Chapter 6

Patterning and Cell Binding Properties of a Protein Photoresist Produced in *E. coli*

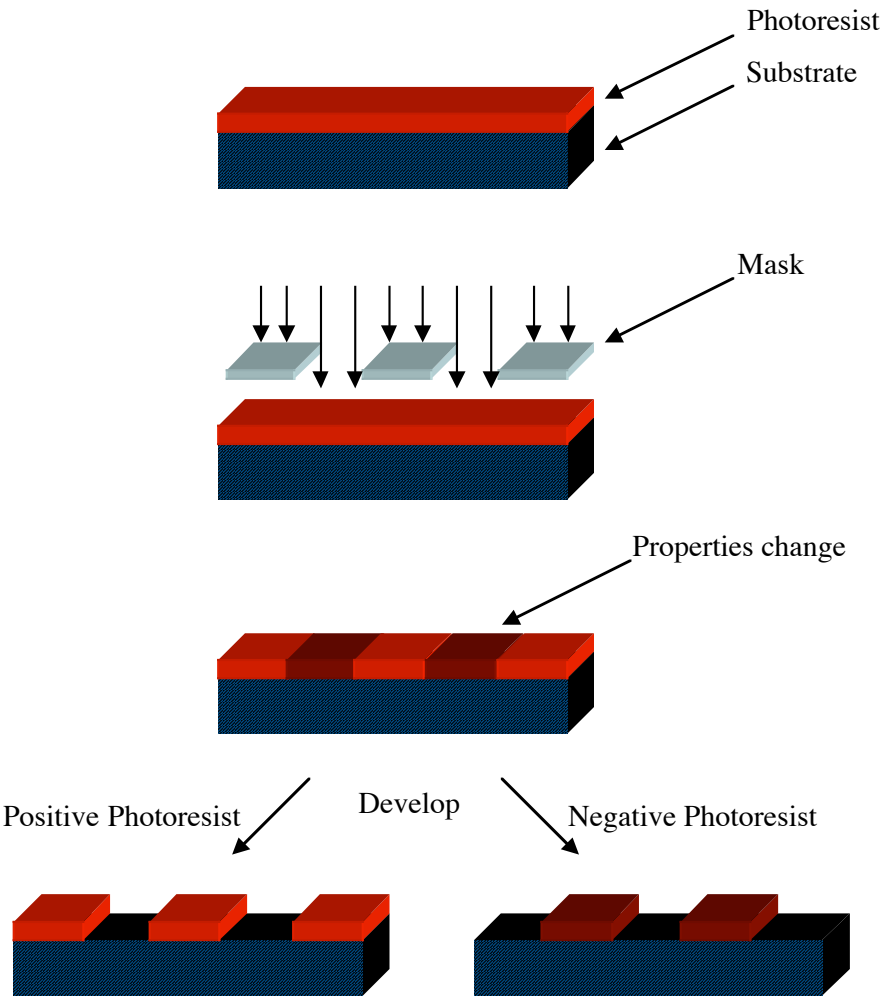
All cell studies were done with Sarah Heilshorn. Dr. Michael Diehl produced the masks for photolithography.

Introduction

Control over organization of proteins on surfaces at microscopic length scales is important to a range of emerging technologies including, but not limited to, biosensors [1, 2], microarray assays [3, 4] and the organization and growth of cells on surfaces [5]. As a result a variety of methods have been developed to address the technical challenges of spatially arraying proteins on surfaces. Microcontact printing and dip-pen methods, both dependent on the controlled application of proteins or ligands, have resulted in arrays proteins with remarkable fidelity. Microcontact printing, popularized by Whitesides and colleges, is simply the transfer of patterns of molecules from an elastomeric template onto an adherent surface [6-8]. Speed, simplicity and biocompatibility are principal assets of this technique, which has demonstrated effectiveness in adsorbing protein layers and creating patterned ligands in self assembled monolayers (SAMS) on silicon or gold [9-13]. Dip-pen lithography is the use of a coated tip, akin to those commonly used in atomic force microscopy, to spot patterns onto surfaces and depends upon similar adhesion mechanisms to microcontact printing [14, 15]. Dip-pen lithography compromises speed and simplicity, with respect to microcontact printing, but can generate features on a significantly smaller scale [16, 17].

Excellent control over feature size, registration, and the high-throughput nature make photolithography an appealing choice for such studies [18]. Indeed many protein patterning studies have been built upon this well established technology [19-23]. Briefly, photolithography is accomplished by spin coating a photoresist onto

