C h a p t e r I I I

CONTROLLED DEGRADATION OF A CELL-ADHESIVE, ELASTOMERIC PROTEIN THROUGH INCORPORATION OF A FLUORINATED AMINO ACID*

III.1 Abstract

The design of biomaterials with controlled mechanical, cell-adhesive, and degradative properties is a common goal in tissue engineering and drug delivery systems. Towards this goal, a series of protein-based biomaterials were synthesized in an engineered bacterial production system. These modular proteins include domains from fibronectin that are known to adhere endothelial cells and elastin-derived repeating units to provide mechanical integrity. Fluorination of the protein by *in vivo* replacement of the amino acid isoleucine with the noncanonical amino acid 5,5,5-trifluoroisoleucine (5TFI) resulted in a tenfold decrease in degradation by the enzyme human leukocyte elastase compared to non-fluorinated protein. However, even after significant fluorination, the materials retain their ability to adhere endothelial cells in a sequence-specific manner. Incorporation of a noncanonical amino acid, without requiring a change in the encoding genetic sequence, represents a novel strategy to tune the rate of degradation of protein-based biomaterials without compromising cell adhesion.

**Manuscript prepared for submission by Sarah C. Heilshorn,* Marissa L. Mock,* and David A. Tirrell.*

**These authors contributed equally to this work.*

III.2 Introduction

Genetic engineering techniques allow the templated production of protein polymers with precisely controlled sequence, molecular weight, and functionality using microbial biosynthesis. Such techniques have been employed in the *de novo* design and synthesis of engineered proteins with a variety of novel physical and biological activities [1-8]. These materials have demonstrated potential in tissue engineering and reconstruction and drug delivery.

Elastin-like domains are of particular interest for engineered, protein-based biomaterials due to their high expression levels, ease of purification, biocompatibility, and tunable mechanical properties [9-14]. The extensive work of Urry and coworkers on the family of elastin-like polypentapeptides $(VPGZG)_x$, where Z is any amino acid, has shown that the hydrophobicity of the biopolymer can be used to tune the lower critical solution temperature (LCST) [15, 16]. Polymers are soluble at temperature below the LCST but phase separate into a polymer-rich coacervate as the temperature is increased. This LCST phase transition allows straightforward purification of elastin-like polymers after biosynthesis using a simple thermal cycling technique.

We have employed this method to purify a set of engineered proteins designed for use as implantable biomaterials. The protein sequences (Figure III-1) are a result of a modular design incorporating domains from fibronectin to adhere endothelial cells [10, 11], which are important for a healthy vasculature, and elastin-like domains to provide mechanical integrity [17, 18]. Sequence **CS5** contains the authentic cell-binding domain, which adheres human umbilical vein endothelial cells (HUVEC) in a sequence-specific

manner [11]. As a negative control, sequence **SC5** contains a scrambled cell-binding domain incapable of promoting sequence-specific HUVEC adhesion [19].

Proteins **CS5** (I=isolecuine) and **CS5-F** (I=5TFI):

M-MASMTGGQQMG-HHHHHHH-DDDDK-{LD-GEEIQIGHIPREDVDYHLYP-G[(VPGIG)$_2$VPGKG(VPGIG)$_2$]$_4$VP}$_3$-LE

T7 tag | His tag | Cleavage site | CS5 binding domain | Elastin-like domain

Proteins **SC5** (I=isolecuine) and **SC5-F** (I=5TFI):

M-MASMTGGQQMG-HHHHHHH-DDDDK-{LD-GEEIQIGHIPREVDDYHLYP-G[(VPGIG)$_2$VPGKG(VPGIG)$_2$]$_4$VP}$_3$-LE

T7 tag | His tag | Cleavage site | Scrambled CS5 binding domain | Elastin-like domain

Figure III-1. Amino acid sequences of the engineered proteins. Each protein has three cassettes of a cell-binding domain interspersed with an elastin-like domain. Protein **CS5** contains the authentic CS5 binding domain[11] while **SC5** contains a negative control, scrambled domain. Proteins **CS5-F** and **SC5-F** are identical to **CS5** and **SC5,** except they are synthesized in medium supplemented with 5TFI.

