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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.

Keywords

self assembly; peptide amphiphile; neurite growth; anisotropic gel; alignment

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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 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 VVIK 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% H₂O for three hours. 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 μ m filter and purified on a Phenomenex C18 Gemini NX column by prep scale reverse phase HPLC running a mobile phase gradient of 98% H₂O and either 2% acetonitrile (HPLC grade, Mallinckrodt) or methanol (HPLC grade, Mallinckrodt) to 100% acetonitrile or methanol, respectively. 0.1% NH₄OH 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 minutes. The solutions were then heated to 80 °C in a water bath for 30 minutes, and then slowly cooled to room temperature. 5 μ L of a 0.1 wt.% PA solution was pipetted onto a carbon film grid (Electron Microscopy Sciences) for 1 minute. The samples were then negatively stained by pipetting 5 μ L 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 hour, 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 μ g/mL) (Invitrogen). Neuronal differentiation was induced by plating P19 cells in non-treated Petri dishes in media containing 5 μ M retinoic acid (Sigma) for 4 days. Neurospheres were collected from the Petri dish and allowed to settle in a centrifuge tube for 10 minutes. Media was removed, then trypsin/EDTA solution was added and the tube was gently agitated for 5 minutes. 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/ μ L. 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 minute 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 obtained from Gibco Life Technology, while reagents and enzymes were obtained from Sigma.

2.5.1. Hippocampal culture—Dissociated hippocampal cultures were prepared from 17 days old mouse embryos following methods described before with some modifications[30]. Briefly, embryos from timed pregnant mouse were taken out using aseptic technique, and the hippocampi from brain were dissected out under ice cold $\text{Ca}^{++}/\text{Mg}^{++}$ -free Hank's Balanced Salt Solution (HBSS). Collected brain tissue was digested by trypsin (0.25%, 20 min at 37°C), and after the digestion was complete, trypsin was neutralized by FBS and Mg^{++} containing media (DMEM, 5% FBS, 1.2 mM MgCl_2 ; solution also contained DNase to breakdown DNA). Trypsin digested tissues were then broken down to single cells by mechanical trituration using fire-polished Pasteur pipettes of decreasing tip diameter, and suspended in medium containing DMEM F-12 and 10% inactivated fetal bovine serum

(FBS). Cells were centrifuged to pellet, re-suspended in serum free culture media (composition as described below) and mixed with PA solution at volume ratio 1:4. Final cell concentration after mixing with PA was 10,000 cells/ μ L and 5,000 cells/ μ L for electrophysiology and immunostaining experiments respectively. Aligned gels with encapsulated hippocampal cells were prepared from the PA cell suspension as described before. Cell nutrition was provided by a serum free neurobasal medium based culture media (containing 2% B27 supplement, 0.5 mM Glutamax™ and 0.5% P/S), and the cultures were maintained in the incubator at 37°C and 5% CO₂. For long-term culture, half of the media was replaced on 14th day and every 7th day thereafter.

2.5.2. Dorsal root ganglion (DRG) culture—Dorsal root ganglions were isolated from mouse embryo (17 days) following method described by Burkey et al.[31], with minor modifications. Harvested DRGs were kept in ice cold Ca⁺⁺/Mg⁺⁺-free HBSS until use. For encapsulation experiments, 2-3 intact DRGs were suspended in 7 μ L of PA solution and pipetted in a 10 μ L pipettor such that each DRG remained well separated from the next inside the pipette tip. Such manipulation allows the DRGs to be separated by several millimeters in the self-assembled gel (aligned and unaligned gels were made as described before). To restrict the free movement of PA scaffold in the culture medium, the scaffold was further embedded in a block of collagen gel (3 mg/mL, rat tail; BD Bioscience) in the center well of glass bottom Petri dish (MatTek corporation). 2 mL of culture medium (DMEM/F12 supplemented with 10% FBS, 2 mM Glutamax, 1% P/S) containing 100 nM nerve growth factor (Life technologies) was added on top of the collagen gel block to support the nutrition and growth of the DRG cells. To assess the migration, the culture was taken out of the incubator at specified time point, and images were acquired in an inverted microscope (Nikon Eclipse TE2000U) under phase contrast optics.

