2 Design and Construction of Photoreactive Artificial Extracellular Matrix Proteins

2.1 Abstract

In vivo, cells integrate a complex set of physical and chemical signals as well as spatial gradients of these signals to maintain proper tissue function. There has been much progress in the artificial replication of these cellular microenvironments by producing substrates with either tailored surface chemistries or regions of variable substrate rigidity, yet there has been minimal advancement in the design of materials that allow for the modulation of both properties simultaneously to study and direct cellular response. The work described here begins to address this challenge through the fabrication of photoreactive artificial extracellular matrix (aECM) proteins with independently tunable chemical and mechanical properties. These aECM proteins consist of two carefully chosen components: an elastin-derived amino acid sequence $(VPGVG)_x$, and a fibronectin-derived cell-binding domain, RGD. A crosslinking site introduced into the $(VPGVG)_x$ sequence dictates the mechanical properties of protein films, while the RGD binding domain mediates cell attachment. The unique construction of the aECM proteins allows both the mechanical (elastic modulus) and chemical (cell-binding domain density) properties of the films to be varied in concert to evaluate the impact of multiple cues on cellular behavior.

aECM proteins were genetically engineered using recombinant DNA technologies and produced in an *Escherichia coli* expression host. Manipulation of an aminoacyl-tRNA synthetase of the bacterial expression strain permitted the incorporation of a non-canonical amino acid, *para*-azidophenylalanine (pN$_3$Phe), within the elastin subunit of the protein. Cell experiments using photocrosslinked aECM proteins demonstrated that HUVEC (human umbilical vein endothelial cells) attachment to these surfaces was

sequence-specific to the adhesive RGD sequence present within the protein. Additionally, micromechanical measurements of aECM films using atomic force microscopy (AFM) were performed to determine the elastic modulus (E) of these materials. The average E obtained for the photocrosslinked RGD protein film investigated was 0.53 ± 0.02 MPa.

2.2 Introduction

The Tirrell laboratory has developed a family of aECM proteins for use as biomaterials in a variety of biomedical applications including vascular graft engineering [1-5] and the development of artificial corneal onlays [6]. aECM proteins are composed of a repeating structural motif derived from elastin coupled to periodically spaced cell-binding domain sequences from fibronectin [7]. Elastin-like multimers of the amino acid sequence (VPGIG) were originally chosen to serve as the backbone of the aECM proteins in vascular graft engineering, as elastin is abundant in the walls of small muscular arteries [8, 9]. These short repeating sequences of elastin have also been shown to reproduce mechanical compliance in the appropriate range for many soft-tissue applications [5].

These proteins have been shown to form films with tunable mechanical properties that also promote cell attachment [4, 10]. In order to achieve a material with tunable mechanical properties, a crosslinking site was included within the construct to serve as a target for one of several chemical crosslinking reactions [10-12]. Variation in the degree of crosslinking allowed the preparation of films with desired mechanical properties. Tensile strength measurements to quantify the mechanical properties of these materials demonstrated elastic moduli that span the range characteristic of native elastins (0.3–0.6 MPa) [10].

To mediate selective endothelial cell adhesion, materials were designed to contain the CS5 cell-binding domain from the IIICS region of fibronectin, as the REDV portion of its sequence is recognized by the $\alpha_4\beta_1$ integrin on endothelial cells and has been shown not to induce binding of fibroblasts, vascular smooth muscle cells, or platelets [13, 14]. Protein constructs were also prepared containing the RGD sequence from the tenth type

III domain of fibronectin, which endothelial cells are known to bind through $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins [15]. Corresponding HUVEC experiments performed on these films established that endothelial cell adhesion to constructed aECM proteins is specific to the presentation of appropriate cell binding domains [3, 4]. Moreover, it was demonstrated that the strength of adhesion to the artificial substrate was retained at shear stresses well above normal physiological values [4]. Further experiments using phase contrast and fluorescence microscopy confirmed that aECM proteins promote HUVEC spreading and proliferation to a confluent cell monolayer [2, 4].

Experiments performed using artificial proteins in the Tirrell laboratory, as well as other reports in the literature, have demonstrated that cells respond to physical and chemical signals as well as gradients of these signals [16-20]. Although there has been much progress in controlling cellular behavior by altering the spatial organization of either chemical or mechanical signals [18, 19], minimal advancement has been made in fabricating substrates that allow for the simultaneous modulation of surface chemistry and substrate mechanics. The ability to reproduce such conditions *in vitro* would provide a novel environment in which to examine the interplay of competing signals affecting cellular response on engineered materials.

To analyze how cells interact with biomaterials of varying properties *in vitro*, we have expanded the aECM protein family through the addition of photoreactive aECM proteins. These proteins contain an elastin-derived amino acid sequence $(VPGVG)_x$, and a fibronectin-derived cell-binding domain, RGD (Figure 2-1). A photocrosslinking site introduced into the $(VPGVG)_x$ sequence dictates the mechanical properties of protein films, while the RGD binding domain mediates cell attachment. A scrambled negative

control protein was also generated by swapping the position of one amino acid in the cell-binding domain (RGD→RDG). Manipulation of the phenylalanyl-tRNA synthetase (PheRS) [21] of the bacterial expression strain permitted the incorporation of the non-canonical amino acid, *para*-azidophenylalanine ($p$$N_3$Phe), within the elastin subunit of the protein. Photochemical crosslinking at $p$$N_3$Phe sites provides control over the elastic modulus of the material that can be altered by adjusting the irradiation dosage at 365 nm or by varying the incorporation levels of $p$$N_3$Phe in the protein. The unique construction of the aECM proteins allows both the chemical (cell-binding domain density) and mechanical (elastic modulus) properties of the films to be controlled independently, thus enabling the investigation of their combined effects on cellular behavior.

