Chapter 5

Efficient Photocrosslinking of an Artificial Extracellular Matrix Protein via *in vivo* Incorporation of Arylazide Functionality

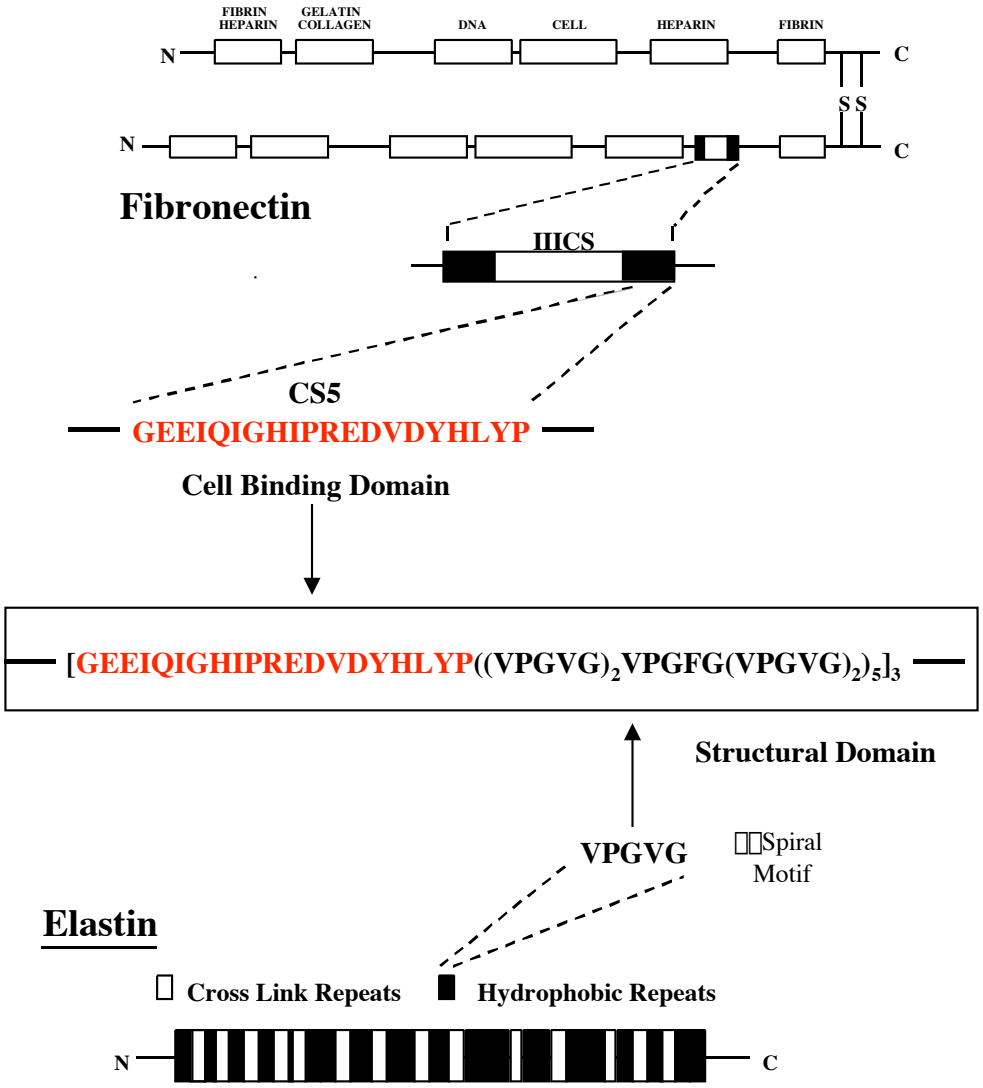
Nandita Sharma created the CS5-ELF construct. A portion of the expression and purification of the CS5-ELF-N_3 (53%) was done with Marrisa L. Mock.

Introduction

Biomolecules are increasingly used in materials applications due to the potential for absolute control of sequence, structure and association properties[1, 2]. Self-assembly and templating in nucleic acids has led to a number of materials with structural control at the molecular level [3] and even molecular machines[4, 5]. Genetic control of protein materials allows precise control of polymer composition and can take advantage of a larger monomer pool than nucleic acids. However, biomolecular synthesis and assembly is generally constrained, with respect to synthetic polymers, by the limited chemical and physical nature of the available monomers. Expansion of the available monomer pool with distinct physical and chemical moieties would greatly increase the scope of biomolecular materials. While materials scientists have long learned lessons from biopolymers, incorporation of natural biologically active motifs within materials to elicit specific biological responses has been more limited. This work incorporates both biologically active sequences and unnatural amino acids within one biomaterial designed as an artificial vascular graft material.