2.5.3. NPC culture—NPCs were cultured as previously described in [32, 33]. Briefly, ventricular zone tissue was dissected out from E13 mouse embryos, were mechanically and enzymatically dissociated into single cells, and plated on non-tissue-culture-treated T75 flask in DMEM/F12 based media (containing 2% B27 supplement, 1% N2 supplement, 2 mM Glutamax™, 1% P/S) supplemented with bFGF (20 ng/mL) and heparin (2 μ g/mL). NPCs were passaged every four days and maintained up to 5 passages. To encapsulate inside the PA gel, undissociated neurospheres (4 days old) were briefly spun down and re-suspended in PA solution by gentle pipetting, taking care not to disrupt the cellular aggregation; aligned and unaligned gels were made from the suspension with intact neurospheres inside. PA gels were further embedded inside a block of collagen gel (3 mg/mL) to prevent their movement in the Petri dish. Cell nutrition was maintained using a culture media having a composition similar to passaging media, but without any growth factor supplementation.

2.6. Immunocytochemical staining

Cells encapsulated in aligned gels were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min at room temperature (RT). Fixed cells were permeabilized and non-specific binding sites were blocked with 0.4% Triton X-100, 10% normal goat serum and 2% BSA in PBS (1 hour, RT). Following permeabilization and blocking, samples were incubated with primary antibody solution for overnight at 4°C. Following primary antibodies were used in present work: Mouse monoclonal Anti- β -III-tubulin, 1:1000 dilution, Abcam; rabbit monoclonal Anti- β -III-tubulin, 1:1000 dilution, Covance; mouse monoclonal anti-microtubule associated protein 2 (MAP2), 1:500 dilution, Sigma; rabbit polyclonal anti-glial fibrillary acidic protein (GFAP), 1:1000 dilution, Abcam; rabbit monoclonal anti-synaptophysin, 1:1000 dilution, Millipore; mouse monoclonal anti-nestin, 1:1000 dilution, BD Bioscience. Primary antibodies were detected with Alexa Fluor®

conjugated secondary antibodies (Life Technologies; 1 hour incubation at RT or overnight incubation at 4°C). Cell nuclei were counterstained with DAPI, Hoechst (Life technologies) or propidium iodide (Sigma).

2.7. Image acquisition and analysis

Fluorescence images were acquired on a Nikon Eclipse TE2000-U inverted microscope, a Zeiss LSM 510 META laser scanning confocal microscope, a Nikon A1R laser scanning confocal microscope, or a Leica SP5 laser scanning confocal microscope. To analyze P19 neurite outgrowth, gels were immunolabeled with antibodies against β -III-tubulin and nuclei stained with Hoechst 34580 (Invitrogen). Gels were imaged on a Zeiss LSM 510 META laser scanning confocal microscope, acquiring z-stacks of randomly selected regions of the gels. Two gels were imaged per condition per experiment. Experiments were done in triplicate. Using the Simple Neurite Tracer plug-in for ImageJ, neurites were traced and measured. Researchers were blinded to the conditions during imaging and tracing.

2.8. Analysis of P19 neurite alignment

Traces of the P19 neurites from the aligned and unaligned gels containing P19-derived neurons were retraced in the ImageJ plug-in NeuronJ. Each neurite was divided into $\sim 8\text{-}\mu\text{m}$ segments and the coordinates for each segment were used to calculate the angle of each neurite segment relative to the direction of the gel, θ_s . An order parameter (OP) was calculated by $OP = \langle 2(\cos^2(\theta_s)) - 1 \rangle$. >100 neurites were measured for each condition in serum-free media experiments. >48 neurites were measured for each condition in experiments with media containing serum.

2.9. NPC neurite outgrowth analysis

Neurospheres were immunolabeled with antibodies against β -III-tubulin. Nuclei were labeled with 100 ng/mL propidium iodide (Sigma). The maximal distance that neurites extended away from the edge of the neurosphere was measured.

