Acta Biomaterialia 9 (2013) 5590–5599

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Design of three-dimensional engineered protein hydrogels for tailored control of neurite growth

Kyle J. Lampe, Alexander L. Antaris, Sarah C. Heilshorn *

Materials Science and Engineering Department, Stanford University, Stanford, CA 94305, USA

ARTICLE INFO

Article history:
Received 4 June 2012
Received in revised form 7 October 2012
Accepted 26 October 2012
Available online 2 November 2012

Keywords:
Hydrogel
Neuron
Neurite
Stiffness
Three-dimensional

ABSTRACT

The design of bioactive materials allows tailored studies probing cell–biomaterial interactions, however, relatively few studies have examined the effects of ligand density and material stiffness on neurite growth in three-dimensions. Elastin-like proteins (ELPs) have been designed with modular bioactive and structural regions to enable the systematic characterization of design parameters within three-dimensional (3-D) materials. To promote neurite out-growth and better understand the effects of common biomaterial design parameters on neuronal cultures we here focused on the cell-adhesive ligand density and hydrogel stiffness as design variables for ELP hydrogels. With the inherent design freedom of engineered proteins these 3-D ELP hydrogels enabled decoupled investigations into the effects of biomechanics and biochemistry on neurite out-growth from dorsal root ganglia. Increasing the cell-adhesive RGD ligand density from 0 to 1.9×10^7 ligands μm^{-3} led to a significant increase in the rate, length, and density of neurite out-growth, as quantified by a high throughput algorithm developed for dense neurite analysis. An approximately two-fold improvement in total neurite out-growth was observed in materials with the higher ligand density at all time points up to 7 days. ELP hydrogels with initial elastic moduli of 0.5, 1.5, or 2.1 kPa and identical RGD ligand densities revealed that the most compliant materials led to the greatest out-growth, with some neurites extending over 1800 μm by day 7. Given the ability of ELP hydrogels to efficiently promote neurite out-growth within defined and tunable 3-D microenvironments these materials may be useful in developing therapeutic nerve guides and the further study of basic neuron–biomaterial interactions.

1. Introduction

Injuries to and diseases of the peripheral and central nervous systems lead to dysfunction and loss of neuronal tissue. The neurons responsible for signal transmission have limited regenerative potential to bridge large tissue defects and are rarely able to re-establish distant axonal connections [1,2]. Extension of long neurites, the collective term for neuronal processes of axons and dendrites, remains a challenge for both endogenous and transplanted neurons [3]. A multitude of biomaterials have been developed to promote neurite growth, including high water content hydrogels formed from both natural and synthetic sources [4]. Natural hydrogels such as collagen and fibrin gels successfully promote extensive neurite out-growth [5–8], but their material properties can vary depending on source location and species [9,10]. This prevents systematic control of the biochemical and biomechanical design parameters known to have an impact on neurite extension. In contrast, engineered material systems enable this design control, allowing investigators to dictate molecular structure, functional bioactivity, and resulting cell–material interactions [11–15].

Elastin-like proteins (ELPs) are produced by recombinant protein synthesis yielding engineered protein polymers made entirely of amino acids. This synthetic strategy dictates exact materials design from the molecule up, providing an orthogonal approach to traditional synthetic polymerization techniques for creating tunable bioactive matrices that can control cell function [16]. The high fidelity transcription and translation processes are a precise and accurate synthesis method resulting in biopolymers with exact specificity of the amino acid monomer sequence and no polydispersity. These proteins are designed by mimicking the useful functionalities found in native matrix proteins. The modular combination of multiple functional peptide sequences enables the rational design of entirely new proteins with specified biomaterial design parameters. In ELPs, repetitive short elastin-like structural sequences (Fig. 1) confer material elasticity and resilience without any known cell interaction domains [17]. Selected bioactive sequences can be interspersed between these elastin-like domains to enable specific cell adhesion interactions [18] and tailored rates of enzymatic degradation [19]. To date these ELPs have been used in cell culture for only two-dimensional (2-D) studies identifying

* Corresponding author. Tel.: +1 650 723 3763; fax: +1 650 498 5596.
E-mail address: heilshorn@stanford.edu (S.C. Heilshorn).

1742-7061/$ - see front matter © 2012 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.actbio.2012.10.033