Depending on the specific medical application, e.g., drug delivery or tissue regeneration, the success of implanted biomaterials will depend on optimization of the *in vivo* degradation characteristics. Often, it is desirable to combine multiple degradation rates in one material. For example, a single system could combine rapid delivery of a pharmaceutical along with sustained release of growth factors for cell infiltration. Protein-based materials are degraded by a class of enzymes called proteases; however, native elastin is resistant to many of these proteases with the notable exception of elastase [20]. Human leukocyte elastase (HLE) is the predominant form of this protease that circulates the body in the blood stream [21]. HLE preferentially cuts after small, hydrophobic amino acids, and previous work in our laboratory showed that HLE prefers to cut after isoleucine in protein **CS5**. We hypothesized that we could alter the

degradation properties of this engineered protein by replacing isoleucine with a noncanonical amino acid.

The introduction of functional groups not contained within the 20 canonical amino acids into proteins is a valuable tool for the protein engineer, providing access to new chemical reactivity [22-26] and physical properties [27-31]. In many cases it is desirable to retain the biological activity of a protein upon introduction of these novel properties, requiring minimal disruption of the active, folded structure and making fluorinated amino acids of special interest. While fluorine is similar in size to hydrogen, the hydrophobicity of the CF_3 group is higher than the CH_3 group due to the low polarizability of fluorine [32], giving fluorination of proteins the potential to dramatically change physical properties of the protein without impairing its biological function [31, 33]. The ability to tune the rate of degradation of a biomaterial by varying the extent of incorporation of a noncanonical amino acid without requiring a change in the encoding genetic sequence would provide powerful control in target applications ranging from drug delivery to tissue engineering.

III.3 Methods

III.3.1 5,5,5-Trifluoroisoleucine (5TFI) synthesis and purification

5TFI was synthesized as previously described [33, 34] with minor modifications. Stereochemical purity was assessed by HPLC on a CROWNPAK CR (+) chiral column (Chiral Technologies, Inc.) with 1% perchloric acid/0.3% trifluoroacetic acid as the mobile phase. The crude product was a mixture of equal parts of the four stereoisomers

of 5TFI (D, L-, D-allo, L-allo-5TFI). It was recrystallized 3 times from 20% aqueous ethanol; in the final crystals only 1.7 % L-allo-5TFI remained.

The mixture of L- and D-5TFI was acetylated. D, L-5TFI (2.0 g, 0.013 mol) was suspended in 7 mL of 2 N NaOH and stirred in an ice bath. Another portion of 2 N NaOH (6 mL) and acetic anhydride (0.6 mL) were added. This addition was repeated 8 times at 2-minute intervals. The resulting clear solution was kept cold and stirred for 2 h before being neutralized with 18 mL of 6 N H_2SO_4. The solution was extracted with ether (3 x 50 mL), and the ether was dried over sodium sulfate and evaporated under vacuum. The yellow oil obtained was recyrstallized from 1:1 ethyl acetate:hexane, yielding 1.5 g white, crystalline N-acetyl-D, L-5TFI (74%).