2.10. Electrophysiology

Whole-cell current-clamp recordings were performed on the hippocampal neurons cultured inside the aligned PA scaffold. The scaffold was securely held inside the recording chamber by a platinum bridge with nylon cross fibers. An upright microscope (Olympus, BX51WI) with long working distance objective was used to visualize the cells under infrared differential interference contrast optics. The extracellular and the internal pipette-filling solution were prepared following previously published recipe[34]. The extracellular solution contained 147 mM NaCl, 2.1 mM KCl, 8.8 mM HEPES, 8.8 mM D-glucose, 1.1 mM CaCl_2 , 1.1 mM MgCl_2 , 2.6 mM pyruvic acid (pH 7.35) and the internal solution contained 144 mM CsCl_2 , 10 mM HEPES, 5 mM Na_2ATP , 1.1 mM EGTA, 0.1 mM CaCl_2 , and 5 mM MgCl_2 (pH 7.4). To access the neuronal soma, PA gel materials on top of the cell bodies were flushed by fluid under positive pressure from a pipette. A second pipette filled with internal solution was used for the recording. Pipette access resistance was 3-5 M Ω and seal resistance was $> 1\text{ G}\Omega$.

2.11. Spinal cord injection of NPCs encapsulated in scaffold

All animal procedures were undertaken in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee approved all procedures. Female Long-Evans rats (8 weeks of age; The Jackson Laboratory, Bar Harbor, ME) were anesthetized using isoflurane. A laminectomy was performed at the T12 level of the spinal cord to expose $\sim 3\text{ mm}$ of the dorsal spinal cord. For injection of NPCs, cells were isolated and cultured as described above (for *in vitro* studies),

using E13 mouse or E15 rat embryos. NPCs were dissociated and suspended in a mixed PA solution of base PA and IKVAV PA (0.4 wt. % and 0.1 wt. %, respectively) at approximately 3,500 cells/ μ L, and loaded into a 10- μ L Hamilton syringe. For manual injection, a 25-gauge needle was attached to the syringe, and the needle was inserted $\sim$ 5 mm along the dorsal spinal cord at a $\sim$ 20-degree angle. As the needle was retracted over a few seconds, 5 μ L of the PA solution was deposited along the needle track. For semi-automated injection, a glass pipette was pulled with a long taper, and the tip cut to $\sim$ 60- μ m inner diameter. The syringe was mounted on a micromanipulator (Sutter Instruments) and the pipette was inserted into the spinal cord at a $\sim$ 20-degree angle a distance of 3.2 mm. As the pipette was slowly retracted at a constant rate of 1 mm/min, a syringe pump (World Precision Instruments) was used to inject the PA solution at a constant rate (1 μ L/min). Six days after injection, the animals were perfused with 4% paraformaldehyde, the cords were harvested, sectioned by cryostat, and immunofluorescently labeled with nestin, β -III-tubulin, and DAPI.

2.12. Statistical analysis

Comparisons between two groups of data were made by using unpaired Student's *t* test. A difference was considered significant when the *P* value was <0.05 . Error bars in the bar graph represent standard error of the mean.

3. Results and Discussion

3.1. Design and characterization of aligned scaffolds

Our first goal was to design a scaffold that could exploit the PA's ability to form aligned gels as well as present bioactive peptide epitopes. Because not all PA molecules are capable of forming aligned gels, we utilized a mixed system composed of a PA that forms aligned gels and another that presents a bioactive epitope. Such mixing of epitope-presenting PAs with PAs lacking epitopes has also been shown to enhance epitope-mediated cell response by improving epitope accessibility on the nanofibers [26, 35]. The PA selected to enable formation of the aligned gel had the peptide sequence VVAEE, and is referred to as the “base” PA because the same sequence was used as the peptide backbone for epitope-presenting PAs. In epitope-presenting PAs, IKVAV or RGDS sequences were conjugated to the termini of the same base sequence. Scrambled controls for these bioactive PAs were designed by altering the order of amino acids in the epitope sequence. Table 1 lists the PAs used in the present study.