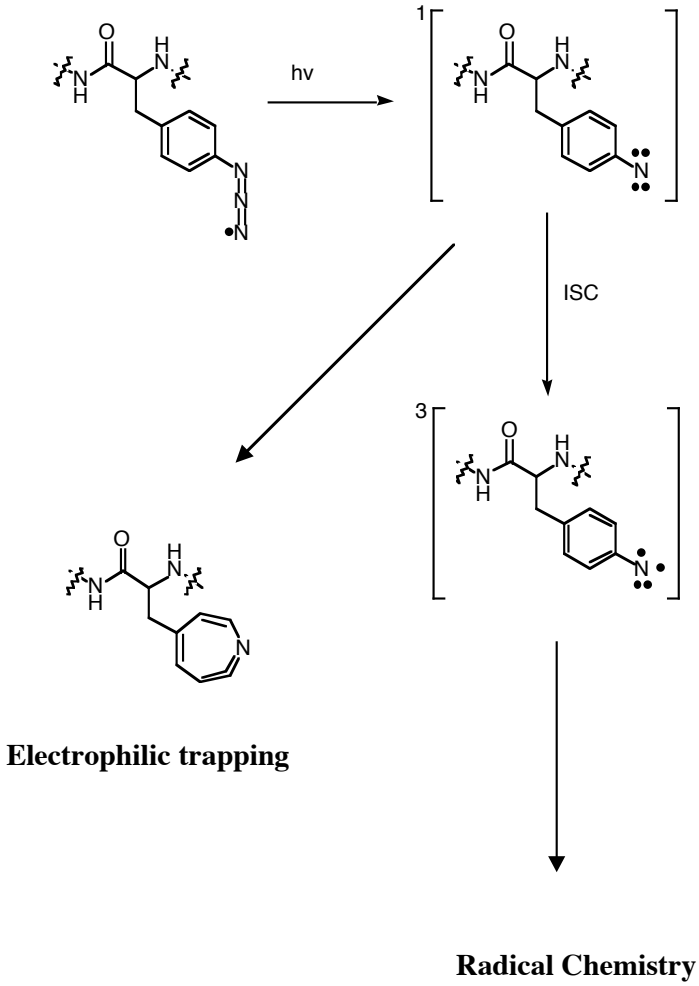
Figure 6.1. Schematic illustration of microfabrication by patterning of photoresists.



a wafer, traditionally silicon, followed by exposure through a mask (Figure 6.1). Exposure changes the properties of the photoresist such that either the exposed or unexposed region can be washed away preferentially by application of appropriate developing solutions. This results in a well-controlled pattern of photoresist with the surface exposed in the interim regions, which theoretically can be controlled down to the wavelength of the incident light. Practically, most photolithographic techniques produce patterns down in the micron to the tenths of micron range [18]. Photolithography based protein patterning has been dependent upon the adsorption of the protein or chemical attachment of synthetic ligands onto the exposed surface, followed by the removal of the exposed photoresist by acetone wash. Unfortunately, these efforts have been challenging due to residual chemicals from processing of photoresist materials. In addition, protein layers formed by adsorption are inherently unstable [24, 25]. As an alternative many groups have turned towards using either synthetic peptides or biomolecules armed with photoreactive functional groups [26-28]. Such methods afford acute control over patterning, allow facile tuning of deposition gradients and, importantly, allow placement of multiple types of molecules in a single pattern [29-32]. While powerful, these techniques require modification of proteins with photoaffinity reagents or attachment of ligands followed by protein adherence, which can complicate the ability to create multiple patterns.

In this study we detail the use of a protein photoresist produced in *E. coli*. Photoresist properties of this protein are imparted by partial replacement of phenylalanine by the photoreactive unnatural amino acid *para*-azidophenylalanine (pN_3Phe) [33-35]. Incorporation of this analog is afforded by expression in the

Figure 6.2. Photodecomposition of arylazides can mediate crosslinking either by electrophillic trapping via the ring expansion product or by the diradical behaviour of the triplit nitrene.