Healthy blood vessels have a mono-layer of endothelial cells coating their luminal surface. These cells secrete autocrine and paracrine signals critical to the regulation of the vascular environment as well as providing a surface which discourages thrombosis [6]. Current synthetic vascular graft materials, Dacron and Teflon being the most common, do not support *in vivo* formation of a healthy endothelial cell layer of endothelial cells and often suffer thrombosis as a result [7-9]. Further, current synthetic graft materials suffer from a mismatch in physical

Figure 5.1. 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.

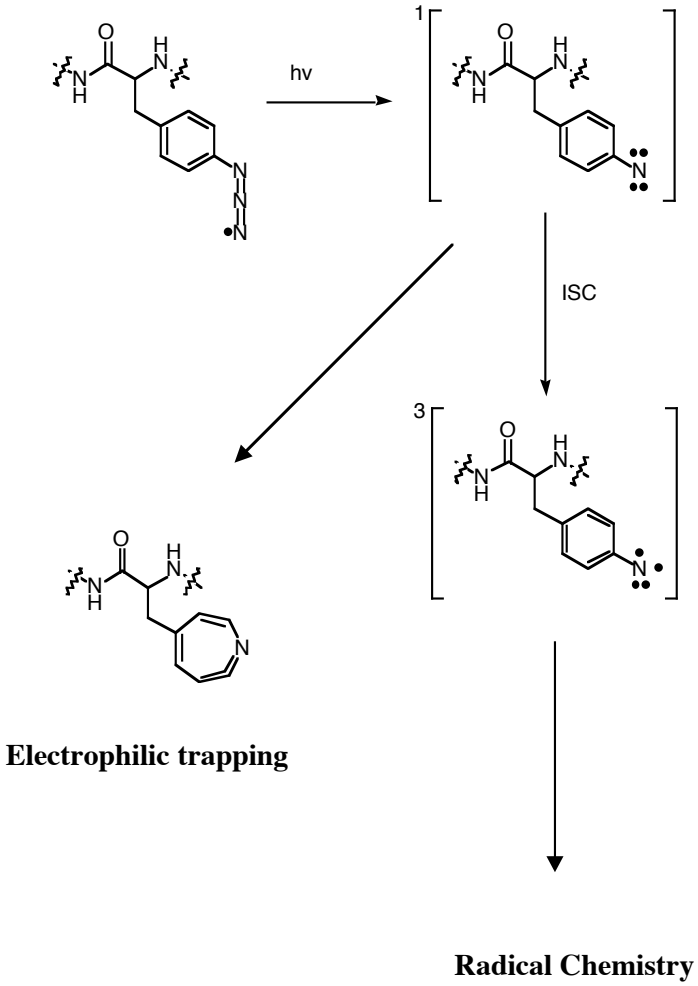


properties with the natural vasculature, which often leads to an over proliferation of smooth muscle cells and occlusion of the vessel [10-12].

In order to meet these challenges our laboratory has developed a series of protein based materials that incorporate cell-binding domains from fibronectin and a structural motif derived from elastin [13-15]. The material used in this study incorporates the CS5 domain from the IIICS region of fibronectin, which has been demonstrated as a site for $\alpha_4\beta_1$ integrin binding (Figure 5.1)[16]. REDV, a subsequence within the CS5 domain, is thought to be the minimal motif for $\alpha_4\beta_1$ integrin binding[17, 18]. Multimers of the pentapeptide VPGVG, derived from elastin, are included to provide appropriate modulus to the material[14, 19]. However, crosslinking is still necessary to provide these materials with the mechanical integrity to withstand the pulsatile stress within the vasculature[20]. Previous reports from this laboratory have utilized introduced lysines that are subsequently chemically crosslinked.

Such an approach suffers from two issues, coherent mixing of the chemical crosslinker into the viscous protein solution needed in order to provide homogeneous material properties and residual activity of the crosslinkers, which can complicate graft acceptance *in vivo* [21]. This work utilizes *in vivo* introduction of a photoactive unnatural amino acid, *para*-azidophenylalanine (pN_3Phe) [22-27] (Figure 5.2), to provide crosslinking sites. Inclusion of this crosslinking amino acid into the backbone allows precise control over crosslinking densities and prevents inhomogeneities in the material as a result of crosslinking. Crosslink density can be manipulated either by changing the number of sites in the primary coding sequence or

Figure 5.2. Photodecomposition of arylazides can mediate crosslinking either by electrophillic trapping via the ring expansion product or by the diradical behaviour of the triplit nitrene.



altering the level of incorporation of the analog through varying the concentration of analog in the expression media. This approach allows flexibility in the casting method, can be photopolymerized in any clear mold, an easy and precise method to control crosslink density and thus vary modulus and freedom from chemicals of any exogenous chemicals which invariably complicates graft production.

