1 INTRODUCTION

1.1 Artificial proteins: definition and background

Genetic engineering technologies have proven extremely useful to biologists. The development of recombinant DNA techniques in the 1970s[1] made it possible to copy genetic material from one organism and insert it into another. While a valuable tool for studying fundamental biology, this also had immediate commercial applications, allowing human proteins like insulin and erythropoietin to be synthesized in large quantities in bacterial cells, improving the shelf-life and pest resistance of crops, and facilitating the production of new vaccines.[2]

Combined with the ability to sequence long pieces of DNA base-pair by base-pair, genetic engineering is even more powerful, since it allows peptides and proteins of nearly any imaginable sequence of natural amino acids to be synthesized in a host organism, such as *E. coli*. These a*rtificial proteins* (also *designed* or *engineered proteins*), made by genetic engineering to include non-natural amino acid sequences, are attractive candidates for creating new materials.[3] Their sequences can be controlled completely, a distinct advantage over synthetic polymers where control is restricted to block, random, or alternating copolymers. Another advantage is their uniformity of chain length, compared with traditional polymer materials that always display some diversity of molecular weight.

The incorporation of modular, repeating structures in artificial proteins can be used to achieve the desirable physical properties characteristic of synthetic polymers, or entirely new features. Early examples of distinctive architectures enabled by protein polymers include β-sheet structures in which the lamellar thickness was controlled by the sequence periodicity,[4] smectic liquid crystal phases of stiff helical rods with uniform molecular weight,[5] and hydrogels that reversibly converted to viscous liquids with pH and temperature changes.[6] Alternatively, artificial proteins may be based on structural proteins like silk.[7] While the scope of properties displayed by natural proteins is large, ranging from collagenous gels of vitreous humor with storage shear modulus of less than 30 Pa[8] to spider silks with elastic modulus 16 GPa,[9] all use the same amide-bond backbone and must be composed from the set of 20 natural amino acid monomers. The chemical diversity of artificial proteins, on the other hand, may be increased with techniques to incorporate non-canonical amino acids.[10,11]

1.2 Artificial proteins as biomaterials

Artificial proteins seem particularly well suited to be used as implantable biomaterials, where the magnitude of potential benefits is large and the amount of material required is often modest. Proteins have the potential to interact intimately with surrounding tissue and promote normal wound healing, whereas metals or synthetic polymers elicit a foreign body reaction when implanted, in which the material is isolated from the body by a fibrous capsule and inflammation persists in the surrounding tissue.[12,13]

More importantly, artificial proteins can be *bioactive*, containing peptide domains from natural proteins that are known to elicit specific biological reactions, such as promoting the adhesion of a particular cell type. In principle, chimeric and engineered artificial proteins could exhibit any combination of the diverse functions of natural proteins including catalysis, binding, signaling, and transport. The ability to hybridize multiple functions in a single protein is a distinct advantage over traditional techniques that require chemical modifications to make materials biocompatible or bioactive. A disadvantage is that certain artificial protein sequences may be immunogenic or may be expressed in low yield in host organisms.

1.3 Applications

Artificial proteins can be made to have both desirable mechanical and biological properties, so they may be attractive candidates for use in the full range of biomaterials applications, from rigid and permanent, like bone implants,[14,15] to soft and degradable, like hydrogels that promote wound healing.[16] For the promise of artificial proteins to be fulfilled, the effects of sequence and architecture on their physical properties must be better understood.

