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The behavior of neuronal cells on tendon-derived collagen sheets as potential substrates for nerve regeneration

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Peripheral nervous system injuries result in a decreased quality of life, and generally require surgical intervention for repair. Currently, the gold standard of nerve autografting, based on the use of host tissue such as sensory nerves is suboptimal as it results in donor-site loss of function and requires a secondary surgery. Nerve guidance conduits fabricated from natural polymers such as collagen are a common alternative to bridge nerve defects. In the present work, tendon sections derived through a process named bioskiving were studied for their potential for use as a substrate to fabricate nerve guidance conduits. We show that cells such as rat Schwann cells adhere, proliferate, and align along the fibrous tendon substrate which has been shown to result in a more mature phenotype. Additionally we demonstrate that chick dorsal root ganglia explants cultured on the tendon grow to similar lengths compared to dorsal root ganglia cultured on collagen gels, but also grow in a more oriented manner on the tendon sections. These results show that tendon sections produced through bioskiving can support directional nerve growth and may be of use as a substrate for the fabrication of nerve guidance conduits.

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1. Introduction

Peripheral nervous system (PNS) injuries result in a decreased quality of life due to factors such as neuropathic pain and oftentimes reduced mobility [1]. Complete nerve transections are a major problem, and on average there are 200,000 incidences every year that require surgical intervention in the United States alone [2]. These peripheral nerve injuries are commonly the result of trauma, such as automobile accidents, burns, explosive injuries, and can be a complication of some surgical procedures. As such, there exists a large clinical need for suitable nerve guidance products for peripheral nerve repair. When the damage is small, the two ends of the nerve stump can often be sutured together end-to-end in order to induce repair. However, this is only possible when the damaged nerve segment is less than several millimeters as tension has been shown to increase scaring and impair regeneration [3–5]. When this is not possible there are a number of other options available, including the gold standard of replacing the damaged portion with a nerve autograft. A limitation of this approach is loss of function at the donor site, while the repaired portion of nerve may not achieve complete functional restoration.

Over the past decade a number of options have emerged for peripheral nerve repair including nerve guidance conduits (NGC). The goal of an NGC is to provide a pathway along which two ends of a sutured nerve stumps can regrow towards each other. This regrowth involves several stages including fluid infiltration, formation of a fibrin matrix and migration of a variety of cell types including Schwann cells [6]. These events can be influenced greatly by the properties of the NGC, such as permeability, stiffness, and degradation rate [7].

A number of nerve guidance conduits are currently available on the market including those comprised of synthetic polymers such as poly(lactide-*co*-glycolic acid) (PLGA), and poly-ε-caprolactone (PCL) [6], as well as natural materials such as animal extracted collagen [8,9] and silk [7,10,11]. Each of these NGCs have differing properties with advantages and disadvantages; a thorough review of FDA-approved NGCs can be found in Kehoe et al. [6].

Many nerve repair products in development make use of guidance strategies such as chemical or physical structures that serve to preferentially induce directional growth. The incorporation of growth factors into these structures generally functions to induce increased proliferation and provide a chemokine gradient along

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which the neuronal cells will grow. Physical features such as channels and grooves are similarly incorporated in order to provide nanotopographical cues which also guide directional growth along their length [12].

These physical structures are often incorporated into both synthetic and natural polymer-based nerve conduits through processing methods such as electrospinning and gel-casting. Electrospinning can produce nano-scale fibers of uniform dimensions that have been shown to guide cell growth [13,14], however this process has been shown to be more difficult with some materials, such as collagen, than synthetic polymers due to the water-insoluble nature of the collagen [15]. Additionally, the collagen fibers produced via this method do not recapitulate the complete native triple-helix and characteristic strength of native collagen fibers [16–18]. Other methods of incorporating aligned structures include extrusion [3], micropatterning using traditional photolithography techniques [19,20], and gel-casting/freeze drying [21]. One additional factor that must be considered when fabricating conduits out of ECM, such as collagen is that the material may shrink in vivo. This may be a result of a number of in vivo processes [22,23] such as crosslinking, or degradation by collagenases, but must be considered as it has the potential to place unwanted tension on the nerve stumps.

Our lab has recently demonstrated a technique, that allows us to create both flat and tubular scaffolds out of tendon-derived collagen that maintains the native collagen fiber structure [24,25]. This is in contrast to more common methods of producing collagen scaffolds that often involve enzymatic or acidic solubilization of the collagen, followed by reformation of a collagen gel into the desired structure. Our fabrication process (Fig. 1) involves taking a block of tendon (such as bovine Achilles tendon), decellularizing the tissue in a sodium dodecyl sulfate solution, and then sectioning the block into thin sheets (5–100 μm thick) using a microtome. These sheets can then be wrapped around rods of various diameters and crosslinked to form tubular conduits (Fig. 2A,B). These conduits can also be constructed with luminal fillers as we have previously shown, such as multiple smaller conduits within the primary NGC [24] or folded sheets of the tendon, in order to provide a three-dimensional surface for nerve regeneration. The benefit of this approach to fabricating collagen conduits is that it maintains the native structure of the collagen, as evidenced by the 67 nm banding pattern (Fig. 2C), as well as the native proteoglycans such as decorin [26], that may be beneficial in promoting axonal growth [27,28].

