3 Lithographic Patterning of Photoreactive Cell-Adhesive Proteins

Reproduced with permission from:

Carrico, I.S., Maskarinec, S.A., Heilshorn, S.C., Mock, M.L., Liu, J.C., Nowatzki, P.J., Franck, C., Ravichandran, G., and D.A. Tirrell. *Lithographic Patterning of Photoreactive Cell-Adhesive Proteins.* Journal of the American Chemical Society, 2007. **129(16)**: 4874-4875.

3.1 Abstract

We describe a novel, simple method for the photolithographic patterning of cell-adhesive proteins. Intrinsically photoreactive proteins are synthesized in *Escherichia coli* through incorporation of the noncanonical, photosensitive 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. We demonstrate the utility of this method of protein patterning by creating stable arrays of fibroblast cells on an engineered protein "photoresist".

3.2 Introduction

Control of the spatial arrangement of proteins on surfaces is essential in a number of emerging technologies, including protein microarrays, biosensors, tissue engineering, and regenerative medicine [1-4]. Patterning is also a powerful tool in cell biology, wherein cell arrays are used to elucidate the factors that mediate migration, proliferation, and cell-cell interactions [5-9]. 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 [10].

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 via site-specific and residue-specific techniques has been reported [11-15]. Here we describe the microbial expression of artificial proteins bearing the photosensitive noncanonical 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 and to support adhesion of mammalian cells through cell-binding domains (CS5 or RGD) derived from fibronectin (Figure 3-1 (A)) [16-19]. These proteins can be crosslinked efficiently upon irradiation at 365 nm. The physical properties of the crosslinked films can be controlled by changing the *p*N_3Phe content, and thin films can be patterned on surfaces via photolithographic techniques. We demonstrate the utility of

the method by creating cell arrays through selective cell attachment to photolithographically prepared protein patterns.

A

CS5: MMASMTGGQQMGHHHHHHHHMG(LDGEEIQIGHIPREDVDYHLYPG((VPGVG)$_2$VPGFG(VPGVG)$_2$)$_5$VP)$_3$LE
T7 tag His tag CS5 cell-binding domain elastin-like repeats

RGD: MMASMTGGQQMGHHHHHHHHDDDDK(LDASFLDYAVTGRGDSPASSKPIAASA((VPGVG)$_2$VPGFG(VPGVG)$_2$)$_5$VP)$_3$)LE
T7 tag His tag RGD cell-binding domain elastin-like repeats

RDG: MMASMTGGQQMGHHHHHHHHDDDDK(LDASFLDYAVTGRDGSPASSKPIAASA((VPGVG)$_2$VPGFG(VPGVG)$_2$)$_5$VP)$_3$)LE
T7 tag His tag scrambled RGD cell-binding domain elastin-like repeats

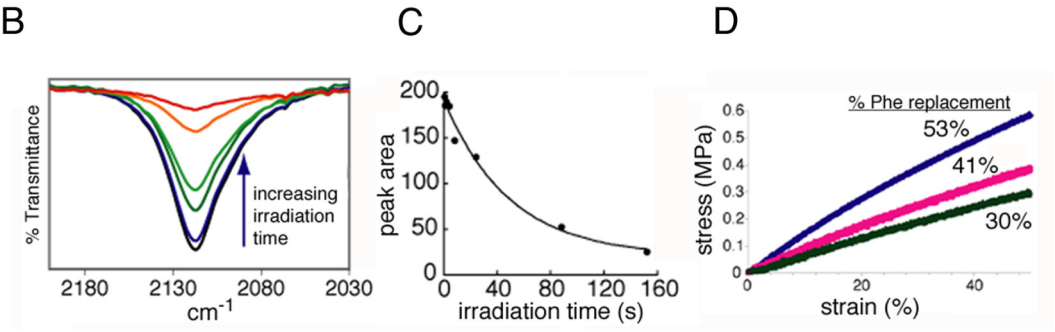

Figure 3-1. Characteristics of aECM-N_3 proteins. (A) Primary sequences of aECM-N_3 variants. (B) FTIR demonstrates loss of the characteristic azide asymmetric stretch as a function of irradiation time of CS5-N_3 films. (C) Peak area versus irradiation time yields a first-order decay with $t_{1/2}$ = 34 sec. (D) Uniaxial tensile testing of irradiated mold-cast films.