Materials and Methods

Materials

pN_3Phe was purchased from either Bachem or Chem-Impex. All other chemicals were purchased and used as obtained from Aldrich. Solvents were purchased and used as obtained from E.M. Science. Zinc selenide crystal was obtained from Wilmad Glass.

Expression of CS5-ELF-F and CS5-ELF-N_3

Bacterial cultures were grown in 19 amino acid (-Phe) minimal medium (see Mat. and Meth. chp 2) with 12mg/L phenylalanine under kanamycin and chloroamphenicol selection. Expression of target protein and T7 RNA polymerase was induced at optical density at 600 nm (OD_{600}) of 0.8-1 by addition of 1 mM IPTG. At this point growth has slowed significantly, presumably due to phe starvation. Ten minutes post-induction either a solution of phenylalanine (to a final concentration of 20mg/L) or solid pN_3Phe was added to obtain the desired final concentration of

analog. Cells were harvested 4 hours post-induction and protein production was monitored by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of the whole cell lysate, using an HRP-conjugated *T7-tag*™ antibody.

Purification of CS5-ELF-F and CS5-ELF-N_3

Cell pellet, produced by spinning down (10,000g, 10 min, 25°C) 1L of expression culture, was resuspended in 20mL of TEN buffer (10 mM Tris, 1 mM EDTA, 0.1 M NaCl) by sonication with probe sonicator, and subsequently frozen. To frozen lysate 1mM PMSF and 10 μg/mL each of DNase and RNase was added. This mixture was agitated for 4 hours (incubator) at 37°C. Target protein was partitioned to the pellet via centrifugation at a temperature above the expected LCST of the protein (22000 g, 60 min, 25°C), and subsequently extracted from the pellet into 4 M urea at 4°C (resuspend with sonication, then stir overnight). This suspension was clarified by centrifugation (22000 g, 60 min, 2°C). The resulting supernatant was dialyzed against cold (4°C) distilled water for several days (12-14KD dialysis tubing; change water every four hours). Precipitate (contaminant protein) formed during dialysis was removed from dialysate by centrifugation (22000 g, 60 min, 2°C) and decantation of supernatant (pure target protein), which was then lyophillized.

^{1}H NMR of CS5-ELF constructs

Purified CS5-ELF proteins were lyophilized completely and dissolved in DMSO-d_6 at 40 mg/mL. Spectra were taken using a 600 Mhz Varian spectrometer with a triax probe at 23°C.

Infrared spectroscopy of CS5-EFL-N_3

Infrared spectra were taken using a Perkin Elmer 1600 series FT-IR. Protein samples were drop cast onto zinc selenide wafers from DMSO. Attenuated total reflectance infrared spectra were taken using cast films irradiated for 30 minutes (see below).

Film preparation and uniaxial tensile testing

Tensile testing was performed using an Instron mechanical tester at 37°C in PBS buffer with a constant strain rate of 10%/minute. Samples were created in teflon molds by drying 10% solutions in DMSO overnight at 50°C. Irradiation was performed with an unfiltered Oriel 100W medium pressure mercury lamp for 30 minutes at a distance approximately 8 inches from the lamp. The samples were removed from the mold, swollen in 4°C water overnight to fully hydrate, cut into testing strips and finally equilibrated in PBS at 37°C. Films were approximately 3mm x 10 mm. Elastic modulus was obtained from the slope of the steepest part of the initial plot. Shear modulus was obtained from the slope of stress versus extension ratio ($\sigma \sim 1/\lambda^2$). Molecular weight between crosslinks was obtained from the ideal network approximation $G = \rho RT/M_c$,where a density of 1.3 g/cm^3, that of collagen, was used. Each protein sample was tested 3-6 times.

