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A bioengineered peripheral nerve construct using aligned peptide amphiphile nanofibers

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ABSTRACT

Peripheral nerve injuries can result in lifelong disability. Primary coaptation is the treatment of choice when the gap between transected nerve ends is short. Long nerve gaps seen in more complex injuries often require autologous nerve grafts or nerve conduits implemented into the repair. Nerve grafts, however, cause morbidity and functional loss at donor sites, which are limited in number. Nerve conduits, in turn, lack an internal scaffold to support and guide axonal regeneration, resulting in decreased efficacy over longer nerve gap lengths. By comparison, peptide amphiphiles (PAs) are molecules that can self-assemble into nanofibers, which can be aligned to mimic the native architecture of peripheral nerve. As such, they represent a potential substrate for use in a bioengineered nerve graft substitute. To examine this, we cultured Schwann cells with bioactive PAs (RGDS-PA, IKVAV-PA) to determine their ability to attach to and proliferate within the biomaterial. Next, we devised a PA construct for use in a peripheral nerve critical sized defect model. Rat sciatic nerve defects were created and reconstructed with autologous nerve, PLGA conduits filled with various forms of aligned PAs, or left unrepaired. Motor and sensory recovery were determined and compared among groups. Our results demonstrate that Schwann cells are able to adhere to and proliferate in aligned PA gels, with greater efficacy in bioactive PAs compared to the backbone-PA alone. *In vivo* testing revealed recovery of motor and sensory function in animals treated with conduit/PA constructs comparable to animals treated with autologous nerve grafts. Functional recovery in conduit/PA and autologous graft groups was significantly faster than in animals treated with empty PLGA conduits. Histological examinations also demonstrated increased axonal and Schwann cell regeneration within the reconstructed nerve gap in animals treated with conduit/PA constructs. These results indicate that PA nanofibers may represent a promising biomaterial for use in bioengineered peripheral nerve repair.

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1. Introduction

It is estimated that at least 200,000 peripheral nerve repairs are performed annually in the United States [1]. Traumatic peripheral nerve injuries represent a particular challenge for reconstructive surgeons due to their strong association with long-term disability and poor functional outcomes. For example, only 7% of patients who sustain a gunshot wound to an extremity with a resultant

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peripheral nerve injury attain normal limb function, compared to 39% of patients who achieve functional recovery in the setting of a gunshot-induced extremity injury without nerve involvement [2]. This poor prognosis is due to the principle of Wallerian degeneration, whereby the nerve segment distal to the site of injury becomes dysfunctional, which in turn leads to atrophy and malfunction of the end organ supplied by the nerve.

Various techniques for nerve repair exist. Direct neurorrhaphy (end-to-end repair) of a transected nerve is an effective means of repair when there is no gap between the cut nerve ends or if that gap is less than 1 cm. This avoids excessive tension over the suture line, which has been shown to negatively affect functional outcomes [3,4]. Complex nerve injuries with gaps of greater than 1 cm can be repaired using interposition autologous nerve grafts. Donor nerve, however, is a limited resource. Moreover, its harvest results in a functional deficit at the donor site and can be associated with donor-site morbidity, such as pain, infection, or neuroma formation [5]. The use of cadaveric nerve allograft has been shown to be effective in the repair of peripheral nerve defects up to 5 cm in length [6]. One advantage to nerve allografts in longer gaps is their ability to provide an internal structure and extracellular matrix that serve as a scaffold to support directional migration of reparative Schwann cells. Cadaveric nerve, however, may be associated with immunologic responses, including graft vs. host disease, or transmission of human pathogens. Recently, improvements have been made in the processing techniques used to prepare cadaveric nerve, resulting in improved removal of immunogenic elements while preserving fascicular architecture [7]. Currently available collagen and poly(lactic-co-glycolic acid) (PLGA) nerve conduits can be effective in the repair of short segment peripheral nerve injuries. Empty collagen conduits, for example, have reported effective repair lengths ranging from 1 cm [8] to less than 3 cm [9]. However, as the length of the defect increases, the efficacy of these empty nerve conduits declines, likely because they are unable to mimic the native internal architecture of the nerve and provide no internal scaffolding to support nerve regeneration.

Due to the shortcomings associated with these approaches to nerve repair, the development of bioengineered nerve conduits that can serve as nerve graft substitutes in the setting of complex nerve injury has been an active area of focus in tissue engineering research. Specifically, the ideal bioengineered nerve construct should not only mimic the aligned architecture of native nerve anatomy to support directional axonal regeneration, but should also provide the vital components of bioactivity found in the native extracellular matrix that facilitate Schwann cell attachment, proliferation, migration, and function.

In this study, we investigate the use of aligned nanofiber gels formed by self-assembling peptide amphiphiles (PA) in peripheral nerve regeneration [10,11]. Because of their unique properties and molecular design, PA nanofibers can mimic the internal fascicular architecture of peripheral nerves, allow for the incorporation of Schwann cells vital for peripheral nerve regeneration, induce cellular and neurite alignment, and guide cell migration [12]. Moreover, the PAs can be engineered to possess bioactivity that is relevant to nerve regeneration. A PA presenting the amino acid sequence IKVAV (derived from laminin) has been shown to induce neural stem cell differentiation, stimulate neurite outgrowth, and lead to functional improvement in acute spinal-cord injury [13,14]. The amino acid sequence RGDS is found naturally on molecules of fibronectin (and other proteins) and PAs bearing this sequence have been shown to promote cellular motility, proliferation, and differentiation *in vitro* [15–21]. Here we present our experience with the use of aligned PAs in both *in vitro* and *in vivo* models of peripheral nerve regeneration.

