Chapter VII. Lithographic patterning of an intrinsically photoreactive cell-adhesive protein

Abstract

We describe a novel, facile method for the production of cell-adhesive photolithographic protein patterns. The procedure is demonstrated using a designed cell-adhesive artificial extracellular matrix protein, produced in *E. coli*, which incorporates the photoreactive noncanonical amino acid *para*-azidophenylalanine (pN$_3$Phe) in a residue-specific manner. Irradiation efficiently decomposes the aryl azide to a reactive intermediate that mediates crosslinking of the polypeptide. Moduli of bulk films provided a direct correlation between number of introduced photoreactive groups and stiffness. Photolithographic protein patterns were produced by standard techniques used in the microelectronics industry, irradiating at 365 nm through a mask and washing away the uncrosslinked "photoresist" with a solubilizing agent, in this case dilute aqueous detergent. The resulting photopatterned aECM protein retains cell-adhesion characteristics and can produce a stable array of endothelial cells in the presence of competing soluble proteins.

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1. Introduction, results, and discussion

Cellular arrays are becoming popular tools for probing cellular physiology to elucidate factors that mediate migration, growth, or cell-cell interactions [1-3]. Cell patterning that relies on the adhesive properties of biomolecules, particularly proteins and peptides derived from the extracellular matrix, has proven most valuable because it mimics the natural cellular environment [4, 5]. Although photolithography holds a preeminent place in microfabrication, optical lithography of proteins has been hampered by the need to use traditional chemical photoresists or modify proteins with photoreactive groups [6]. To circumvent these issues, new techniques have emerged that allow direct placement of adhesive proteins or peptides. Microcontact printing, part of the soft lithography approach developed by Whitesides and colleagues [7], consists of pattern transfer from an elastomeric membrane "stamp" to surfaces of self assembled monolayers. Alternatively, dip-pen nanolithography (DPN) uses a tip, akin to those commonly used in atomic force microscopy, to spot adhesive patterns onto prepared surfaces [8]. DPN compromises the speed and simplicity of microcontact printing for a significant decrease in accessible feature size. Despite these advances, the hallmarks of photolithography -- high registry, throughput, and fidelity -- remain attractive. However, proteins are not intrinsically photoreactive. Recently, several labs have reported the incorporation of photoreactive noncanonical amino acids into proteins in a site-specific [9, 10] or residue-specific fashion [11]. Here we describe the creation of protein polymers with multiple copies of the photosensitive noncanonical amino acid *para*-azidophenylalanine (*p*N_3Phe), which permits efficient photocrosslinking. Further, we demonstrate that thin films of this protein, dubbed artificial extracellular matrix protein

with aryl azides (aECM-N_3), can be patterned on a surface using traditional photolithographic techniques. Finally, we demonstrate the utility of the method by creating cell patterns through selective endothelial cell attachment to the aECM-N_3 protein, which contains a known endothelial cell-binding domain.

The noncanonical amino acid was introduced in a residue-specific manner, which permits gram scale production of protein, introduction of *p*N_3Phe at many sites, and facile tuning of the level of incorporation [12]. This method of incorporation replaces the natural amino acid, phenylalanine, with the unnatural analog by competition at the aminoacyl-tRNA synthetase, the enzyme responsible for charging the amino acid to its cognate tRNA(s) [13]. The phenylalanyl-tRNA synthetase used for this study was a previously characterized mutant known to accept a number of phenylalanine analogs including *p*N_3Phe [11]. The aECM-N_3 construct belongs to a family of artificial proteins designed in our laboratory to combine desirable mechanical properties, resulting from an elastin-like repeat, with the ability to selectively adhere endothelial cells through a cell-binding domain derived from the CS5 region of fibronectin (Figure 1a) [14, 15]. Protein was expressed in a phenylalanine-auxotrophic *E. coli* strain in media supplemented with *p*N_3Phe and purified by taking advantage of the inverse temperature phase behavior of proteins including elastin-like repeats [16]. The extent of *p*N_3Phe incorporation in the homogeneous protein product was determined by ^{1}H NMR (Figure 1b).

