Chapter I. Introduction

1. Protein engineering approach to biomaterials

The majority of currently implanted biomaterials were initially developed for use in other industries before being adopted by the medical profession to fulfill specific surgical needs. Common examples of these materials include titanium, stainless steel, and a variety of synthetic polymeric materials. While many of these materials perform well, especially given the fact that they were not designed for biomedical use, other implants have suboptimal patency due to their limited biocompatibility.[1] To address this issue, the *de novo* design of new biomaterials specifically for use in implantable devices is an important area of research.

A novel approach to creating new biomaterials is the design and synthesis of artificial polypeptides using recombinant protein technology.[2-4] DNA sequences encoding modules of naturally occurring proteins can be spliced together to form artificial proteins that combine desirable properties from natural proteins. Advantages of this synthesis strategy versus traditional synthetic polymers include exact control of the monomer sequence and molecular weight, the ease of creating multiple variants of block copolymers, and the ability to include modules that confer bioactivity. Potential disadvantages include the potential for low yields and difficult purification schemes required for these types of syntheses, although technology is quickly being developed to address these issues for protein pharmaceuticals. Another shortcoming is the limitation of available monomer, or amino acid, building blocks available to the biological protein synthesis machinery. To alleviate this constraint, researchers are developing new

methods to incorporate noncanonical amino acids into *in vitro* and *in vivo* protein synthesis systems.[5-7]

2. Small-diameter vascular grafts

The initial motivation for this project was to demonstrate the applicability of protein engineering to the design of new biomaterials for the treatment of cardiovascular disease. Specifically, I sought to develop and characterize artificial proteins that would have potential as small-diameter (<6 mm inner diameter) vascular grafts. Arteries that fall into the small-diameter category include the coronary artery that supplies blood to the heart and peripheral arteries that supply blood to the lower limbs.

When a small-diameter artery is blocked, a number of potential surgical remedies are available to the surgeon including stents, angioplasty, and bypasses. When a bypass is deemed necessary to direct blood flow around the blocked segment, the surgeon's first choice of bypass material is autologous vessel, i.e., veins or arteries harvested from the patient. However, in many circumstances, this is not a feasible option due to multiple bypass surgeries or other complicating factors such as diabetes. In these cases, bypasses are constructed from synthetic polymeric materials, most commonly ePTFE, expanded poly(tetrafluoroethylene), and PET, poly(ethylene terephthalate), better known by their trade names Gore-Tex and Dacron. While bypasses constructed from these synthetic materials perform well in areas of high blood flow, they often fail within a few years of implantation in regions of low blood flow such as small-diameter arteries.[8-10]

To address this obvious medical need, many research groups are investigating the underlying causes of graft failure and developing alternative strategies to produce more

successful graft materials. Two important limitations of the current synthetic graft materials have been identified: i) a mismatch in mechanical properties between the synthetic graft and the host tissue and ii) an inability of the synthetic graft to promote endothelial cell adhesion to the inner lining of the graft. Both ePTFE and PET are much stiffer and less compliant than natural blood vessels. This mismatch in mechanical properties is thought to stimulate the over-proliferation of smooth muscle cells, which surround blood vessels and provide the pumping action.[11] When these smooth muscle cells over-divide, they pinch off the graft and restrict blood flow. To address this issue, some researchers are investigating the use of polyurethanes as graft materials because they are more flexible and compliant.[12-14]

Endothelial cells line the lumen of blood vessels and express several autocrine and paracrine molecules that regulate many of the processes used by blood vessels to maintain optimum functionality.[15,16] In addition, endothelial cells play a major role in the immune system by regulating leukocyte migration into the perivascular tissue.[17] Both ePTFE and PET grafts are unable to support the development of an endothelial cell monolayer after implantation. To promote endothelial cell adhesion to the graft surface, a variety of strategies have been investigated including pre-seeding with endothelial cells,[18-20] pre-clotting with plasma,[21,22] treatment with various cytokines and antibodies,[23,24] incorporation of microspheres containing therapeutics,[25,26] and pre-incubation with albumin[27] or extracellular matrix proteins.[28-30] While many of these strategies have demonstrated moderate success in *in vitro* and even small clinical trials, no single strategy has gained wide spread acceptance as a proven method of improving small-diameter graft patency.