RGD-N_3
MMASMTGGQQMGHHHHHHHHDDDDK(LDASFLDYAVTG<u>RGD</u>SPASSKPIAASA((VPGVG)$_2$VPGFG(VPGVG)$_2$)$_5$VP)$_3$)LE
T7 tag His tag RGD cell-binding domain elastin-like domain

RDG-N_3
MMASMTGGQQMGHHHHHHHHDDDDK(LDASFLDYAVTG<u>RDG</u>SPASSKPIAASA((VPGVG)$_2$VPGFG(VPGVG)$_2$)$_5$VP)$_3$)LE
T7 tag His tag scrambled RDG domain elastin-like domain

Figure 2-1. Photoreactive aECM protein sequences. Each protein contains a T7 tag, a heptahistidine tag, a cell-binding domain, and an elastin-like repeat.

2.3 Materials and Methods

2.3.1 Cloning of photoreactive aECM proteins

The genes encoding the aECM proteins were constructed using the cloning scheme shown in Figure 2-2 [22]. To obtain plasmids harboring these genes, a plasmid containing the desired elastin-like repeat and a different domain (SC5) was used to perform a cell-binding domain swap (pEC2-SC5-ELF_5, cloned by Dr. Marissa Mock) [23]. In order to increase the efficiency of the swap procedure, the SC5-ELF_5 insert was

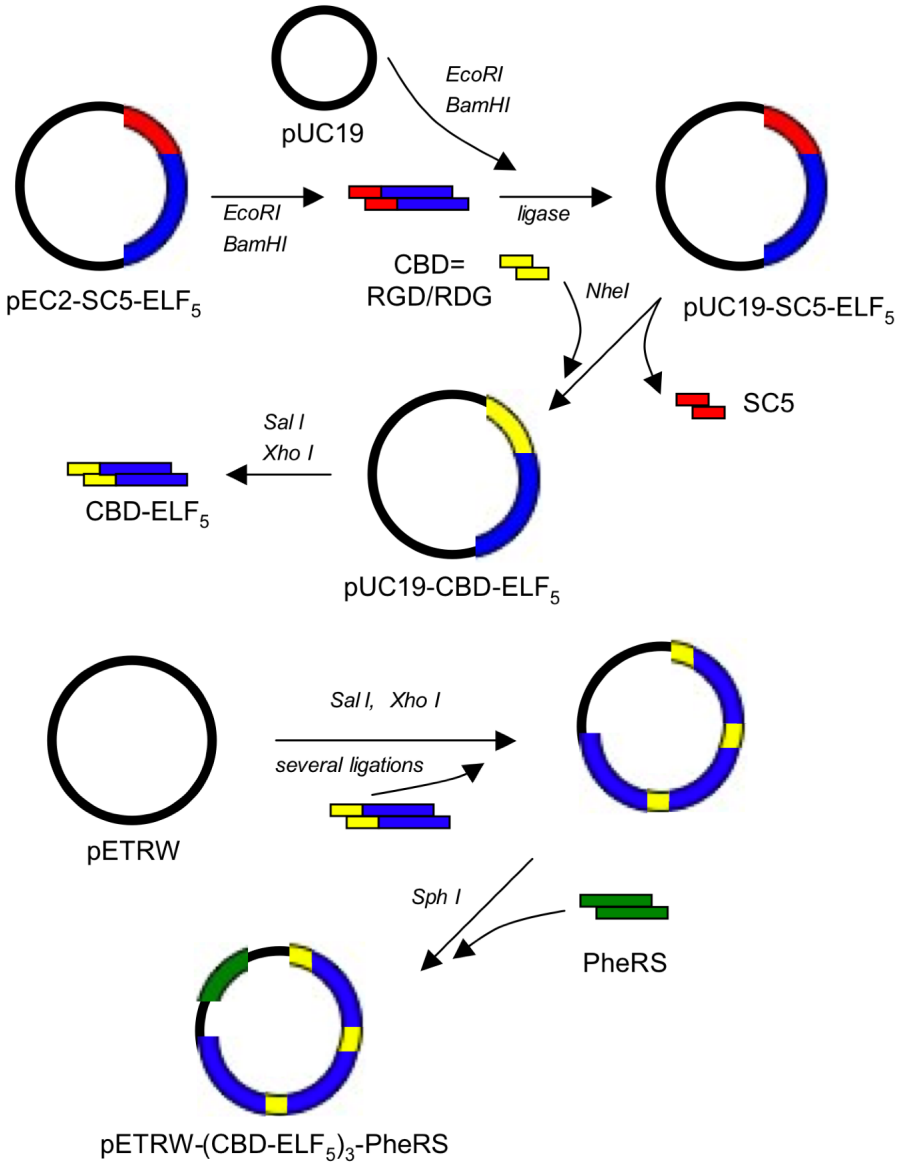

Figure 2-2. Schematic of the protocol for cloning aECM proteins.

