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Variations in rigidity and ligand density influence neuronal response in methylcellulose–laminin hydrogels

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ABSTRACT

Cells are continuously sensing their physical and chemical environment, generating dynamic interactions with the surrounding microenvironment and neighboring cells. Specific to neurons, neurite outgrowth is influenced by many factors, including the mechanical properties and adhesive signals of the growth substrata. In designing biomaterials for neural regeneration, it is important to understand the influence of substrate material, rigidity and bioadhesion on neurite outgrowth. To this end, we developed and characterized a tunable 3-D methylcellulose (MC) hydrogel polymeric system tethered to laminin-1 (MC-x-LN) across a range of substrate rigidities (G^* range = 50–565 Pa) and laminin densities. Viability and neurite outgrowth of primary cortical neurons plated within 3-D MC hydrogels were used as cell outcome measures. After 4 days in culture, neuronal viability was significantly augmented with increasing rigidity for MC-x-LN as compared to control non-bioactive MC; however, neurite outgrowth was only observed in MC hydrogels with complex moduli of 565 Pa. Varying LN while maintaining a constant MC formulation (G^* = 565 Pa) revealed a threshold response for neuronal viability, whereas a direct dose-dependent response to LN density was observed for neurite outgrowth. Collectively, these data demonstrate the synergistic play between material compliance and bioactive ligand concentrations within MC hydrogels. Such results can be used to better understand the adhesive and mechanical factors that mediate neuronal response to MC-based, tissue-engineered materials.

1. Introduction

Multiple factors influence cellular adhesion and migration on natural or synthetic supports, including the physical properties and density of adhesive ligands. Non-fouling materials (e.g. polyethylene glycol, dextran, methylcellulose hydrogels) provide a backbone to link specific biological motifs to control cellular adhesion, migration and infiltration [1–4]. With many of these polymeric substrates, control over substrate rigidity is possible through modifications of the polymeric matrix properties. Consequently, the relationship of matrix structure and substrate rigidity to cell adhesion, migration and spreading has been studied in a variety of cellular systems [5–7]. Of particular interest are observations that cell behavior on similar materials varies based on cell type. For instance, fibroblasts spread and migrate on more rigid substrates [8,9], whereas among neuronal phenotypes, more compliant substrates have been shown to support longer neurite outgrowth compared to more rigid materials [10–13]. In addition to material properties, specific combinations of ligand modality and density are essential for successful cell spreading, migration and neurite outgrowth [6,7,14–16].

A number of in vitro neurite outgrowth studies have utilized polymeric hydrogel systems to assess neurite outgrowth in 3-D as the matrix structure and mechanical properties are unique to each specific material [1,4,10,15]. Non-fouling polymers provide structural support while enabling covalent coupling of bioadhesive cues to generate designer 3-D scaffolds. Methylcellulose (MC) is one such non-fouling hydrogel system in which the mechanical properties can be tuned to a range of rigidities by modifying the solution concentration, ionic strength and/or molecular weight. Moreover, the thermoresponsive property of MC is appealing for neural tissue-engineering applications, particularly for situations where the implanted material needs to conform to irregularly shaped lesions/defects (e.g. traumatic spinal cord and brain injuries) [17–20]. We previously reported successful functionalization of MC with the extracellular matrix protein (ECM) laminin-1 (LN) via two different conjugation schemes [1,18]. Due to the ease of manipulating mechanical properties, biofunctionalization and the potential for use in neural tissue engineering, we chose to evaluate

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the neuronal response to alterations in mechanical and ECM tethering densities within MC hydrogels.

In vivo, cellular behavior is directed through numerous interactions a cell encounters with its external environment (i.e. cell–cell, ECM, growth factors, cytokines, etc.). LN is of particular interest for neuronal behaviors as this 800 kDa heterotrimeric protein stimulates cell-signaling pathways for adhesion, neuritogenesis and survival (see Ref. [21] for review). Additionally, LN and LN-derived active moieties have been incorporated within polymeric matrices to study neurite outgrowth [10,11,22,23]. However, questions still remain on how substrate material, rigidity and incorporation/density of bioactive moieties such as LN in a 3-D MC matrix may interact to affect neurite outgrowth.

In this study we evaluated the neuronal response to variations in both MC matrix compliance and LN ligand density. Specifically, we focused on investigating the relationship between viscoelastic properties of MC and the tethering density of LN in relation to neuronal viability and neurite outgrowth. The knowledge gained from this study may be applied toward developing design criteria for MC-based neural tissue-engineered constructs capable of eliciting and supporting neurite outgrowth.

