50 °C and irradiated with a 100 W mercury lamp for 30 min. 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 × 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 5564 with a 5 N load cell. Films were extended at a rate of 10% length/minute. Each protein sample was tested 3–6 times.

6.3.4 Photolithographic Patterning of aECM-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 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 covered dropwise with a 100 mM solution in pyridine of mPEG-SPA-2000 (MW = 2000 Da,

Nektar Therapeutics, San Carlos, CA). After 12 h, the PEGylated coverslips were rinsed under streams of water 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 aECM-N_3 in DMSO that had been centrifuged for 1 min at 14,000 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 via 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.

6.3.5 Cell Culture

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

6.3.6 Cell Patterning

Patterned coverslips were placed in a 24-well plate, and HUVECs were added at a 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 three 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 three times with 1 mL PBS before blocking with 0.5 mL of a 10% BSA solution for 30 min at room temperature. Afterwards, 0.25 μL of a monoclonal anti-T7 tag 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, Carlsbad, CA) was incubated with the samples for 1 h in the dark. Labeled samples were washed with 1 mL of water for 10 min with agitation followed by 1 mL of water for 5 min without agitation. The samples were then incubated with 1 mL of DAPI solution (0.3 μM in PBS) for 5 min at room temperature. Samples were rinsed three times with 1 mL of water and mounted to a glass slide using a filtered mounting solution of 1:1 PBS:glycerol and clear fingernail polish as a sealant.

6.3.7 Cell Spreading

mPEG-SPA-5000 (100 mg) was dissolved in an excess of propargyl amine (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): δ = 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).[19] 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 2.3–2.7 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 min at room temperature, and rinsed three times. Coverslips with PEGylated RGD-N_3 or RDG-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, Cambrex BioSciences) and seeded in a total volume of 3 mL per well at a density of 5.0×10^3 cells/cm^2. For quantification of spread area, pictures were obtained 30 min post-seeding using 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 in total were examined in at least 4 independent experiments.

6.3.8 Atomic Force Microscopy

Topographical scans of RDG-N_3 protein patterns on PEGylated coverslips were obtained with an AutoProbe M5 atomic force microscope (Park Scientific Instruments, Woodbury, NY) in constant-force contact mode, using pyramidal tips (0.58 N/m, Veeco DNP-S). Imaging was performed dry or 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.

6.4 Results and Discussion

Preparation of aECM-N_3 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.[20] The PheRS used for this study was a previously characterized mutant with relaxed substrate specificity.[20] Proteins were expressed in a Phe-auxotrophic *E. coli* strain grown in cultures supplemented with *p*N_3Phe and purified by taking advantage of the temperature-dependent phase behavior of proteins with elastin-like repeats.[21] Incorporation efficiency was determined by integration of the aromatic proton signals in the ^{1}H NMR spectra of

the purified proteins; the extent of Phe replacement varied from 13% to 53%, depending on the concentration of *p*N_3Phe in the expression medium (see Supporting Information).

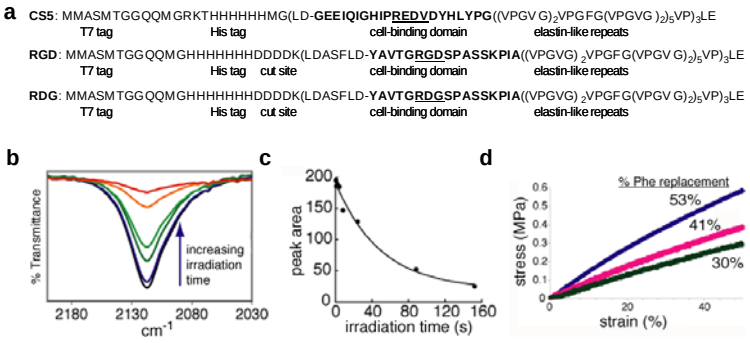

Figure 6.1 Response of aECM-N_3 to irradiation. (a) Primary sequences of aECM-N_3 family. (b) FTIR demonstrates loss of the characteristic azide asymmetric stretch as a function of irradiation time of CS5-N_3 films. (c) Peak area vs. irradiation time yields a first-order decay with $t_{1/2}$=34 sec. (d) Uniaxial tensile testing of irradiated mold-cast films.