first isolated by enzymatic digestion with *EcoRI* and *BamHI* (New England Biolabs, Ipswich, MA) and cloned into a high copy pUC19 plasmid to create pUC19-SC5-ELF_5. Next, synthetic oligonucleotides (Qiagen, Valencia, CA) encoding the RGD and RDG domains were annealed, phosphorylated, and ligated using T4 DNA ligase (Roche Applied Science, Indianapolis, IN) into a pEC2 plasmid to produce pEC2-RGD and pEC2-RDG (this step was omitted from the cloning figure). To remove the SC5 domain from the plasmid for the domain exchange, the SC5-containing plasmid was digested with *Nhe I* (New England Biolabs) and the digestion mixture was separated using gel electrophoresis (1% agarose). The digested plasmid was excised from the gel and the SC5 domain was discarded. To complete the domain exchange, pEC2-RGD and pEC2-RDG plasmids were digested with *Nhe I* to isolate the target domains. These domains were gel purified (1% agarose) and ligated into the *Nhe I* sites in the digested pUC19-SC5-ELF_5 plasmid to create pUC19-RGD-ELF_5 and pUC19-RDG-ELF_5. The ligation was allowed to proceed for 1.5 hours at 25°C, and the ligation mixture was subsequently transformed into *E. coli* XL1-Blue cells (Stratagene, Wilmington, DE). Transformed cells were plated on ampicillin (Sigma, St. Louis, MO) selection plates for overnight growth. Transformants were grown in 2 x YT liquid cultures, miniprepped (QIAprep Spin Miniprep Kit, Qiagen), and screened using a *Nhe I* test digestion to confirm the presence of the inserted domains. All samples were sent for sequencing (Laragen, Los Angeles, CA) using designed primers (Qiagen) to validate the orientation of inserted domains.

To obtain RGD-ELF_5 and RDG-ELF_5 cassettes for the next ligation step, pUC19-RGD-ELF_5 and pUC19-RDG-ELF_5 plasmids were digested with *Sal I* and *Xho I* (New England Biolabs) and the reaction mixtures were purified by gel electrophoresis (1%

agarose). The cohesive ends produced by *Sal I* and *Xho I* are sequence compatible, enabling the insertion of several gene cassettes into the plasmids. This procedure maintains a single *Xho I* site necessary for the insertion of additional DNA cassettes.

Using this method, RGD-ELF_5 and RDG-ELF_5 cassettes were ligated into an *Xho I*-digested pETRW expression vector (designed by Ralf Weberskirch). This expression vector contains a bacteriophage T7 promoter that allows for gene expression upon induction with isopropyl-1-β-D-thiogalactosidase (IPTG, Calbiochem, San Diego, CA), a heptahistidine tag, and an enterokinase cleavage site (amino acid sequence DDDDK in Figure 2-1). The ligation mixture was incubated at 16°C for 12 hours and transformed into *E. coli* XL1-Blue cells. Transformed cells were plated onto kanamycin (Sigma) selection plates for overnight growth. Transformants were grown in liquid cultures of 2 x YT and miniprepped. A test digestion using restriction enzymes *BglII* (New England Biolabs) and *Xho I* was performed to confirm the presence of the inserted cassettes. Since the insertion of three cassettes is required for each construct, the ligation procedure was repeated several times to generate the plasmids, pETRW-(RGD-ELF_5)$_3$ and pETRW-(RDG-ELF_5)$_3$. The sequence of the inserted cassettes was confirmed by sequencing using designed primers (Qiagen).

To create the final plasmids, the gene encoding a mutant *E. coli* phenylalanyl-tRNA synthetase (Ala294Gly) was isolated from a vector kindly provided by Dr. Peter Kast [21]. This mutant phenylalanyl-tRNA synthetase (PheRS) allows the incorporation of *p*N_3Phe in place of phenylalanine when protein expression is induced in a phenylalanine auxotrophic expression host [24]. A cell stock containing this vector was miniprepped and digested with *Sph I* (New England Biolabs) to isolate the PheRS gene.

The PheRS gene was gel purified (1% agarose) and ligated into *Sph I*-linearized pETRW-(RGD-ELF_5)$_3$ and pETRW-(RDG-ELF_5)$_3$ plasmids. The ligation mixture was incubated at 16°C for 12 hours and transformed into *E. coli* XL1-Blue cells. The cells were plated on kanamycin selection plates for overnight growth. Colonies were picked from the transformation plate and grown in 2 x YT media. The liquid cultures were miniprepped and screened for gene insertion using a test digestion with *Sph I.* All samples were sent for sequencing using designed primers (Qiagen). This cloning procedure produced both pETRW-(RGD-ELF_5)$_3$-PheRS and pETRW-(RDG-ELF_5)$_3$-PheRS plasmids. These plasmids were then transformed into a phenylalanine auxotrophic derivative of *E. coli,* designated AF-IQ [25], for protein expression under chloramphenicol (Sigma) and kanamcyin selection. When expressed in the presence of *p*N_3Phe, these DNA constructs produced proteins RGD-N_3 and RDG-N_3.

2.3.2 Protein expression and purification

Expression of RGD-N_3 and RDG-N_3 proteins was performed using a medium shift procedure to allow for the incorporation of *p*N_3Phe [12]. Briefly, overnight starter cultures were inoculated in 5 mL of 2 x YT media containing kanamycin and chloramphenicol. The turbid overnight cultures were then used to inoculate 1 L expression cultures of M9 minimal media (-phenylalanine) supplemented with 80 mg of phenylalanine and antibiotics. The cultures were grown at 37°C in a shaking incubator to an optical density (OD_{600}) > 1.0. Expression was induced with 1 mM IPTG for 10 minutes to allow for functional copies of PheRS to be synthesized before the cultures were centrifuged (10 min, 5000*g*, 4°C). The cell pellets were washed twice in 250 ml of