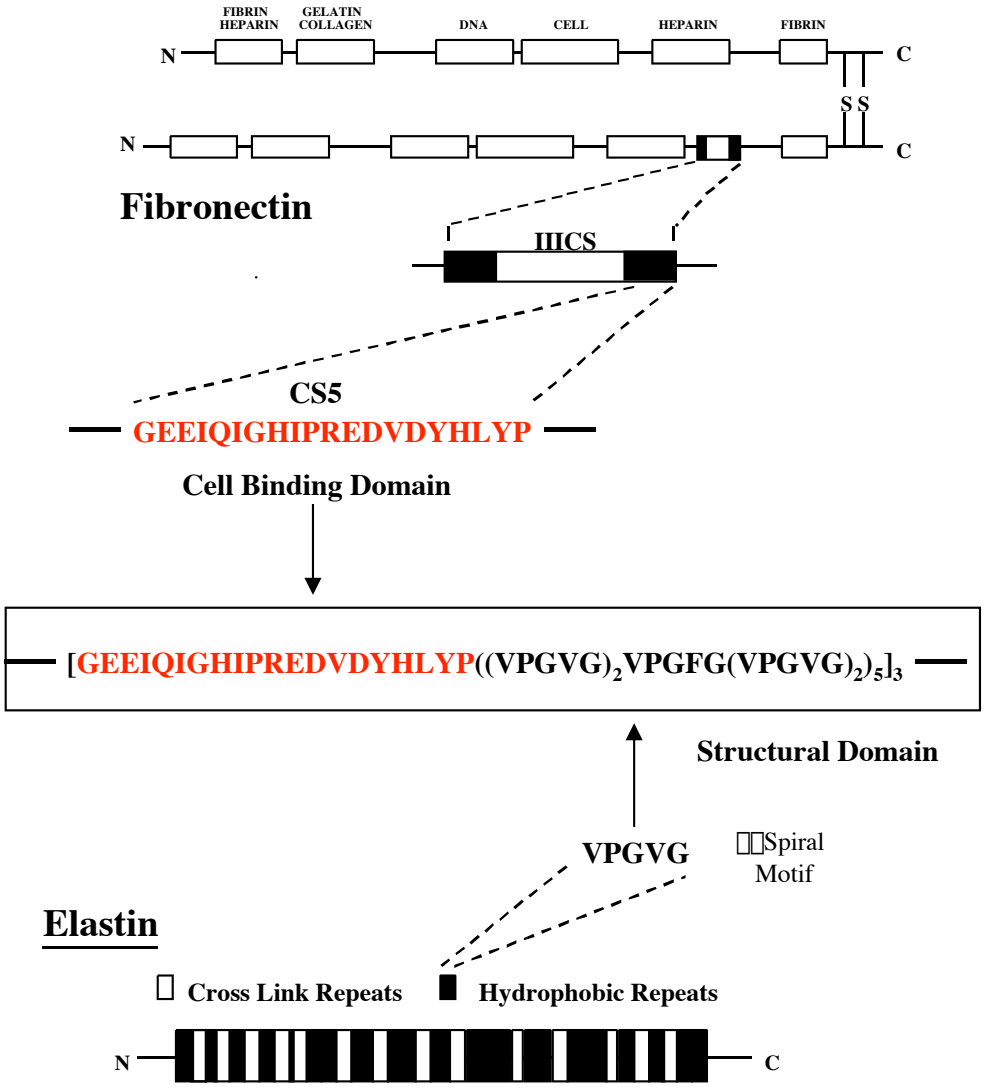


presence of the analog from *E. coli* auxotrophic for phenylalanine and armed with a plasmid born copy of a mutant phenylalanyl-tRNA synthetase [36-38]. The azide decomposes upon exposure to irradiation (Figure 6.2), the same sources used for microelectronics production, to produce an arylnitrene that mediates crosslinking within the material and to the surface. Standard microelectronics photolithography sources use 365 nm light, which will not damage natural protein or cellular constituents [39, 40]. Our studies use a designed artificial extracellular matrix protein [41-43] armed with pN_3Phe. This protein incorporates a CS5 cell binding domain derived from the IIICS region of fibronectin [14, 44, 45] and a structural domain composed of repeats of elastin-like $(VPGVG)_2VPGFG(VPGVG)_2$ (Figure 6.3) [46]. Development of the pattern on a variety of surfaces is afforded by simple washing in methyl sulfoxide. Resultant protein patterns demonstrate high spatial fidelity and support endothelial cell attachment via an internal integrin-binding domain.

Materials and Methods

Diethyldiaminotriethoxy silane (DEDA) was purchased from Gelest. Polyethylene glycol N-hydroxy succinimidyl esters were purchased from Shearwater polymers. All other chemicals were purchased from Aldrich and used as received.

Figure 6.3. Extracellular matrix construct is a polypeptide copolymer composed of CS5 cell binding domains, from the IIICS region of elastin, and elastin-like structural domains composed primarily of VPGVG repeats.



Preparation of DEDA modified surfaces

Glass coverslips were cleaned by sonication in potassium hydroxide saturated ethanol for 15 minutes. The resultant slides were washed briefly with water and allowed to react in a solution of Ethanol/5% AcOH/DEDA (93:5:2) for 15 minutes. The modified surfaces were washed thoroughly with deionized water and blown dry under a stream of argon.

Preparation of PEG modified surfaces

DEDA modified slides were treated with 200 mM solution of PEG_{2000}-NHS ester (PEG_{2000} corresponds to a PEG of approximately 2kD) in pyridine for at least 1 hour. The surfaces were then washed with pyridine and ethanol and subsequently dried under a stream of argon.

Spin coating CS5-ELF proteins onto modified surfaces

Solutions of CS5-ELF proteins in DMSO (1.25 weight %) expressed and purified as described in chapter 3, were centrifuged at 14,000 rpm for 5 minutes to sediment any particulate matter. The surface of treated 22x22 mm coverslips was completely covered with the 18 μL of CS5-ELF solutions by dragging the solution across the surface with the disposable tip of a pipettman. Slides covered with protein solutions spun at 2000 rpm for 100 seconds demonstrated an even coating. DMSO was removed from films by drying in a 50° oven for 1-2 minutes, producing a thin film indistinguishable from an untreated slide by eye.

Photolithography of CS5-ELF coated slides

Photolithography of protein films on DEDA and PEG coated glass was performed on a Karl-Zuss mask aligner. A mask of chrome on quartz was prepared using standard techniques (negative photoresist, chrome deposition, stripping) [18] from a transparency printed at 3000 dots per inch resolution. Stripping of photopatterned protein surfaces was found to proceed to best in DMSO solutions as determined by phase contrast examinations of resulting patterns. DEDA surfaces were stripped by smooth agitation of the surface in room temperature DMSO for 30 minutes. PEG modified surfaces were stripped by sonication in room temperature DMSO.