The goal of the present study was to evaluate the suitability of collagen sections created through our fabrication process for neuronal applications such as NGCs. Aligned growth, proliferation and adherence of various neural-related cell types were assessed.

2. Materials and methods

2.1. Section fabrication

Tendon sections were fabricated as previously described [24]. Briefly, we decellularized bovine Achilles tendon (Blood Farm, Groton, MA) in a 1% w/v sodium dodecyl sulfate (SDS) (Sigma, St. Louis, MO) solution for 48 h and rinsed the tendon overnight to remove any residual SDS. The sections were frozen at −20 °C and sectioned at 50 μm thick using a cryomicrotome. The sections were placed on poly-lysine coated microscope slides (Polysine, Thermo Scientific, Billerica, MA) and then rinsed in deionized H_2O (diH_2O) for 1 h to remove any of the embedding material. For adhesion and proliferation assays, the tendon sections were placed on 22 mm diameter glass coverslips (Electron Microscopy Sciences, Hattsfield, PA). All samples were sterilized by soaking in 70% ethanol, rinsing 3× with PBS, followed by 1 h of UV irradiation. Then, for specified experiments tendon samples, collagen gel-coated wells, or tissue culture poly styrene (TCPS) wells were coated in poly-D-lysine (PDL) (MW 70,000–150,000, Sigma) by filling the well with a 0.01 mg/ml solution overnight and rinsing with phosphate buffered saline (PBS).

2.2. Collagen sample preparation

For adhesion, proliferation and DRG experiments collagen gel samples were prepared using Culturex 3-D Culture Matrix™ Rat Collagen I (Trevigen, Gaithersburg, MD). The gel was prepared in a 12-well tissue culture plate following the manufacturer's directions. For each plate, 250 μl of a 1 mg/ml neutralized collagen gel was pipetted into each well, and the plate placed in a 37 °C humidified incubator for 1 h. Each well was then rinsed three times with PBS and either used as-is or coated with PDL as described above.

2.3. Scanning electron microscopy

The 50 μm tendon samples were produced via sectioning and dehydrated in graded ethanol (10%, 20%, 50%, 95%, 100% v/v). The samples were sputter coated with Pt–Pd using a Cressington 208HR sputter coater (Watford, England) and imaged using at 6.0 kV in a Zeiss FESEM Ultra55 (Oberkochen, Germany).

2.4. Schwann cell culture

Rat Schwann cells (CRL-2768, ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagles Medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) and Penicillin-Streptomycin (10,000 units Penicillin, 10,000 μg Streptomycin/mL) (Life Technologies) in a 37 °C humidified incubator at 5% CO_2. Cell morphology and alignment were observed via immunofluorescence by fixing the cells in 10% neutral buffered formalin (Sigma) for 10 min and then permeabilized with 0.1% (v/v) Triton X-100 (Sigma) for 5 min. After blocking with 1% (w/v) bovine serum albumin solution for 30 min, the cells were stained with rhodamine-phalloidin (Sigma) for 30 min, counterstained the cell nuclei with 4′,6-diamidino-2-phenylindole (DAPI) and observed via fluorescence microscopy.

2.5. Schwann cell adhesion assay

For adhesion determination, Schwann cells were plated onto the samples (tendon, PDL-tendon, collagen, collagen-PDL, TCPS and PDL TCPS) in 12 well plates at a concentration of 100,000 cells in 50 μl per well. Each substrate and time point was assayed in quadruplicate using cells of the same passage number. At a given time (0.5 h, 1 h or 2 h) the wells were rinsed gently with PBS and aspirated to remove any non-adherent cells. To each well, 1 ml of media was added with 100 μL AlamarBlue stock solution (Life Technologies). We then incubated the cells for 5 h at 37 °C. Following incubation, aliquots of the media

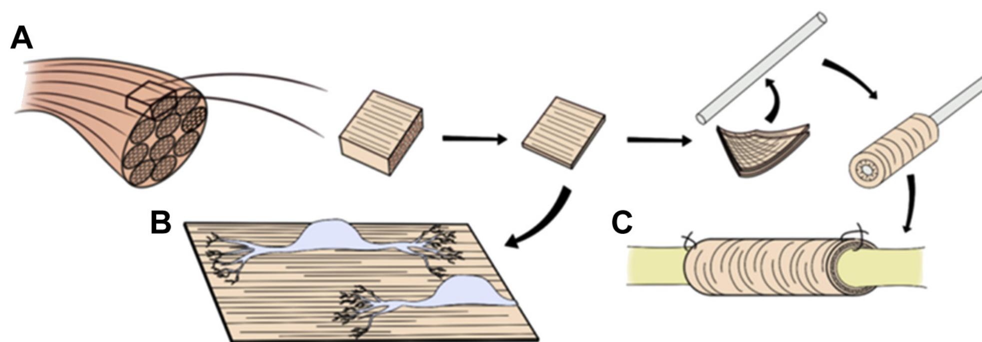

Fig. 1. Scheme showing fabrication and use potential of NGCs. A) Process use to fabricate the tendon sections, involving removing a block from the bulk tendon, sectioning the decellularized block and then wrapping multiple sheets around a tubular rod. B) Illustration of the directional alignment of DRG along the collagen fibers. C) Illustration of the multilayer tubular structures fabricated from the collagen sheets used as an NGC.

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