0.9% chilled NaCl by shaking, and resuspended in newly prepared M9 minimal media (without phenylalanine) to a volume of 1 L. The cultures were then supplemented with 80 mg of phenylalanine (positive control) or solid *p*N_3Phe (250 and 350 mg, Bachem, Bubendorf, Switzerland). The cultures were left to incubate, shaking at 37°C for 15 minutes before a second induction with 1 mM IPTG. After 4 hours of protein expression, the cells were harvested by centrifugation (10 min, 10,000*g*, 4°C). The pelleted cells were resuspended in 20 mL of TEN buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl) and frozen at –80°C. The cell pellets were defrosted for 4 hours at 37°C with 10 μg/ml of deoxyribonuclease I, 10 μg/ml of ribonuclease A, and 50 μg/ml of phenylmethylsulfonyl fluoride. The target proteins were centrifuged at a temperature above the expected lower critical solution temperature (LCST, 60 min, 25,000*g*, 25°C) and then were extracted from the pellets into 4 M urea at 4°C [26]. The suspensions were clarified by centrifugation below the expected LCST (60 min, 25,000*g*, 2°C) and the supernatants were loaded into dialysis tubing (VWR, West Chester, PA, MWCO: 12–14,000 Da) and dialyzed against filtered deionized water at 4°C for 3 days. The dialysis products were centrifuged (60 min, 25,000*g*, 2°C) and the supernatants containing the target proteins were frozen and lyophilized.

2.3.3 aECM protein characterization

2.3.3.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The expression and purification of photoreactive aECM proteins was monitored by gel electrophoresis using previously described protocols [4].

2.3.3.2 Liquid chromatography-mass spectrometry

Lyophilized samples of RGD-N_3 and RDG-N_3 proteins (less than 200 μg) were submitted for molecular weight analysis at the Protein/Peptide MicroAnalytical Laboratory in the Beckman Institute at Caltech.

2.3.3.3 Amino acid analysis

Purified protein samples (< 10 μg) along with a solid sample of *p*N_3Phe were sent for amino acid analysis at the Molecular Core Facility at the University of California, Davis.

2.3.3.4 ^{1}H NMR analysis

^{1}H NMR analysis of purified RGD-N_3 and RDG-N_3 proteins was performed to determine the level of *p*N_3Phe incorporation. Samples were prepared at a concentration of 1 mM in DMSO-d_6 (Cambridge Isotope Laboratories, Andover, MA) and analyzed using a 600 MHz ^{1}H NMR (Varian) at Caltech.

2.3.4 Preparation of photocrosslinked films for cell studies and mechanical testing

2.3.4.1 Irradiation setup

Dry protein films were irradiated using unfiltered UV light (365 nm) from a high-pressure arc lamp (Oriel Q, 100 watts at 5 amps; measured intensity = 1.5 mW/mm^2). The lamp was allowed to warm up for at least fifteen minutes before use.

2.3.4.2 Substrate preparation for cell spreading experiments

Substrates for cell spreading experiments were prepared following a protocol developed by former lab members, Drs. M.L. Mock and J.C. Liu [23, 27]. Circular glass coverslips (12 mm diameter, Corning, Corning, NY) were sonicated for 25 minutes in a saturated solution of potassium hydroxide in ethanol. Coverslips were rinsed individually with a stream of ethanol followed by a stream of distilled water and dried. Clean coverslips were spotted with 8 µl of a 12.5 mg/ml solution of RGD-N_3 or RDG-N_3 and were spin-coated for 100 seconds at 1400 rpm on a Specialty Coating Systems model P-6000 spin coater. Protein-coated slides were dried at 50°C for 4 hours before a 60 second exposure to UV light to allow for sub-quantitative photocrosslinking. The protein films were then reacted with alkynyl-mPEG-SPA-5000 in a Cu(I)-catalyzed azide-alkyne [3+2] cycloaddition reaction in phosphate buffered saline (PBS) 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 [28]. Substrates were then rinsed for 1 hour in 1 mM EDTA, for 12 hours in 0.05% SDS, and for 2 hours in distilled water. The prepared substrates were adhered to the bottom of a well in a 6-well tissue culture plate by applying sterilized vacuum grease around the back edge of the coverslip. All wells had been previously blocked with a 0.2% solution of bovine serum albumin (BSA) in PBS. The covalent attachment of PEG molecules to the surface of protein films was performed to deter nonspecific cell attachment observed in preliminary experiments using these proteins.

The alkynyl-mPEG-5000 used in the [3+2] cycloaddition reaction was produced by dissolving 100 mg of mPEG-SPA-5000 (Nektar, Huntsville, AL) in an excess of propargylamine. The reaction mixture was left to stir overnight, and then was then poured

into 200 ml of ether. The precipitated product was collected by centrifugation and analyzed by ^{1}H NMR. The degree of PEGylation on protein surfaces was evaluated by X-ray photoelectron spectroscopy (XPS).

2.3.4.3 Substrate preparation for AFM measurements

RGD-N_3 substrates for AFM measurements were prepared by dissolving 10 mg of protein in 100 µl in water at 4°C. The solution was centrifuged (14,000*g*, 5 min) and the supernatant was added to the surface of base-cleaned (saturated potassium hydroxide in ethanol) 12 mm diameter glass coverslips. Samples were spin-coated for 30 seconds at 7000 rpm at 4°C, and left to dry at 4°C before exposure to UV light for 5 minutes. Uncrosslinked, soluble protein was removed by rinsing the substrate with water.

2.3.5 Cell maintenance

HUVEC (BioWhittaker, Walkersville, MD) were maintained in a 37°C, 5% CO_2 humidified incubator. The cells were grown in Endothelial Growth Medium-2 (EGM-2, 2% serum, BioWhittaker), which was replaced with new media every two days. Near confluent HUVEC cultures were passaged non-enzymatically by treatment with 0.61 mM EDTA (Gibco, Grand Island, NY). Only cell passages 2–10 were used for experiments; no differences between cell passages were noted.