N-acetyl-D, L-5TFI was enzymatically deacetylated to give pure L-5TFI. The acetylated amino acid (1.5 g, 0.0098 mol) was dissolved in 400 mL of 100 mM KH_2PO_4 (pH 7). Acylase I from porcine kidney (Sigma, 10 mg) was added, and the reaction was incubated at 37°C and followed by thin layer chromatography (4:1:1 n-butanol:acetic acid:water) with ninhydrin detection. When the concentration of free amino acid was no longer increasing (3 – 4 days), the reaction was acidified to pH 5 with concentrated HCl, filtered through a 0.22 μm filter, acidified further to pH 2, and extracted with ethyl acetate (3 x 75 mL). Evaporation of the ethyl acetate layer gave N-acetyl-D-5TFI. The aqueous layer was evaporated under vacuum, and the residue was taken up in methanol and filtered to remove a large portion of salt. The methanol was evaporated and the residue was dissolved in 100 mL of 0.1 N HCl and applied to an ion exchange column (Dowex 50WX4-100, Sigma). L-5TFI was eluted with 1 N NH_4OH and obtained as a white powder upon evaporation of the eluent under vacuum (150 mg, 10%). The final

product, by HPLC, is 2.7 % L-allo-5TFI and 3.2 % D-amino acid (a mixture of D-5TFI and D-allo-5TFI, which do not separate under the HPLC conditions employed).

III.3.2 Protein expression and purification

Proteins **CS5** and **SC5** were expressed as previously described [11]. To express proteins **CS5-F** and **SC5-F**, a competent isoleucine auxotrophic derivative of *E. coli* strain BL21(DE3), designated AI (*E. coli* B F^- *ompT hsdS*(r_B^- m_B^-)*gal dcm* λ(DE3) *ilvD691*), constructed in our laboratory [33] and harboring the plasmid pLysS (Qiagen), was transformed with, respectively, the plasmids pET28-CS5 and pET28-SC5 [17] to yield strains AI-pET28-CS5 and AI-pET28-SC5. To express proteins from these strains, a culture was grown overnight in 2xYT medium and used to inoculate 1 L of M9AA medium supplemented with the antibiotics chloramphenicol and kanamycin. At an OD_{600} of 0.8-1.0, the M9AA cultures were induced by adding 1 mM IPTG. After 20 additional minutes of growth, the cells were washed twice with 0.9% NaCl and resuspended in M9 medium containing 19 amino acids (excluding isoleucine) to a final volume of 1 L. The cultures were supplemented with 400 mg/L of 5TFI (effectively 100 mg/L of L-5TFI) and grown for 2 h. Fluorinated proteins were purified by Ni-affinity chromatography using Qiagen Ni-NTA agarose resin. Purity was assessed by SDS-PAGE and Western blotting with anti-T7 tag-horseradish peroxidase conjugate antibody (Amersham). Level of 5TFI incorporation was assessed by amino acid analysis at the University of California, Davis Molecular Structure Facility (Beckman 6300 amino acid analyzer).

3.2. Tryptic digest/MALDI

Purified proteins **CS5-F** and **SC5-F** were incubated with trypsin (50 mM ammonium bicarbonate buffer, overnight, room temperature). The proteolysis products were purified by eluting from a C18 ZipTip (Millipore) with 75:25 acetonitrile:0.1% trifluoroacetic acid, spotted on an analysis plate at 4°C, and analyzed by MALDI-TOF mass spectrometry on an Applied Biosystems Voyager DE Pro instrument.

III.3.3 LCST measurement

The LCST of proteins **CS5** and **CS5-F** was measured at 10 mg/ml in phosphate buffered saline (PBS), pH 7.4, by increasing the temperature at a rate of 30°C/h and measuring the percent transmission (measured in volts) at 300 nm on an Aviv model 62DS spectrophotometer (Lakewood, NJ).

III.3.4 Analysis of elastase degradation

For quantification of full-length chains, the degradation reaction was carried out at 37°C for 3 days in sodium borate buffer, pH 8, with 0.22 μM human leukocyte elastase (HLE, Elastin Products Company, Owensville, MO) and 100 μM protein. Samples were taken at 0, 1, 3, 6, 12, 24, 48, and 72 h and diluted with an equal amount of 2x SDS-sample buffer with β-mercaptoethanol and frozen at -20°C. Samples were boiled for 5 min, run on a 12% Tris-tricine gel at 150 V for 1 h, and transferred to poly(vinylidene fluoride) (PVDF) membrane for Western blot analysis using an anti-T7 tag-horseradish peroxidase conjugate antibody (Amersham) with a 10 second exposure. Densitometry was performed on Western blots using Image J (National Institutes of Health freeware image analysis program) to quantify the amount of whole-length protein remaining at

each time point. Control curves run in parallel indicated a linear relationship between protein concentration and Western blot intensity for both **CS5** and **CS5-F** (supporting information, Figure III-8).