3. Artificial extracellular matrix proteins - Design strategy

Our lab has approached the problem of small-diameter vascular grafts from a different perspective. Using our expertise at employing bacterial genetic engineering to design and synthesize polypeptides having pre-determined and precisely-controlled molecular architectures, we designed a new family of artificial extracellular matrix (aECM) proteins with two initial design criteria: i) matched physical compliance with the arterial wall and ii) establishment of an endothelial cell monolayer.[31] In order to achieve these goals, the molecules we design include two prominent features. First, a mammalian elastin analog was chosen to provide control over mechanical properties. Second, cell-binding domains derived from the naturally occurring extracellular matrix protein human fibronectin were chosen to support endothelial cell attachment and spreading.

The reasons for choosing an elastin-like sequence to provide physical integrity were several-fold. First, elastin is found in the naturally-occurring extracellular matrix of small-diameter arteries.[32] Second, elastin-like polymers can be readily formed into coatings and films and easily purified due to their lower critical solution temperature (LCST) behavior.[33] Also, the repeating polypentapeptide GVGVP has undergone extensive biocompatibility testing including tests for mutagenicity, toxicity, antigenicity, pyrogenicity, and thrombogenicity.[34] Finally, repetitive elastin-like polymers have been shown to exhibit a wide range of mechanical properties that are similar to those demonstrated by the arterial wall.[33] By decreasing the compliance mismatch between the vascular graft and the arterial matrix, common modes of graft failure such as endothelial

damage and peri-anastomotic smooth muscle cell proliferation can be minimized.[11] Based upon the extensive work of Urry and coworkers, we have focused on the elastin analog VPGIG. By substituting a more hydrophobic residue into the peptide, the predicted LCST is reduced from 25°C to 10°C allowing for easy handling at convenient temperatures.[35,36]

Two cell-binding domains, CS5 and RGD, have been included in the aECM proteins discussed in this work. The CS5 cell-binding domain includes residues 90-109 of the type III connecting segment (IIICS) of fibronectin.[37] The minimum active sequence of the CS5 domain was determined to be REDV which targets the $\alpha_4\beta_1$ integrin.[38-41] Hubbell and coworkers have demonstrated that human umbilical vein endothelial cells (HUVEC) will attach and spread on the hexapeptide GREDVY immobilized on glycophase glass while human foreskin fibroblasts, human vascular smooth muscle cells, and human blood platelets will not.[42] Their work also demonstrated that endothelial cell binding to REDV grafted materials could be inhibited by soluble REDV peptides. The RGD cell-binding domain is derived from the tenth type III domain of fibronectin[43-45] and serves as a ligand for the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins.[46,47] The RGD cell-binding domain promotes cell spreading of a variety of cell types and has been much studied by the cell biology and biomedical engineering community.

Urry and coworkers first suggested the combination of elastin peptides with cell-binding domains and were able to display increased endothelial cell spreading on GRGDS peptides inserted into the GVGVP elastin sequence.[48,49] The REDV binding domain appears to be more selective than the central cell-binding domain of fibronectin, which carries the RGD sequence.[41] For example, smooth muscle cells also express the

$\alpha_v\beta_3$ integrin and have been shown to adhere to the RGD sequence.[50] Therefore, the careful selection of the cell-binding domain in terms of ligand affinity offers a method to selectively adhere cells expressing particular integrins. Although previous attempts by Urry and coworkers to show increased endothelial cell adhesion to REDV peptides inserted into a GVGVP motif were unsuccessful,[51] it is also known that cellular adhesion is dependent upon ligand conformation.[52,53] We hypothesize that this previous attempt did not present the REDV peptide in the correct context for cellular recognition. Therefore, we have included the entire CS5 binding domain into our artificial extracellular matrix proteins in an attempt to allow the domain sufficient flexibility to achieve a bioactive conformation.

4. Artificial extracellular matrix proteins - Previous results

Several artificial extracellular matrix proteins incorporating the above peptide domains have been designed and synthesized by our laboratory.[31,54-57] Analogs including various ratios of elastin to cell-binding domain have been developed as well as versions containing lysine residues for site-specific crosslinking. Most aECM proteins include a T7 tag to aid in identification and purification. The T7 tag is an eleven amino acid peptide that occurs at the natural amino terminus of the bacteriophage T7 major capsid protein and is used as an epitope tag to follow target proteins using immunological methods. The expression method has been optimized to yield large quantities of protein using *E. coli* strain BL21(DE3)pLysS under control of a bacteriophage T7 promoter. A simple, reproducible purification procedure selectively precipitates contaminating proteins below the lower critical solution temperature of the target polymer.