Results and Discussion

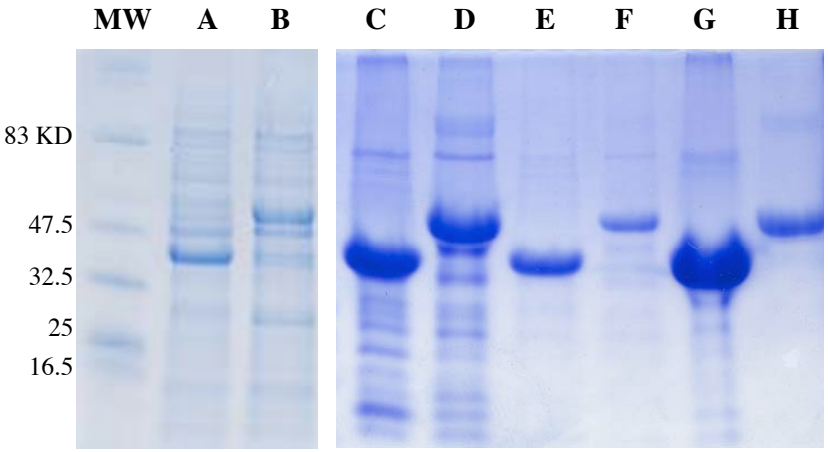
Protein expression and purification

Expression of CS5-ELF-F typically gave 60 mg/L of expression media, whereas expression of CS5-ELF-N_3 typically yielded 40-50 mg/L. The azide content of the CS5-ELF-N_3 construct was easily variable by altering the concentration of analog in the expression media (Table 5.1). Maximum incorporation percentage, using the non-media shift method, in the presence of the A294G PheRS mutant was 53%. This demonstrates that the phenylalanine exhaustive method, which relies upon the consumption of phenylalanine before induction and addition of pN_3Phe, while much easier can still achieve high incorporation rates (53% vs. 67% for media shift procedure). The lower solution critical temperature nature of these constructs allows the facile purification of these constructs to homogeneity (Figure 5.3). ^{1}H NMR of the purified protein also demonstrates purity and allows accurate quantitation of analog incorporation (Figures 5.4 and 5.5).

Infrared spectroscopy of CS5-EFL-N_3

Kinetics of azide decomposition was obtained by tracking the disappearance of the arylazide asymmetric stretch at 2130 cm^{-1}. Such measurements demonstrate a rapid consumption of azide when irradiated with an unfiltered ultraviolet source (Figure 5.6a). More than 90% of the signal was abolished within the first 70 seconds.

Figure 5.3. Tris Tricine SDS-PAGE (9%) analysis of expression results. Electophoresis run at 150 volts for 45 minutes with no provision for cooling. Both gels were run side by side, many lanes were removed for clarity.



A: Whole cell lysate from media supplemented with Phe
B: Whole cell lysate from media supplemented with pN_3Phe
C: Pellet produced from centrifugation of dialysate (Phe)
D: Pellet produced from centrifugation of dialysate (pN_3Phe)
E: Pellet produced warming of cleared dialysate (Phe)
F: Pellet produced warming of cleared dialysate (pN_3Phe)
G: E taken up in DMSO
H: F taken up in DMSO

Table 5.1. Percent of phenylalanine replaced by pN_3Phe as a function of pN_3Phe in the growth medium. Protein harvested 4 hours post induction. Percent incorporation analyzed by ^1H NMR.

Concentration of pN_3Phe in Expression Media (mg/L)	% phenylalanine replaced by pN_3Phe
250	53
100	41
75	30
50	19
25	13

Figure 5.4. Expanded 600Mhz ^{1}H NMMR of CS5-ELF-F (a) and CS5-ELF-N_3 (b) in DMSO-d_6.