a

MMASMTGGQQMGRKTHHHHHHMG[LDGEEIQIGHIPREDVDYHLYGP((VPGVG)$_2$VPGFG(VPGVG)$_2$)$_5$]$_3$LE

CS5 domain — Elastin sequence

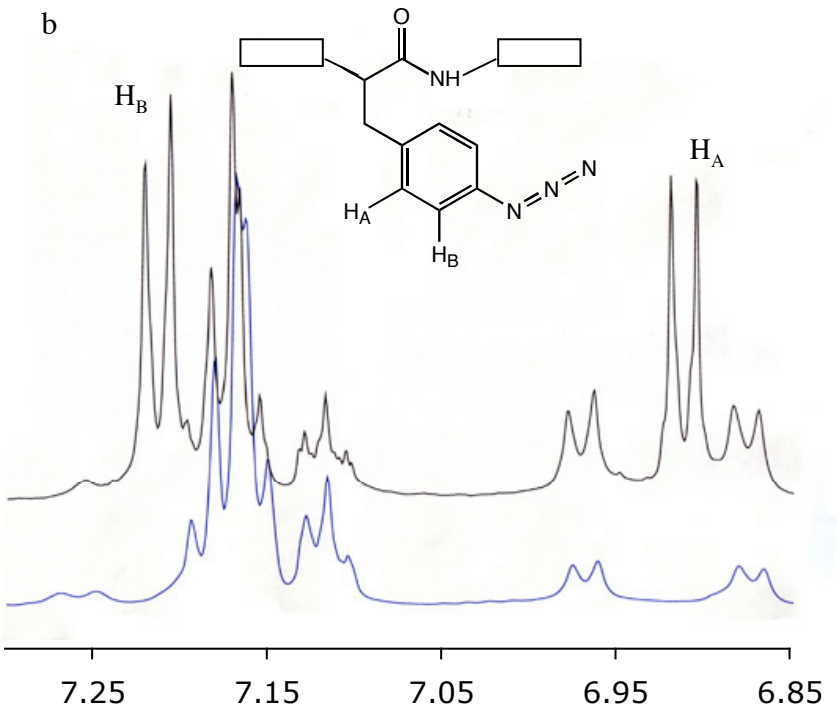

Figure 1. Design and production of aECM-N_3. (a) Design of the aECM-N_3 construct includes a cell-binding domain (the CS5 region of fibronectin) and a structural domain (the VPGVG elastin-like repeats with internal phenylalanine sites for incorporation of *p*N_3Phe). (b) Expanded ^{1}H NMR region (6.85 - 7.30ppm) of both the aECM construct expressed in the presence of phenylalanine (bottom spectrum) and expressed in the presence of 250 mg/L *p*N_3Phe (top spectrum). Spectra are identical except for the two extra doublets in the top spectrum from the aromatic protons of *p*N_3Phe. 600 MHz spectrum were taken on 1 mM samples in DMSO-d_6 at 23°C.

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 asymmetric stretch at 2130 cm^{-1}. To directly mimic the anticipated photolithography, measurements were performed on thin films of aECM-N_3 spun cast directly onto a zinc selenide wafer and irradiated using a standard mask aligner with a quartz wafer in place of the mask. Azide loss under these conditions was rapid (Figure 2a), following first order kinetics with a half-life of 34 seconds (Figure 2b). It is noteworthy that none of the other IR resonances was noticeably altered under these conditions, implying that irradiation at 365 nm specifically affects the aryl azide. The elastic modulus of aECM-N_3 films, determined through uniaxial tensile testing of extensively irradiated bulk films under physiological conditions (37°C, pH 7.4), was found to directly correlate with the level of azide incorporation (Figure 2c). Calculations indicate an intermolecular crosslinking efficiency between 31% and 43% [17].