For quantification of the number of cleaved peptide bonds, the degradation reaction was carried out at 37°C in sodium borate buffer, pH 8, with 0.22 μM HLE and 50 μM protein under constant mixing. The extent of reaction was characterized using 2,4,6-trinitrobenzene sulfonic acid at 4°C to quantify the number of N-termini in solution at 4 h.

III.3.5 Cell adhesion

Human umbilical vein endothelial cells (HUVEC, Bio Whittaker) were maintained in a 37°C, 5% CO_2 humidified environmental chamber. The cells were grown in Endothelial Growth Medium-2 (5% serum, Bio Whittaker), which was replaced every two days. Near confluent HUVEC cultures were passaged non-enzymatically by treatment with 0.61 mM EDTA (Gibco). Passages 2-5 were used.

Solutions of engineered proteins (1 mg/ml in PBS) and fibronectin (10 μg/ml in PBS) were adsorbed onto tissue culture polystyrene overnight at 4°C. Surfaces were rinsed with PBS, blocked with 0.2% heat-inactivated bovine serum albumin (BSA fraction V, Sigma) for 30 minutes, and rinsed.

HUVEC in suspension were labeled with a 5-μM solution of calcein acetoxymethyl ester (Molecular Probes) in serum-free Endothelial Basal Medium (EBM, Cell Applications, San Diego, CA) at room temperature for 30 min. Cells were rinsed twice and resuspended in EBM at 2.67×10^5 cell/ml. Cells (150 μl/well) were added to

adsorbed protein substrates in 96-well plates and incubated for 30 min. A solution of 21% w/w Percoll™ (Sigma) and PBS was added (200 μl/well) and plates were centrifuged at 100*g* for 10 min. Non-adherent cells were removed using harvesting frames (Molecular Devices) with the filters removed. PBS (100 μl/well) was added and fluorescence was measured using a Perkin Elmer HTS 7000 Bio Assay Reader at an excitation of 485 nm and emission of 538 nm. A cell adhesion index was calculated as the fluorescence reading of a test well divided by the fluorescence reading of HUVEC attached to fibronectin subjected to 1 *g*. The detachment force applied was estimated to be 26 pN using Archimedes' theorem [19].

III.4 Results and discussion

III.4.1 Protein synthesis and characterization

Using an engineered bacterial strain, the genetic message encoding the **CS5** protein can be alternatively read to produce protein **CS5-F**, a fluorinated version of **CS5**. The high isoleucine content of the **CS5** protein permits extensive fluorination through incorporation of the noncanonical amino acid 5TFI, which has demonstrated levels of isoleucine replacement from 85 – 93% in bacterial systems [33]. 5TFI was prepared as previously reported [33, 34] and used to express proteins **CS5-F** and **SC5-F** with yields of 0.83 mg/g and 0.71 mg/g wet cell mass, respectively. Nickel column purification yielded 5.4 mg pure **CS5-F** and 3.6 mg pure **SC5-F** from 1 L shake-flask fermentations. In contrast, proteins **CS5** and **SC5** express well and are easily purified using the thermal cycling technique to provide multi-gram quantities [17, 19]. Typical yields are 2-5 g pure

protein from 10 L batch fermentations. Further optimization is required to synthesize the fluorinated proteins above milligram yields.