3.3 Materials and Methods

3.3.1 Cloning of aECM-N_3 constructs

Synthetic oligonucleotides encoding the CS5 cell-binding domain were annealed, phosphorylated, and ligated into pEC2 to produce pEC2-CS5 [20]. An oligonucleotide encoding the elastin-like repeat (VPGVG)$_2$VPGFG(VPGVG)$_2$ was similarly ligated into pUC19 (New England Biolabs, Ipswich, MA) between the *Eco*RI and *Bam*HI sites. The insert was excised with *Ban*I and self-ligated to form multimers. The multimerization mixture was ligated into *Ban*I-linearized pEC2-CS5. Transformants with the pentamer

sequence were selected, digested with *Xho*I and *Sal*I, and ligated into a modified pET28a plasmid (Novagen, San Diego, CA). This step was repeated twice to obtain the final $[CS5(ELF)_5]_3$ construct under control of the T7 promoter. A similar cloning strategy was used for the RGD and RDG constructs. The *pheRS** 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 [21]. The final plasmids were designated pNS-CS5-ELF [22], pSM-RGD-ELF, and pSM-RDG-ELF.

3.3.2 Protein expression and purification

aECM-N_3 proteins were expressed in a phenylalanine auxotrophic *E. coli* strain, termed AF-IQ [23], 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 x YT medium and used to inoculate 1L of M9AA medium supplemented with antibiotics kanamycin and chloramphenicol (Sigma). At an OD_{600} of 1.0, expression of the target proteins and the T7 RNA polymerase was induced by adding 1 mM isopropyl-b-D-thiogalactoside (IPTG). After 10 minutes, 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 350 mg of *p*N_3Phe and grown for 4 hours. Protein expression was monitored by SDS-PAGE and Western blotting using an anti-T7 tag horseradish peroxidase conjugated antibody (Novagen). Cells were harvested after 4 hours by centrifugation (10,000g, 10 min, 25°C), and the cell pellets were resuspended in 20 mL of TEN buffer (10 mM Tris, 1 mM EDTA, 0.1 M NaCl) by sonication and

subsequently frozen. The frozen lysate was treated with 1 mM phenylmethylsulfonylfluoride and 10 μg/mL of both DNase and RNase. The resulting mixture was agitated for 4 hours at 37°C and later centrifuged at a temperature above the expected lower critical solution temperature (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. The resulting suspension was clarified by centrifugation (22000*g*, 60 min, 2°C), and the supernatant was dialyzed in cold (4°C) distilled water for 3 days, frozen, and lyophilized. Typical experiments yielded 40 mg of protein per liter of culture.

3.3.3 Mechanical testing

Samples were prepared in Teflon molds by drying 10% aECM-N_3 solutions in DMSO overnight at 50°C followed by irradiation with a 100 W mercury lamp for 30 minutes. Samples were removed from the molds, swollen in 4°C water overnight, cut into test strips, and finally equilibrated in PBS at 37°C. Films were approximately 3 mm x 10 mm. Tensile testing of equilibrated films was performed at 37°C in phosphate buffered saline at pH 7.4. Films were extended at a rate of 10% length/minute using an Instron-5542 (Instron Corporation, Canton, MA) with a 5N load cell. The data was acquired using the Instron Series IX Software for Windows (Merlin version).

3.3.4 Photolithographic patterning of aECM-N_3 proteins

Passivated glass slides were prepared by treatment with a silane. Standard glass microscope slides (Corning) were immersed in a solution of concentrated H_2SO_4 for 1 hour. After a thorough washing with water, slides were placed into a boiling solution of

$NH_4OH/H_2O_2/H_20$ (1:1:5) for 30 minutes. The slides were gently shaken in a 1% (v/v) solution of 2-[methoxy(polyethylenoxy)propyl] trimethoxysilane (Gelest) in toluene for 30 minutes. The slides were rinsed immediately with toluene, followed by a stream of methanol and water. The functionalized slides were then cured at 100°C for 30 minutes. Each PEO-modified slide was cut into 4 similarly sized pieces using a diamond cutter, and individual substrate pieces were covered dropwise with 10 μL of a 12.5 mg/mL solution of aECM-N_3 in DMSO that had been centrifuged for 1 minute at 14,000 rpm to remove particulates. Substrates were spun for 30 seconds at 1000 rpm on a Specialty Coating Systems model P-6000 spin coater. Protein-coated slides were dried at 50°C for 4 hours. Exposure of protein to sunlight was avoided until photolithography was complete. Protein-coated slides were irradiated for 60 seconds at 365nm using a Karl Suss mask aligner under a chrome-on-quartz mask prepared by Dr. Michael Diehl via chrome deposition and stripping from a 3000 dpi transparency. Irradiated substrates were washed for 4.5 hours in 6 M guanidine hydrochloride to remove soluble protein from the masked regions. Protein-patterned substrates were then rinsed for 5 minutes in filtered water and sterilized by an ethanol rinse before use.