Understanding the response of the photoactive 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 asymmetric stretch at 2130 cm^{-1} (Figure 6.1b).[22] Measurements were performed on thin films of CS5-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 6.1c). 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 absorbs above 310 nm.[23]

Elastic moduli of irradiated CS5-N_3 films were determined by uniaxial tensile testing under simulated physiological conditions (Figure 6.1d). As expected, the elastic modulus correlated with the extent of *p*N_3Phe incorporation. Irradiated CS5-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).[11] 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.[24,25]

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. CS5-N_3 was readily patterned, but cell behavior on photocrosslinked CS5-N_3 films was not specific to the CS5 sequence, which prompted us to focus on RGD-N_3. Protein films created by spin coating 10% solutions of aECM-N_3 in dimethyl sulfoxide directly on poly(ethylene glycol) (PEG)-coated glass coverslips were clear and homogeneous by optical microscopy. Protein films were irradiated for 30 s at 365 nm through a chrome-on-quartz mask. Stripping of the masked areas was accomplished by washing in mild aqueous detergent (0.05% sodium dodecylsulfate).

Fluorescence immunolabeling with an anti-T7-tag antibody showed that the protein was localized only within the irradiated areas of the pattern. Films prepared from protein lacking *p*N_3Phe formed no detectable patterns even after prolonged exposure times. Atomic force microscopy (AFM) of RGD-N_3 patterns spun at 1000 rpm revealed uniform features that were 467 or 750 nm thick when the films were dry or hydrated, respectively. Protein patterns stored either dry or in aqueous solutions were stable for weeks. Investigation of the material properties of thin photocrosslinked films is ongoing.

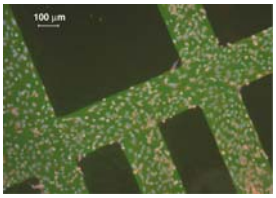

Figure 6.2 Fluorescence microscopy of HUVECs attached to photopatterned CS5-N_3. Immunostaining with anti-T7 (green) demonstrates colocalization of aECM protein and cells (stained with nuclear stain (blue) and cytoskeletal stain (red)).

To create cell arrays, human umbilical vein endothelial cells (HUVECs) were plated on CS5-N_3 patterns in the absence of serum. After 6 h of incubation, the medium was supplemented with 2% serum. After 24 h of incubation, HUVECs exhibited well-spread morphologies and had proliferated to confluence exclusively within the patterned areas (Figure 6.2). HUVEC monolayers in the interior of the protein regions were indistinguishable from monolayers grown on fibronectin coatings; however, cells positioned along the CS5-N_3 pattern edges were elongated parallel to the pattern border,

consistent with previous studies.[26] Ongoing studies are being done to replicate these results with RGD-N_3.

HUVEC patterns were stable in serum for 48 h after reaching confluence, consistent with known behavior of PEG coatings as cell-resistant backgrounds.[27] At longer times, cells began growing beyond the pattern at the corners, presumably in concert with synthesis and secretion of ECM proteins.

To determine whether cells specifically recognize the RGD cell-binding domain, we compared HUVEC spreading after 30 min of incubation on uniformly photocrosslinked RGD-N_3 and RDG-N_3 films (Figure 6.3). Nonspecific interaction with aECM-N_3 films was reduced by covalent attachment of PEG (see the Materials and Methods section). The distribution of HUVEC spread areas on RGD-N_3 films closely matches that on fibronectin. HUVECs spread significantly less well on RDG-N_3 and resemble cells on the negative control protein bovine serum albumin (BSA).

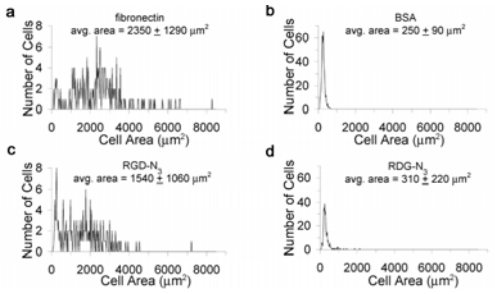

Figure 6.3 HUVEC spread areas on (a) fibronectin, (b) BSA, (c) RGD-N_3, (d) RDG-N_3 demonstrate sequence-specificity of cell spreading.

6.5 Conclusion

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 interactions, and geometric shape. Applications in medical devices, tissue engineering, and array technologies are readily imagined.