To confirm the replacement of isoleucine with 5TFI, the engineered proteins were digested with the protease trypsin to yield protein fragments of predicted sequence, which were then analyzed by MALDI-TOF mass spectroscopy (Figure III-2). The peak at approximately 2576 Da has been assigned to two proteolytic fragments consisting of residues 136-161 and 262-286. These identical fragments contain five potential isoleucine replacement sites. Accordingly, the higher mass peaks are assigned to fragments with incorporation of one, two, three, four, and five 5TFI residues, each with a shift of 53.88 Da corresponding to the mass difference between isoleucine and 5TFI. Subsequent amino acid analysis reported 5TFI replacement of 82% of isoleucine residues

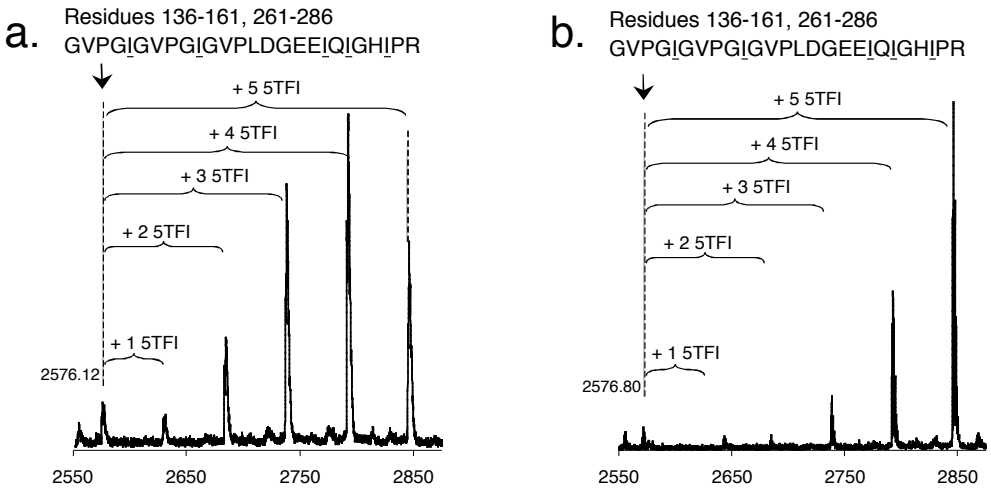

Figure III-2. MALDI-TOF of tryptic digest fragments of a) CS5-F and b) SC5-F. The expected mass of the unsubstituted peptide fragments comprising residues 136-161 and 261-286 = 2576. Peaks are apparent at masses expected for replacement of 1 through 5 isoleucines with 5TFI.

in **CS5-F** and 92% in **SC5-F**. This is consistent with results from previous studies demonstrating high incorporation efficiency of 5TFI into recombinant proteins [33].

We were interested in the effect of fluorination on the thermodynamic phase behavior of these proteins, as they are commonly purified through thermal cycling [7, 9]. As discussed above, proteins with elastin-like domains are known to exhibit an inverse temperature transition that is affected by the identity of the amino acid in the Z position [15, 16, 30, 35]. Relative to the most common pentapeptide repeat in bovine and porcine elastin, VPGVG [36, 37], the LCST is lowered when the amino acid occupying the Z position is more hydrophobic than valine and raised when Z is more hydrophilic. As expected, introducing the highly hydrophobic amino acid 5TFI into the Z position of the elastin-like domain results in a decrease of the LCST by more than 20°C (Figure III-3).