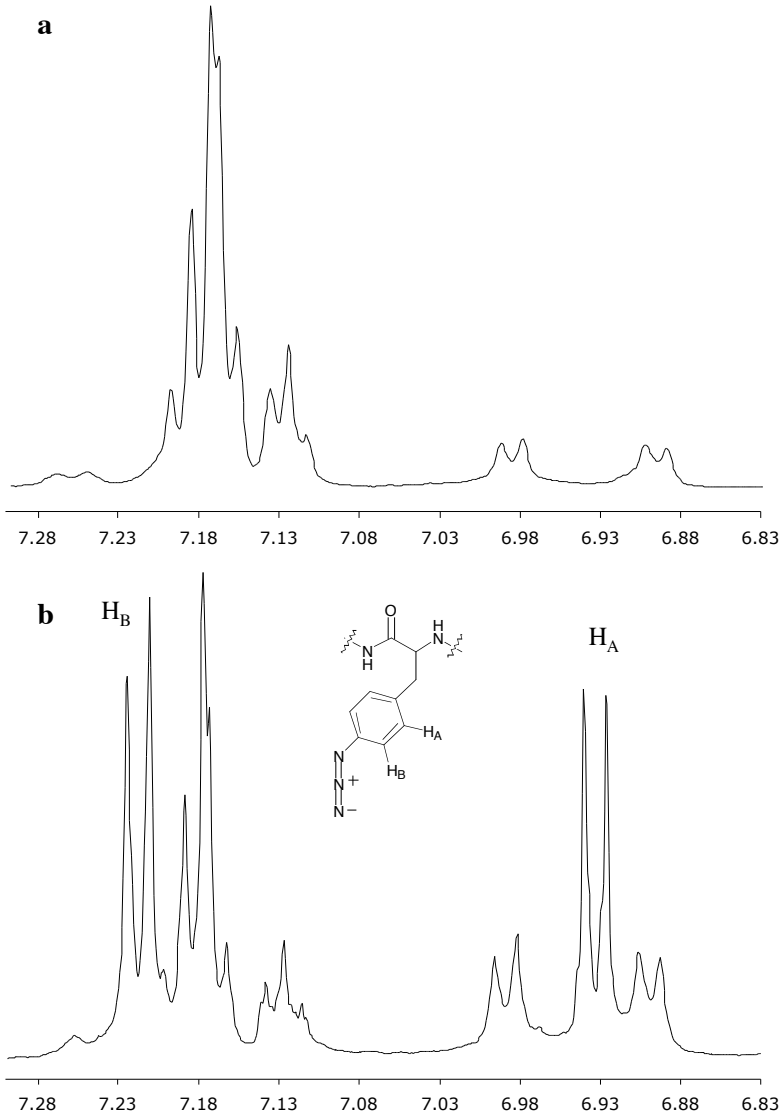
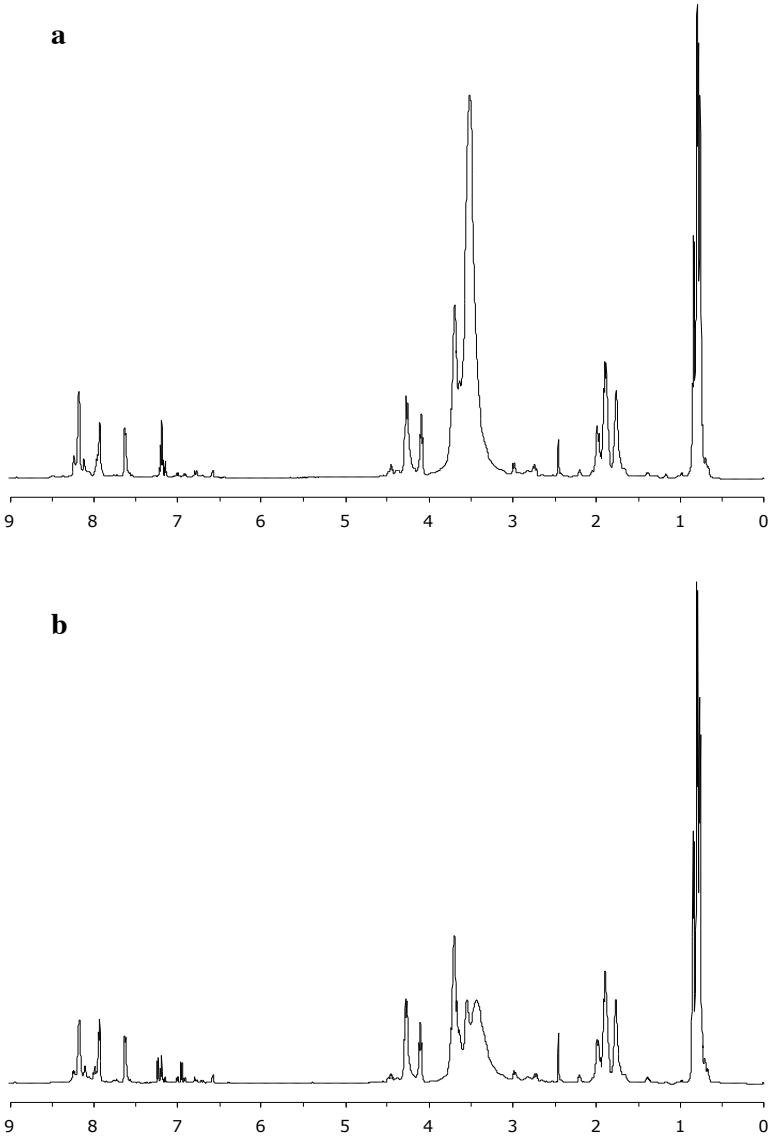


Figure 5.5. Full 600 Mhz ^{1}H NMR of CS5-ELF-N_3 (53%) and CS5-ELF-F.