2.3.6 Cell spreading experiments

Photocrosslinked substrates (RGD-N_3 and RDG-N_3 proteins) were adhered to the bottom of a 6-well tissue culture plate using vacuum grease as described above. For a

positive control, fibronectin (10 μg/ml solution in PBS, Millipore, Billerica, MA) was adsorbed overnight in a 6-well plate at 4°C. For a negative control, heat-inactivated BSA (2 mg/ml solution in PBS) was adsorbed overnight in a 6-well plate at 4°C. All substrates were blocked with a 0.2% BSA solution for 30 minutes at room temperature and rinsed three times with PBS before cell seeding. HUVEC were resuspended in endothelial cell basal media (EBM-2, BioWhittaker) and added to each well at a density of 5.0×10^3 cell/cm^2 and placed in a humidified incubator (total volume = 3 ml). After 30 minutes, the plates were removed from the incubator and cells were imaged using a 10x phase contrast objective on a Nikon Eclipse TE 300 inverted microscope. Images were captured on a Sony CCD color video camera equipped with MetaMorph® imaging software (Molecular Devices, Sunnyvale, CA). The cells were manually traced using Image J provided by the National Institutes of Health to quantify the spread area. For each substrate, approximately 200 cells were traced in 4 independent experiments.

2.3.7 AFM equipment

Images and force curves of photocrosslinked protein films were obtained using a Park Scientific Instruments M5 atomic force microscope located in Aeronautical Engineering at Caltech. Samples were imaged using pyramidal-tipped triangular silicon nitride cantilevers (Veeco, Plainview, NY) with a listed spring constant of 0.58 N/m. To collect force curves from indented samples, a spherical tip with an attached SiO_2 particle (600 nm diameter, Novascan, Ames, IA) was used. The spring constant for this assembly was determined by calibrating it against two reference cantilevers and was calculated to be 0.25 N/m.

2.3.8 AFM measurements: film thickness and force curves

2.3.8.1 Film thickness measurements

Photocrosslinked RGD-N_3 films were scratched along the length of the coverslip with a pair of fine forceps to reveal the underlying glass substrate. The revealed surface was confirmed to be glass, based on its linear force profile when indented. The edge of this scratch was used as a reference point for imaging the film thickness. Scans of 30 μm x 30 μm were performed and the film thickness was recorded. The reported thickness was calculated by averaging measurements obtained at several locations ($n \geq 5$).

2.3.8.2 Force curves

Indentation measurements were obtained on a hydrated RGD-N_3 sample at room temperature according to a previously described protocol [12]. The glass microsphere-cantilever assembly was placed above the location where the sample thickness was measured (identified using a coupled optical microscope and visual markers) to associate indentation values with a known film thickness. The tip was then indented into the protein sample. The force and the z-displacement were recorded. Force curves were generated by plotting the loading force (nN) versus the z-displacement (nm). The indentation range was set relative to the contact point (-150 nm, +1350 nm), thereby limiting the deformation force to ~ 20–30 nN. The same spot was indented repeatedly, and the indentation-retraction cycles were 10 seconds in duration (tip speed = 3 μm/sec). Measurements were performed at several spots (> 1 mm apart) on the substrate.

2.4 Results and Discussion

2.4.1 Characterization of aECM proteins

2.4.1.1 SDS-PAGE

Protein expression and purification of newly constructed aECM proteins were tracked using SDS-PAGE (Figure 2-3). Aliquots of cultures representing equal numbers of cells (determined by the OD_{600}) were collected before and after induction (t = 0 and t = 4 hours) and resuspended in 4 M urea. These aliquots, along with samples of the collected pellets and supernatants from the LCST purification process, also prepared in 4 M urea, were loaded on a 12% polyacrylamide gel and size fractionated by electrophoresis (135 V, 75 min). The presence of the aECM proteins is apparent throughout the expression and purification; the purified target RGD-N_3 and RDG-N_3

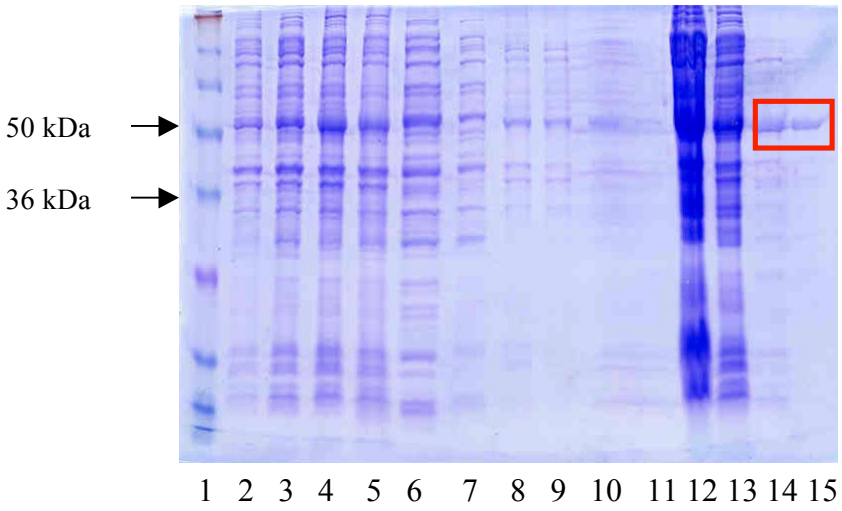

Figure 2-3. SDS-PAGE analysis of the purification of aECM proteins. Lane 1 of the gel contains the molecular weight marker; lanes 2 and 3 contain the pre-induction samples (t = 0 hr); lanes 4 and 5 contain the post-induction samples (t = 4 hr); lanes 6 and 7 contain the pellets of the first LCST cycle; lanes 8 and 9 contain the supernatants of the first LCST cycle; lanes 10 and 11 contain the supernatants of the second LCST cycle; lanes 12 and 13 contain the pellets of the second LCST cycle; and lanes 14 and 15 contain purified RGD-N_3 and RDG-N_3 proteins (red box), respectively.

proteins were detected near the calculated molecular weight of 42.99 kDa (red box). Previous work indicates that aECM proteins run at higher apparent molecular weights than predicted [27]. For 1 L cultures grown to a final OD_{600} = 1.20, approximately 45 mg of each protein was recovered following purification.