Mixed solutions of the base and epitope-presenting PAs (4:1 by weight) were thermally annealed to induce the formation of aligned nanofiber domains. Base PA molecules alone formed high aspect ratio ribbon-like assemblies, with twists at regular intervals along the long axis of the nanostructure (Fig. 1A); similar to the base PA, the annealed mixed PA solutions maintained a dominant “twisted ribbon” nanostructure (Fig. 1B). These mixed solutions formed robust gels when pipetted through a saline solution containing 25 mM CaCl_2 (Fig. 1C) and scanning electron microscopy (SEM) micrographs revealed that the gels were composed of aligned nanofiber bundles (Fig. 1D). Although SEM demonstrates the fiber alignment over a macroscopic length scale on the surface of the gels, it is difficult to examine fiber spacing in the interior of the gel using this technique. To assess the spacing between the fibers, resin-embedded scaffolds were examined under transmission electron microscopy (TEM) (Fig. 1E). Thin sections of the scaffold, parallel to the direction of its long axis, revealed that nanofibers are separated by large spaces of tens to hundreds of nanometers in width. The inter-fiber spaces are occupied by water giving the scaffold its highly hydrated character, and is possible due to the low concentration of PA ($\sim$ 1% w/v) used to form the gels.

3.2. Neurite growth and orientation in aligned scaffolds

The epitope-presenting aligned scaffolds were tested for their ability to promote neurite outgrowth and orientation using P19 embryonal carcinoma cells treated with retinoic acid to differentiate them into neurons [29]. Cells suspensions in culture media were mixed with thermally treated PA solutions at a ratio of 1:4 (v/v) to have a final cell concentration of 5,000 cells/ μ l, and the base PA and bioactive PA concentrations of 0.8% and 0.2% (w/v), respectively. Pipetting the cell suspension in a CaCl_2 -containing gelling solution (150 mM NaCl, 3 mM KCl, 25 mM CaCl_2) resulted in encapsulation of the cells in the rapidly formed aligned gel. The gelling solution was immediately replaced with media (serum-free media was used to prevent the potentially confounding effects of serum protein adsorption to the nanofibers), cultured for two days and stained for β -III-tubulin to examine neurite growth.

After two days of culture in the scaffolds, a fraction of the cells had developed neurites. Cells in scaffolds containing the IKVAV PA were approximately three times more likely to develop neurites than in scaffolds composed only of the base PA; in IKVAV PA scaffolds $7.7 \pm 0.7\%$ of cells produced neurites, compared with $2.5 \pm 0.5\%$ and $3.9 \pm 0.8\%$ of cells in the base PA and VVIAK PA scaffolds, respectively (Fig. 2A). Neurite formation in gels with RGDS epitope and scrambled RSDG control, however, was not significantly different than base PA gels. The greater number of neurites observed in IKVAV PA gels is likely related to the increased percentage of cells expressing β -III-tubulin (79%) in IKVAV PA gels, significantly more than in all of the other conditions, with percentages near 60%. Thus, enhancement of neurite development in IKVAV PA scaffolds may be related to IKVAV's ability to promote neuronal differentiation [25].

In addition to an enhancement in neurite formation, the average neurite length in the presence of PA fibers with IKVAV epitopes ($31.2 \pm 1.1 \mu\text{m}$) was significantly higher compared with the scrambled VVIAK epitope ($23.9 \pm 0.6 \mu\text{m}$) and base PA ($23.9 \pm 0.4 \mu\text{m}$) controls (Fig. 2B). Neurite outgrowth was also promoted by PA fibers with RGDS epitopes, with 23% higher average neurite length ($31.3 \pm 1.2 \mu\text{m}$) than in the presence of the scrambled RSDG epitope. The extent of enhancement in neurite growth following epitope incorporation matches with previous observations using different materials [10, 36] and demonstrates the usefulness of the strategy in aligned PA scaffolds.