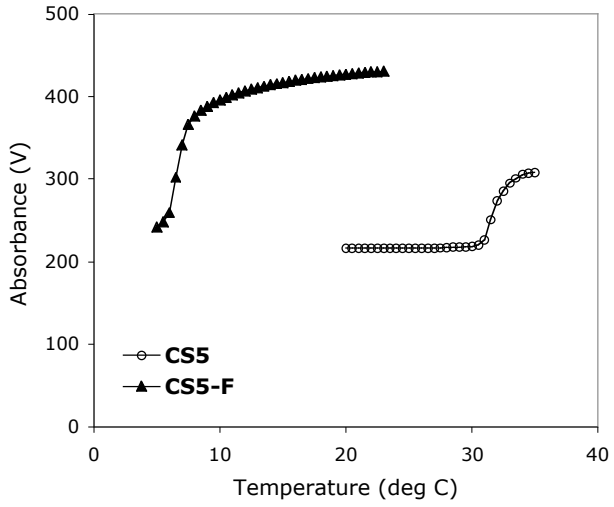

Figure III-3. Replacement of 82% of the isoleucine residues in CS5 with a fluorinated amino acid (CS5-F) decreases the LCST by 20°C, as evidenced by the turbidity of a 10 mg/ml in PBS solution, pH 7.4.

Therefore, the LCST of elastin-like proteins can be tuned by incorporation of fluorophilic 5TFI side chains, which may aid in optimization of thermal cycling purification techniques.

III.4.2 Protein degradation

Previous work in our laboratory has identified isoleucine as the favored HLE cut-site in these engineered, elastomeric proteins (S. C. Heilshorn, P. J. Nowatzki, T. Yamaoka, and D. A. Tirrell, manuscript in preparation). Therefore, incorporation of 5TFI into the isoleucine position was explored as a method to enhance HLE resistance. Due to the low expression levels of **CS5-F**, full kinetic analysis of the degradation reaction on the fluorinated protein was not possible. However, previous research in our laboratory has demonstrated that degradation of **CS5** follows traditional Michaelis-Menten kinetics with a catalytic constant, k_{cat}, of 0.033 s^{-1} and a Michaelis constant, K_m, of 2451 μM (supporting information, Figure 1II-7). Using these parameters, it is possible to predict the degradation rate of **CS5** and **CS5-F**, assuming that only the peptide bonds following isoleucine residues can be cleaved. The actual number of cleaved bonds in a reaction mixture can be quantified using 2,4,6-trinitrobenzene sulfonic acid to detect the concentration of N-termini. After 4 h of HLE degradation, 90% fewer peptide cleavages were observed on the fluorinated protein compared to the non-fluorinated protein (Figure III-4). These experimental results were in good agreement with the values predicted using the Michaelis-Menten model, which supports the assumption that HLE can only cleave peptide bonds following isoleucine in the fluorinated elastomer.

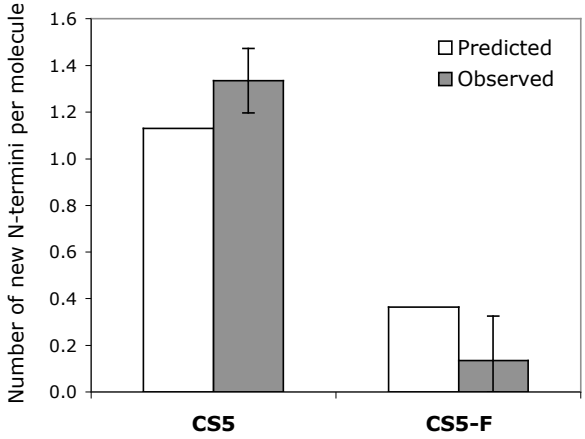

Figure III-4. HLE degradation of **CS5-F** produces 90% fewer new N-termini per original molecule than **CS5** after 4 h of reaction. Observed data represent two independent experiments, both testing three replicates of each substrate; error bars represent one standard deviation. Predicted data are based on the assumption that the reactions follow Michaelis-Menten kinetics and only the peptide bonds following isoleucine residues can be cleaved.