A residual signal was not consumed even after hours, but at these time points an additional peak at 2030 cm^{-1} appeared, indicating other photodecomposition products within the protein (data not shown). Fortunately, ATR-IR indicates complete consumption of the azide on both sides of the film (Figure 5.6b). These results suggest that secondary photoproducts could interfere with the proteins ability to perform in the designed manner. Fortunately, lithography of these proteins (Chapter 4) indicates that consumption of the azide will occur when irradiating at 365+ nm ensuring no damage to native protein functionality as tryptophan, tyrosine and phenylalanine absorption coefficients drop off precipitously after 300nm.

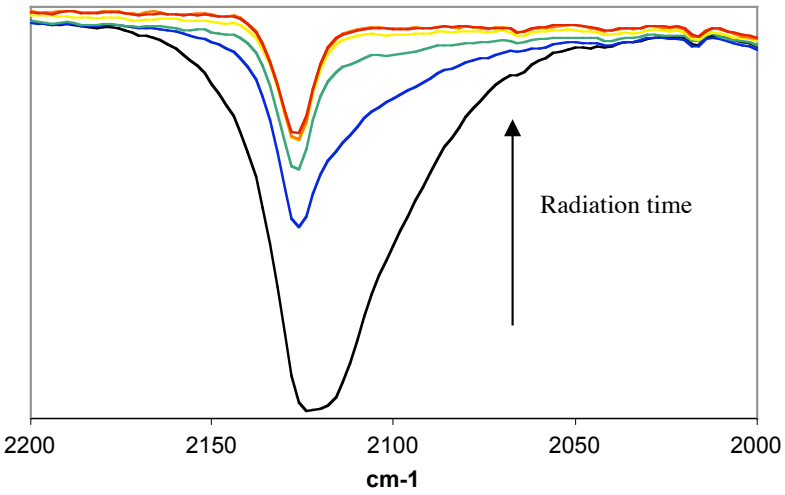
Film production

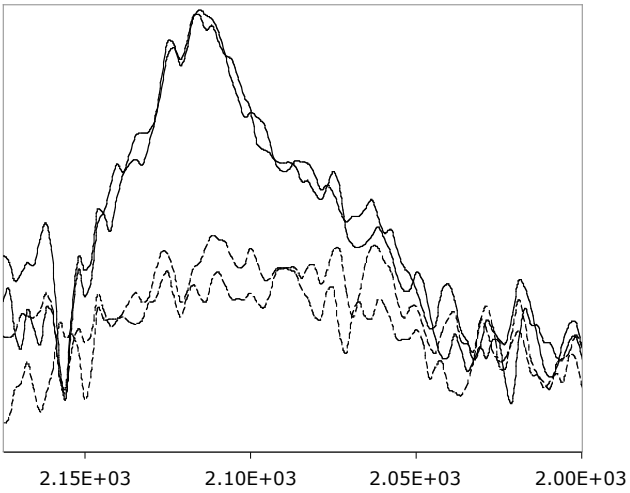
All irradated CS5-ELF-N_3 films remained clear and coherent after swelling in water and DMSO. However, as the azide composition decreased films became noticeably more tacky and less stiff. Irradiated CS5-ELF-N_3 with 13 and 19% phe replacement were too weak to remove from the mold without damage and thus were not used for mechanical testing (Table 5.2). Non-irradiated CS5-ELF-N_3 and CS5-ELF-F although clear after irradiation, slowly became incoherent when swelled in cold water.

Mechanical testing

Uniaxial tensile testing was used to probe the designed material properties of these aECM constructs. Films composed of irradiated CS5-ELF-F protein as well as

Figure 5.6. (a) Transmission infrared spectrum of CS5-ELF-N_3 (53%) film on ZnSe before irradiation (solid), after 10 sec (dashed) and 20 sec (dotted). Exposures of 70 sec to 490 sec are overlaid on one another. Attenuated total reflectance infrared spectrum of both sides of CS5-ELF-N_3 (53%) (b) before (2 upper spectrum; solid lines) and after irradiation (two bottom spectrum; dotted lines).





