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Physical and biological regulation of neuron regenerative growth and network formation on recombinant dragline silks

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ABSTRACT

Recombinant spider silks produced in transgenic goat milk were studied as cell culture matrices for neuronal growth. Major ampullate spidroin 1 (MaSp1) supported neuronal growth, axon extension and network connectivity, with cell morphology comparable to the gold standard poly-lysine. In addition, neurons growing on MaSp1 films had increased neural cell adhesion molecule (NCAM) expression at both mRNA and protein levels. The results indicate that MaSp1 films present useful surface charge and substrate stiffness to support the growth of primary rat cortical neurons. Moreover, a putative neuron-specific surface binding sequence GRGGL within MaSp1 may contribute to the biological regulation of neuron growth. These findings indicate that MaSp1 could regulate neuron growth through its physical and biological features. This dual regulation mode of MaSp1 could provide an alternative strategy for generating functional silk materials for neural tissue engineering.

1. Introduction

Central nervous system regeneration remains one of the most challenging tasks in regenerative medicine. Neural engineering strategies have been explored for aiding nerve regeneration, including implanted artificial extracellular matrixes to support neural cell grafts [1,2], nerve guidance conduits for promoting glial cell migration and axon outgrowth [3] and controlled delivery systems for growth factors and suppressing inhibitory scar formation [4]. These approaches provide control of the microenvironment of neural cells by presenting biophysical, topographical and biochemical cues to assist neuronal adhesion, extension and connectivity. To support these applications, it is necessary to have suitable biomaterials that promote optimal neuronal growth. Accordingly, a variety of biocompatible materials have been explored in primary neuronal cultures, including extracellular matrix proteins [5] and synthetic polymers [6]. However, there are limited options for neural-compatible materials due to the insufficient understanding of neuron–material interactions.

It is well-known that the physical properties of the matrix play an important role in neuronal growth. Extensive studies have shown that substrate stiffness can modulate axon outgrowth, as neuronal cells display a preference to a particular range of mechanical stiffness [7–10]. This effect is thought to interact with the contractile forces generated by the cytoskeletal components such as the microtubule and actin filaments of the axon during different stages of axonal outgrowth, including extension, retraction and elongation [8,11]. In addition, positive surface charge is necessary for neuronal surface adhesion to the substrate [12]. Surface coatings with positively charged poly-peptides such as poly-lysine are routinely used to facilitate neuronal adhesion to synthetic materials.

Specific cell-interacting motifs are necessary for cells to sense the surroundings and convert the information to modulate cellular behavior. Neurons have neural specific cell adhesion molecules (CAMs), such as NCAM, that are involved in mediating neuronal cell–cell adhesion [13]. As one of the first adhesion molecules extensively characterized [14]. NCAM has been shown to mediate cell–cell binding by homophilic interactions [15] as well as interacting with a variety of other cellular proteins such as receptors [16], membrane–cytoskeletal components [17] and the cellular prion protein (PrP) [18]. NCAM-mediated cell adhesion is found to be a critical step for triggering signaling events that lead to neurite outgrowth [19].

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Considering these various needs for neuronal growth, a useful biomaterial for neural engineering should provide suitable surface binding cues as well as appropriate physical properties. Traditional cell culture matrices include chemically synthesized polymers (e.g. poly-lysine, poly-ethylene glycol, poly-lactic acid) as well as naturally extracted extracellular matrix proteins from mammalian organs and tissues (e.g. collagen, fibronectin, laminin, Matrigel™). While synthetic polymers may possess higher versatility in material fabrication for specific needs, they usually lack biologically active sites to communicate with cells and assist their growth, and thus do not mimic the *in vivo* environment very well. On the other hand, naturally extracted extracellular matrix proteins are usually bioactive and have positive influences on cell growth. However, they suffer from batch-to-batch variations and generally have more undefined components due to the lack of standardized extraction protocols [20,21]. There is a need for new sources of matrices for tissue engineering that could overcome both the limitations of synthetic and naturally extracted materials. Recently, the excellent material properties of silk proteins originated from silkworms and spiders, have drawn increased attention from tissue engineers to investigate their potential as biomaterials for tissue regeneration [22,23].

Silk fibroins are attractive biomaterials due to their tunable mechanical properties and biocompatibility. Using silkworm (*Bombyx mori*) silk-based biomaterials, we have previously identified the optimal ranges of physical properties, such as surface charge and mechanical stiffness, to support the growth of primary rat cortical neurons [9,10]. Recombinant silk–elastin proteins have also been shown to support neuronal growth when combined in appropriate ratios [12]. Furthermore, spider silk has been used to repair nerve defects [24] and a recombinant spider silk mimic supported the growth of neuron stem cells in both 2D and 3D cultures [22].

Spider silks represent a unique group of fibrous proteins that contain multiple basic protein motifs which control various structural and functional aspects of the protein. Typical orb-weaving spider produces seven types of silks [25]. Among them, dragline silk, the lifeline of spider, displays the most extraordinary mechanical properties with both high tensile strength and elasticity, it is known as one of the toughest materials by weight in the world. Despite being an ancient material, the molecular nature of dragline silk has only started to be unraveled over the last two decades [25,26]. Previous studies have identified that dragline silk is comprised by two proteins: major ampullate spidroin 1 (MaSp1) and major ampullate spidroin 2 (MaSp2) [27,28]. MaSp1 contains two distinct motifs: a poly-alanine region that forms tightly packed beta-sheet crystalline that contribute to the tensile strength of dragline silk; a GGX motif (X = L, Y, Q, A), which forms glycine II helix to connects crystalline regions in an amorphous matrix and may provide elasticity. MaSp2 also contains poly-alanine regions, but is distinct from MaSp1 due to a GPGXX motif (X = G, Q, Y) which forms beta-spirals and is responsible for dragline silk's high elasticity [29]. In the native spider silk sequence, these motifs were repeated hundreds of times to form proteins with molecular sizes of more than 300 kDa [25]. Besides the main repetitive sequences, spider silk also contains a highly conserved terminal domain [30,31] which likely plays a role in protein aggregation and fiber formation [32] and to control protein solubility when the secreted protein is stored in the silk gland at high concentrations [33].