The original and primary focus of our group's research into bioactive artificial proteins has been in devising new materials for vascular grafts. The quest for an effective synthetic blood vessel reaches back decades, but success has been limited.[17,18] Especially challenging from a clinical perspective are small

diameter (< 5 mm) vascular grafts that are indicated, for example, to replace the major peripheral arteries that supply the legs and arms. The preferred surgical approach to replace an occluded or diseased peripheral artery is to use a similar-diameter vein harvested from the same patient, but the supply and quality of appropriate veins is limited. Commonly used synthetics, expanded polytetrafluoroethylene (ePTFE / "Gore-Tex") and woven poly-ethylene terephthalate ("Dacron"), work reasonably well for large diameter vascular grafts (> 5 mm), but fail at small diameters.[18-20]

The reasons synthetic polymer grafts fail are thought to be twofold. One, they do not support the regeneration or maintenance of endothelial cells, which coat all mammalian blood vessels and are essential for their health.[21] Two, the rigidity of synthetic polymer grafts, which distend roughly 1% during a blood pressure cycle versus ~10% for native vessels, causes abnormal shear stresses and consequent abnormal cellular function. Specifically, smooth muscle cells react by over-proliferation (intimal hyperplasia) and block the synthetic blood vessel over time.[17,22]

Many groups have attempted to design functional small diameter vascular grafts, typically addressing either the mechanical or biological needs of such materials.[18,23] Compliant grafts have been made from elastomeric synthetic polymers like polyurethane,[24,25] while efforts to support an endothelial cell monolayer have included pre-seeding of existing synthetic grafts,[26,27] modifying the surface properties by plasma treatment,[28] or adsorption of bioadhesive molecules like fibronectin.[29] Several biofunctionalized polyurethane graft

designs have been made[30-32] in an attempt to address both biological and mechanical requirements. Tissue engineering may eventually produce working blood vessels,[33-35] although the techniques are costly, and matching the mechanical properties of native tissue will require considerable development. Our group envisions hybrid artificial proteins that effect both blood vessel-like flexibility and the ability to adhere endothelial cells in a single material.

More recently, we have investigated using artificial proteins of the type originally designed for vascular grafts in other biomaterials applications. One possibility is to use flexible, signal-bearing artificial proteins to direct stem cell fate in neurorestorative implants.[36] Synthetic corneal onlays, a reversible alternative to ablative refractive vision surgeries such as LASIK, are another possible application. While this is a more recent challenge in biomaterials engineering, similar issues have arisen as with polymeric vascular grafts. Synthetic materials do not promote the adhesion of the epithelial cells necessary to maintain the biology of the cornea,[37] while the complexity of adapted biological materials like collagen lead to immune reactions and abnormal cell growth.[38] There is a need for transparent, stable, elastic materials that adhere epithelial cells, and artificial proteins of the type designed for use in vascular grafts appear well-suited to this application.

1.4 Design strategy

The research described in this thesis is based on a family of artificial proteins with a common design. Their architecture is analogous to block

copolymers, with cell-binding peptide domains alternating with larger flexible domains that govern the mechanical properties (Figure 1). Both domains are biomimetic: the cell-binding domains are cloned from portions of the human protein fibronectin, which adheres to a wide range of integrin cell-surface receptors and extracellular matrix molecules, and the flexible domain is derived from mammalian elastin, which is a large component of mechanically responsive tissues like skin, tendons, and blood vessels. Because these proteins capture key features of the extracellular matrix, we refer to them as artificial extracellular matrix proteins ("aECM" or "aE").

One cell-binding domain engineered into the artificial proteins is RGD (Arg-Gly-Asp), from the tenth type III module of fibronectin, which mediates adhesion to a variety of cell types through several integrin receptors.[39] Its prolific ability to adhere cells has brought about its use in dozens of biomaterials.[16,40,41] The other cell-binding domain used here is CS5, from the alternatively spliced type III connecting segment region of fibronectin, which is known to adhere to the integrin $\alpha_4\beta_1$.[42] It was observed that human endothelial cells attach and proliferate on immobilized GREDVY peptides (a portion of the CS5 sequence), while human fibroblasts, smooth muscle cells, and platelets do not,[43] so CS5-bearing proteins may be useful in vascular grafts.[44]

