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Aligned neurite outgrowth and directed cell migration in self-assembled monodomain gels

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ABSTRACT

Regeneration of neural tissues will require regrowth of axons lost due to trauma or degeneration to reestablish neuronal connectivity. One approach toward this goal is to provide directional cues to neurons that can promote and guide neurite growth. Our laboratory previously reported the formation of aligned monodomain gels of peptide amphiphile (PA) nanofibers over macroscopic length scales. In this work, we modified these aligned scaffolds specifically to support neural cell growth and function. This was achieved by displaying extracellular matrix (ECM) derived bioactive peptide epitopes on the surface of aligned nanofibers of the monodomain gel. Presentation of IKVAV or RGDS epitopes enhanced the growth of neurites from neurons encapsulated in the scaffold, while the alignment guided these neurites along the direction of the nanofibers. After two weeks of culture in the scaffold, neurons displayed spontaneous electrical activity and established synaptic connections. Scaffolds encapsulating neural progenitor cells were formed *in situ* within the spinal cord and resulted in the growth of oriented processes *in vivo*. Moreover, dorsal root ganglion (DRG) cells demonstrated extensive migration inside the scaffold, with the direction of their movement guided by fiber orientation. The bioactive and macroscopically aligned scaffold investigated here and similar variants can potentially be tailored for use in neural tissue regeneration.

1. Introduction

Many nervous system disorders involve damage or degeneration of axon tracts [1]. Structurally, these tracts are composed of highly ordered and aligned bundles of axons, which are present not only in nerves and the spinal cord, but also throughout the brain, forming complex networks of connectivity. Though damaged axon tracts in the central nervous system fail to recover spontaneously, chronically injured axons retain their regenerative capacity for a prolonged period [2]. Attempts to promote the growth of injured axons across spinal cord lesions have resulted in partial recovery of motor and sensory functions [3–5]. Despite these promising demonstrations in animal models, translation into effective therapies remains elusive and advancements in technologies for axonal regeneration are required.

This need has led to significant interest in the development of scaffolds capable of promoting highly aligned, dense axon growth to repair damaged or degenerated axon tracts [6–11]. One approach is to introduce alignment of fibers in the scaffold, since neurite growth cones are capable of following topographical features in a process called contact guidance [12,13]. Aligned features have been incorporated into scaffolds using a variety of methods, including electrospinning [7,8], directional freezing and drying [11], magnetic fields [6], processed tissue [9], and oriented filamentous viruses [10]. Frequently, the materials used in these constructs are biologically inert polymers, requiring subsequent incorporation of adhesion-promoting proteins or peptides to promote cell attachment and growth. Reported procedures for aligning fibers, such as

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the application of high voltage or directional freezing, are not compatible with maintaining cell viability, making it difficult or impossible to encapsulate cells within the fiber network of the scaffold during the alignment process. Several studies have shown improved functional recovery after cell transplantation in animal models of spinal cord injury [14,15]. The inability to encapsulate cells in aligned scaffolds with current technologies prevents the exploitation of potential benefits from combining cell transplantation with directional cues from an aligned system.

Peptide-based self-assembled materials have emerged as attractive candidates for regenerative medicine, possessing a number of desirable characteristics, such as the facile incorporation of bioactive epitopes and their inherent biocompatibility [16]. Peptide amphiphiles, composed of an unbranched alkyl chain linked to a peptide segment, can be designed to self-assemble into high aspect ratio nanofibers capable of gelation at low concentrations upon screening of the charged amino acids in physiological solutions [17]. Our group has extensively studied PAs as a platform for applications that include bone, cartilage, enamel, nervous tissue regeneration, angiogenesis for ischemic disease, drug delivery, and cancer therapeutics [5,18–23]. Recently, we have reported a method to create monodomain gels composed of highly aligned bundles of nanofibers [24]. Thermal treatment of isotropic PA solutions induces the formation of large plaque-like structures at elevated temperatures, which upon cooling break apart into bundles of multiple PA nanofibers. The aqueous solutions obtained through this pathway are lyotropic liquid crystals with birefringent microdomains of aligned bundles. When these liquid crystalline solutions are pipetted into $CaCl_2$-containing physiological saline, they form string-like gels that remarkably have single monodomain orientation of the bundles over macroscopic distances. Furthermore, cells suspended in the liquid crystalline solution prior to gel formation can be encapsulated within bundles of aligned nanofibers throughout the macroscopic gel. Previous work in our laboratory has demonstrated the capability of using ECM-derived peptide epitopes on PA nanofibers such as RGDS and IKVAV to elicit desired cellular responses [25,26]. For example, PAs displaying the laminin-derived peptide IKVAV induced differentiation of neural progenitor cells into neurons [25], enhanced neuronal viability [27] and promoted neurite growth [28]. When delivered to an acutely injured mouse spinal cord, IKVAV PA gels reduced gliosis and induced axonal regeneration across the lesion, leading to functional motor recovery [5].

We report here the design of a bioactive aligned scaffold using co-assembly of epitope-bearing and unfunctionalized PA molecules with the aim of creating a material with several features critical for neuroregenerative applications. The effects of IKVAV and RGDS epitope presentation and fiber alignment on neurite outgrowth were explored on a variety of encapsulated neuronal cell types. We examined the scaffold's ability to support neuronal action potential generation and synapse formation and explored the possibility of cell transplantation in the spinal cord using the aligned scaffold. Additionally, we investigated the potential of these scaffolds to control the directional migration of cells.