non-irradiated CS5-ELF-N_3 were not coherent when swelled in DMSO or cold water, whereas irradiated CS5-ELF-N_3 films proved quite stable. Tensile testing was amenable only to films of 30% azide incorporation or higher; films of lower azide content would tear easily upon mechanical manipulation. CS5-ELF-N_3 with 53%, 41% and 39% incorporation gave consistent stress versus strain curves, yielding elastic moduli of 1.39 ± 0.09, 0.94 ± 0.09, and 0.53 ± 0.1(MPa) and shear moduli of 0.53 ± 0.02, 0.38 ± 0.04, and 0.22 ± 0.03(MPa) respectively (Figure 5.7). These physical moduli compare well with chemically crosslinked films of a similar sequence [13, 14] and are nicely spaced within the values reported for native elastin (shear modulus 0.3 - 0.6 MPa) [28]. Molecular weight between crosslinks can be estimated, assuming an ideal network, from shear moduli. Treatment of the data reveal molecular weight between crosslinks (M_c) of 6,310, 8,815 and 15,227 respectively. Division of the respective polymers by the number of incorporated azides gives a theoretical molecular weight between crosslinks. Comparisons of theoretical and calculated M_c's give crosslinking efficiencies, the percentage of pN_3Phe sites that give rise to productive chemical crosslinks, of 43%, 41% and 31%, remarkable given reports of similar photoactive reagents usually only give ~30% efficiency and the requirements for a productive chemical crosslink in a polymer setting (Table 5.2). Efficiency may be partly attributable to the high azide content of the dry films. Solvents, particularly water, are known to decrease the efficiencies of trapping the aryl nitrene [29].

Figure 5.7. Representative stress vs. strain curves for photocrosslinked CS5-ELF-N_3 films incorporating various levels of pN_3Phe at 37°C in PBS pulling at a constant strain rate of 10%/minute.

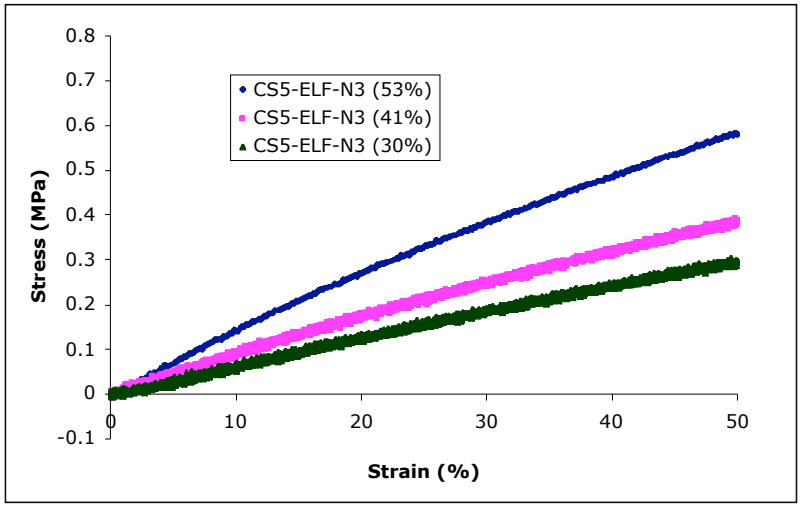


Table 5.2 Shear and elastic modulus, MW between crosslinks and photocrosslinking efficiency as a function of percent Phe replacement by pN_3Phe.

% phenylalanine replaced by pN_3Phe	Elastic Modulus (Mpa)	Shear Modulus (Mpa)	M_c kDa	Efficiency %
53	1.39 ± 0.09	0.53 ± 0.02	6.310	43
41	0.94 ± 0.09	0.38 ± 0.04	8.815	41
30	0.53 ± 0.10	0.22 ± 0.03	15.227	31
19	n.d.	n.d.		
13	n.d.	n.d.		

Conclusion

We have developed a method for photochemical crosslinking of protein polymers by introduction of a photoactive unnatural amino acid into the backbone. Moduli produced are easily variable by azide incorporation and encompass the target range, that of the native material. Produced materials have had no chemical treatment whatsoever, which should alleviate difficulties that come from implantation of traditional biomaterials. Such a technique could be easily extended to encompass other biomaterials particularly drug delivery applications and bioadhesives.

References

1. Seeman, N. C., *DNA in a material world*. Nature, 2003. **421**(6921): p. 427-431.

2. van Hest, J. C. M. and D. A. Tirrell, *Protein-based materials, toward a new level of structural control*. Chemical Communications, 2001(19): p. 1897-1904.

3. Seeman, N. C. and A. M. Belcher, *Emulating biology: Building nanostructures from the bottom up*. Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**: p. 6451-6455.

4. Yan, H., et al., *A robust DNA mechanical device controlled by hybridization topology*. Nature, 2002. **415**(6867): p. 62-65.

5. Mao, C. D., et al., *A nanomechanical device based on the b-z transition of DNA*. Nature, 1999. **397**(6715): p. 144-146.

6. Ross, R., *The pathogenesis of atherosclerosis - a perspective for the 1990s*. Nature, 1993. **362**(6423): p. 801-809.

7. Eberhart, A., et al., *A new generation of polyurethane vascular prostheses: Rara avis or ignis fatuus?* Journal of Biomedical Materials Research, 1999. **48**(4): p. 546-558.