To test whether the enhancement of neurite outgrowth was maintained in the presence of serum, we repeated the neurite outgrowth assay in serum-containing media. We observed that the presence of serum increased average neurite lengths (Fig. 2C-F), and maintained the trend of longer neurite lengths in IKVAV PA scaffolds ($68.7 \pm 5.3 \mu\text{m}$) relative to both the base PA scaffolds ($43.2 \pm 6.4 \mu\text{m}$) and VVIAK scaffolds ($47.6 \pm 5.9 \mu\text{m}$). Since IKVAV epitopes in the scaffold enhanced both the number and length of neurites, we selected IKVAV PA for further experiments to study the aligned bioactive scaffold.

The role of nanofibers in directing neurite outgrowth was investigated by comparing the alignment of neurites in aligned and isotropic (i.e. unaligned) PA gels. Neurites were traced and divided into short segments. The angle (θ_s) between each segment and the long axis of the scaffold was measured and plotted in a polar histogram with 10-degree bins (Fig. 2G and H). In the aligned gels, the majority of the neurite segments were within 20 degrees of the scaffold direction, whereas in isotropic gels the segments were distributed equally in all directions. To further quantify the neurite alignment, an orientation parameter (OP; defined as $\text{OP} = \langle 2(\cos^2(\theta_s)) - 1 \rangle$), was calculated for each gel; perfect alignment would have an OP value of 1 and random alignment would have an OP value of 0. In aligned scaffolds of the base PA and IKVAV PA, OPs of neurites were 0.76 and 0.62, respectively, indicating a strong propensity to grow in the direction of the scaffold. Since the IKVAV PA cannot form aligned gels without being mixed with a PA with this capability (as with the base PA), the

slight reduction in the OP in base/IKVAV PA aligned gels was expected. In isotropic gels of the base PA and IKVAV PA, however, the measured OP values were ~ 0 indicating the absence of any preferential alignment. These results demonstrate that the fiber alignment in the gel acts as a potent directional cue for neurite growth.

To test whether the fiber alignment could also direct neurite growth of primary neurons, mouse embryonic hippocampal cells were cultured in aligned IKVAV gels. One remarkable feature of this culture inside the gels was the very low number of astrocytes; visualized by staining against astrocyte-specific glial fibrillary acidic protein (GFAP), only a few astrocytes, scattered in the gel, could be found after seven days in culture (Fig. 3A). In contrast, when the hippocampal cells were cultured on glass surfaces for seven days, approximately 20% of cells were positive for GFAP, demonstrating that the PA scaffold appears to restrict astrocyte growth; this observation is in agreement with previous reports of suppressed glial proliferation and development by IKVAV PA gels [5, 25]. Encapsulated neurons also produced an extensive amount of neurite growth throughout the gel and, similar to P19 neurons, the neurites extended predominantly in the direction of the scaffold (Fig. 3A). To investigate the relationship between neurites and the nanofibers inside the scaffold, we examined resin embedded sections by TEM. Sections taken along the long axis of the scaffold revealed that neurites were predominantly parallel to the direction of the nanofibers (Fig. 3B). The long, anisotropic spaces between nanofibers afford sufficient room and directional guidance for neurite growth, while still providing many nanofibers to adhere to in close proximity to a given neurite.

3.3. Electrical activity and synapse formation of neurons

We next investigated whether the neurons cultured inside the scaffold could functionally mature to conduct information by generating action potentials. Hippocampal neurons were cultured for up to two weeks in IKVAV PA scaffolds and whole-cell patch-clamp recording was performed in current clamp mode from one and two weeks old neurons. Spontaneous action potentials were observed at both time points examined (Fig. 3C), although the amplitude of the action potential substantially increased in the second week, matching with a time-course of developmental maturation observed in two-dimensional culture on glass coverslips [37]. To test whether the neurons were connected by synapses within the scaffold, cultures were stained for the dendritic marker MAP2 (microtubule associated protein 2) and the pre-synaptic axon terminal marker synaptophysin. We observed many synaptophysin-positive punctae distributed on the dendritic tree, suggesting formation of synapses (Fig. 3D). The electrophysiological recording and immunostaining together suggest that neurons inside the scaffold form a connected network of mature neurons, capable of propagating electrical information. With the ability to promote extensive neurite growth in the direction of the aligned nanofibers, along with the capability to form synaptic connections, this scaffold has the potential to propagate electrical signals in a desired direction.

