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Lithographic Patterning of Photoreactive Cell-Adhesive Proteins

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Control of the spatial arrangement of proteins on surfaces is essential in a number of emerging technologies, including protein microarrays, biosensors,[1] tissue engineering, and regenerative medicine.[2] 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.[3] 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.[4]

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 via site-specific[5] and residue-specific techniques has been reported.[6] 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[7] and to support adhesion of mammalian cells through cell-binding domains (CS5 or RGD) derived from fibronectin (Figure 1A).[8] These proteins can be cross-linked efficiently upon irradiation at 365 nm. The physical properties of the cross-linked 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.

aECM-N_3 variants were expressed in *Escherichia coli* cultures supplemented with *p*N_3Phe (Supporting Information). 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.[9] This method results in statistical decoding of phenylalanine (Phe) codons placed at regular intervals in the coding sequence.[9] 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.[10] 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 (Supporting Information).

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 1B).[11] 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 1C). It is noteworthy that none of the other infrared bands was 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 cross-linking in protein and nucleic acid systems.[12]

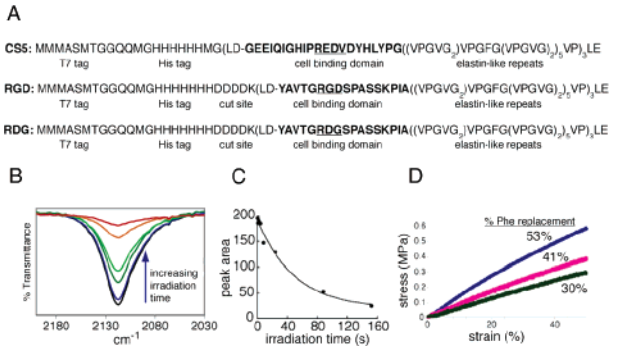

Figure 1. Characteristics of aECM-N_3 proteins. (A) Primary sequences of aECM-N_3 variants. (B) FTIR demonstrates loss of the characteristic azide asymmetric stretch as a function of irradiation time of CS5-N_3 films. (C) Peak area versus irradiation time yields a first-order decay with $t_{1/2} = 34$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

Elastic moduli of irradiated CS5-N_3 films were determined by uniaxial tensile testing under simulated physiological conditions (Figure 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, values near the range characteristic of elastins (0.3−0.6 MPa).[7] 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 cross-linking. 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.[13]

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)-

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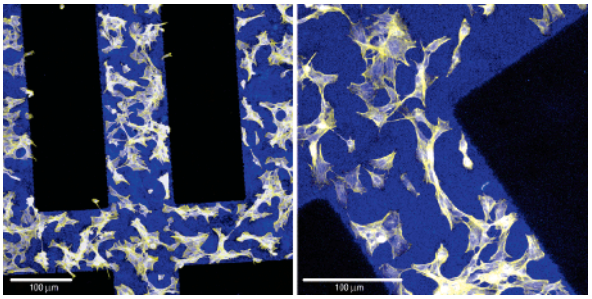

Figure 2. Confocal microscopy of Rat-1 fibroblasts attached to photopatterned RGD-N_3. Immunostaining with anti-T7 (blue) demonstrates colocalization of aECM-N_3 protein and cells (stained with rhodamine phalloidin (yellow)). Scale bars represent 100 μm.

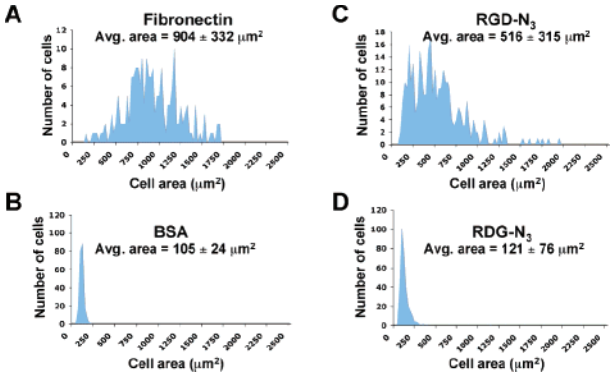

Figure 3. Rat-1 fibroblast cell spread areas on (A) fibronectin, (B) BSA, (C) RGD-N_3, and (D) RDG-N_3. RGD-N_3 supports sequence-specific cell spreading.

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. Noncontact 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 (Supporting Information).

To create cell arrays, Rat-1 fibroblasts were deposited on RGD-N_3 patterns in the absence of serum. After 4 h of incubation, the unattached cells were removed by mild washing with phosphate buffered saline (PBS), revealing cell patterns (Figure 2). 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 (Supporting Information).[14]

To determine whether cells specifically recognize the RGD cell-binding domain, we compared cell spreading on uniformly photo-cross-linked RGD-N_3 and RDG-N_3 (sequence-altered, negative control) films (Figure 3). After 4 h 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).

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 cross-linked 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.

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Supporting Information Available: Methods for cloning, protein expression and purification, patterning, and cell studies. AFM and phase contrast images are also provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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