2.4.1.2 Liquid chromatography-mass spectrometry (LC-MS)

LC-MS was performed to verify the molecular weight of the purified aECM proteins. The spectra of both RGD-N_3 and RDG-N_3 proteins showed a peak at ~ 42,990 amu, which indicates the presence of the purified proteins containing phenylalanine (Figure 2-4). The observed masses of the proteins are within 0.01% of the calculated mass. The broader peak observed in the spectra represents the distribution of proteins with varying levels of *p*N_3Phe incorporation. The calculated molecular weight of proteins containing 100% incorporation of *p*N_3Phe incorporation is ~ 43.73 kDa.

2.4.1.3 Amino acid analysis

Amino acid analysis was performed to determine the amino acid composition of the purified RGD-N_3 and RDG-N_3 proteins. The HPLC (high performance liquid chromatography) spectra of the hydrolyzed protein samples displayed peaks corresponding to amino acids present in the designed proteins (data not shown). The data was analyzed by dividing the amount of each recovered amino acid by the total recovered for all amino acids to generate a mole percent. These values were then compared to theoretical values calculated using the known amino acid sequence (Table 2-1). Only a small variation in the theoretical and observed values was detected for most amino acids

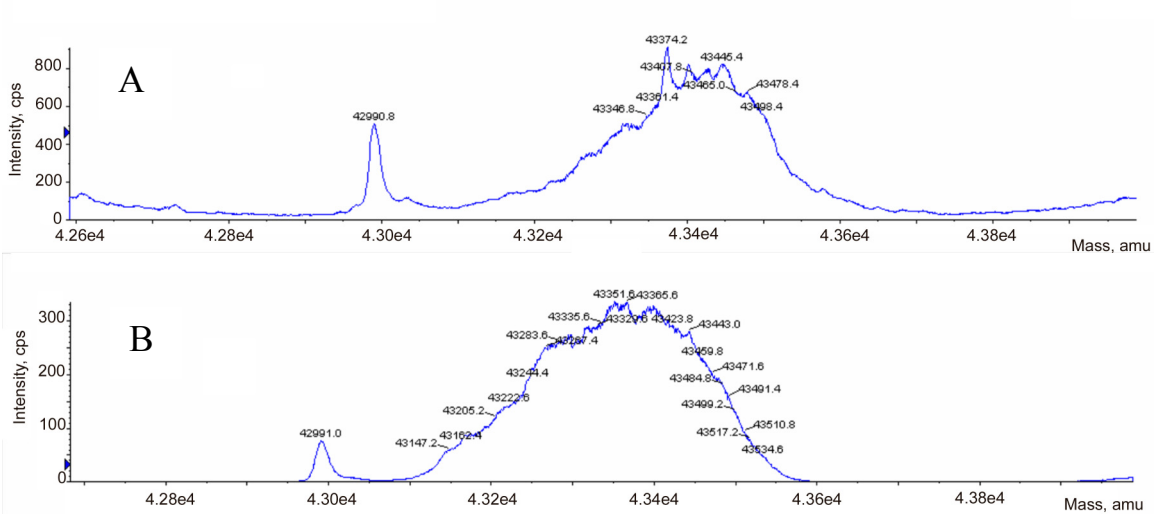

Figure 2-4. LC-MS spectra of purified (A) RGD-N_3 and (B) RDG-N_3 proteins. In each spectrum, the peak at 42.99 kDa represents protein containing 100% phenylalanine, and the broader peak represents proteins containing varying amounts of *p*N_3Phe.

(less than 1 mol%). The deviation in the theoretical and observed mol% for phenylalanine residues is the result of partial replacement of this residue by *p*N_3Phe.

2.4.1.4 ^{1}H NMR Analysis

The incorporation of *p*N_3Phe into proteins was determined by comparing the ^{1}H NMR spectra of proteins expressed in the presence of phenylalanine and *p*N_3Phe. The spectra of the two proteins are identical except for the presence of two additional doublets in the spectra of the proteins expressed in *p*N_3Phe (^{1}H NMR: δ 7.26 (d, J = 8.4 Hz, 2H), 6.98 (d, J = 7.8, 2H)). These peaks result from the aromatic protons of *p*N_3Phe, and the area under these peaks was integrated to give an incorporation percentage. The level of *p*N_3Phe incorporation into proteins was modulated by varying the concentration of *p*N_3Phe in the expression media. RGD-N_3 and RDG-N_3 cultures expressed in the presence of 250 mg/L and 350 mg/L of *p*N_3Phe yielded incorporation percentages of 15% and 51%, respectively.

Table 2-1. Theoretical and measured amino acid compositions of RGD-N_3 and RDG-N_3 proteins. The deviation in the theoretical and observed mol% for phenylalanine residues is the result of partial replacement of this residue by *p*N_3Phe.