3.4. Neural progenitor cell response to aligned scaffolds

While primary neurons demonstrated the scaffold's ability to promote and align neurite growth, a similar response from neural stem cells would have greater potential for regenerative applications. Previous work in our laboratory demonstrated that PAs with the IKVAV epitope were capable of promoting differentiation of neural progenitor cells (NPCs) into neurons [25]. To investigate whether the aligned scaffold could be used to differentiate NPCs into neurons and guide their neurite extension, mouse embryonic NPC neurospheres were encapsulated in both aligned and unaligned gels. β -III-tubulin staining of seven day-old cultures demonstrated differentiation of many progenitors to neurons, even without the supplementation with neuron-differentiation-promoting growth factors (e.g. BDNF) to the serum-free media. In both the aligned and unaligned IKVAV epitope containing gels, many

of the cells on the periphery of the neurospheres in contact with the PA differentiated into neurons (Fig. 4A and B). However, the distance that neurites extended from the neurospheres was nearly three times longer in the aligned gels ($386 \pm 39 \mu\text{m}$) compared with the unaligned gels ($137 \pm 16 \mu\text{m}$) (Fig. 4C). The greater neurite growth in aligned gels can partially be explained by the ease of extension in a linear direction; in comparison to the randomly oriented neurite growth in the unaligned gels, neurites in aligned gels projected in a single direction. In contrast to the observed differentiation in IKVAV PA gels, significantly fewer cells on the neurosphere surface differentiated into neurons in control VVIKAK gels and neurite outgrowth was minimal, suggesting the specificity of the IKVAV epitope activity (Fig. 4D).

3.5. Cell transplantation and aligned scaffold formation in the spinal cord

We have also explored the possibility of forming an aligned scaffold encapsulating NPCs *in situ* within the spinal cord. A PA solution containing NPCs was injected into adult rat spinal cord along a linear needle track. On histological examination of the spinal cord six days post-injection, the scaffold could be easily identified by the dense distribution of cells (Fig. 5A and C) and the positive staining to nestin (a marker for neural stem cells) (Fig. 5B and D). As seen in Fig. 5B and D, the nestin-positive cell processes were locally aligned to the scaffold direction, although some variability in the scaffold thickness and overall contour resulted from the manual operation of the injection process.

To create a more uniform scaffold in the spinal cord, we developed a semi-automated micromanipulator based technique, where a syringe pump was used to inject the PA solution at a constant rate (1 $\mu\text{L}/\text{min}$), while the pipette was slowly retracted (1 mm/min). This technique resulted in much greater uniformity in the diameter of the PA gel (Fig. 5E), while the alignment of neuronal processes in the gel was maintained (Fig. 5F). These experiments demonstrate the possibility of transplanting cells, encapsulated within an aligned gel, in neural tissue.

The formation of an aligned material directly within nerve tissue was possible due to a combination of favorable properties of the PAs used here—their ability to achieve long-range alignment of nanofibers following an application of shear to the liquid crystalline PA solutions and their capacity to instantly gel upon contact with Ca^{2+} -containing extracellular fluid. Previous research from our group demonstrated that a PA gel presenting IKVAV epitopes was capable of inducing axonal growth across a spinal cord injury leading to partial recovery of locomotor function [5]. Adding the positive benefits of cell transplantation and fiber alignment presented here potentially could improve the recovery further. Such a combinatorial approach is particularly attractive, since cell delivery has been effective at promoting axonal regeneration in the treatment of spinal cord injury [14, 15]. Additionally, approaches using materials with aligned features have demonstrated their potential in promoting directional axonal growth across a spinal cord lesion [7, 11].