3.3.5 Cell culture

Rat-1 fibroblasts (ATCC, Manassas, VA) were maintained in a 37°C, 5% CO_2 humidified environmental chamber. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM, 10% fetal calf serum, Gibco), which was replaced every two days. Near-confluent Rat-1 cultures were passaged by treatment with trypsin (0.05% trypsin/EDTA) at 37°C for 2–5 minutes (Invitrogen) and resuspended in 2 mL of serum-

free DMEM. Cells were then centrifuged (1050 rpm, 3 min), and resuspended in fresh serum-free DMEM at a concentration of 1.0 x 10^6 cells/mL.

3.3.6 Cell patterning

Patterned substrates were placed in a 6-well plate, and Rat-1 fibroblasts (6.0 x 10^5 cells/substrate, ~ 1.0 x 10^5 cells/cm^2) were deposited on the patterned surfaces in a total of 3 mL of serum-free DMEM. After 4 hours of incubation, phase contrast images were captured with a Nikon Eclipse TE 300 microscope. Fluorescence images were acquired using a laser scanning confocal microscope consisting of a confocal system (Nikon C-1) combined with an inverted optical microscope (Nikon TE-2000-U). To fix and fluorescently label cell patterns, the substrates were washed three times with PBS before treatment with chilled acetone at -20°C for 5 minutes. The substrates were again rinsed with PBS and blocked with a 10% BSA solution for 30 minutes at room temperature. Afterwards, 1 μL of a monoclonal anti-T7 tag primary antibody (Novagen) was added and allowed to incubate at room temperature for 1.5 hours. Individual substrates were then transferred to a standard Petri dish (VWR) containing a solution of 0.1% BSA and placed on a waver to wash for 10 minutes. A secondary antibody/phalloidin solution composed of 425 μL PBS, 62.5 μL secondary antibody (Cy2-labeled anti-mouse, 0.5 mg/mL, Chemicon), and 12.5 μL rhodamine phalloidin (6.6 μM, Invitrogen) was added per sample for 1 hour in the dark. Labeled samples were washed in 0.1% BSA for 10 minutes on a waver. The samples were then rinsed three times with PBS without agitation, and mounted with a glass coverslip using 25 μL of Biomeda gel/mount

(Biomeda Corporation, Foster City, CA) solution. The samples were dried at room temperature for 1 hour and sealed with clear fingernail polish.

3.3.7 Cell spreading

Cell spreading experiments were performed on uniformly crosslinked films to determine whether the nature of cell attachment to protein-coated surfaces is sequence-specific to the RGD cell-binding domain. RGD-N_3 and RDG-N_3 substrates were prepared by spin coating 10 μL of a 12.5 mg/mL protein solution at 1000 rpm for 30 seconds onto base-cleaned (saturated potassium hydroxide in ethanol) coverslips (12 mm diameter). Substrates were dried at 50^{o}C overnight and uniformly irradiated for 60 seconds using an unfiltered Oriel 100W medium pressure mercury lamp. Substrates were rinsed with water for several minutes before being sterilized by an ethanol rinse.

For the positive control, 1 mL of a 10 μg/mL fibronectin solution in PBS was adsorbed overnight in a 6-well plate at 4^{o}C. The wells were rinsed three times with PBS, and blocked with a 2 mg/mL BSA solution for 30 minutes at room temperature and rinsed three times. For the negative control, 1 ml of a 2 mg/mL BSA solution in PBS was adsorbed overnight in a 6-well plate at 4^{o}C. The wells were rinsed three times with PBS, and blocked with a 2 mg/mL BSA solution for 30 minutes at room temperature and rinsed three times. RGD-N_3 and RDG-N_3 substrates were adhered to the bottom of a BSA-blocked well by applying sterilized vacuum grease around the edge of the coverslips. Rat-1 cells were resuspended in serum-free media and seeded in a total volume of 3 mL per well at a density of 2.0 x 10^3 cells/cm^2. For quantification of spread area, pictures were obtained 4 hours 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 3 independent experiments.