IR determination of photodecomposition kinetics

A 10% CS5-ELF-N_3 solution in DMSO was spun onto a ZnSe crystal at 2000 rpm for 99 seconds. The resulting film was dried for 2 minutes at 50°C. Infrared spectra were taken of the irradiated film at various time points until the azide signal disappeared. Irradiations were performed under the exact same conditions that patterning was performed. Irradiation was performed through a quartz slide of the exact same thickness as the mask used.

Endothelial cell maintenance

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 0.61mM EDTA solution (Gibco, Grand Island, NY). Cells between passages 3 and 8 were used for all experiments.

Imaging endothelial cell attachment by phase contrast

All slides were sterilized by immersion in a 75% ethanol solution in water for at least two minutes, and then subsequently dried using canned air. At this point the samples were placed in sterile 6 well polystyrene culture plates. Samples were blocked with a 2% heat denatured BSA in PBS for 30 minutes. 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. For cell spreading assays, a density 8.4×10^4 cell/cm^2 was used. Cell spreading experiments were typically samples every 30 minutes after 1 hour for 3 hours. Cell patterning experiments were typically examined after 2 hours and again after 4-12 hours.

Cell fixation, staining and probing with T7 antibody

Each well of a 6-well plate was washed with 2 mL PBS (3x). Fixation was accomplished by application of 1mL ice-cold acetone to the washed wells for exactly 1min. Subsequently the wells were once again washed with 2 mL PBS (3x). Blocking was effected by applying 2 mL 10% BSA solution to the wells for 30 minutes at room temperature. After this incubation 0.2 μL of anti-T7 primary antibody (Novagen) was added and allowed to incubate at room temperature for at least 6 hours. These samples were then washed with 2 mL PBS (3x, five minutes each) without agitation. A secondary antibody/phalloidin solution composed of 862

□L PBS, 100 □L 2° antibody (cy2 labelled anti-mouse, Molecular Probes) and 38 □L Phalloidin (Molecular Probes) was incubated with the samples in the dark for 1 hour. Samples underwent subsequent washes with PBS, 2 mL, 2 mL 10 minutes with agitation, and 2 mL for 5 minutes without agitation. Finally the samples were incubated with 1mL of DAPI solution (0.3 □M in PBS) for 5 minutes at room temperature. Samples were then rinsed again with 2 mL PBS (3x) and mounted via standard protocol.