8. Bos, G. W., et al., *Small-diameter vascular graft prostheses: Current status*. Archives of Physiology and Biochemistry, 1998. **106**(2): p. 100-115.

9. Conte, M. S., *The ideal small arterial substitute: A search for the holy grail?* Faseb Journal, 1998. **12**(1): p. 43-45.

10. Kobashi, T. and T. Matsuda, *Fabrication of compliant hybrid grafts supported with elastomeric meshes*. Cell Transplantation, 1999. **8**(5): p. 477-488.

11. Rabkin, E. and F. J. Schoen, *Cardiovascular tissue engineering*. Cardiovascular Pathology, 2002. **11**(6): p. 305-317.

12. Nerem, R. M. and D. Seliktar, *Vascular tissue engineering*. Annual Review of Biomedical Engineering, 2001. **3**: p. 225-243.

13. Panitch, A., et al., *Design and biosynthesis of elastin-like artificial extracellular matrix proteins containing periodically spaced fibronectin cs5 domains*. Macromolecules, 1999. **32**(5): p. 1701-1703.

14. Welsh, E. R. and D. A. Tirrell, *Engineering the extracellular matrix: A novel approach to polymeric biomaterials. I. Control of the physical properties of artificial protein matrices designed to support adhesion of vascular endothelial cells*. Biomacromolecules, 2000. **1**(1): p. 23-30.

15. Heilshorn, S. C., et al., *Endothelial cell adhesion to the fibronectin cs5 domain in artificial extracellular matrix proteins*. Biomaterials, 2003. **24**(23): p. 4245-4252.

16. Humphries, M. J., et al., *Identification of an alternatively spliced site in human-plasma fibronectin that mediates cell type-specific adhesion*. Journal of Cell Biology, 1986. **103**(6): p. 2637-2647.

17. Massia, S. P. and J. A. Hubbell, *Vascular endothelial-cell adhesion and spreading promoted by the peptide redv of the iiics region of plasma fibronectin is mediated by integrin alpha-4-beta-1*. Journal of Biological Chemistry, 1992. **267**(20): p. 14019-14026.

18. Mould, A. P., et al., *The cs5 peptide is a 2nd site in the iiics region of fibronectin recognized by the integrin alpha-4-beta-1 - inhibition of alpha-4-beta-1 function by rgd peptide homologs*. Journal of Biological Chemistry, 1991. **266**(6): p. 3579-3585.

19. Urry, D. W., et al., *Elastomeric polypeptides as potential vascular prosthetic materials*. Abstracts of Papers of the American Chemical Society, 1988. **196**: p. 143-PMSE.

20. Nerem, R. M., et al., *Hemodynamics and vascular endothelial biology*. Journal of Cardiovascular Pharmacology, 1993. **21**: p. S6-S10.

21. Jayakrishnan, A. and S. R. Jameela, *Glutaraldehyde as a fixative in bioprostheses and drug delivery matrices*. Biomaterials, 1996. **17**(5): p. 471-484.

22. Datta, D., et al., *A designed phenylalanyl-trna synthetase variant allows efficient in vivo incorporation of aryl ketone functionality into proteins*. Journal of the American Chemical Society, 2002. **124**(20): p. 5652-5653.

23. Kirshenbaum, K., I. S. Carrico, and D. A. Tirrell, *Biosynthesis of proteins incorporating a versatile set of phenylalanine analogues*. Chembiochem, 2002. **3**(2-3): p. 235-237.

24. Chin, J. W., et al., *Addition of p-azido-l-phenylaianine to the genetic code of escherichia coli*. Journal of the American Chemical Society, 2002. **124**(31): p. 9026-9027.

25. Tabb, J. S., J. V. Vadgama, and H. N. Christensen, *Characterization of para-azidophenylalanine as a system-l substrate and a photoaffinity probe*. Federation Proceedings, 1986. **45**(6): p. 1940-1940.

26. Escher, E., et al., *Para-azido-l-phenylalanine peptides .1. Synthesis of peptide ligands for chymotrypsin and aminopeptidases*. Israel Journal of Chemistry, 1974. **12**(1-2): p. 129-138.

27. Escher, E. and R. Schwyzer, *Para-nitrophenylalanine, para-azidophenylalanine, meta- azidophenylalanine, and ortho-nitro-para-azido-phenylalanine as photoaffinity labels*. Febs Letters, 1974. **46**(1): p. 347-350.

28. Abbot, W. M. and R. P. Cambria, *Biological nad synthetic vascular prostheses*. 1994, New York: Gurne and Straton.

29. Fleming, S. A., *Chemical reagents in photoaffinity labelling*. Tetrahedron, 1995. **51**(46): p. 12479-12520.