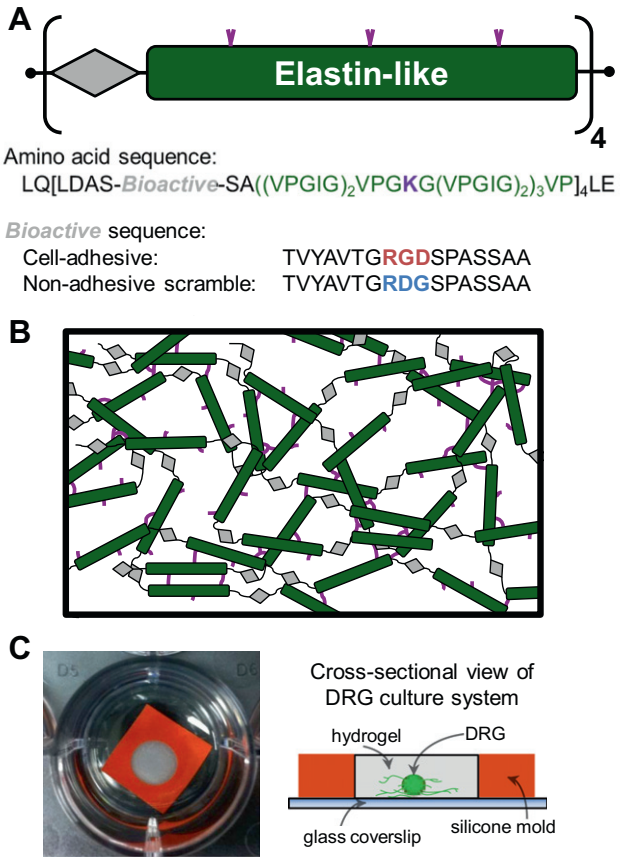

Fig. 1. Schematic of ELP design and hydrogel network. (A) ELPs were designed with modular repeats of bioactive (grey diamond) and elastin-like (green bar) sequences. The bioactive domains were either an extended RGD sequence to confer the engineered protein with cell adhesion ligands or a non-adhesive scrambled RDG sequence. (B) The elastin-like structural domains included lysines (purple) for site-specific crosslinking with primary amine-reactive crosslinkers yielding a 3-D hydrogel network. (C) Photograph (left) and schematic (right) of ELP crosslinked into an amorphous, semi-translucent hydrogel using a 5 mm silicone mold (orange) to encapsulate a single DRG cluster and culture the system within a 24-well plate.

the effect of cell-adhesive ligand density on neurite out-growth of PC12 cells, a model neuron-like cell line [20].

While traditional cell culture utilizes stiff, planar, 2-D substrates, recent results have highlighted the importance of dimensionality for neuronal culture, with three-dimensional (3-D) matrices critically affecting neural metabolic activity, growth, and phenotype [21,22]. Much has been learned regarding neural cell–cell and cell–material interactions by studying cells seeded on biomaterials in two dimensions [8,23–25] and in some cases allowing for migration into the material (termed 2.5-D) [26]. However, in two dimensions cells must reorganize their integrin cell surface receptors and cytoskeleton to adapt to the planar presentation of receptor ligands, leading to distinct dynamic and spatial differences in the distribution of cell–cell and cell–matrix interactions compared with three dimensions [27–29]. In a 3-D environment cells are no longer restricted to a polarized, planar geometry, as they sense and respond to stimuli from multiple directions with differentially distributed traction forces [30]. In the case of neurons dimensionality plays a major role in neurite extension, retraction, branching, and maturation into axons and dendrites. As a result, neurons cultured in 3-D versus 2-D environments display strikingly distinct morphologies, with 3-D cultures resulting in neuritic geometries more representative of those which occur in vivo [21]. These data strongly suggest that the development of biomaterials for neural regenerative applications requires 3-D analysis and, therefore, tunable materials that allow for cell–material interactions in all three dimensions.

Biomechanics and cell-adhesive ligand density are two critical regulators of neurite out-growth that can be tuned to create extracellular matrix (ECM)-like microenvironments. First, the stiffness of a supporting matrix is known to be a major factor determining the behavior of cultured neural cells [22–25,31]. Brain and spinal cord matter are some of the most compliant human tissues, and as such it has been hypothesized that compliant hydrogels are most well suited to induce neural regeneration [2]. In many cases significant improvements in neurite growth [22,24,25,31] and neuronal differentiation from neural stem cells (NSCs) [23,32] were reported for biomaterials with the lowest stiffness tested. Others have shown enhanced NSC proliferation and differentiation at intermediate stiffnesses that closely match the mechanical properties of native neural tissue [22,23]. In addition to stiffness, independent control of cell-adhesive ligand density is a well-known important design parameter controlling cell function [13,24,33]. The RGD ligand has been specifically incorporated into a number of biomaterials and tuned to examine location- and concentration-dependent effects on neurite length and branching [20,24,34–36]. Cells may respond to these biomechanical and biochemical cues in a context-dependent manner [24], thus having independent control of each parameter within the same biomaterial can enable systematic evaluation of cell–material interactions.

Here our goal was to design a family of ELP hydrogels to promote out-growth of neurites in a 3-D environment and to better understand the effects of biomaterial design parameters on the rate, length, and density of neurite growth. These amorphous 3-D matrices allowed viable encapsulation and extensive neurite growth from explanted chick dorsal root ganglia (DRG), a commonly studied tissue composed of neurons and glia found at the interface between the central and peripheral nervous systems. Individual neurites were surrounded by matrix and grew in multiple focal planes, thus demonstrating the three-dimensionality of the system. Through the use of an in-house-designed, automated image processing algorithm we show that the biomechanical and cell-adhesive biochemical parameters of ELP hydrogels both have profound effects on the neurite length distribution, longest neurite out-growth distance, and total out-growth. Tuning the cell-adhesive RGD density from 0 to 1.9×10^7 ligands μm^{-3} while maintaining a constant modulus of 1.5 kPa resulted in a roughly two-fold increase in total DRG neurite out-growth over 7 days in 3-D culture. In contrast, the tuning of initial elastic modulus from 0.5 to 2.1 kPa by adjusting the matrix crosslink density while maintaining a constant 1.9×10^7 RGD ligands μm^{-3} inhibited neurite out-growth across all time points tested. These results demonstrate the usefulness of ELP hydrogels to present systematically controlled and defined 3-D microenvironments to neuronal cultures, thereby allowing multi-functional and independent control over the cell-adhesive and biomechanical properties that influence cell behavior.

2. Materials and methods

2.1. Elastin-like polypeptide synthesis and purification

ELPs were designed and synthesized as previously reported with a cell-adhesive, fibronectin-derived extended RGD sequence or a non-adhesive RDG scrambled sequence (Fig. 1) [20]. Briefly, the encoding plasmid was transformed into the BL21 (DE3) strain of *Escherichia coli*. Cultures were grown to an OD_{600} of 0.8. Protein production was induced under the T7-lac promoter with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4–6 h. Cell pellets were harvested and lysed by sonication in TEN buffer (0.1 M NaCl,