To examine the time course of degradation of **CS5** and **CS5-F**, Western analysis was used to determine the amount of full-length, intact protein remaining after HLE exposure for various times, and densitometry was employed to quantify the percent of full-length protein remaining at each time point (Figure III-5). Incorporation of 5TFI into the elastin-like protein significantly inhibited elastase activity. At 6 h, 46% of **CS5-F** remained intact, compared to 0% of **CS5**. Furthermore, full-length **CS5-F** was still detectable after 24 h exposure to HLE. Similar to the analysis performed above, the degradation reaction rates can be predicted using the Michaelis-Menten parameters for peptide cleavage after isoleucine residues. Using the simple assumption that each peptide cleavage results in the loss of one full-length protein chain, the predicted degradation rates for **CS5** and **CS5-F** are in good agreement with the observed values.

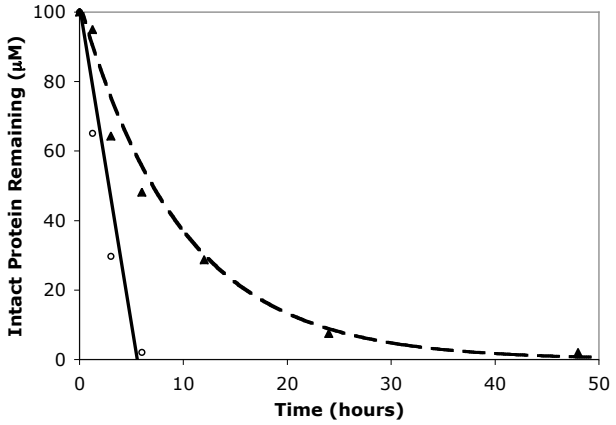

Figure III-5. HLE degradation of protein was monitored by Western blot and quantified using densitometry analysis of **CS5** (○) and **CS5-F** (▲) protein. No full-length **CS5** was observed after 6 h, while nearly half of **CS5-F** is still intact. The rate of chain degradation was predicted for **CS5** (dashed line) and **CS5-F** (solid line) using Michaelis-Menten parameters for the cleavage of peptide bonds after isoleucine residues.

III.4.3 Endothelial cell adhesion

We also investigated the ability of the fluorinated proteins **CS5-F** and **SC5-F** to promote cell adhesion. **CS5** was previously reported to be adherent to HUVEC in a sequence-specific manner [11]. Such adhesion is mediated through interactions with the REDV minimal binding sequence within the CS5 cell-binding domain. We wished to confirm that sequence-specific HUVEC adhesion would not be compromised by significant fluorination of the elastin-like regions. The ability of these engineered proteins to adhere HUVEC was examined using a buoyant centrifugation assay (Figure III-6). At a detachment force of 24 pN, protein **CS5-F** exhibited HUVEC adhesion greater than that of protein **CS5** and negative control proteins **SC5-F** and **SC5**, which contain scrambled cell-binding domains. Adhesion to fibronectin, which contains multiple cell-binding domains, was included as a positive control.

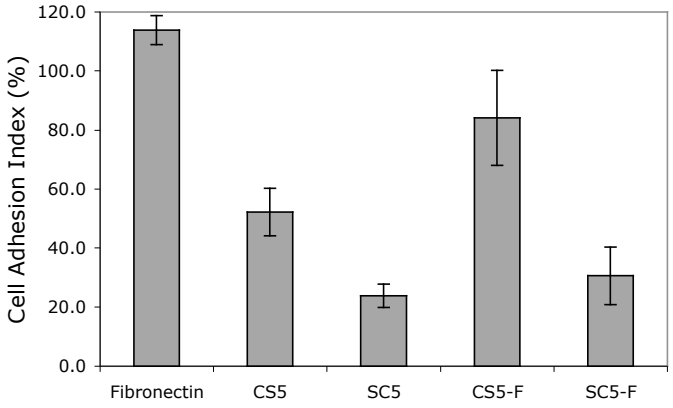

Figure III-6. Percent of HUVEC remaining adherent to adsorbed fibronectin and engineered proteins after 10 min exposure to 24 pN detachment force (100 *g*) relative to HUVEC remaining on adsorbed fibronectin after 10 min exposure to 0.24 pN (1*g*). Data represent three independent experiments in which six replicates of each substrate were tested; error bars represent one standard deviation.