Amino Acid	Theoretical mol%	aECM-RGD mol%	aECM-RDG mol%
Ala (A)	4	4.7	4.5
Arg (R)	0.7	0.4	0.8
Asn (N)	0	0	0
Asp (D)	2.8	3.4	3.1
Cys (C)	0	0	0
Glx (Q and E)	0.7	1.5	1.3
Gly (G)	32.6	32.3	32.2
His (H)	1.4	1.5	1.5
Ile (I)	0.7	1	1
Leu (L)	1.4	2	1.9
Lys (K)	0.9	1.2	1.1
Met (M)	0.9	1.1	0.8
Phe (F)	3.7	0.9	1.4
Pro (P)	17.2	16.7	16.8
Ser (S)	3.3	3.1	3.1
Thr (T)	0.9	1.1	1
Trp (W)	0	0	0
Tyr (Y)	0.6	0.4	0.8
Val (V)	28.3	28.8	28.8
Total	100.1	100.1	100.1

2.4.2 PEGylated and photocrosslinked aECM films for cell studies

To create samples appropriate for cell spreading studies, RGD-N_3 and RDG-N_3 protein films were first sub-quantitatively photocrosslinked with UV light before undergoing a Cu(I)-catalyzed azide-alkyne [3+2] cycloaddition reaction with alkynyl-mPEG. The alkynyl-mPEG was synthesized through the reaction of an activated ester form of the PEG molecule with propargylamine. The reaction occurred with 50% conversion, and the product was analyzed using ^{1}H NMR (data not shown). The degree of PEGylation was quantified using XPS by comparing the carbon/nitrogen ratio before and

after the PEGylation reaction. The XPS data indicated that an average of 1–2 PEG molecules per protein chain were successfully attached (data not shown).

2.4.3 Cell spreading results

To examine the sequence-specific nature of HUVEC spreading on the newly constructed aECM proteins, a series of cell spreading experiments was performed on uniformly photocrosslinked RGD-N_3 and RDG-N_3 proteins. HUVEC attained a greater measured cell area and exhibited well-spread morphologies on RGD-N_3 films as compared to the RDG-N_3 films. Cell areas achieved on the RGD-N_3 samples mimic those results obtained for the positive control of fibronectin, whereas the cell areas measured on the RDG-N_3 samples matched the areas measured on the negative control of BSA (Figure 2-5). The observation that HUVEC spread on RGD-N_3 films and not on RDG-N_3 films

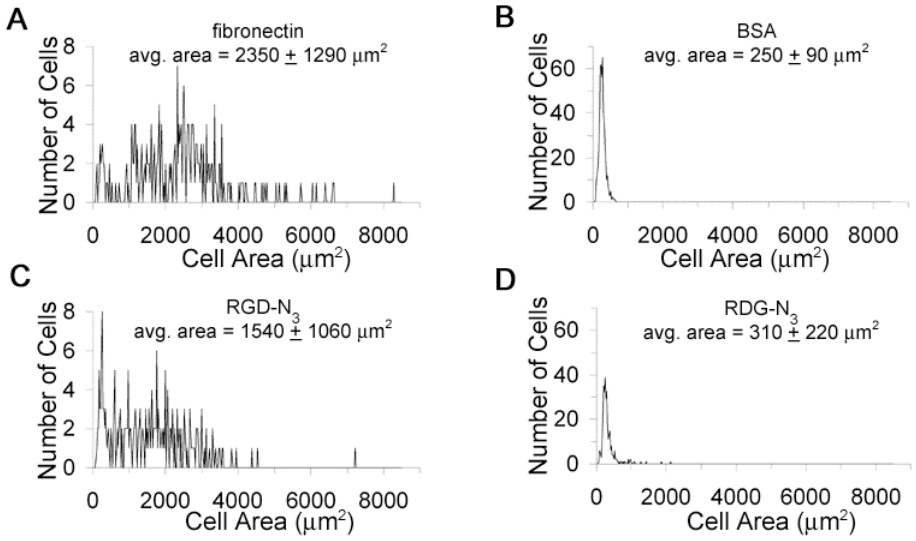

Figure 2-5. HUVEC spread areas on (A) fibronectin; positive control, (B) BSA; negative control, (C) RGD-N_3, and (D) RDG-N_3. The observation that HUVEC spread well on RGD-N_3 films and not on RDG-N_3 films indicates that cells recognize the RGD cell-binding domain specifically.

indicates that cells recognize the RGD cell-binding domain specifically. Moreover, the ability of HUVEC to recognize and spread on the RGD-N_3 protein suggests that photocrosslinking reactions that form the film do not render the RGD domain inactive.

2.4.4 AFM results: film thickness and elastic moduli measurements

2.4.4.1 Film thickness measurements

Thin films of photocrosslinked RGD-N_3 protein appeared smooth and uniform when imaged by AFM. An average film thickness of 205.5 ± 9.3 nm was obtained for dry samples (Figure 2-6), and a film thickness of 371 nm was acquired for a hydrated sample. Film thicknesses across the length of the sample did not deviate more than ± 10% from the average. Films were made using a sample of RGD-N_3 protein with 51 % *p*N_3Phe incorporation.