3.3.8 Atomic force microscopy

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

3.4 Results and Discussion

3.4.1 Expression and characterization of aECM-N_3 proteins

aECM-N_3 variants were expressed in *E. coli* cultures supplemented with *p*N_3Phe. Incorporation of *p*N_3Phe into the recombinant proteins relies on activation of the photosensitive amino acid by the phenylalanyl-tRNA synthetase (PheRS) of the bacterial expression host. The PheRS used for this study was a previously characterized mutant with relaxed substrate specificity [24]. This method results in statistical decoding of phenylalanine (Phe) codons placed at regular intervals in the coding sequence [24]. Proteins were expressed in a Phe auxotrophic *E. coli* strain and purified by exploiting the temperature-dependent phase behavior of proteins that contain elastin-like repeats [25]. Incorporation efficiency was determined by integration of the aromatic proton signals in

the ^{1}H NMR spectra of the purified proteins (Figure 3-2); the extent of Phe replacement varied from 13% to 53%, depending on the concentration of *p*N_3Phe in the expression medium (Figure 3-3).

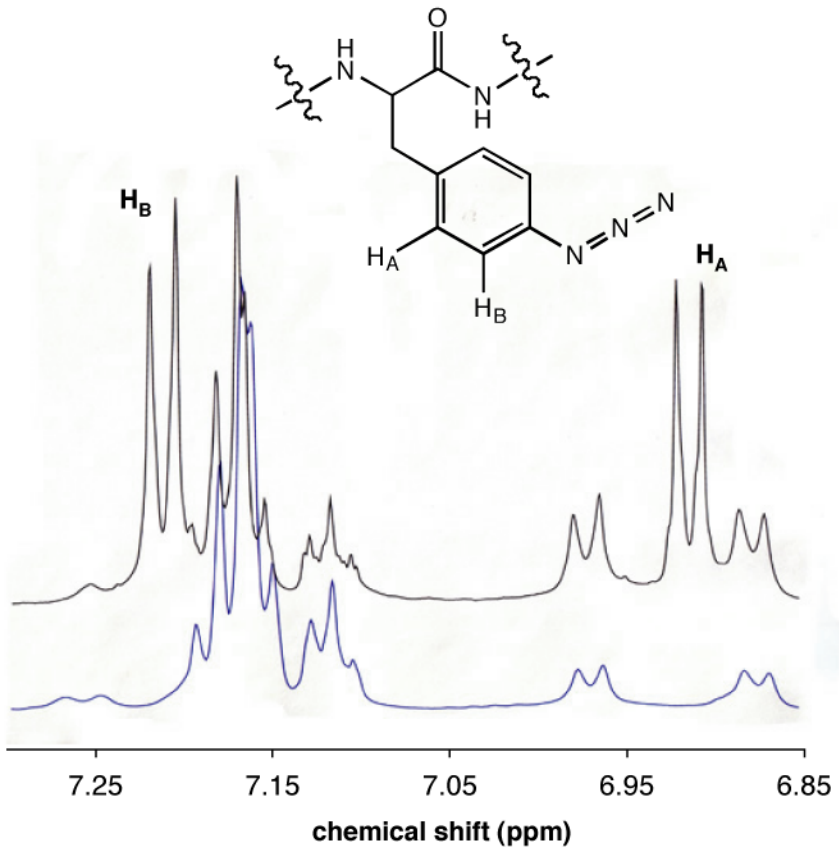

Figure 3-2. Representative ^{1}H NMR spectra (6.85–7.30 ppm region) of an aECM-N_3 construct expressed in media supplemented with phenylalanine (bottom spectrum) or with 250 mg/L *p*N_3Phe (top spectrum). Spectra are identical except for two additional doublets in the top spectrum assigned to the aromatic protons of *p*N_3Phe; integration indicates replacement of 53% of the phenylalanine residues by *p*N3Phe. 600 MHz spectra were taken on 1 mM samples in DMSO-d_6 at 23°C.

