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Designer Protein-Based Scaffolds for Neural Tissue Engineering

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Abstract— **A key attribute missing from many current biomaterials is the ability to independently tune multiple biomaterial properties without simultaneously affecting other material parameters. Because cells are well known to respond to changes in the initial elastic modulus, degradation rate, and cell adhesivity of a biomaterial, it is critical to develop synthetic design strategies that allow decoupled tailoring of each individual parameter in order to systematically optimize cell-scaffold interactions. We present the development of a family of biomimetic scaffolds composed of chemically crosslinked, elastin-like proteins designed to support neural regeneration through a combination of cell adhesion and cell-induced degradation and remodeling. Through use of a modular protein-design strategy, a range of biomaterials is created that allows independent tuning over the initial elastic modulus, degradation rate, cell adhesivity, and neurite outgrowth. By combining these engineered proteins into composite structures, biomaterials are created with 3D patterns that emerge over time in response to cell-secreted enzymes. These dynamic 3D structures enable the delivery of multiple drugs with precise spatial and temporal resolution and also enable the design of biomaterials that adapt to changing scaffold needs.**

I. Introduction

The treatment of spinal cord injuries presents a particularly difficult challenge in medicine due to the inability of the central nervous system to spontaneously regenerate after injury [1]. Upon injury, native cells and proteins within the spinal cord create an inhibitory environment that leads to scarring instead of regeneration [2]. This is in direct contrast to the physiological response of the peripheral nervous system in which cells are rapidly recruited to clear debris from the injury site and to produce molecules that stimulate regeneration [3]. Despite the challenges presented by spinal cord nerves, they have been shown to partially regenerate with proper stimulation [4], and it is thought that a combinatorial approach involving scaffolds supplemented with cells, proteins, and growth factors that mimic the restorative environment of the peripheral nervous system will lead to success [5].

Before this combinatorial approach can be successful an appropriate material must be selected for construction of the scaffold. We propose assembling this scaffold using a three-dimensional porous matrix comprised of cross-linked, synthetic, protein polymers. With careful design this protein scaffold is biocompatible, is biologically active, has tunable mechanical properties, offers controlled biodegradation, and can be made using a controlled synthesis technique. Scaffolds such as these have been previously considered for applications such as small-diameter vascular grafts [6,7].

Specifically, the engineered synthetic proteins used in this application are composed of alternating structural and bioactive sequences. The structural sequences are similar to repeated sequences from elastin, a fibrous protein found in connective tissue that is known to provide both elasticity and resilience [6]. The bioactive sites consist of peptide sequences that have been previously shown to mimic actions performed by proteins in the extracellular matrix environment. Two types of bioactive sites that are included are cell binding domains and protease cleavage sites. Cell binding domains, such as the RGD domain of fibronectin, can offer the scaffold a capacity to bind growing neurons to its surface [8]. The inclusion of protease cleavage sites can allow the growing neurons, which secrete serine proteases from their axonal growth cones, to remodel and grow through the scaffold as they extend their neurites [9]. Currently we have demonstrated that the proteolytic degradation rate of these protein-based biomaterials can be tuned independently from the initial elastic modulus and the cell adhesivity [10]. Furthermore, we have demonstrated that these biomaterials are compatible with *in vitro* neuronal culture, and variations in RGD domain density are positively correlated with neuronal adhesion and neurite outgrowth [10].

II. Experimental Results

A. Protein Design

Protein-based materials often offer inherent biocompatibility, can be degraded into non-cytotoxic fragments, and possess similar properties to native tissue, greatly facilitating their use as engineered cell scaffolds. Furthermore, protein-polymers are synthesized using genetic templates, which allow for precise molecular-level control over polymer content and, therefore, degradation rate. The specific amino acid compositions of our engineered proteins represent a modular repeating structure consisting of alternating active and structural domains. The structural domains are based upon a well-known sequence borrowed from the protein elastin, that imparts properties of mechanical resilience and elasticity. The intermittent active domains contain short amino acid sequences previously selected through analysis of a four-amino acid peptide library to exhibit varying susceptibility to cleavage by the enzymes, tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) [11]. These enzymes are members of the serine protease family, are primarily produced *in vivo* by endothelial and neuronal cells, and have