The elastin-like domains contain multimers of pentapetides with sequence $(VPGXG)_n$, where X is any amino acid, extensively studied by Urry et al.[45] These peptides are derived from hydrophobic repeating sequences in mammalian elastin, rich in valine, glycine, proline, and alanine. Crosslinked peptides of this

type replicate many of the mechanical properties of native elastin.[45,46] They display what is known as a lower critical solution temperature (LCST) in water, in which the proteins are soluble at low temperatures, but separate into an aggregated phase when the temperature is raised above their characteristic LSCT.[45]

Despite their ability to aggregate, elastin and elastin-like peptides do not have a well-defined folded three dimensional structure either above or below the LCST; they are rich in β-turn or β-sheet-like secondary structure,[47,48] but are extraordinarily mobile.[49,50] The structure-function relationship of elastin has long been a contentious issue and a subject of considerable research. The classical view is that elastin is like a solvent-swollen rubbery network whose elasticity derives from the architecture of its crosslinks.[49,51,52] However, models of elastin based on crystallographic evidence of closely related molecules[53,54] have induced others to suggest extended secondary-structural motifs which provide an entropic restoring force when distorted under strain.[45,55,56] Neither model can account for all of elastin's observed properties,[57] but recent experiments that examine elastin-like peptides using solid-state NMR[58,59] describe the distribution of torsional angles, while molecular simulations[57,60] have pointed out the importance of hydration in controlling elasticity.

Because of their unique thermosensitive behavior, elastin-based peptides have been incorporated into artificial proteins designed for a wide range of materials applications,[61] including soft tissue augmentation,[62] drug delivery,[63,64] immunosorbent assays,[65] tumor cell targeting,[66] nanoporous materials,[67]

switchable-permeability membranes,[68] protein purification,[69] and heavy metal remediation.[70] The well-characterized peptide VPGVG has been shown to be broadly biocompatible in toxicity, antigenicity, pyrogenicity, thrombogenicity, and mutagenicity tests, and does not result in fibrous capsule formation when implanted.[71] In light of these encouraging mechanical and biological properties, our laboratory chose $(VPGIG)_n$ as a structural basis for artificial extracellular matrix proteins. The substitution of isoleucine (I) for valine (V) in the native-like sequence lowers the LCST to near 10 °C[72] and allows the resulting protein to be easily purified by temperature cycling at laboratory conditions.[73]

1.5 Previous results

The first artificial extracellular matrix proteins designed and synthesized by our laboratory (Figure 1.1A) contained alternating CS5 and $(VPGIG)_n$ domains, which promoted the adhesion of endothelial cells when adsorbed to glass.[73] To allow proteins of this type to be chemically crosslinked into insoluble networks, the sequence was modified to include N- and C-terminal lysine residues (Figure 1.1B). At the same time, a T7 antibody recognition tag was appended, as it facilitated identification during purification and increased protein expression levels. This material was chemically crosslinked with glutaraldehyde into stable matrices with tensile properties similar to native elastin.[74] These proteins were also crosslinked with hexamethylene diisocyanate, a less toxic[75] lysine-reactive molecule; mechanical properties and swelling of the resulting matrices are characterized in Chapter 2 of this thesis. When adsorbed

to surfaces, proteins of this type support endothelial cell adhesion at physiologically relevant shear rates.[76]