6.6 Acknowledgments

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6.7 Supporting Information

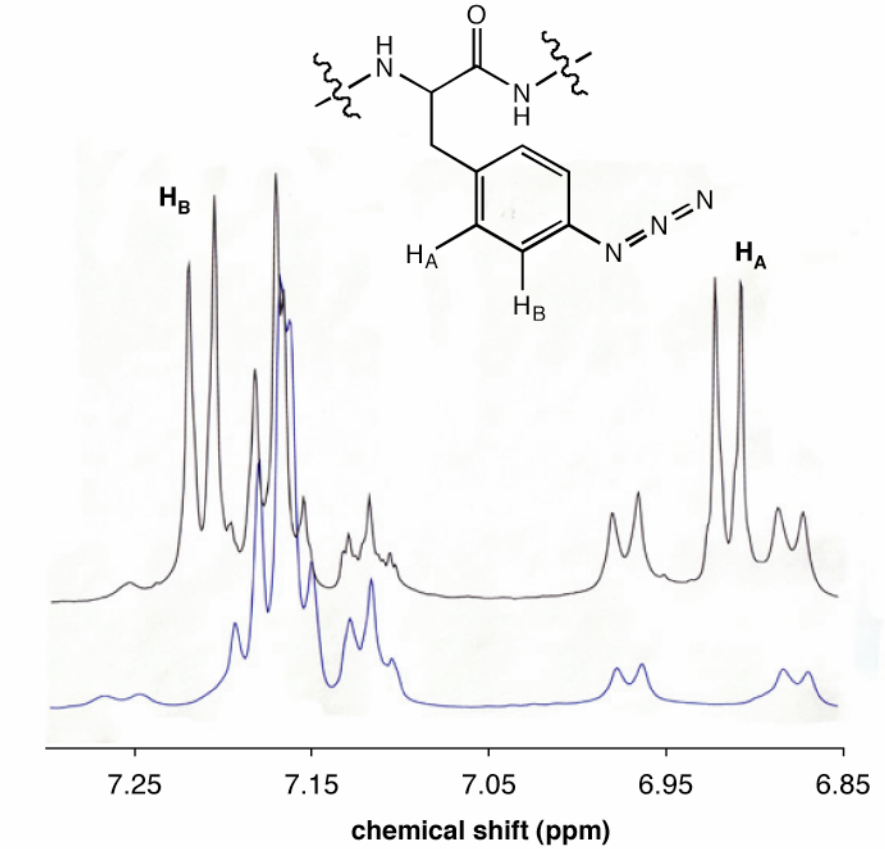

Figure 6.4 ^{1}H NMR spectrum of the CS5-N_3 construct expressed in media supplemented with phenylalanine (bottom spectrum) or with 250 mg/L *p*N_3Phe (top spectrum). Spectra are identical except for two additional doublets in the top spectrum resulting from the aromatic protons of *p*N_3Phe; integration indicates 53% *p*N_3Phe incorporation. 600 MHz spectra were taken on 1 mM samples in DMSO-d_6 at 23 °C.

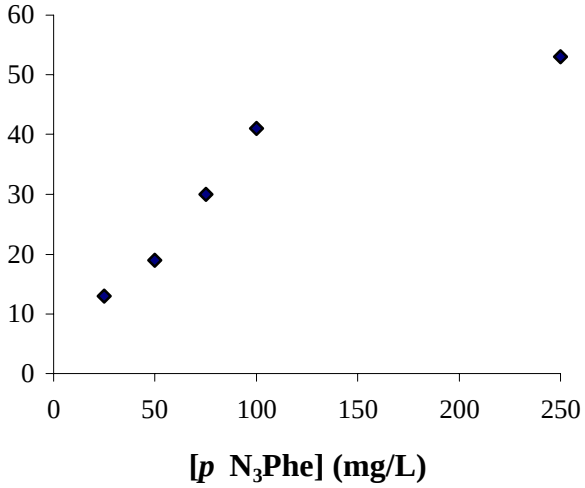

Figure 6.5 Incorporation of *p*N_3Phe into CS5-N_3 as a function of concentration in the expression medium.

