1 Introduction

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1.1 Designing functional biomaterials

The design of functional biomaterials that elicit specific cellular behavior constitutes a major challenge for the fields of tissue engineering and materials science. Efforts to develop such materials have principally involved the design of scaffolds and hydrogels to mimic the dynamic interactions between cells and the extracellular matrix *in vivo* [1, 2], and the incorporation of extracellular adhesion ligands and growth factors into engineered materials has proven effective in directing cellular response in many applications [3-5]. Innovative methods to fabricate "smart," or stimuli-responsive, biomaterials that react to cell-mediated processes or to environmental changes in pH or temperature have allowed materials to play more interactive roles in tissue morphogenesis [6, 7]. In order to construct materials that promote specific cellular fates, it is essential to assert greater control over both the structural properties and biochemical characteristics of these materials.

In this context, the use of protein-based biomaterials provides a uniquely powerful approach to the control of macromolecular structure and function. The growing ease of expression of recombinant protein polymers promises to expand the use of protein-based materials, both in the investigation of basic cellular processes and in therapeutic applications. Advances in microfabrication technology and controlled release systems have also expanded the possibilities for control of the spatial and temporal patterns in which proteins can be presented to cells [8]. These methods have yielded important new insights into the mechanisms by which cells sense and respond to signals, and have enhanced substantially the repertoire of techniques available for materials design.

In comparison with other areas of protein engineering, biomaterials design presents unique challenges because of the crucial role of mechanical behavior in determining success or failure in materials applications. Materials design often begins with consideration of mechanical behavior – identifying and optimizing the properties needed to produce stable platforms for tissue replacement or regeneration. The biological properties of the material are then modified to enhance tissue specificity, cellular activity and biocompatibility.

1.2 The role of mechanical properties in biomaterials design

1.2.1 Elastin-like polypeptides

Materials for use in tissue engineering must provide structure and support to regenerating tissue while accommodating cellular infiltration and homeostasis. A logical strategy for the design of synthetic materials with the requisite mechanical properties utilizes structural motifs derived from extracellular matrix proteins [9]. Artificial proteins incorporating elastin-like polypeptides represent a class of materials whose physical properties are remarkably similar to those of native elastin. Moreover, these proteins are tunable at the genetic level to match the requirements of specific applications [10, 11]. For example, repeats of elastin-like polypeptides (ELP) with the amino acid sequence $(VPGZG)_x$, where Z is any amino acid except proline, have been developed for use in vascular grafts. Lysine residues have been encoded at regular intervals in such protein sequences to allow for crosslinking by difunctional electrophiles. Variation in the stoichiometric ratio of crosslinker and protein enabled the preparation of films with moduli spanning the range reported for native elastins [11, 12].

Similar ELPs have been engineered to function as injectable scaffolds for cartilaginous tissue repair [13]. Thermally responsive ELPs were designed to exhibit liquid-like behavior at room temperature, permitting simple suspension of chrondrocytes; however, upon reaching physiological temperatures (*in situ* upon injection), the ELP mixture aggregates into a stiff gel-like coacervate. Cells become entrapped within an elastic three-dimensional matrix with mechanical properties comparable to those reported for collagen and hyaluronan-based scaffolds commonly used to promote cartilage regeneration [13]. Since the physical properties of ELPs are genetically encodable, the potential exists for the formulation of specialty ELPs to augment healing in specific cartilaginous tissues that are prone to injury.

1.2.2 Crosslinking mechanisms

Another approach to modulating the mechanical properties of protein-based materials involves the use of defined crosslinking reactions. To facilitate site-specific solid-state crosslinking using both UV and visible light photoinitiators, Nagapudi and coworkers functionalized the lysine residues of elastin-like polypeptide repeats with acrylate moieties [14]. Electrospinning of these proteins into fibers followed by photoirradiation produced highly extensible networks appropriate for cell culture. Elbjeirami et al. reported a similar enhancement in the mechanical properties of collagen gels through lysyl oxidase (LO) mediated crosslinking. Gels seeded with LO-transfected vascular smooth muscle cells exhibited nearly a two-fold increase in both the elastic modulus and tensile strength as compared to scaffolds containing mock-transfected cells [15].