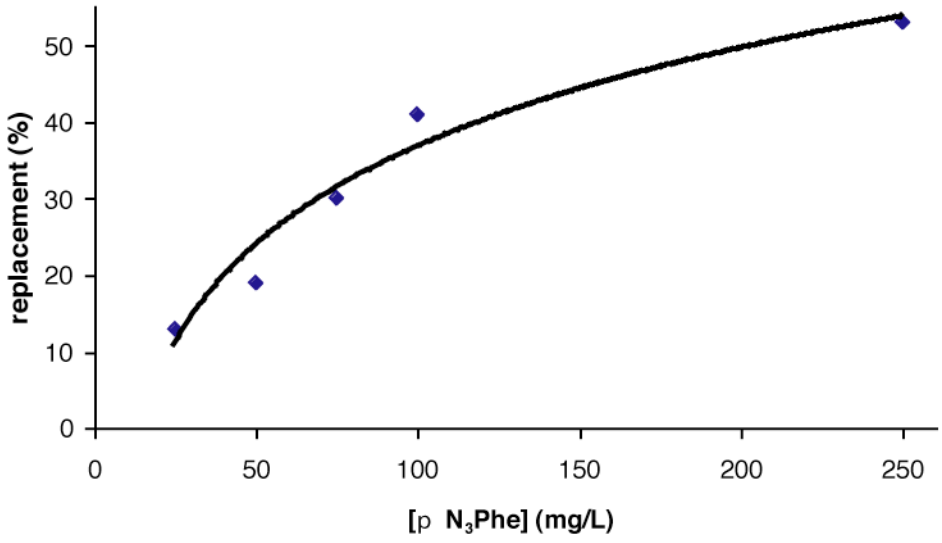

Figure 3-3. Incorporation of *p*N_3Phe into the CS5-N_3 protein as a function of concentration in the expression medium.

3.4.2 Kinetics of photodecomposition

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 asymmetric stretch at 2130 cm^{-1} (Figure 3-1 (B)) [26]. 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 s (Figure 3-1 (C)). 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 the other canonical amino acids. Aryl azides have been used previously to effect photochemical crosslinking in protein and nucleic acid systems [27, 28].

3.4.3 Mechanical testing

Elastic moduli of irradiated CS5-N_3 films were determined by uniaxial tensile testing under simulated physiological conditions (Figure 3-1 (D)). 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, values near the range characteristic of elastins (0.3–0.6 MPa) [16]. Replacement of less than 20% of the encoded Phe residues produced films too weak to test, and films made without *p*N_3Phe yielded no evidence of crosslinking. The capacity to vary the modulus by altering the *p*N_3Phe concentration in the expression medium is an attractive feature of this method, as recent work has highlighted the role of mechanical transduction mechanisms in mediating the physiology of adherent cells [29, 30].

3.4.4 Cell patterning

To investigate the potential of photoreactive proteins as substrates for studies of cell adhesion and growth, we created patterns of adherent fibroblasts on proteins patterned by photolithography. Protein films created by spin coating 12.5 mg/mL solutions of RGD-N_3 in dimethylsulfoxide directly on poly(ethylene oxide) (PEO)-coated glass slides were clear and homogeneous by optical microscopy. Protein films were irradiated for 60 s at 365 nm through a chrome-on-quartz mask. Stripping of the masked areas was accomplished by washing in 6 M guanidine hydrochloride.

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. Non-contact atomic force microscopy (AFM) of RGD-N_3 patterns revealed uniform protein films. Films spun at 1000 rpm were 467 or 750 nm thick when imaged dry or hydrated, respectively. (Figure 3-4).