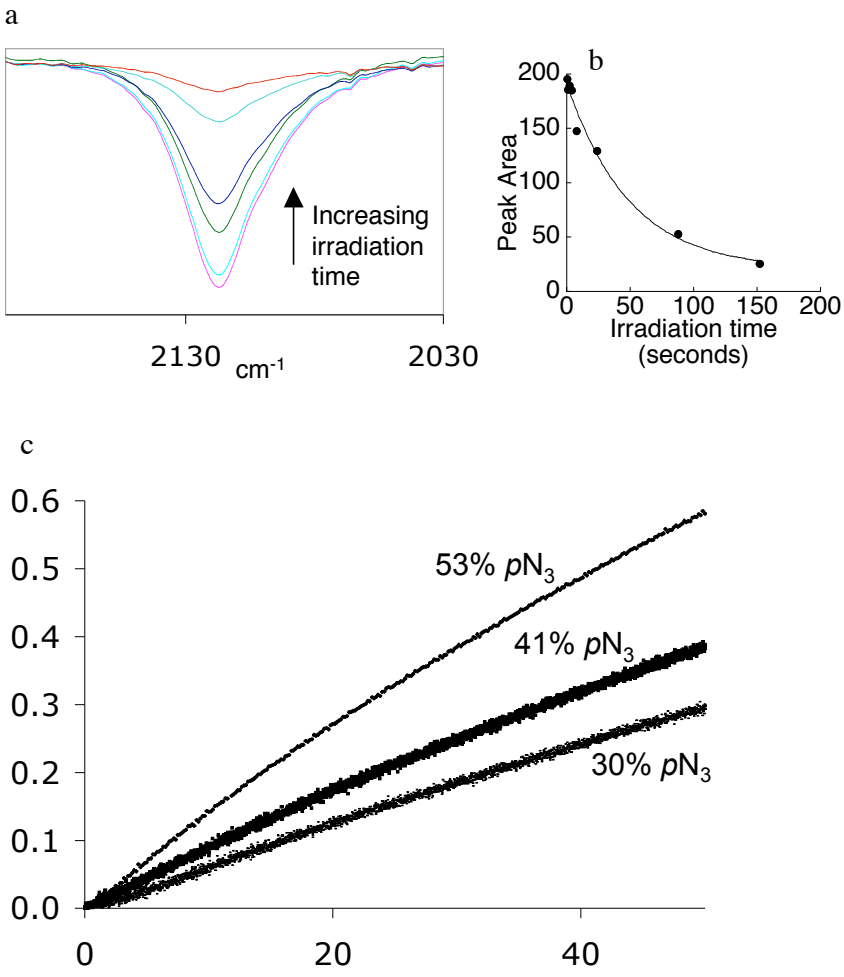

Figure 2. Characterization of azide decomposition by infrared spectroscopy and modulus of resultant films. (a) FT-IR spectrum of the aryl azide asymmetric stretch of aECM-N_3 films as a function of irradiation time. Irradiation times are 0 (bottom), 0.5, 8, 24, 88 and 124 seconds (top). Thin films were spin coated at 2000 rpm for 99 seconds directly onto zinc selenide wafers from a 10% solution in DMSO and dried at 50°C for 2 minutes. All irradiations were performed at 365 nm through quartz slide. (b) Peak area versus irradiation time yields a first order exponential decay with a rate constant of 0.02 sec^{-1} ($t_{1/2}$=34 sec). (c) Linear tensile testing of solvent-cast aECM-N_3 films with variable *p*N_3Phe incorporation. The elastic modulus scales with the number of *p*N_3Phe residues incorporated per protein construct. Samples were created in PTFE molds by drying 10% aECM solutions in DMSO overnight at 50°C. Tensile testing of equilibrated films was performed at 37°C in phosphate buffered saline at pH = 7.4. Samples were extended at a rate of 10% length/minute.

Protein films created by spin coating a solution of protein in methylsulfoxide directly onto the aminated glass coverslips were clear and homogeneous by optical microscopy. Dry films were irradiated at 365 nm through a chrome mask on quartz using a standard mask aligner. Efficient stripping was accomplished by rinsing with 0.05% aqueous sodium dodecylsulfate. The resolution in the protein layer is sufficient to visualize the rough edges of the protein features, which are the result of a mask generated from a 3000 dpi print (Figure 3c). Use of a traditional chemical photoresist produces features with similar edge roughness. Pattern development as a function of irradiation time is evident (Figure 3a-c) and plateaus after approximately 30 seconds. The photocrosslinked pattern retained the ability to participate in biological recognition by an appropriate antibody, as evidenced by fluorescent immunolabelling of the irradiated protein (Figure 4).