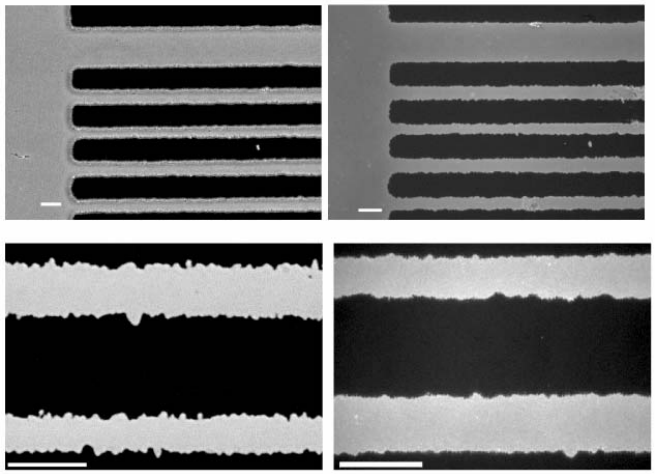

Figure 6.6 Phase contrast microscopy images of the chrome mask used in photopatterning (left panels) compared with fluorescence microscopy images of CS5-N_3 patterns (right panels). Scale bars represent 50 μm. In the top panels (lower magnification), the protein pattern could be visually matched to precisely the region on the mask that created it. The bottom panels (higher magnification) show two separate regions with features of similar sizes.

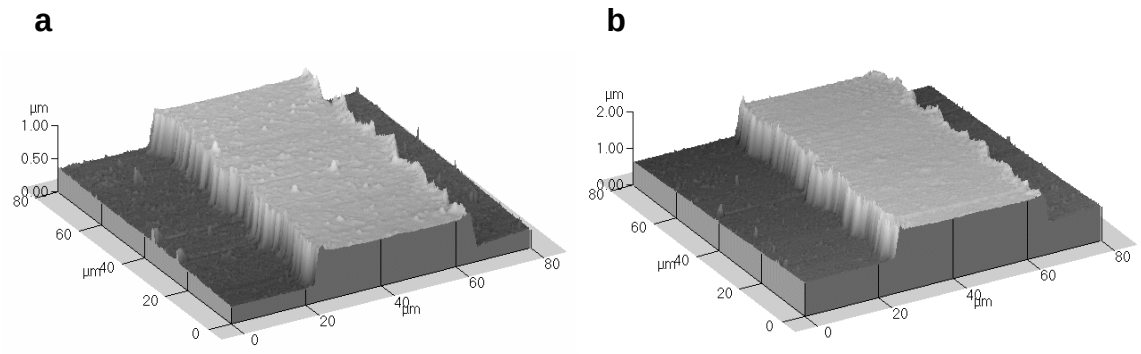

Figure 6.7 AFM images of patterned RGD-N_3. The images were recorded on (a) dried or (b) hydrated aECM-N_3 patterned films in constant-force contact mode with an autoprobe M5 atomic force microscope.

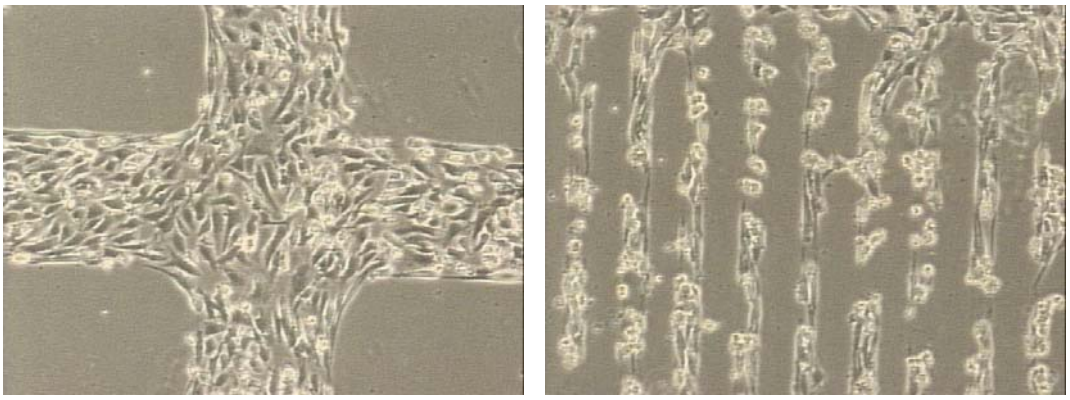

Figure 6.8 Phase contrast microscopy of HUVECs attached to photopatterned CS5-N_3.

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