2. Materials and methods

2.1. PA synthesis

PAs were prepared by standard fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis, either manually or on an automated peptide synthesizer (Applied Biosystems 433A or CEM Discover System). Rink resin (Novabiochem) was used to make the base, RGDS, and RSDG PAs. Pre-derivatized Wang resins (Novabiochem) were used to make the IKVAV and VVIAK PAs. Fmoc-protected amino acids were purchased from Anaspec Inc. After synthesis of the peptide segment, a palmitic acid was added to the N-terminus. The PAs were cleaved from the resin and deprotected using a solution of 95% trifluoroacetic acid (TFA), 2.5% triisopropyl silane (TIS) and 2.5% H_2O for 3 h. The cleavage solution was drained into a round bottom flask and the resin was rinsed several times with DCM. All liquid was removed using rotary evaporation, and the PA residue was washed with cold diethyl ether and poured into a fritted filter. After the diethyl ether passed through the filter, the PA flakes were rinsed again with diethyl ether, allowed to dry.

For purification, the PAs were dissolved in water with 0.5 M ammonium hydroxide, adjusting the pH to 9–10 with additional ammonium hydroxide as needed. The solution was passed through a 0.22 μm filter and purified on a Phenomenex C18 Gemini NX column by prep scale reverse phase HPLC running a mobile phase gradient of 98% H_2O and either 2% acetonitrile (HPLC grade, Mallinckrodt) or methanol (HPLC grade, Mallinckrodt) to 100% acetonitrile or methanol, respectively. 0.1% NH_4OH was added to all mobile phases to aid PA solubility. HPLC fractions were checked for the correct compound using electrospray ionization mass spectroscopy (ESI-MS), rotary evaporated to remove acetonitrile and methanol, and lyophilized (Labconco, FreezeZone6) at a pressure 0.03 torr.

2.2. Transmission electron microscopy (TEM)

2.2.1. TEM of PAs

Samples for conventional TEM microscopy were prepared from 0.1 wt.% PA solution dissolved in a salt solution (150 mM NaCl and 3 mM KCl) and adjusted to a pH of 7.2–7.4 by adding NaOH. In mixed PA systems, solutions of the PAs were mixed and bath sonicated for 15 min. The solutions were then heated to 80 °C in a water bath for 30 min, and then slowly cooled to room temperature. 5 μL of a 0.1 wt.% PA solution was pipetted onto a carbon film grid (Electron Microscopy Sciences) for 1 min. The samples were then negatively stained by pipetting 5 μL of a 2 wt.% uranyl acetate solution two times and allowed to dry. Samples were imaged using a FEI Spirit TEM.

2.2.2. TEM of resin-embedded gels

Aligned scaffolds with cells encapsulated were fixed in modified Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) by overnight incubation. Samples were post-fixed in osmium tetroxide and stained en-bloc by uranyl acetate solution. Stained samples were dehydrated by incubation in a graded series of ethanol. Following dehydration, ethanol was replaced with propylene oxide, and finally the samples were embedded in resin (Embed 812, Electron Microscopy Sciences) through intermediate exchange steps. 70 nm sections of resin-embedded scaffold were prepared (sectioned parallel to the direction of scaffold long-axis) using a Leica Ultracut UCT ultramicrotome. Sections were post stained with lead citrate and uranyl acetate for enhanced contrast and micrographs were obtained on Tecnai Spirit G2 microscope (FEI) operating at 120 kV.

2.3. Scanning electron microscopy (SEM)

Aligned PA scaffolds were fixed with 2.5% glutaraldehyde in PBS (1 h, RT), followed by dehydration in a graded series of ethanol concentration. Finally, ethanol was removed from the sample using a critical point dryer (Tousimis Samdri-795) in order to minimize the loss of structural details. Dried scaffolds were adhered to carbon conductive tabs (Ted Pella Inc.) and were coated with a thin film (14 nm) of osmium metal using an osmium plasma coater (Filgen, OPC-60A). Images were acquired using a Hitachi S-4800 Field Emission Scanning Electron Microscope operating at an accelerating voltage of 5 kV.

2.4. P19 cell culture

P19 embryonal carcinoma cells were cultured similarly as MacPherson et al. [29]. P19 cells were cultured in media composed of α-MEM (Gibco), 7.5% newborn calf serum (Lonza), 2.5% fetal bovine serum (Gibco), penicillin (100 units/mL) and streptomycin (100 μg/mL) (Invitrogen). Neuronal differentiation was induced by plating P19 cells in non-treated Petri dishes in media containing 5 μM retinoic acid (Sigma) for 4 days. Neurospheres were collected from the Petri dish and allowed to settle in a centrifuge tube for 10 min. Media was removed, then trypsin/EDTA solution was added and the tube was gently agitated for 5 min. Cells were dissociated by triturating the neurospheres, and then media was added to inactivate the trypsin. Cells were centrifuged and the pellet resuspended in media to a concentration of 25,000 cells/μL. Cells were mixed (1:4) with PA solution. The PA/cell solution was pipetted through a gelling solution (150 mM NaCl, 3 mM KCl, 25 mM $CaCl_2$) for 1 min to induce gelation, then the gelling solution was removed and media was added to the dish. Cells encapsulated in the scaffolds were cultured for 2 days.

2.5. Neuron culture

Cells for primary cultures of hippocampus, dorsal root ganglion (DRG) and neural progenitor cells (NPCs) were obtained from CD1 mouse embryo. Dissection and animal handling were carried out in accordance with Public Health Service Policy on Humane Care and Use of Laboratory Animals and in compliance with protocol approved by Institutional Animal Care and Use Committee. Unless specifically mentioned, all saline solutions and culture medium supplements were