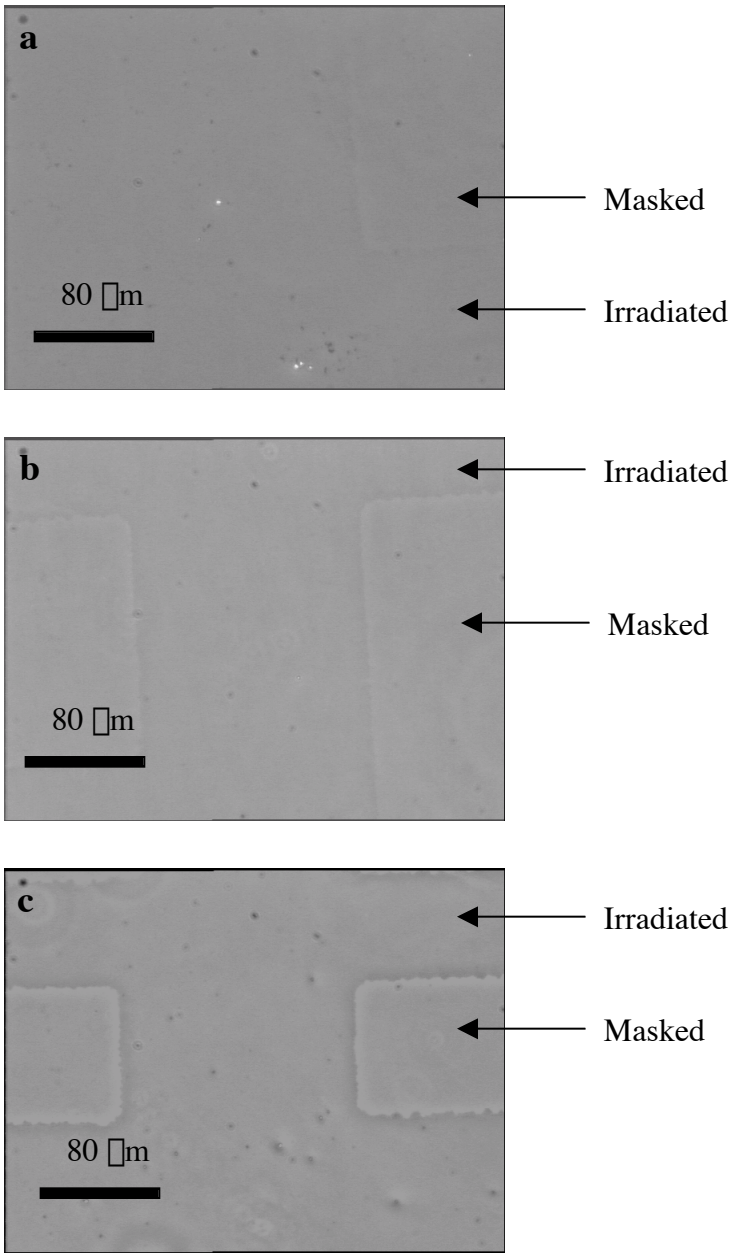

Figure 3. Optical and physical characterization of photopatterned aECM-N_3 films. Optical image of features irradiated through a mask for different lengths of time. Films were prepared by spin coating a 10% aECM-N_3 solution onto aminated glass at 2000 rpm for 99 seconds and drying at 50°C. Lithography was accomplished using a standard Carl-Zuss mask aligner at 365 nm. Gentle agitation in DMSO stripped non-irradiated regions. Features from (a) a slide irradiated for 1 second were very faint, (b) films photopatterned for 5 seconds yielded more pronounced features, and (c) slides irradiated for 30 seconds or longer resulted in higher contrast patterns.

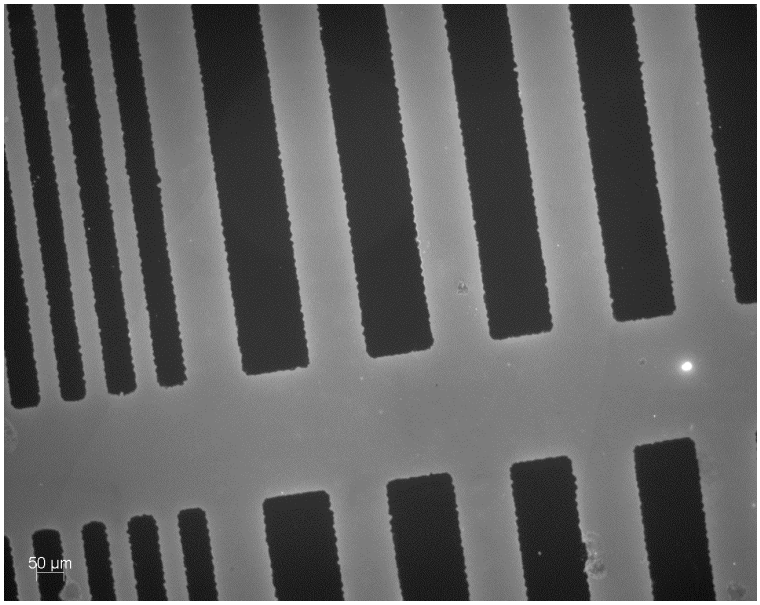

Figure 4. Fluorescence microscopy of photopatterned aECM-N_3. Pegylated glass slides were prepared by treatment of aminated glass with 200 mM solution of PEG_{2000}-benzotriazole carbonate in pyridine for 3.5 hours. The PEGylated slides were spin coated with aECM-N_3, dried, irradiated for 30 seconds, and washed overnight in 0.05% SDS to produce well-defined protein features on a non-adhesive background. aECM-N_3 was detected by primary anti-T7-tag antibody followed by Cy-2 conjugated secondary antibody.