These results suggest that 5TFI incorporation into these artificial proteins does not inhibit sequence-specific HUVEC binding. Therefore, fluorination of this engineered protein can successfully alter the thermodynamic behavior and proteolytic susceptibility without impairing the desired biological activity. While the observed increase in HUVEC adhesion to the fluorinated protein **CS5-F** relative to the non-fluorinated protein **CS5** is interesting, these results require further investigation. Ongoing research in our laboratory has shown similar context-dependence of adhesion strength to engineered proteins containing the CS5 domain.

We have demonstrated the ability to control the rate of proteolysis of elastin-like biomaterials through the incorporation of a noncanonical amino acid. From a single genetic message, two protein-based materials with varying degree of fluorination were created. Fluorination of the engineered elastomer retards elastase degradation of the

protein while also altering the thermodynamic phase behavior. The ability of the material to adhere endothelial cells through the sequence-specific interaction with the CS5 cell-binding domain is unaffected. Residue-specific incorporation of noncanonical amino acids into proteins is an additional tool for the biomedical engineer in the attempt to precisely control the material properties and biological activity of protein-based biomaterials.

III.5 Supporting information

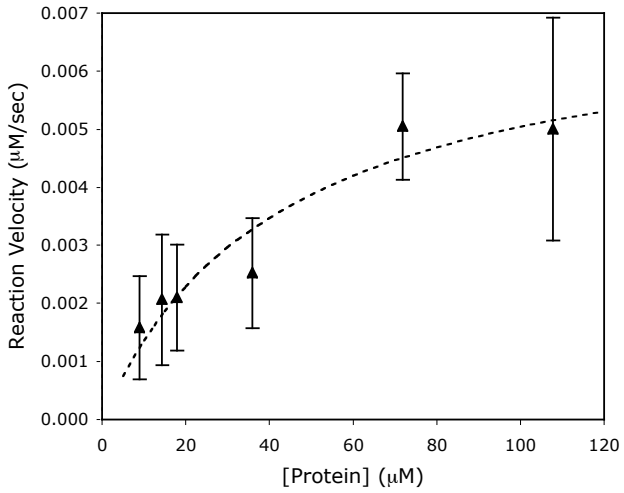

Figure III-7. Kinetic analysis of HLE degradation of **CS5**. Error bars represent a 90% confidence interval. The dashed line represents a best fit of the observed data to the Michaelis-Menten kinetic model.

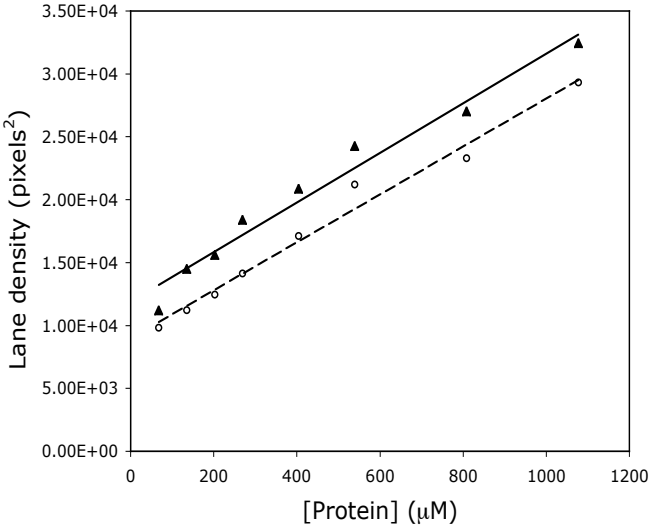

Figure III-8. Control curves for densitometry analysis of Western blots for **CS5** (○) and **CS5-F** (▲) with best-fit lines. Band intensity showed a linear increase with concentration up to 1 mM for both proteins.

III.6 References

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