3.6. Cell migration in aligned scaffolds

In order to understand peripheral neuronal response to the aligned PA environment, next we encapsulated intact embryonic dorsal root ganglia (DRGs) in aligned IKVAV PA scaffolds (Fig. 6A). To facilitate repeated observation from the same DRG, the scaffold was immobilized on the culture dish by embedding it within a collagen gel. Interestingly, the DRG cells showed extensive migration in the scaffold, with their direction guided by the fiber alignment (Fig. 6B and C). By one week, cells were found to have migrated up to several hundreds of micrometers from their initial location in the explant. Time-lapse observation revealed that the majority of cells migrated by extending leading processes in the direction of the nanofiber alignment, followed by nuclear movement and retraction of

the trailing process, similar to cell migration observed in other three-dimensional matrices (Video S1) [38]. Some of these migrating cells stained positive for the neuron-specific marker β -III-tubulin, suggesting that either neurons migrated from the DRG or that undifferentiated cells migrated before differentiating into neurons (Fig. 6D). Even though cells traveled long distances within the PA gel, very little migration was observed in the surrounding collagen gel. However, when placed at the end of an aligned PA scaffold such that part of the DRG remained exposed to the surrounding collagen matrix (Fig. 6E), we observed directional cell migration into the aligned PA gel as well as radial migration into the anisotropic collagen matrix. Minimal cell migration into the surrounding collagen gel when completely encapsulated inside the PA gel could be due to the formation of a PA-collagen interface less favorable for cell migration. As expected, DRGs placed inside an unaligned PA gel showed very little cell migration, further confirming that nanofiber alignment is a critical requirement for promoting cell motility (Fig. 6F).

Although DRG cell migration has been previously shown on aligned electrospun surfaces [39], facile encapsulation of DRGs in self-assembled, aligned PA gels enabled anisotropic migration in a 3D environment. The aligned scaffolds may facilitate directional migration in a manner similar to their guidance of neurite extension, as the projection of the leading process in migratory cells employs similar molecular mechanisms as growing neurites [40]. However, migratory cells undergo the additional step of nucleokinesis, where the nucleus moves into the leading process, followed by retraction of the trailing process [40]. The significant cell migration out from the DRG demonstrates that the aligned gels provide an adequate physical environment for these additional processes to occur. Though reports of migration from DRG typically focus on Schwann cell migration [39], neuronal migration from DRG has also been reported [41]. Our observation that some of the migrating cells were β -III-tubulin-positive suggests the scaffold is capable of supporting neuronal migration, though this may have been facilitated by preceding Schwann cell migration. Neuronal or neuroblast migration is abundant in the developing nervous system [40], and is an ongoing process in the brain regions that remain neurogenic in the adult; for example, neuroblasts from the subventricular zone migrate along the rostral migratory stream to repopulate the olfactory bulb [42]. The ability of the aligned PA scaffolds to guide neuronal migration opens the potential for new regenerative approaches that guide implanted or endogenous cells from one destination to another.

4. Conclusions

We have shown that hand-fabricated macroscopically aligned gels containing encapsulated neurons and bioactive peptide amphiphile fibers have the capacity to guide the growth of oriented neurites and the direction of cell migration. The neurite growth observed in a variety of neuronal cell types suggests the potential use of this scaffold in treating conditions ranging from spinal cord injury to peripheral nerve repair using transplanted cells. The unique capacity to form aligned scaffolds directly within neural tissue, concomitant with cell transplantation, creates new opportunities in tissue regeneration that depend on specific cell or process orientation. The control of directional cell migration by the scaffold also opens the possibility of their use in repopulating cells in regions of acute neuronal loss, for example as a result of stroke.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Characterization of the aligned PA scaffold. (A, B) TEM image of the base PA alone (A) and after mixing with the IKVAV PA (B). (C) An aligned PA scaffold prepared in a bath of CaCl_2 -containing salt solution inside a cell culture dish. (D) SEM images of the aligned nanofiber structure at lower and higher magnification. (E) TEM image of a resin-embedded aligned gel sectioned parallel to fiber orientation reveals the fiber spacing.