As a proof of concept, we demonstrate the power of this technology by creating stable cell patterns on non-adhesive polyethylene glycol coated coverslips. Human umbilical vein endothelial cells (HUVEC) were incubated on processed slides for 6 hours in serum-free medium followed by 24 hours in the presence of serum prior to fixation and immunochemical labeling (Figure 5). Cells appear well-spread and are specifically located within the patterned protein features. Cell viability and adhesion occur at levels consistent with that of adsorbed surfaces of similar aECM proteins containing only natural amino acids [18], further indicating that photocrosslinking does not significantly impair protein properties. Additionally, the micropatterned cell arrays were stable over 48 hours in the presence of serum and did not display any toxic effects over 6 days.

The ability to take advantage of ubiquitous microelectronics microfabrication technology to create patterns from proteins expressed in *E. coli* provides a facile method to create biocompatible protein patterns. This technique provides a means to adjust the moduli of the resultant films, which is not readily accomplished with widely used protein adsorption or stamping methods. A controlled variation in exposure time or intensity would allow the easy production of gradients in protein concentration or film modulus. Multiple-protein patterns could be accessed through several cycles of exposure and stripping. Indeed, the use of intrinsically photoreactive proteins provides many powerful new tools for the study and production of biomaterials.

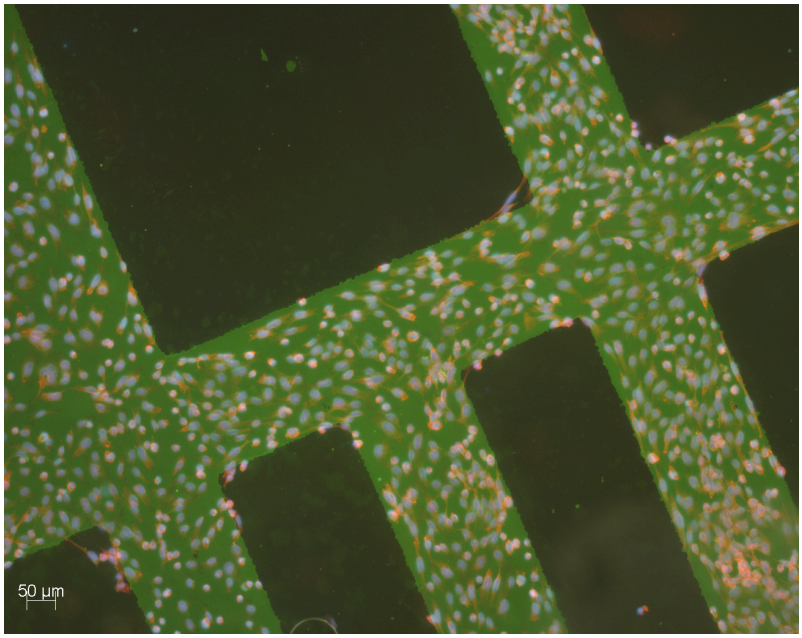

Figure 5. Fluorescence microscopy of HUVEC attached to photopatterned aECM-N_3. Cells were plated onto the prepared surfaces in serum-free medium and allowed to incubate 6 hours prior to incubation with serum-supplemented medium. After 24 hours the cells were fixed with acetone and labeled with phalloidin (actin cytoskeletal stain, shown in red), DAPI (a nuclear stain, in blue), and primary anti-T7-tag antibody followed by Cy-2 conjugated secondary antibody (for aECM-N_3 detection, in green).

2. References and notes

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12. Typical experiments yielded 40 mg of protein per liter of culture. Tuning was achieved by altering the amount of pN_3Phe in the growth media. Incorporation percentages between 13 and 53% were easily obtained.
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17. Assuming an ideal elastic network, the molecular weight between crosslinks can be determined from the elastic modulus ($G=pRT/M_c$; where density is taken to be that of collagen $1.3 g/cm^3$). Knowledge of the molecular weight between

crosslinks and the number of introduced aryl azides allows estimation of the number of intramolecular crosslinks.

18. Cell viability was measured using a standard Wst-1 assay and cell adhesion was quantified using the buoyant centrifugation method described in Liu, J. C.; Heilshorn, S. C.; Tirrell, D. A. *Biomacromolecules* **2004**, *5*, 497-504.