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been linked to physiological roles in thrombolysis (blood clot dissolution) and extracellular matrix (ECM) degradation. For each enzyme, sequences were chosen to generate predicted fast degrading (t1, u1), medium degrading (t2, u2), and slow degrading (t3, u3) protein-polymers. An additional active domain, with a predicted limited vulnerability to both tPA and uPA cleavage, containing the well-known integrin binding RGD sequence from the natural ECM protein fibronectin, was also selected for synthesis. Prior to characterization, all protein-polymers were expressed in *Escherichia coli* bacteria and purified using a temperature cycling protocol [12,13]. The design of these engineered proteins includes cell adhesion sequences to enable neuronal attachment as well as sequences sensitive to cleavage by urokinase plasminogen activator (uPA), a protease locally secreted from the tips of growing neurites, to enable highly localized and tunable degradation properties. *In vitro* studies using the model PC-12 neuronal-like cell line show that the crosslinked proteins support tunable cell adhesion and neuronal differentiation. Increasing the density of RGD peptides present in the protein substrates leads to increased cell adhesion and more extensive neurite outgrowth. These engineered proteins offer the ability to independently tailor the mechanics, degradation properties, and cell adhesivity of scaffolds for the study of central nervous system regeneration.

B. Independent Tuning of Initial Elastic Modulus and Biodegradation Rate

Unlike hydrogel scaffolds that utilize peptide enzymatic cleavage to induce biodegradation, these protein-polymer scaffolds enable independent tuning of the initial mechanical properties and the subsequent biodegradation kinetics. These engineered proteins are produced using recombinant techniques and chemically crosslinked into highly swollen hydrogels with controllable mechanical properties. By altering the stoichiometric ratio of crosslinker present during the reaction, the extent of crosslinking and hence the initial elastic modulus of the scaffold can be tailored. Because these crosslinking sites are present within the elastin-like domains and do not involve the site for proteolytic cleavage, the initial extent of crosslinking is completely decoupled from the initial density of proteolytic cleavage sites. By altering the sequence of amino acids flanking the protease recognition site, the Michaelis-Menten kinetics of the degradation reaction can be tailored. For example, through a modest 3% change in the chemical identity of three otherwise identical engineered proteins, we can modify the protease substrate specificity resulting in tunable changes in protease degradation half-life over two orders of magnitude. Under constant uPA or tPA exposure, the designed scaffolds exhibit systematic variation of scaffold lifetime, from being fully degraded within a single day to showing no noticeable degradation within a full week. Blending of these proteins with three distinct cleavage rates enables the formation of an entire family of scaffolds with exquisite fine-tuning of biodegradation rate.

C. Dynamic Scaffolds with 3D Adaptive Patterns

By arranging polymers with widely different and controlled degradation rates within a single, composite hydrogel material, patterns can be enzymatically triggered to emerge over time in response to biologically relevant proteases. Additionaly, the material released during pattern formation can be modified to serve as a drug delivery vehicle. Here we demonstrate the synthesis and characterization of polymers with highly tunable degradation rates, the evolution of three-dimensional structures within composite polymeric materials, and the use of spatial and temporal patterns to deliver fluorochromic molecules with distinct delivery profiles.

D. Cell-Scaffold Interactions

In vitro studies using the model PC-12 neuronal-like cell line, primary rat neural progenitor cells, and primary rat striatum neurons indicate that crosslinked protein scaffolds containing the RGD domain from fibronectin support cell adhesion, neuronal differentiation, and neurite extension. In a 2D culture model, we demonstrate direct control over the cell adhesivity and neurite outgrowth by tuning the density of RGD peptides within the scaffold. In a 3D culture model, we demonstrate that cells survive the chemical crosslinking procedure with high viability to form a homogeneous distribution of cells throughout the scaffold. Confocal imaging demonstrates that these 3D culture scaffolds support neuronal differentiation and neurite extension.

III. CONCLUSION

The overall goal of this research is to use synthetic proteins to fabricate a regenerative scaffold that can be systematically tailored to obtain the optimal stimuli required for spinal cord nerve regeneration.

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