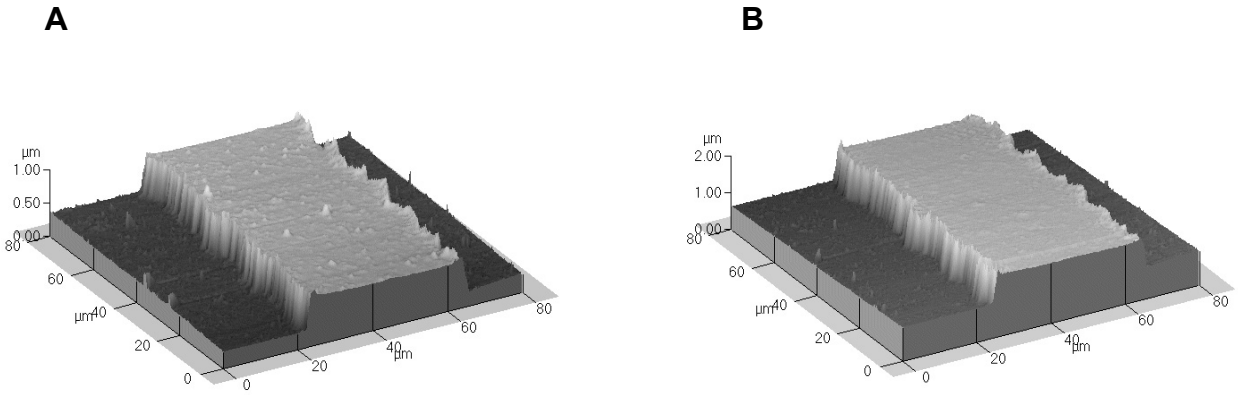

Figure 3-4. AFM images of patterned RGD-N_3. The images were recorded on (A) dried or (B) hydrated patterned films in constant-force mode with an autoprobe M5 atomic force microscope (Park Scientific).

To create cell arrays, Rat-1 fibroblasts were deposited on RGD-N_3 patterns in the absence of serum. After 4 hr of incubation, the unattached cells were removed by mild washing with phosphate buffered saline (PBS) revealing cell patterns (Figure 3-5). Cell monolayers in the interior of the protein regions were indistinguishable from monolayers grown on fibronectin coatings; however, cells positioned along the RGD-N_3 pattern edges were elongated parallel to the pattern border, consistent with previous studies [31].

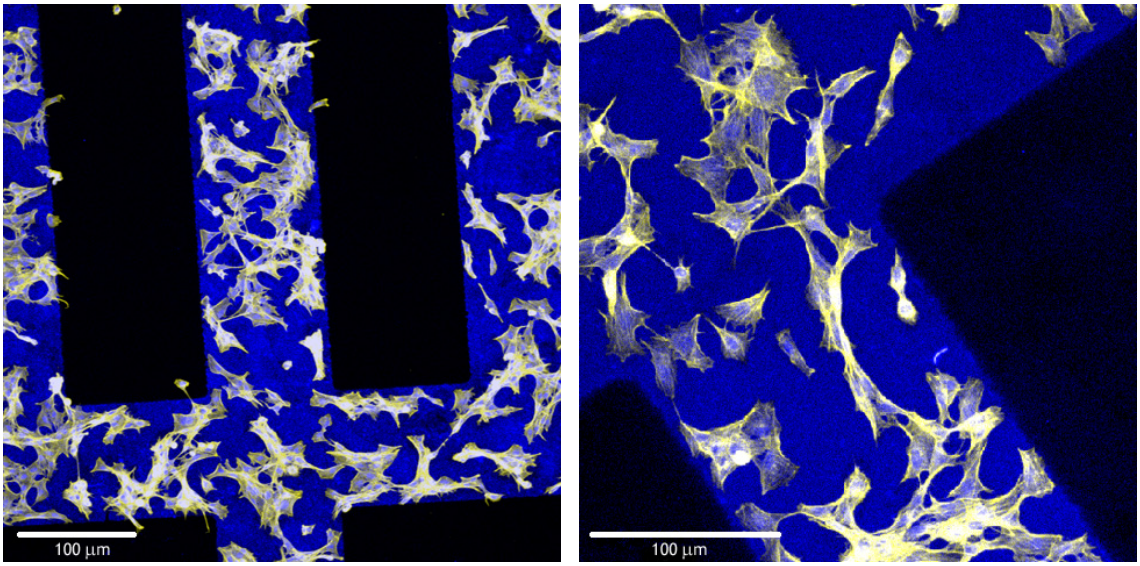

Figure 3-5. Confocal microscopy of Rat-1 fibroblasts attached to photopatterned RGD-N_3. Immunostaining with anti-T7 (blue) demonstrates colocalization of aECM-N_3 protein and cells (stained with rhodamine phalloidin (yellow)). Scale bars represent 100 µm.

3.4.5 Cell spreading

To determine whether cells specifically recognize the RGD cell-binding domain, we compared cell spreading on uniformly photocrosslinked RGD-N_3 and RDG-N_3 (sequence-altered, negative control) films (Figure 3-6). After 4 hr of incubation, Rat-1 cells spread well on RGD-N_3 films, although the extent of spreading was reduced in comparison to that observed on the fibronectin control. In contrast, cells did not spread on RDG-N_3 and resembled cells plated on bovine serum albumin (BSA).