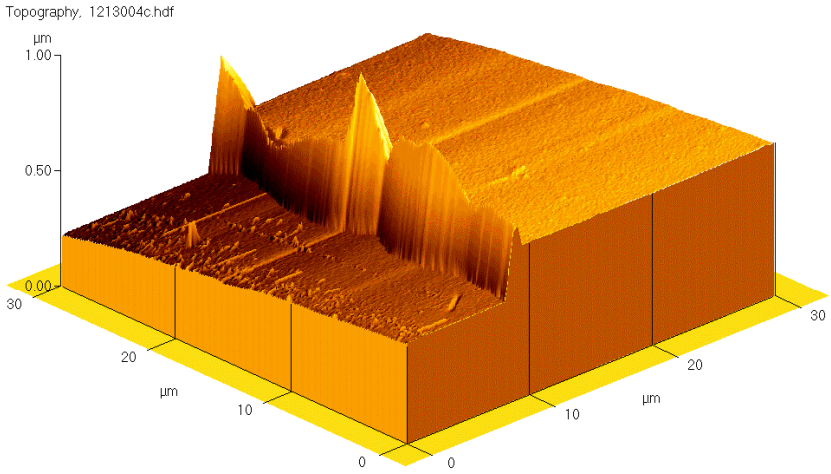

Figure 2-6. AFM topography scans of photocrosslinked RGD-N_3 protein. The film (right side of image) was scratched to reveal the underlying glass substrate (left side of image). The scales are not zeroed, but have been exaggerated in order to highlight the height difference between the glass substrate and the sample. The sharp spikes in the image are artifacts of the scratching procedure. The average height of this dry film was 205.5 ± 9.3 nm.

2.4.4.2 Elastic moduli measurements

A representative loading force curve obtained by nanoindentation of a photocrosslinked RGD-N_3 protein film (*p*N_3Phe content = 51%) is shown in Figure 2-7. The elastic modulus of the sample was extracted from the acquired data by using a model developed by Dimitriadis and coworkers [29]. This model describes the indentation profile of linear-elastic films of finite height using a spherical indenter, which corresponds to the experimental conditions in this study. The following equation summarizes the model assuming a Poisson ratio ν = 0.5 (incompressible; a sensible assumption for biological and rubbery materials):

$$(1) \qquad F = \frac{16E}{9} R^{1/2} \delta^{3/2} \left[1 + 1.133\chi + 1.283\chi^2 + 0.769\chi^3 + 0.0975\chi^4 \right]$$

where F is the loading force, E is the elastic modulus, and δ is the indentation depth obtained using a rigid sphere of radius R. The first segment of the equation is derived from the classical Hertz contact model [30]. Additional terms have been added to the classical equation to form the Dimitriadis model which accounts for the finite height (h) of the sample, where χ is:

$$(2) \qquad \chi = \sqrt{R\delta} / h$$

In this study, R corresponds to the radius of the glass bead attached to the cantilever, and the film height is the value measured during AFM topography scans.

In order to extract E, equation (1) was fitted to the obtained AFM indentation data according to a previously described protocol [12]. The indentation depth (δ) was calculated by subtracting the tip deflection from the total (z) displacement. The loading force (F) values used in the calculation were obtained by first identifying the contact point (Figure 2-7), the point where the indenter makes initial contact with the sample.

The contact point of the loading curve was assigned visually since the "snap-to-contact" point, or initial point, was easily identifiable. Force values beyond the contact point represent actual indentation data and were used in the calculation.

Using the data collected from the force curves, the elastic modulus was calculated at each data point. A single value of E was assigned to the sample by averaging the model-predicted moduli from 15 nm to 10% strain. The average E obtained for this RGD-N_3 sample was 0.53 ± 0.02 MPa, which lies in the range of values obtained using similar photocrosslinkable aECM proteins [12].

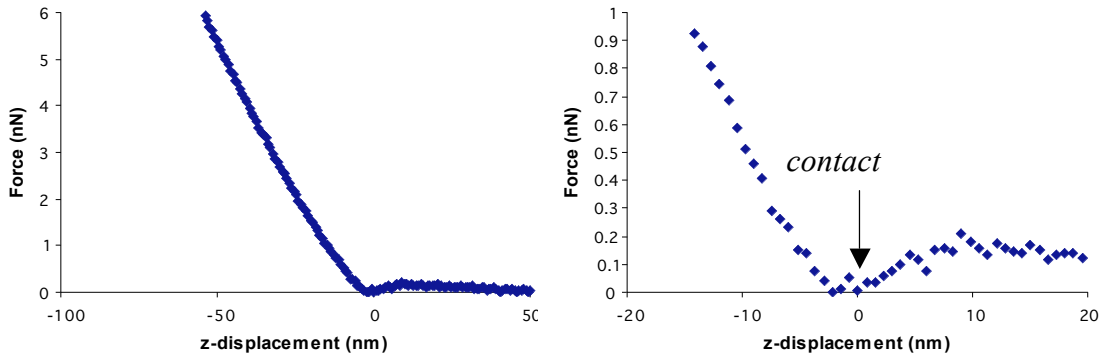

Figure 2-7. Representative loading indentation profiles for thin films of photocrosslinked RGD-N_3 protein. The loading force data is plotted against the z-displacement (indentation depth). The plot on the left shows the nature of the loading curve for an entire experiment, and the plot on the right is a magnified view to show the contact point assignment. These measurements were obtained on hydrated samples.

2.5 Conclusions

The construction and characterization of photoreactive aECM proteins has been successfully completed. SDS-PAGE, LC-MS, ^{1}H NMR, and amino acid analysis confirmed the molecular weight and composition of the designed proteins. Varying the amount of *p*N_3Phe added to the expression media altered the incorporation level of

pN_3Phe into proteins. HUVEC attachment to photocrosslinked films was found to be specific to the presence of the RGD cell-binding domain, and the elastic modulus of a hydrated protein thin film was acquired by nanoindentation. The average E obtained for the RGD-N_3 sample investigated was 0.53 ± 0.02 MPa.

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