1.2.3 Controlled degradability of structural scaffolds

Scaffold degradation can be synchronized with cellular repair such that materials can be replaced by functional tissue over a time interval that minimizes structural destabilization. The use of recombinant proteins in biomaterials offers this additional design advantage, since proteins can be engineered to incorporate amino acid sequences that are susceptible to selected cellular proteases. Halstenberg and coworkers functionalized poly(ethylene glycol) (PEG)-based hydrogels with an engineered artificial protein containing two plasmin degradation sites, a heparin binding site, an RGD binding motif to promote cell adhesion, and repeating amino acid sequences based on fibrinogen and antithrombin III [16]. The resulting hydrogel facilitated sequence-specific cellular adhesion through the RGD domain, possessed serine-protease degradability, and exhibited mechanical integrity adequate to sustain cellular growth *in vitro* on a timescale comparable to those of normal wound healing processes. Similarly, specific elastase target sequences were cloned into protein polymers to allow for programmed turnover of these matrices by cell-mediated processes [17, 18]. The elastase cut site was strategically placed within the artificial protein to allow for liberation of a hexapeptide known to promote cellular proliferation and wound healing responses.

1.2.4 Effect of mechanical properties on cellular behavior

In addition to their crucial role in providing structural support, the mechanical properties of biomaterials have been shown to impact cellular behavior [19]. The efficacy of several silk and collagen based substrates to support chrondrogenesis of cultured human mesenchymal stem cells (hMSC) was found to be influenced primarily by scaffold

degradation rates rather than by chemical composition [19]. Samples of crosslinked collagen and slowly degrading silks promoted cell differentiation and matrix deposition whereas uncrosslinked collagen samples collapsed prematurely and were unable to support significant cartilaginous tissue formation.

This observation, in concert with others that demonstrate the connection between substrate mechanics and cellular response, has prompted investigators to examine the use of spatial variation in mechanical properties as a design strategy for engineered materials. Gray and coworkers produced fibronectin-coated polymeric substrates containing micropatterned square islands of increased stiffness and observed that cells initially plated uniformly over the surface reposition themselves on the rigid islands of the substrate over the course of several days [20]. Mechanically-directed migration has also been observed on gradient-compliant photocrosslinkable polyacrylamide-based hydrogels [21]. For vascular smooth muscle cells cultured on these gradient substrates, preferential migration was exhibited toward the region of highest elastic modulus, whereas cells distributed on uniformly compliant substrates displayed no such directional movement. As indicated by the authors, the substantial accumulation of cells on stiffer portions of the substrate suggests that the detailed nature of the gradient pattern may be important in controlling the extent of cell migration [21, 22]. More recently, mechanical gradients have been used to manipulate other cellular processes including attachment and spreading [23].

1.3 The role of biochemical properties in biomaterials design

Protein-based materials offer special advantages for providing biochemical instructions to guide cellular behavior, in that the identity and presentation of bioactive ligands may be engineered to elicit various cellular phenotypes. In establishing qualitative design strategies for the fabrication of biomaterials, three general approaches have emerged to induce cellular behavior and guide tissue morphogenesis using both naturally and artificially derived proteins: altering the identity of adhesive proteins and peptide domains; modulating the density and spatial presentation of proteins and peptide domains; and optimizing the temporally controlled presentation of proteins and peptide domains.

1.3.1 Choice of adhesive proteins and peptide domains

A dominant theme in biomaterials research over the past decade has addressed the judicious inclusion of adhesion ligands that interact with specific cellular receptors. Heilshorn and coworkers have reported artificial extracellular matrix (aECM) proteins in which elastin-like sequences alternate with CS5 cell binding domains derived from fibronectin to enable adhesion of human umbilical vein endothelial cells (HUVEC) through the $\alpha_4\beta_1$ integrin [24]. Incorporation of the CS5 domain facilitated sequence-specific adhesion to aECM proteins and sustained cellular attachment under dynamic stresses characteristic of the vascular system. In a companion study, Liu et al. were able to increase both the rate and extent of cell spreading and attachment on similar aECM proteins by replacing the CS5 ligand with the well-characterized RGD cell binding domain [25]. HUVEC formed focal adhesions and normal stress fibers on the RGD-

modified surfaces through the $\alpha_v\beta_3$ integrin, demonstrating that cellular response to aECM proteins can be modulated by the careful selection of cell binding domains that foster distinct ligand-receptor interactions. In a related approach, the adhesive pentapeptide YIGSR was incorporated into a dually modified polyurethaneurea-based material designed by Jun and West to promote endothelialization through specific adhesion receptors [26]. The second modification involved the use of PEG chains as soft segments in the polymer to limit nonspecific platelet and protein adhesion.