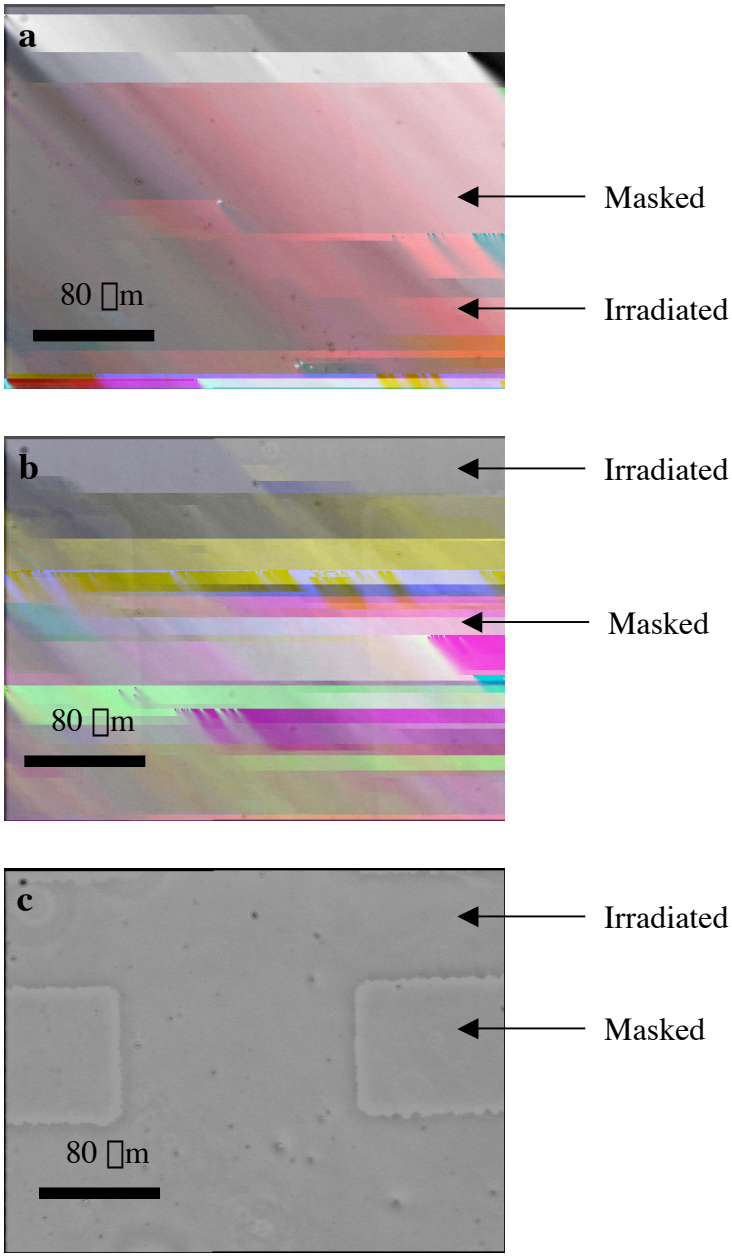
Results and Discussion

Phase contrast imaging of protein patterns

Spin coated CS5-ELF-N_3 (53%), CS5-ELF-N_3 with 53% replacement of phe by pN_3Phe as determined by ^{1}H NMR, films on DEDA derivatized slides demonstrate irradiation-dependent solubility as demonstrated by phase contrast images of the DMSO washed films (Figure 6.4). Films patterned for 1 second (a) demonstrate marginally discernable contrast, whereas contrast builds in with longer irradiation time (b and c) a result of thicker layers of retained protein film. Irradiation greater than 30 seconds does not improve contrast consistent with IR studies indicating complete consumption of azide at this time. Films composed of CS5-ELF-F or a similar elastin construct without phenylalanine did not produce any patterns under the same conditions (30 sec photopattern, 30 minutes DMSO wash).

Figure 6.4

Figure 6.4. Phase contrast images of photopatterned CS5-ELF-N_3 (53%). Irradiation of dry film for 1 (a), 10 (b) and 30 (c) seconds at 365nm through chrome/quartz maskfollowed by washing in DMSO for 1 hour to strip away uncrosslinked reagions.



Infrared detection of azide photolysis kinetics

Infrared spectroscopy allows us to track the ablation of the azide as a response to irradiation under conditions identical to those used in other experiments within this study. Irradiations were performed in the same mask aligner behind quartz of identical grade and thickness to that of the mask used for patterning. First order kinetics can be roughly drawn from the data, which is rough primarily because getting equivalent infrared signals over long time periods was difficult. The azide is very sensitive to 365 nm light as the signal is completely destroyed within a few minutes of irradiation in the mask aligner (Figure 6.5). This is somewhat surprising due to the relatively small absorbance of simple arylazides at this wavelength. Efficiency at 365nm is very important as using this wavelength prevents any unwanted damage to the protein via excited states of tryptophan or tyrosine [47].

Cell attachment to CS5-ELF-N3 constructs

Initial cell attachment studies on films incorporating various levels of pN_3Phe unexpectedly revealed a positive correlation to azide content. HUVEC demonstrate a markedly stronger affinity for CS5-ELF-N_3 (53%) films than for CS5-ELF-F films by centrifugal assay [42]. Further, stepwise decrease of azide content generates a corresponding decrease in the rate HUVEC adhesion (Figure 6.6 a-c). The results corresponded to images taken at all observed time points. Additionally, destruction of the azide by irradiation resulted in an attenuation of cell spreading activity (Figure 6.7 a,b). Taken together these results indicate strongly that HUVEC are responsive to

Figure 6.5. Photolysis of incorporated pN_3Phe followed by FT-IR. (a) FT-IR spectrum of preparted CS5-ELF-N_3 surfaces as a function of time. A 10% weight solution of the protein was spun onto a ZnSe crystal. From bottom to top: 0, 0.5, 8, 24, 88 and 124 seconds of irradiation. (b) Plot ln[A]/$[A]_0$ (based on peak area) versus time.