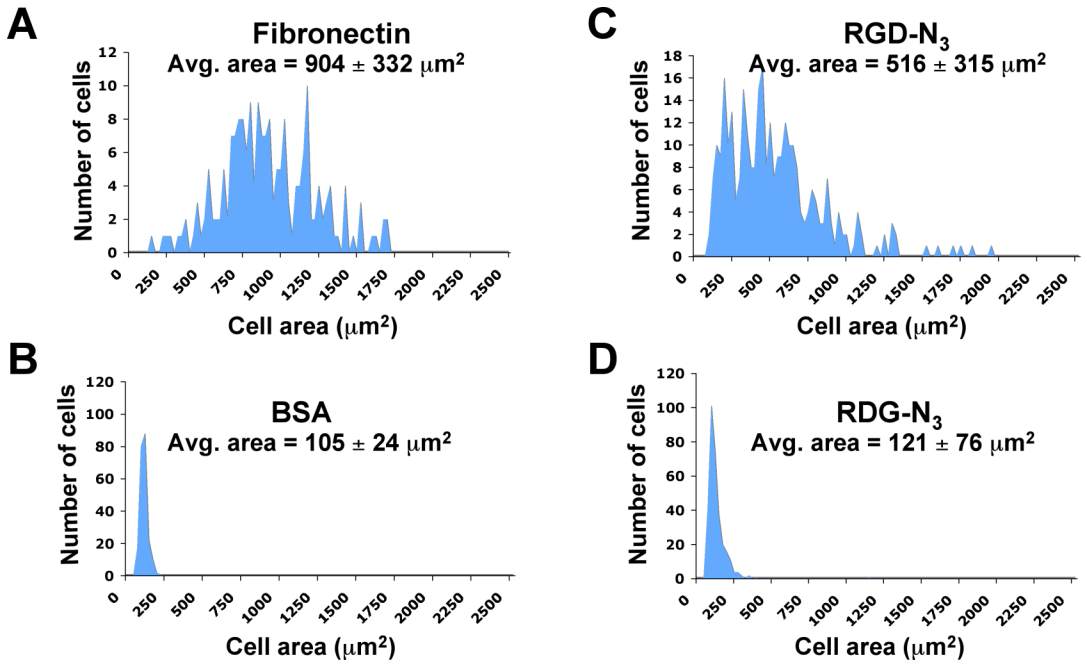

Figure 3-6. Rat-1 fibroblast cell spread areas on (A) fibronectin, (B) BSA, (C) RGD-N_3, and (D) RDG-N_3. RGD-N_3 supports sequence-specific cell spreading.

3.5 Conclusions

The availability of intrinsically photoreactive proteins enables new approaches to protein patterning. 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.

3.6 Acknowledgements

We thank Michael Diehl, Alireza Ghaffari, and Nandita Sharma for helpful discussion, and Kechun Zhang for help in preparing the patterned substrates. Support was

provided by the NSF Center for the Science and Engineering of Materials at Caltech, NIH 62523 and EB 01971, an NIH predoctoral fellowship to SAM, a Whitaker Graduate Fellowship to JCL, and the Joseph J. Jacobs Institute for Molecular Engineering for Medicine.

3.7 References

1. Ekins, R.P., *Multi-Analyte Immunoassay.* Journal of Pharmaceutical and Biomedical Analysis, 1989. **7**(2): 155-168.
2. Tan, W., and T.A. Desai, *Layer-by-layer microfluidics for biomimetic three-dimensional structures.* Biomaterials, 2004. **25**(7-8): 1355-1364.
3. Templin, M.F., et al., *Protein microarray technology.* Trends in Biotechnology, 2002. **20**(4): 160-166.
4. Christman, K.L., V.D. Enriquez-Rios, and H.D. Maynard, *Nanopatterning proteins and peptides.* Soft Matter, 2006. **2**(11): 928-939.
5. Voldman, J., *BioMEMS – Building with cells.* Nature Materials, 2003. **2**(7): 433-434.
6. Shim, J., et al., *Micro- and nanotechnologies for studying cellular function.* Current Topics in Medicinal Chemistry, 2003. **3**(6): 687-703.
7. Corey, J.M., and E.L. Feldman, *Substrate patterning: an emerging technology for the study of neuronal behavior.* Experimental Neurology, 2003. **184**: S89-S96.
8. Rozkiewicz, D.I., et al., *Covalent microcontact printing of proteins for cell patterning.* Chemistry–a European Journal, 2006. **12**(24): 6290-6297.