2. Materials and methods

2.1. Rat Schwann cells cultures

Cells were purchased from ATCC (cell line RT4-D6P2T) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). Cells were cultured to confluence, treated with 0.25% trypsin, centrifuged, and resuspended in PBS for a final concentration of 3500 cells/μl for WST-1 proliferation assays and 10,000 cells/μl for immunocytochemistry (ICC).

2.2. Backbone-PA and bioactive PA gels

In order to synthesize the backbone-PA (consisting of only palmitoyl-VVAAEE-NH_2), the molecule was dissolved in aqueous 150 mM NaCl, 3 mM KCl, and NaOH solution to obtain a final 1 wt% solution at a pH between 7.2 and 7.4. In order to fabricate mixtures containing epitope-bearing PAs IKVAV and RGDS (consisting of palmitoyl-VVAAEE-NH_2 with appended peptides, either RGDS (Arg-Gly-Asp-Ser) or IKVAV (Ile-Lys-Val-Ala-Val)), the backbone-PA was dissolved in aqueous 150 mM NaCl, 3 mM KCl, and NaOH solution to obtain a 1.33 wt% solution at a pH between 7.2 and 7.4. The epitope-containing PA was then dissolved with the same solution to obtain a 1 wt% solution at a pH between 7.2 and 7.4. The 1.33 wt% PA solution was then mixed in a 3:1 ratio with the 1% bioactive epitope solution creating a final bioactive PA solution of 1 wt% PA and 0.25 wt% bioactive epitope. The three resulting solutions (backbone-PA, RGDS-PA, and IKVAV-PA) were then heated to 80 °C for 30 min in a water bath and left in the bath for slow cooling to 37 °C overnight [10,11].

2.3. Combining backbone-PA and bioactive PA with Schwann cells

Following completion of the slow cooling, each of the PA solutions was mixed with Schwann cell suspension in a 4:1 ratio. A final cell density of 3500 cells/μl was used for the WST-1 proliferation assays and 10,000 cells/μl for immunocytochemistry studies.

2.4. Backbone-PA and bioactive PA-cell gel fabrication

A 200 μl volume of gelling solution consisting of 20 mM $CaCl_2$, 150 mM NaCl, and 3 mM KCl was pipetted onto a glass slide to form a thin film. The PA-Schwann cell suspensions were then pipetted in 4 μl aliquots onto the thin film, instantly forming a string-like gel, with cells encapsulated within. These cell-embedded gels were maintained free floating in DMEM with 10% FBS in culture plates and incubated at 37 °C. Medium was changed every 72 h.

2.5. Collagen gels

Volumetric ratios of 1 μl HEPES, 10 μl 10XPBS, 87 μl Collagen 10 mg/mL, and 2 μl Schwann cell PBS suspension were combined and mixed gently. Final cell density of 3500 cells/μl was used for the WST-1 proliferation assays and 10,000 cells/μl, for immunocytochemistry studies. The mixture was then pipetted into a culture well (either a 96-well plate or 2-welled microscopy slides), and after 1 h of curing and solidification at 37 °C, medium was added gently to the wells and changed every 3 days.

2.6. WST-1 proliferation assay

To evaluate the viability of cells within the gels, WST-1 reagent (Roche Applied Science, Indianapolis, IN, USA) was used for each PA-cell and collagen gel–cell complex. WST-1 assay has been widely used to measure cell proliferation. Aligned PA-cell gels along with collagen-cell gels were fabricated through the aforementioned methods at a final cell density of 3500 cells/μl. Gels were then cultured in 96-well plates for various time periods (days 1, 7, 14, and 21). Following each time point, gels were incubated for 90 min in medium containing 10% WST-1 assay reagent. Following incubation, 100 μL of the conditioned medium were then transferred to a 96-well reading plate, and absorption values were read in a spectrophotometer at 450 nm. Measurements were repeated in sets of four.

2.7. Immunocytochemistry

PA-cell gels along with collagen-cell gels were fabricated through the aforementioned methods at a final cell density of 10,000 cells/μl. These gels were then cultured for 48 h in 2-well microscopy slides, followed by fixation with 10% formalin and staining with mouse anti-vinculin monoclonal antibody (Abcam, Cambridge, MA), followed by FITC-conjugated anti-mouse secondary antibody (Abcam). Actin filaments were stained with rhodamine phalloidin (Life Technologies, Carlsbad, CA). Specimens were then viewed with confocal microscopy (Confocal SP1 MP-Inverted, Leica Microsystems Inc., Buffalo Grove, IL) using oil magnification, and images were recorded.

2.8. Preparation of PLGA tube

Poly (lactide-co-glycolide acid) (PLGA) (75:25 mol ratio of D, L-lactide to glycolide, 0.76 dL/g, Lakeshore Biomaterials, Birmingham, AL) tubes were created using a modified gas foaming technique initially described by Mooney et al. [22]. PLGA microspheres were prepared with dichloromethane in water emulsion technique. A 1:7 weight ratio mixture of PLGA microspheres and NaCl particles (38–63 μm) were loaded into the annular gap of two glass tubes having an outside diameter of 3 mm

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