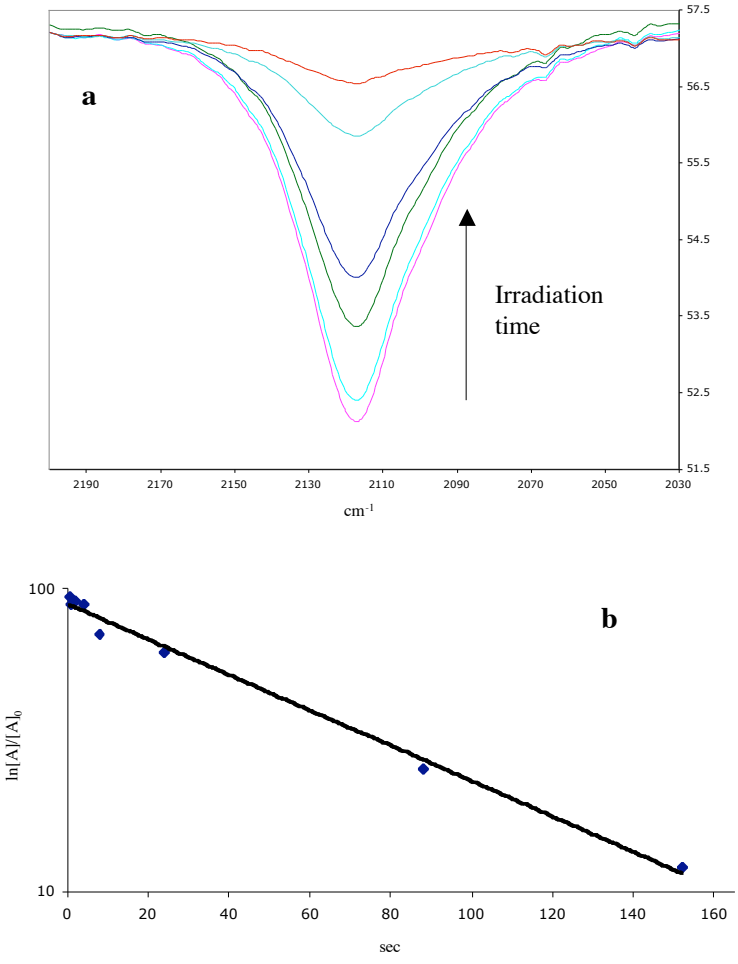


Figure 6.6. Representative phase contrast images of HUVECs 2 hours after plated on CS5-ELF-N_3 containing different levels of azide. Spun coat proteins were irradiated for 1 sec without a mask and washed for 1 hour in DMSO CS5-ELF-N_3 (13%) (a) demonstrate no spreading of cells after two hours whereas CS5-ELF-N_3 (30%) (b) demonstrate a small amount of spreading and CS5-ELF-N_3 (53%) demonstrate mostly spread HUVECs.

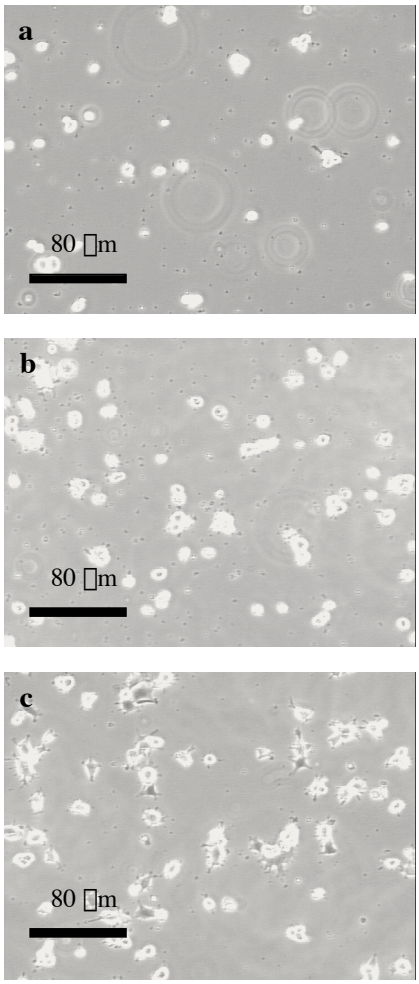
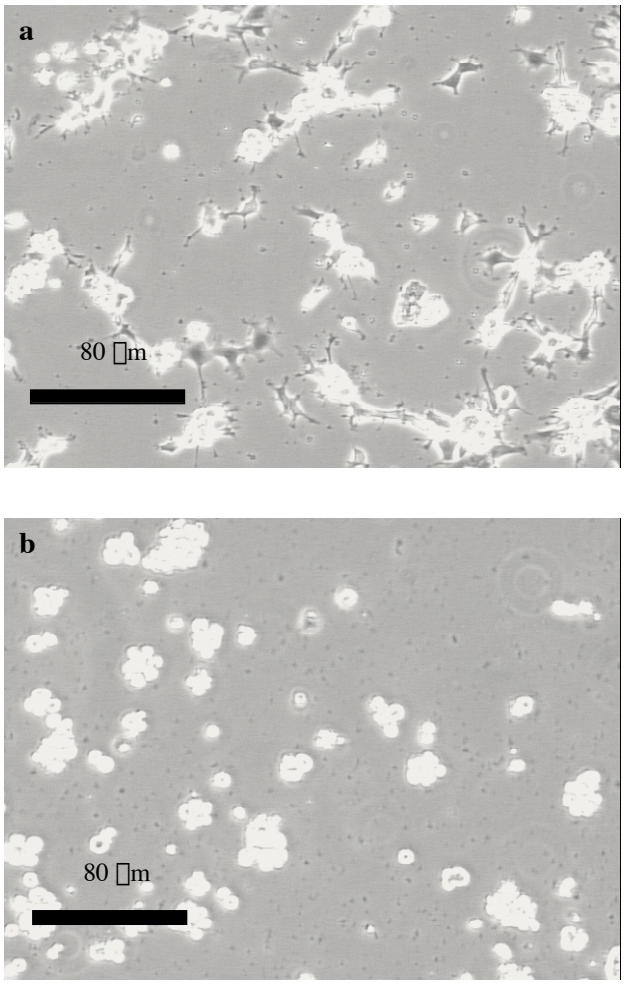


Figure 6.7. Representative phase contrast images of HUVECs on CS5-ELF-N_3 (53%). 1 second irradiation (a) demonstrates high levels of cell spreading whereas 30 second irradiation (b) reduces the amount of cellular adhesion.



azide content of CS5-ELF proteins, with a possibility that they are also responsive to the effect of photodecomposition on the construct.