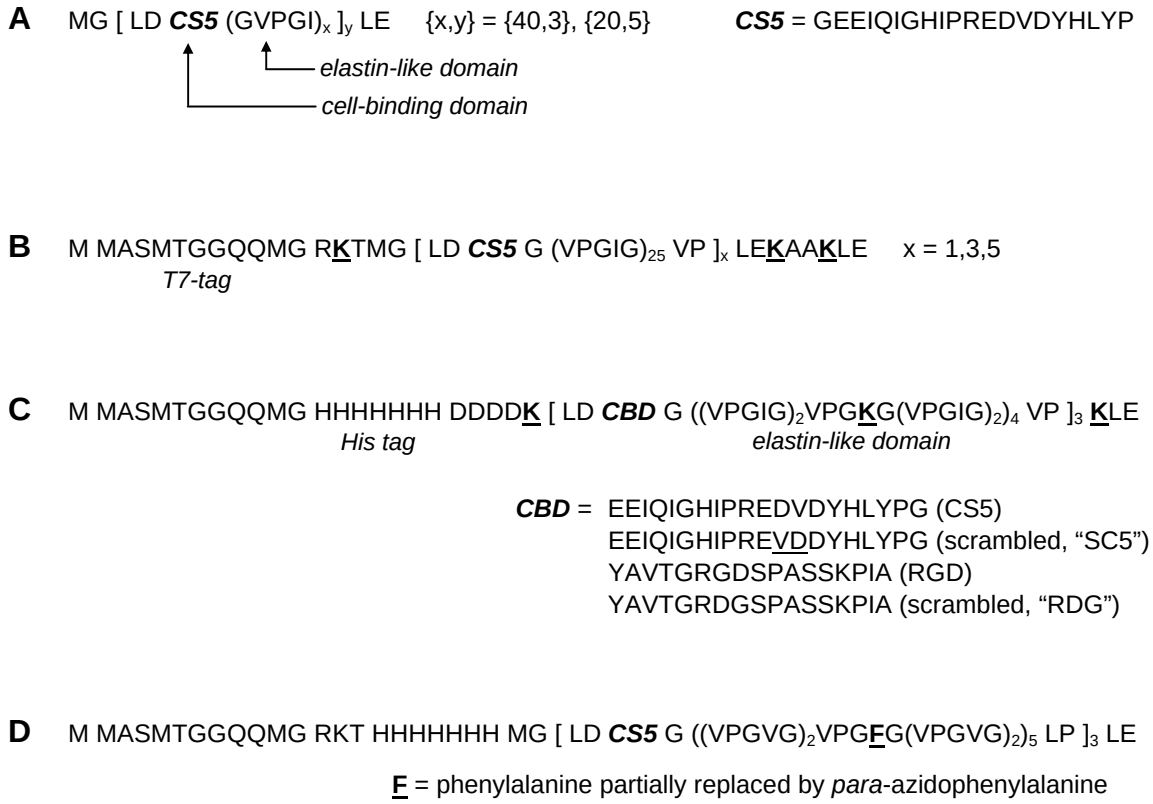

Figure 1.1 Amino acid sequences of artificial extracellular matrix (aECM) proteins, with the functions of component peptides identified. Crosslinked films of (B) are characterized in Chapter 2. Proteolytic degradation of (B) and (C) is studied in Chapter 3. Chapter 4 reports the performance of corneal onlays made from (C) with RGD cell-binding domains. The synthesis and characterization of mechanically tunable thin films of (D) are described in Chapter 5.

The next generation of aECM proteins added two features: one, an increased density of lysine side chains, spaced throughout the elastin portion of

the protein, to facilitate highly lysine-specific crosslinking with using N-hydroxysuccinimidyl esters; two, native and sequence-scrambled versions of the CS5 and RGD cell binding domains, so that the sequence-specificity of cell binding could be demonstrated (Figure 1.1C). Binding of endothelial cells to the CS5 and RGD domains was confirmed.[77] The binding to CS5 appeared to be made less robust by the lysines in the elastin-like domain.[78] Mechanical properties of crosslinked films of these third generation proteins again spanned the desired range.[79] Chapter 3 examines the sequence-dependence of proteolytic degradation of soluble and crosslinked aECM proteins of this (1.1C) and the previous (1.1B) designs. Chapter 4 describes the *in vivo* performance of corneal onlays, permanent contact lenses, made from crosslinked RGD-based aECM proteins of this third type.