9. Suh, K.Y., et al., *A simple soft lithographic route to fabrication of poly(ethylene glycol) microstructures for protein and cell patterning.* Biomaterials, 2004. **25**(3): 557-563.

10. Blawas, A.S. and W.M. Reichert, *Protein patterning.* Biomaterials, 1998. **19**(7-9): 595-609.

11. Kirshenbaum, K., I.S. Carrico, and D.A. Tirrell, *Biosynthesis of proteins incorporating a versatile set of phenylalanine analogues.* Chembiochem, 2002. **3**(2-3): 235-237.

12. Zhang, K.C., M.R. Diehl, and D.A. Tirrell, *Artificial polypeptide scaffold for protein immobilization.* Journal of the American Chemical Society, 2005. **127**(29): 10136-10137.

13. Chin, J.W., et al., *Addition of a photocrosslinking amino acid to the genetic code of Escherichia coli.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(17): 11020-11024.

14. Chin, J.W., et al., *Addition of p-azido-L-phenylaianine to the genetic code of Escherichia coli.* Journal of the American Chemical Society, 2002. **124**(31): 9026-9027.

15. Bose, M., et al., *The incorporation of a photoisomerizable amino acid into proteins in E-coli.* Journal of the American Chemical Society, 2006. **128**(2): 388-389.

16. Di Zio, K. and D.A. Tirrell, *Mechanical properties of artificial protein matrices engineered for control of cell and tissue behavior.* Macromolecules, 2003. **36**(5): 1553-1558.

17. Heilshorn, S.C., et al., *Endothelial cell adhesion to the fibronectin CS5 domain in artificial extracellular matrix proteins.* Biomaterials, 2003. **24**(23): 4245-4252.

18. Heilshorn, S.C., J.C. Liu, and D.A. Tirrell, *Cell-binding domain context affects cell behavior on engineered proteins.* Biomacromolecules, 2005. **6**(1): 318-323.

19. Liu, J.C., S.C. Heilshorn, and D.A. Tirrell, *Comparative cell response to artificial extracellular matrix proteins containing the RGD and CS5 cell-binding domains.* Biomacromolecules, 2004. **5**(2): 497-504.

20. Cantor, E.J., PhD Thesis, 1997, University of Massachusetts, Amherst.

21. Kast, P., *Pkss – a 2nd-Generation General-Purpose Cloning Vector for Efficient Positive Selection of Recombinant Clones.* Gene, 1994. **138**(1-2): 109-114.

22. Sharma, N., PhD Thesis, 2001, University of Massachusetts, Amherst.

23. Yoshikawa, E., et al., *Genetically-Engineered Fluoropolymers – Synthesis of Repetitive Polypeptides Containing P-Fluorophenylalalanine Residues.* Macromolecules, 1994. **27**(19): 5471-5475.

24. Ibba, M., and D. Soll, *Aminoacyl-tRNA synthesis.* Annual Review of Biochemistry, 2000. **69**: 617-650.

25. Welsh, E.R., and D.A. Tirrell, *Engineering the extracellular matrix: A novel approach to polymeric biomaterials. I. Control of the physical properties of artificial protein matrices designed to support adhesion of vascular endothelial cells.* Biomacromolecules, 2000. **1**(1): 23-30.

26. Pretsch, E., P. Buhlmann, and C. Affolter. *Structure Determination of Organic Compounds: Tables of Spectral Data* (W. Fresenius, J.F.K. Huber, E. Pungor, G.

A. Rechnitz, T. West, eds.), 2000, Verlag Berlin Heidelberg New York: Springer-Verlag.

27. Costas, C., et al., *RNA-protein crosslinking to AMP residues at internal positions in RNA with a new photocrosslinking ATP analog.* Nucleic Acids Research, 2000. **28**(9): 1849-1858.

28. Cleary, M.A., P.S. Pendergrast, and W. Herr, *Structural flexibility in transcription complex formation revealed by protein-DNA photocrosslinking.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(16): 8450-8455.

29. Engler, A., et al., *Substrate compliance versus ligand density in cell on gel responses.* Biophysical Journal, 2004. **86**(1): 617-628.

30. Ingber, D.E., *Mechanosensation through integrins: Cells act locally but think globally.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(4): 1472-1474.

31. Zhang, S.G., et al., *Biological surface engineering: a simple system for cell pattern formation.* Biomaterials, 1999. **20**(13): 1213-1220.