Cell patterning based on cell preference to non-irradiated CS5-ELF-N3 surfaces

The above phenomenon was exploited to provide films of patterned cellular adhesion. Thus cells demonstrated a marked preference for the masked, non-irradiated, regions of patterned CS5-ELF-N_3 (53%) films (Figure 6.8). The effect diminished stepwise on films made from CS5-ELF-N_3 (30%) and CS5-ELF-N_3 (13%), again confirming a direct link between azide content and HUVEC adhesion.

Cell patterning on stripped surfaces

Demonstration of a true cell adhesive photoresist required the deposition of the CS5-ELF-N_3 on surface that demonstrates non-adhesiveness towards both the protein construct as well as the HUVEC. Polyethylene glycol modified surfaces provide excellent deterrence to cell adhesion [7, 48], and are prepared by reaction of N-hydroxy succinimidyl esters with DEDA. Onto this surface the protein construct was photopatterned for 30 seconds and subsequently stripped by sonication in DMSO for 10 minutes. Phase contrast microscopic examination of HUVEC 6 hours after plating on patterned CS5-ELF-N_3 (53%) provided clear patterns of cellular adhesion with a small amount of extension beyond protein islands (Figure 6.9). Confirmation of colocalization of the CS5-ELF-N_3 and HUVEC was obtained by fluorescence microscopy, which highlighted cells with actin and nucleic acid staining and allowed visualization of CS5-ELF-N_3 through immunostaining the T7 tag (Figure 6.10).

Figure 6.8. Representative phase contrast images of HUVECs plated upon CS5-ELF-N_3 (53%) irradiated through a mask for 30 seconds demonstrate markedly better cell adhesion to features (nonirradiated regions) versus irradiated regions.

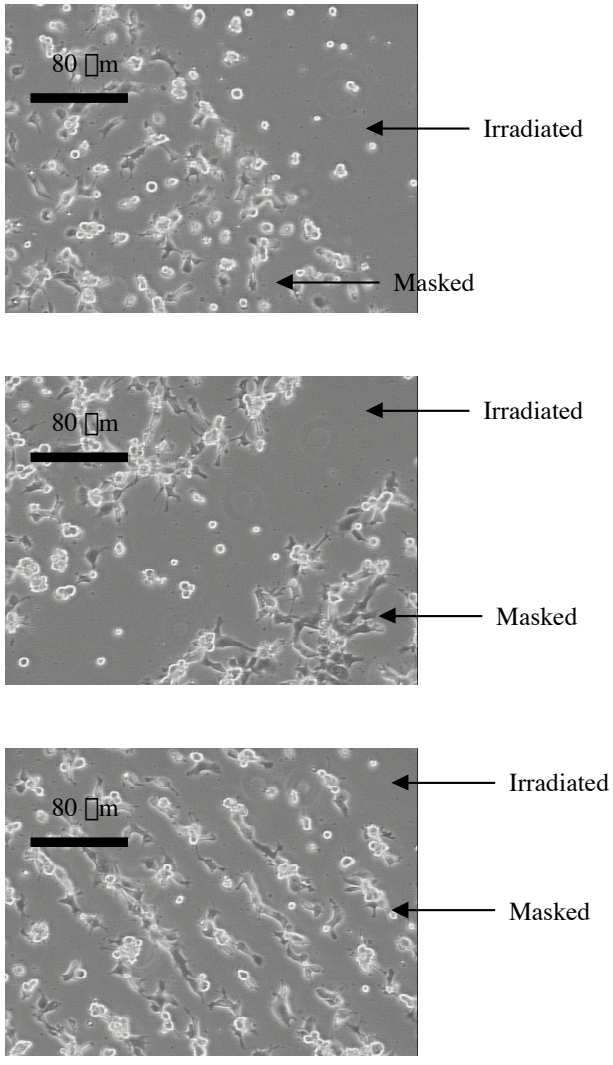


Figure 6.9. HUVEC patterning 6 hours after seeding on "protein resist" CS5-ELF-N_3 (53%) patterned on polyethylene glycol coated slides. Lithography was performed for 30 seconds with no preexposure.