Targeting the adhesion of a particular cell type to a material surface has also been achieved by using a non-integrin based ligand. Gobin et al. grafted the elastin-derived amino acid sequence VAPG, known to bind to a peripheral membrane receptor on smooth muscle cells, into photopolymerizable hydrogels based on acrylate-terminated derivatives of PEG [27]. Inclusion of the VAPG sequence promoted the specific adhesion of vascular smooth muscle cells while deterring adhesion of endothelial cells, fibroblasts, and platelets.

1.3.2 Density and presentation of active protein and peptide domains

Cellular processes including adhesion, migration, and proliferation are strongly influenced by the surface density as well as the spatial distribution of bioactive domains [8, 28]. Gaudet and coworkers recently demonstrated that fibroblast spreading, motility, and contractility could be modulated by varying collagen surface densities. Interestingly, as collagen surface density was increased, cell spreading did not increase as expected, but rather decreased, suggesting that beyond a certain collagen surface density fibroblasts interact differently with the underlying substrate [29]. This transition density or threshold

concentration matched the average calculated density of cell surface integrin receptors, a result that supports the notion that increased cell spreading is precluded by saturation of available cell surface integrin receptors.

Heilshorn and coworkers investigated the importance of cell-binding domain context on cell adhesion and spreading on engineered aECM proteins containing crosslinking sites located either within the interior or within the terminal portions of the protein chains [30]. Terminally crosslinked aECM proteins supported robust cell spreading and adhesion. However, crosslinking at sites within the repeating units of the protein resulted in a notable decrease in cellular response, suggesting that protein modification remote from the putative receptor-binding sequence could result in conformational changes affecting ligand affinity or accessibility. In order to probe the effects of protein conformation and context on cell adhesion, the Mrksich group developed a generalized model system that allows for control of the density, patterning, and orientation of immobilized engineered fusion proteins presented on a surface [31]. The engineered protein of interest comprises the 10^{th} domain of fibronectin (for cell adhesion) fused to the serine esterase cutinase which reacts to form a covalent adduct to a phosphonate ligand presented on tri(ethylene glycol)-terminated self-assembled monolayers (SAMs). This immobilization strategy minimizes unwanted protein-substrate contacts, and offers considerable versatility as a method for screening protein-cell interactions.

Control over the spatial presentation of protein ligands is an especially valuable tool for guiding morphogenetic processes that rely on gradient patterns for proper development. Although the influence of soluble gradients on cellular behavior has been

extensively characterized, cellular response to immobilized gradients has only recently been investigated. By using microfluidic devices, substrate-bound gradients of extracellular signaling molecules can be formed on scales relevant for cell studies [32, 33]. In introducing this technology, Whitesides and coworkers demonstrated that the axonal polarity of rat hippocampal neurons could be oriented along increasing surface density gradients of adsorbed laminin [34]. Kapur et al. observed that axonal specification and extension of pheochromocytoma cells could also be guided along an immobilized gradient of nerve growth factor in a manner akin to that observed with a gradient of the solubilized protein [35]. The surface gradient resulted in morphologically thicker neuronal processes as compared to those treated with the soluble growth factor, suggesting that cells may activate different response mechanisms depending on the method of gradient presentation.

In order to preserve gradient fidelity for long term cell studies, Gunawan and coworkers covalently immobilized counter gradients of collagen I and laminin on carboxy-terminated SAMs using active-ester linking chemistry [36]. The expression profiles of two cell cycle progression markers could be directly controlled by the local ratio of extracellular matrix proteins.