A photosensitive aECM protein was also made by incorporating the non-canonical amino acid *para*-azidophenylalanine[80] into phenylalanine sites regularly placed within the elastin-like domains, as were lysines in the chemically crosslinkable protein (Figure 1.1D). The ability to photocrosslink artificial proteins may aid their application as biomaterials, but it also provides opportunities for developing cell culture substrates with patterns of mechanical properties, described in Chapter 5, which can be used to understand mechanosensitive cell behavior. This design was also used as a protein photoresist, allowing cells to be simply and controllably patterned on surfaces; this work is described in the Appendix.

1.6 Thesis organization and description of contributions

This thesis reports progress in developing biomaterials using crosslinked matrices of artificial extracellular matrix (aECM) proteins. I have focused on physical characterizations of these materials, which, along with research in our laboratory into their biological activity, has elucidated important principles for protein-based design and brought the aECM proteins from the drawing board through to implantation in mammals.

A primary objective of this work was to devise crosslinking systems to stabilize the aECM proteins into matrices with elastic properties similar to human blood vessels. Chapter 2 describes the crosslinking of aECM proteins with isocyanate-functional molecules to give highly extensible, uniform hydrogel films whose density and elastic moduli are similar to native elastin. The crosslinker concentration was observed to affect both the modulus and swelling characteristics of the matrices. I wrote this chapter and performed all the experiments.

Another key property of biomaterials implants that must be well understood is their susceptibility to degradation *in vivo*. While elastin in nature has an exceptionally long half-life of tens of years,[62] proteolytic enzymes are up-regulated when tissues are remodeled, e.g., at implantation sites, and the chemical properties of the aECM proteins may affect their degradation in other ways. The susceptibility of two types of aECM proteins, in both soluble and crosslinked form, to elastase degradation is measured in Chapter 3. Small sequence changes to the protein were observed to powerfully affect degradation

rate, a result that will inform the design of future protein-based materials to best fit the materials requirements of their applications. The paper was principally written by Dr. Sarah Heilshorn, a member of our laboratory, who began work on the project in the laboratories of Tetsuji Yamaoka at the Kyoto Institute of Technology. Sarah did all experiments involving soluble proteins, while all the work on crosslinked films was done by me. Specifically, I devised a crosslinking system to match physical properties between the two aECM architectures, characterized their degradation by mechanical testing, and analyzed the results using rubber network assumptions.

The elasticity and cell-binding capabilities of the aECM made them easily adaptable for use as corneal onlays, or permanent contact lenses. Chapter 4 reports *in vivo* testing in rabbit corneas of transparent onlay lenses made by crosslinking aECM proteins containing RGD cell-binding domains. A majority of this chapter was written by me, including the introduction, discussion, and conclusions sections, and the description of the lenses themselves. I made and characterized the lenses, although the aECM protein was originally cloned by Julie Liu and the crosslinking technique was adapted from one developed by Dr. Kathy Di Zio, members of the Tirrell laboratory. The surgeries and *in vivo* characterizations, and their descriptions for the manuscript, were performed at the University of California at San Francisco by Dr. Marsha Cheung and Dr. Daniel Schwartz. Substantial portions of the experimental and results section texts are taken from U.S. Patent Application 11/040,130, by the same authors as the manuscript.

Chapter 5 describes our attempt to fashion systems that can be used to understand cell response to biomaterials with coordinating mechanical and biological signals. Recently, cells have been observed to react to the stiffness of the substrate on which they are characterized.[81-83] We used aECM proteins that incorporate a photoreactive, non-canonical acid to make single films with patterned mechanical properties. The elastic properties of the films were determined with an AFM nanoindentation technique, the validity of which was confirmed by bulk sample measurements and finite element simulations. I wrote the chapter and performed all measurements and analysis it describes, while Christian Franck (Graduate Aeronautics Laboratories, California Institute of Technology) did the finite element modeling and provided substantial expertise and insight throughout. Chapter 5 makes reference to a yet-unpublished paper from our group, which I had a minor role in writing; it is included in an Appendix for convenience.

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