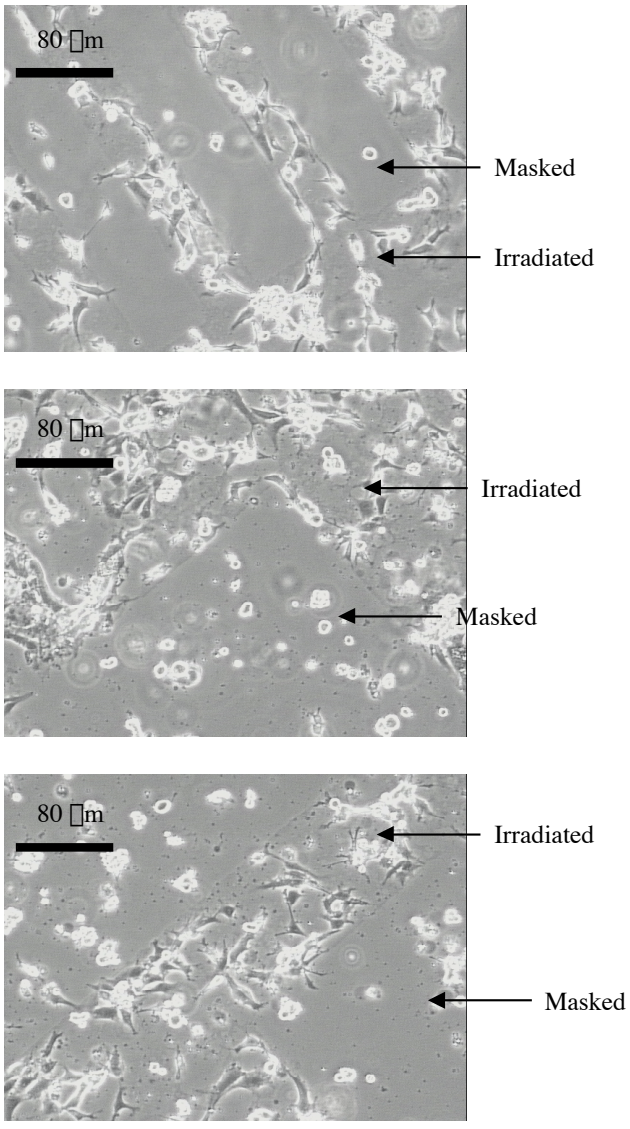
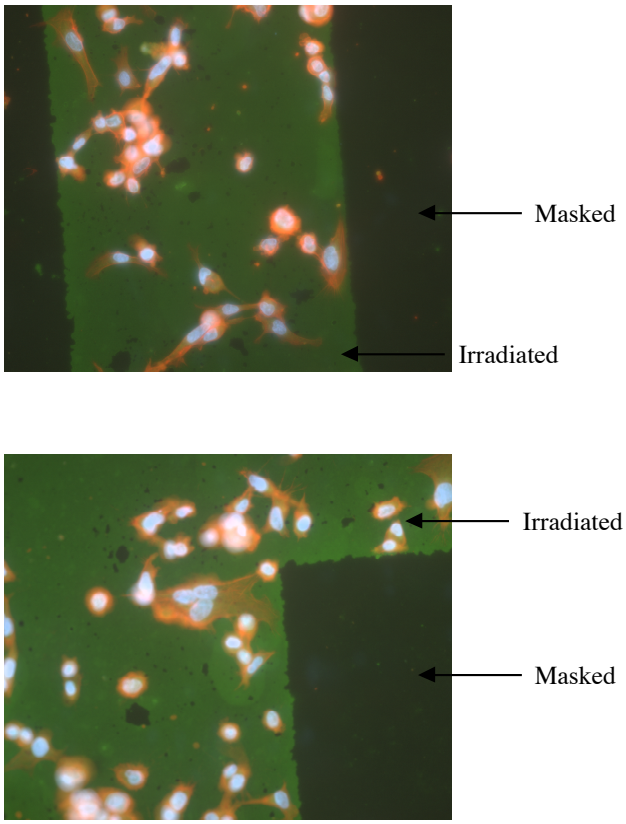


Figure 6.10. Fluorescent HUVEC array images. Actin staining by Phallodin (shown in red), nucleic acid staining by DAPI (shown in blue) and T7 antibody staining of CS5-EFL-N3 (shown in green). HUVEC plated on CS5-ELF-N_3 (53%) (spun on PEG slides) photopatterned for 30 seconds and stripped by sonication in DMSO for 1 hour. Cells fixed 6 hours after plating.



Conclusion

This report demonstrates the use of a protein armed with pN_3Phe as a bioactive photoresist. The protein construct, spun on a variety of surfaces, is sensitive to irradiation under standard conditions commonly used in the microelectronics industry and widely available. Patterns at the micron level are simple to produce using pure DMSO as a developer. Kinetics of azide decomposition at 365 nm demonstrate complete decomposition pN_3Phe within minutes, although 10 seconds irradation is sufficient for clean pattern formation. Further, we have shown that this material retains its bioactive nature. Cells demonstrate responsiveness not only to the presence of the material, but also, unexpectedly, to the level of azide content. This phenomenon is presumably responsible for the cells preference for non-irradiated islands within the protein film. It is also plausible that irradiation damages the cell binding domains within the material. However, both the cells preference for high azide content as well as the observation that irradiation does not noticeably damage the T7 epitope argue for the former explanation. In addition we have demonstrated patterning based on stripping non-irradiated protein from PEGylated surfaces. HUVEC's dramatic preference for the CS5-ELF construct, versus PEGylated surfaces, is obvious from phase contrast images. Irradiated sections of protein presumably polymerize and crosslink to the methyl-terminated PEG providing a stable cell adhesive pattern. Thus this construct contains two methods for patterning, one based upon preference for high azide content and another resulting from stripping to non-adhesive polyethylene glycol modified surface. This system has

straightforward advantages in that it is remarkably simple. Photoreactive protein production is very similar to standard protein expression, and can in theory be extended to any protein that can be expressed in *E. coli*. Film casting is a simple matter of spin coating and brief drying. Finally, patterning is accomplished via standard microelectronics technique and developing involves washing in DMSO. Thus, this method has proven exceptionally straightforward and is an effective as a method for patterning proteins in a bioactive fashion.

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