Three-dimensional, freestanding films of aECM proteins were created by crosslinking with glutaraldehyde,[31] bifunctional isocyanates,[58] or bifunctional NHS-esters.[55] Mechanical testing of these films produced stress-strain profiles consistent with elastic behavior. The elastic moduli were tunable by genetically dictating the molecular weight between crosslinks and by modifying the stoichiometric amount of crosslinker incorporated into the films. Using these techniques, films were created with elastic moduli of the same order of magnitude as those demonstrated by native elastin.[31,55,58]

Initial investigations into the biocompatibility of the aECM proteins were conducted *in vitro* using human umbilical vein endothelial cells (HUVEC). These experiments verified that aECM proteins were not cytotoxic and could promote cell spreading and proliferation.[54] Furthermore, HUVEC adhesion was increased on aECM proteins with higher densities of the CS5 cell-binding domain, suggesting that HUVEC adhesion may be directly mediated by interactions with the CS5 amino acid sequence.[54]

5. Artificial extracellular matrix proteins - Current results

The objective of this thesis project was to further characterize the applicability of aECM proteins as small-diameter vascular grafts. Towards this goal, a primary objective was to determine if HUVEC adhesion to aECM proteins was caused by sequence-specific interactions with the cell-binding domain. Chapter 2 describes the assays I developed to assess HUVEC adhesion and the application of these protocols to confirm HUVEC interactions with the CS5 cell-binding domain contained within aECM proteins. Chapter 2 also describes the development and application of several *in vitro* assays to probe the biocompatibility of aECM proteins. These include the *limulus* amoebocyte lysate assay

to quantify lipopolysaccharide contamination, ELISAs for tissue plasminogen activator and plasminogen activator inhibitor-1 secretion, and the construction and testing of a flow chamber to measure cell adhesion strength against opposing shear stress.

These cell spreading and adhesion assays were then employed in Chapter 3 to compare HUVEC adhesion to aECM proteins containing the CS5 and RGD cell-binding domains. I provided technical assistance and consultation to Julie Liu, who performed the majority of the work involving the RGD cell-binding domain.

Chapter 4 represents a collaboration between Julie Liu and myself to better understand the importance of context, i.e. amino acid choice distal to the cell-binding domain, in affecting cell spreading and adhesion. Ms. Liu and I contributed equally to the assay development, data collection, and data interpretation for this chapter.

The importance of amino acid choice in modifying aECM resistance to protease degradation was explored in Chapter 5. This work began in the laboratory of Dr. Tetsuji Yamaoka at the Kyoto Institute of Technology during a summer research program sponsored by the National Science Foundation and was completed upon my return to Caltech. I was assisted in this endeavor by Paul Nowatzki, who provided expertise in the measurement of bulk film mechanical properties.

Another strategy to modify aECM resistance to protease degradation involves the incorporation of noncanonical amino acids into the primary amino acid sequence at known degradation sites. This strategy was employed in Chapter 6 by the incorporation of 5,5,5-trifluoroisoleucine (5TFI) into an aECM protein. 5TFI synthesis, protein expression and purification, and analysis of 5TFI incorporation were performed by

Marissa Mock and subsequent protein characterization for solubility, degradation, and cell adhesion was performed by myself.

Chapter 7 relates the incorporation of another noncanonical amino acid, *para*-azidophenylalanine (*p*N_3Phe), into an aECM protein. The azide moiety allows photoactivated crosslinking of the aECM protein into three-dimensional films and two-dimensional patterns. Protein synthesis and characterization were performed by Isaac Carrico, the photopatterning protocol was developed by Dr. Carrico, Marissa Mock, and myself, and I performed cell culture on the resulting protein patterns.

Finally, chapter 8 presents evidence that the cell-binding domains incorporated into aECM proteins are still capable of cell recognition after being crosslinked into films. HUVEC adhesion to the aECM films could be tuned by modifying the elastic modulus or the cell-binding domain density of the material. Preliminary evidence also suggests HUVEC can respond to substrate modulus by modifying the amount of matrix metalloproteinase-2 that is secreted and activated. These results suggest that aECM films may be useful not only as small-diameter vascular grafts, but also as well-characterized substrates for studies that seek to understand the biochemical and biomechanical signals initiated by cell-matrix contacts.

6. References

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