1.3.3 Temporally controlled presentation of active proteins and peptide domains

The growth and repair of many tissues is coordinated through the temporally controlled liberation of soluble signals such as growth factors [5]. Design of materials to regulate these dynamic processes has centered on the fabrication of controlled release systems with defined dose and delivery kinetics for the purpose of eliciting localized and

measurable cellular responses. Richardson and coworkers developed a polymeric scaffold that allows for the coordinated and sequential release of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) for use in therapeutic angiogenesis [37]. The two growth factors were incorporated into a single material by mixing pre-encapsulated PDGF microspheres with a particulate polymer mixture containing lyophilized VEGF before processing into a porous matrix. Mixing VEGF with polymer particles resulted in rapid release of this factor, initiating angiogenesis, while slow degradation of microspheres released PDGF to support vessel growth and maturation. Toward the same goal, Ehrbar et al. covalently attached a recombinant version of VEGF containing a protease sensitive cleavage site to fibrin gel matrices, allowing the rate of growth factor release to be regulated by local cellular enzymatic activity [38]. Cell-demanded release of VEGF from remodeled fibrin scaffolds increased the formation of structurally intact and morphologically regular vascular beds in embryonic chicken chorioallantoic membranes.

Karageorgiou and coworkers have reported covalent coupling of active growth factors to materials to allow for induction of cellular responses that require delivery of the factor for prolonged periods of time [39]. Immobilization of bone morphogenetic protein-2 (BMP-2) on silk scaffolds was found to be more effective in inducing osteogenic differentiation of bone marrow stromal cells than addition of soluble BMP-2 to the growth medium [40].

1.4 Outline of thesis

The goal of this thesis is to characterize the cellular response to both biological and mechanical cues presented in the form of artificial extracellular matrix (aECM) proteins. 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 the protein films, while the RGD binding domain mediates cell attachment. Crosslinking is facilitated through the incorporation of the noncanonical amino acid, *para*-azidophenylalanine (*p*N_3Phe), into the elastin backbone. Irradiation of protein films with ultraviolet light initiates photocrosslinking within the material that can be tuned by adjusting the irradiation dosage or by controlling the extent of *p*N_3Phe incorporation in the protein. Chapter 2 outlines the design and construction of this protein as well as a negative control protein. Marissa Mock initiated the cloning work, and I completed the genetic assembly of both proteins and performed the expression and characterization. The cell spreading experiments were performed in collaboration with Julie Liu. I received technical advice from Paul Nowatzki regarding use of the AFM. I conducted the AFM work and wrote the chapter.

Chapter 3 demonstrates an application of the photoreactive aECM proteins using photolithographic techniques to generate protein patterns for directed cell attachment. Isaac Caricco, Sarah Heilshorn, and Marissa Mock originally developed this project, demonstrating the ability to pattern cells using another photoreactive aECM protein containing a CS5 binding domain. I developed a new photopatterning method to pattern RGD-based proteins and used these surfaces to create cellular arrays. I also demonstrated

that attachment to these protein patterns is sequence-specific to the RGD domain. Issac Caricco performed the mechanical characterization, and Christian Franck and Paul Nowatzki obtained the AFM data.

Chapter 4 focuses on the use of the proteomic technique BONCAT (bio-orthogonal noncanonical amino acid tagging), to identify newly synthesized proteins that are expressed when cells are deposited on aECM proteins and fibronectin. Jennifer Hodas and Daniela Dieterich designed and synthesized the disulfide tag (DST) used in the BONCAT experiments, and Jennifer Hodas developed the BONCAT DST protocol. Dr. Janek Szychowski synthesized the azidohomoalanine used in the experiments. I made modifications to the provided protocol in order to conduct cell spreading experiments, and all of the mass spectrometry work was performed at the Proteomic Exploration Laboratory at Caltech. I also conducted mRNA microarray analysis to quantify the extent of differential gene expression that results when cells are deposited on aECM proteins compared to fibronectin. Following mRNA extraction and purification, all microarray chips were prepped and scanned by the Millard and Muriel Jacobs Genetics and Genomics Laboratory at Caltech. I also performed cell spreading experiments on aECM proteins and fibronectin. Lastly, I analyzed the collected data and wrote the chapter.

Chapter 5 describes the tracking and quantification of cellular traction forces in three-dimensions using laser scanning confocal imaging and digital volume correlation. Time-lapse confocal imaging of migrating 3T3 fibroblasts on fibronectin (FN)-modified polyacrylamide gels of varying thickness revealed significant in-plane (x, y) and normal (z) displacements, demonstrating that cells exert forces in all three dimensions when exploring their extracellular environment. All of this work was performed in

collaboration with Christian Franck, an aeronautics graduate student in Professor Ravichandran's Laboratory at Caltech.

1.5 References

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