2. Methods

2.1. Methylcellulose–laminin tethering scheme

MC ($M_w \sim 38$ and 40 kDa; Sigma–Aldrich, St. Louis, MO) hydrogels were prepared in Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen, Carlsbad, CA) according to a dispersion technique previously reported [19]. Tethering of LN (Invitrogen, Carlsbad, CA) to MC was accomplished via the photosensitive conjugation reagent *N*-sulfosuccinimidyl-6-[4′-azido-2′-nitrophenylamino] hexanoate (sulfo-SANPAH; Pierce Biotechnology, Inc., Rockford, IL). Briefly, LN (200 μg ml^{-1}; Invitrogen) was incubated with a 0.5 mg ml^{-1} sulfo-SANPAH solution in the absence of light for 2.5 h. Residual unreacted sulfo-SANPAH was removed with microcentrifuge filters (NanoSep, 50000 MWCO; Pall Corporation, Port Washington, NY). LN-SANPAH (200 μg ml^{-1}) was reconstituted and thoroughly mixed on ice with MC. A thin layer of the MC+LN-SANPAH mixture was then cast onto a glass slide and exposed to UV light for 4 min (100 W, 365 nm; BP-100AP lamp, UVP, Upland, CA) to initiate the photoconjugation reaction. Upon completion of the photoconjugation reaction, unbound LN was rinsed away using D-PBS supplemented with 0.1% Tween-20 followed by three rinses with D-PBS. MC tethered to LN is referred to as MC-x-LN; unmodified MC is referred to as MC. For cell culture assays, we used MC tethered to bovine serum albumin (BSA, MC-x-BSA; Sigma) to control for any effects of the conjugation procedures. MC-x-BSA was generated according to MC-x-LN procedure, in which the molar equivalent amount of BSA was substituted for LN. Conjugation of LN to MC was quantified via a dot-blot immunoassay [18].

2.2. Dynamic rheological characterization

Rheological analyses were performed on a Bohlin CVO rheometer (Bohlin, East Brunswick, NJ) with a parallel plate configuration (0.5 mm thick and 12 mm in diameter). To examine the mechanical integrity of the MC hydrogels at physiological temperatures, a frequency sweep from 0.05 to 5 Hz was performed on each sample group (n = 6–8 per group) under constant low-amplitude oscillatory shear strain (0.5%) upon equilibration to 37 °C. We evaluated MC samples of varying polymer M_w (38 and 40 kDa), MC concentration in DPBS (6.75, 7.8 and 9.0%), MC at various stages of the tethering scheme (UV-irradiated MC and MC-x-BSA), and varied protein tethering densities (20, 200 and 400 μg ml^{-1}). The complex moduli over a 0.05–5 Hz frequency sweep were compared to determine differences in rigidity across groups.

2.3. Cortical neuron isolation and dissociation

Cortices from embryonic day 18 (E18) Sasco Sprague–Dawley rats (Charles River, Wilmington, MA) were dissected and dissociated according to protocols approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee. This well-established protocol was modified from the Brewer laboratory [24] and optimized for cortical neuron cultures. Briefly, cortices were extracted from E18 fetuses, rinsed twice with Hank's balanced salt solution (HBSS; Invitrogen), and placed in a hibernation solution consisting of L-15 supplemented with 2% B-27 (Invitrogen). Cortices were then stored at 4 °C until dissociation and plating (maximum of 7 days post-dissection). Dissociation of the cortical tissue began with rinsing the tissue three times with cold HBSS, followed by incubation in trypsin (0.25%) + EDTA (1 mM; Invitrogen) at 37 °C for 10 min. Following removal of trypsin, cortices were rinsed twice with HBSS then placed in HBSS supplemented with DNase (0.15 mg ml^{-1}; Sigma) and vortexed for 30 s. Remaining tissue fragments were removed and the resulting cell suspension was centrifuged at 180*g* for 3 min. The cell pellet was resuspended in Neurobasal medium supplemented with 2% B-27 and 0.5 mM L-glutamine (neuronal medium; Invitrogen) and placed on ice until the cells were plated. Cells were plated within 30 min of dissociation with no reduction in viability, as measured with a standard trypan blue exclusion viability assay.

2.3.1. 3-D neuronal cultures within MC

Dissociated primary cortical neurons were mixed on ice with MC to achieve the desired MC formulation and a plating density of 3.5×10^6 cells ml^{-1}. After thoroughly mixing the cell–MC solution, the solution was transferred into custom microwell chambers to attain a 300 μm thick hydrogel (150 μl volume). The plated cell–MC solution was allowed to gel at 37 °C for 45 min in a tissue culture incubator (37 °C, 5% CO_2 and 95% relative humidity). Upon gelation, neuronal medium was added on top of the hydrogel. Experimental groups consisted of varying MC formulations and LN tethering densities (n = 4–7 trials in triplicate). Additional, control cultures were also plated on poly-L-lysine-coated polystyrene to control for potential variations in viability between dissection/dissociation procedures. All cultures were maintained in a tissue culture incubator with neuronal medium exchanges every other day until the experimental endpoint.

2.4. Viability and neurite outgrowth

At 4 days post-plating, viability was assessed with calcein AM (Invitrogen) and ethidium homodimer-1 (EthD-1; Sigma). Briefly, cultures were rinsed with PBS and then incubated with PBS containing 2 μM calcein AM and 4 μM EthD-1 for 30 min. Upon removal of the calcein AM/EthD-1 solution and subsequent rinsing, cellular viability was examined with a confocal laser scanning microscope (LSM 510; Zeiss, Thornwood, NY). Three random 100 μm thick z-stacks for each construct were captured and analyzed with LSM Image Browser (Zeiss) to record viability and neurite outgrowth. Each confocal z-stack was projected into a 2-D image to facilitate cell counting for cell viability quantification; the total number of calcein AM cells was divided by the total number of calcein AM and EthD-1 cells. The cell density within each 3-D culture was calculated from total cell count data (i.e. calcein AM+EthD-1 cells) divided by the confocal z-stack volume. Viability data were normalized to MC-x-BSA controls. From each 2-D