Fig. 2.

Neurite outgrowth of P19 neurons in aligned PA scaffolds. Neurons were cultured for two days inside aligned scaffolds under serum-free conditions. (A) The percentage of P19-derived cells with neurites, and (B) average neurite lengths ($n > 100$) in scaffolds containing IKVAV, RGDS, and their respective scrambled epitopes. (C) Neurite length for cells cultured in serum-containing media ($n > 48$). (D-F) Representative images of neurons in aligned scaffolds, stained for β-III-tubulin (green) and Hoechst (blue). * $P < 0.01$. # $P < 0.05$. ^ $P < 0.001$. (G,H) Polar histograms showing the angles of P19 neurites relative to the direction of the scaffold composed of the base PA (G) and IKVAV PA (H); the red and black lines depict neurite growth in aligned and unaligned gels, respectively.

Fig. 3.

(A) Hippocampal neurons cultured in an IKVAV PA aligned scaffold for seven days and stained for β -III-tubulin (green), GFAP (red), and DAPI (blue) revealing extensive, aligned neurite growth. (B) TEM micrograph of a hippocampal neurite inside an aligned gel showing its parallel orientation to the aligned PA nanofibers. (C) Whole-cell patch-clamp recording from gel-encapsulated neurons cultured for 8 days (top trace) and 13 days (bottom trace) shows spontaneous action potentials. (D) A hippocampal neuron cultured inside an IKVAV PA scaffold for 13 days and stained for dendritic marker MAP2 (red) and synaptic marker synaptophysin (green). [Arrow indicates the direction of fiber alignment]

Fig. 4.

Neurite growth from differentiated NPCs in PA scaffolds. (A-B) Intact neurospheres were encapsulated in an (A) aligned or (B) unaligned IKVAV PA gel, cultured for 7 days and stained with anti- β -III-tubulin antibody (green) and propidium iodide (red). (C) Comparison of the maximal distance extended by neurites from the neurosphere in aligned and unaligned IKVAV PA gels. (D) Similar comparison of neurite extension between aligned IKVAV and control (VVIK) PA gels. (* $P < 0.001$, $n > 15$ neurospheres).

Fig. 5. Transplantation of NPCs encapsulated in an IKVAV PA aligned scaffold in the rat spinal cord. (A,B) Sagittal section of a rat spinal cord six days after *in situ* formation of an aligned scaffold (dashed line outlines the perimeter of the scaffold); encapsulated NPCs are visualized by staining against nestin (green; blue: DAPI). (C,D) Enlarged views of sections indicated by the squares in A and B. (E,F) Uniform scaffold formation by micromanipulator-assisted needle retraction and syringe pump injection (green: β -III-tubulin, red: fluorescent PA, blue: DAPI).

Fig. 6. Scaffold alignment guided cell migration. Dorsal root ganglions (DRG) were encapsulated in IKVAV PA aligned scaffolds, which were further embedded in a block of collagen gel. Phase contrast images of a representative DRG after 1 day (A) and 6 days (B) of culture. (C) A magnified view of the region indicated by arrowhead in image (B). (D) 3D Reconstruction from a confocal image stack of β -III-tubulin stained DRG, cultured for 6 days inside aligned scaffold. Arrow points to the original DRG location and dotted lines indicate the scaffold boundary. *Inset*: Enlarged view of a section from the region indicated by the rectangle. DRG cells migration when (E) placed at the end of the aligned scaffold (demarcated by dotted lines) or (F) encapsulated in an unaligned PA scaffold.

Table 1
List of PAs and their peptide sequences

PA	Sequence
Base PA	Palmitoyl-VVAAEE-NH ₂
IKVAV PA	Palmitoyl-VVAAEEEEGIKVAV-COOH
VVIAK PA	Palmitoyl-VVAAEEEEGVVIAK-COOH
RGDS PA	Palmitoyl-VVAAEERGDS-NH ₂
RSDG PA	Palmitoyl-VVAAEERSDG-NH ₂