In order to investigate the mechanisms behind the interaction between neuron cells and the silk matrices, and potentially expand the candidates pool of silk matrices for neuron culture, recombinant MaSp1 and MaSp2 proteins based on *Nephila clavipes* dragline silks were produced in this study. The two silk proteins were studied for their ability to support the growth of rat cortical neurons in comparison to poly-L-lysine as well as to *B. mori* silk coatings that have been routinely used in our lab for neuronal growth.

2. Materials and methods

2.1. Recombinant spider silk protein expression and purification

MaSp1 and MaSp2 were purified and analyzed according to published procedures by Tucker et al. [34]. Briefly, goat milk was collected and defatted before pumping through a tangential flow filtration system with 750 KDa and 50 KDa membrane to obtain clarified and concentrated solution with recombinant spider silks. The spider silk proteins were precipitated by ammonium sulfate from remaining milk proteins, washed with dH_2O and lyophilized. Protein purity was tested by Western blots using αM5 as primary antibody and AP conjugated anti-rabbit antibody as secondary antibody.

2.2. Regenerated silkworm silk preparation

The procedure to prepare lyophilized silkworm silk from *B. mori* cocoons was previously described [35]. Briefly, cocoons were degummed by boiling 60 min in Na_2CO_3 solution (20 mM) to remove sericin. Silk fibroin was dissolved in LiBr solution (9.3 M) at 60 °C for a final concentration of 20 wt%. This solution was dialyzed against water using Slide-a-Lyzer dialysis cassettes (Pierce, MWCO 3500) for 72 h. The aqueous silk solution was lyophilized to obtain dried silk fibroin.

2.3. Peptide synthesis

Peptide (GRGGLAAAGRGGLAAAGRGGLGY) carrying the putative NCAM binding sequence GRGGL was synthesized by FMOC chemistry at the Tufts core facility. Half of the peptide was labeled by FITC-AHA (fluorescein-5-aminohexylacrylamide) at the N-terminus for neuron surface coatings. All peptides were purified to 95% pure by HPLC and molecular weight confirmed by MALDI-TOF mass spectrometry.

2.4. Silk film preparation

Lyophilized silks (MaSp1, MaSp2 and silkworm silk) were dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) to prepare a 2 wt% solution, For cell culture, 150 μL of solution were applied to each well in 24-well tissue culture plates and dried completely in a laminar flow hood. For dynamic mechanical analysis, silk films were cast on polydimethylsiloxane (PDMS) molds instead of tissue culture plastic for easy peeling. Silk films were annealed by submerging the samples in 90% methanol for 30 min and washing with ethanol followed by Dulbecco's phosphate buffered saline (DPBS) three times and the allowed to dry completely.

2.5. Primary cortical neuronal culture

Primary cortical neurons from embryonic day 18 (E18) Sprague Dawley rats (Charles River, Wilmington, MA, USA) were plated on 24-well plates with different silk substrates described previously. The brain tissue isolation protocol was approved by Tufts University Institutional Animal Care and Use Committee and complies with the NIH Guide for the Care and Use of Laboratory Animals (IACUC # B2011-45). Control wells were coated with 1 mg/mL poly-L-lysine (M_r = 75,000–150,000D, Sigma–Aldrich, St. Louis, MO, USA) according to Sigma's procedure. For synthetic GRGGL peptide coatings, the peptide solution with varied concentrations were added to each well and incubated in room temperature overnight. The solution was removed by aspiration and plates were thoroughly rinsed by DPBS before cell seeding. Cells were plated at a density of 250,000 cells per well (125,000 cells/cm^2) and cultured in NeuroBasal media (Invitrogen, Carlsbad, CA, USA) supplemented with B-27 neural supplement, penicillin/streptomycin (100 U/ml and 100 μg/mL), and GlutaMax™ (2 mM) (Invitrogen). Cells were cultured in an incubator (Forma Scientific, Marietta, OH, USA) with 37 °C, 100% humidity and 5% CO_2 for up to 7 days *in vitro* (DIV).

2.6. Cell viability assay and image analysis

Cell viability was assessed using the LIVE/DEAD Viability Assay Kit (Invitrogen). Briefly, culture media was replaced with Dulbecco's phosphate-buffered saline (DPBS; Invitrogen) containing calcein AM (4 μM) and ethidium homodimer-1 (2 μM). After a 30 min incubation at 37 °C, the cells were changed into fresh culture media, and viewed under a fluorescence microscope (Leica DM IL; Leica Microsystems, Wetzlar, Germany) equipped with a digital camera (Leica DFC340 FX).

Fluorescence images were acquired using excitation at 470 ± 20 nm and emission at 525 ± 25 nm for live cells, and excitation at 560 ± 20 nm and emission at 645 ± 40 nm for dead cells, respectively. Images were analyzed using NIH ImageJ software. Thresholds for positive live staining and positive dead staining were selected for each image. Particles of positive staining with sizes in-between 10–50 μm were counted as individual cells.

To quantify neuronal growth, a custom-made Image J plugin based on NeurphologyJ [36] was used. Cell soma and neurites were separately identified based on their different intensity thresholds, and simultaneously measured for their numbers and morphological features.