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Engineered neural tissue for peripheral nerve repair

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ABSTRACT

A new combination of tissue engineering techniques provides a simple and effective method for building aligned cellular biomaterials. Self-alignment of Schwann cells within a tethered type-1 collagen matrix, followed by removal of interstitial fluid produces a stable tissue-like biomaterial that recreates the aligned cellular and extracellular matrix architecture associated with nerve grafts. Sheets of this engineered neural tissue supported and directed neuronal growth in a co-culture model, and initial *in vivo* tests showed that a device containing rods of rolled-up sheets could support neuronal growth during rat sciatic nerve repair (5 mm gap). Further testing of this device for repair of a critical-sized 15 mm gap showed that, at 8 weeks, engineered neural tissue had supported robust neuronal regeneration across the gap. This is, therefore, a useful new approach for generating anisotropic engineered tissues, and it can be used with Schwann cells to fabricate artificial neural tissue for peripheral nerve repair.

1. Introduction

Cellular and extracellular matrix (ECM) alignment is a common feature of biological tissues, with anisotropy being critical to function in many instances. Examples include the collagen fibres that orientate in response to force vectors and strengthen ECM in musculoskeletal tissues, and the tracts of aligned cells present within the nervous system. Furthermore, failure to recreate the aligned cellular and/or ECM architecture of native tissues is a limitation in clinical repair and reconstruction, with scarring and poor restoration of mechanical properties being common [1]. For this reason, much research into tissue engineering and biomaterials has focused on the development of anisotropic scaffolds to confer orientation on cells and to provide the mechanical properties associated with aligned ECM [2–4].

One tissue where the use of an aligned cellular substrate would potentially be of great benefit for clinical repair is peripheral nerve. Neuronal regeneration and functional recovery are problematic following nerve damage and, where there is a long (>3 cm) defect, the current clinical gold standard treatment involves using a nerve autograft which has limited availability and results in donor site morbidity [5]. Shorter gaps can be bridged using hollow conduits or primary repair, and decellularized allograft tissue is also available [6]. The nerve autograft contains aligned Schwann cells which support and guide regenerating neurites from the proximal to the distal side of the repair site, and recreating this anisotropic 3D cellular architecture is the focus of much research in the area of peripheral nerve repair [7–9].

A range of approaches are available for achieving anisotropic engineered tissues including the use of aligned fibres or channels, patterned surfaces, electrical and magnetic fields, mechanical loading and gradients of physical and chemical cues to organize engrafted and/or infiltrating cells. Promising recent approaches that have been used to generate anisotropic cellular substrates to support nerve regeneration in experimental nerve injury models include the use of Schwann cell-seeded aligned fibres made from synthetic polymers [10], Schwann cells seeded within longitudinally porous cross linked collagen scaffolds [11], differentiated adipose-derived stem cells seeded within decellularised nerve tissue [12], neural stem cells aligned on the luminal surface of patterned polylactide tubes [13], and strips of Schwann cell-seeded poly-3-hydroxybutyrate [14]. In all these cases the anisotropic structure was achieved through the use of a pre-aligned scaffold into which cells were seeded.

In an alternative approach to the conventional tissue engineering paradigm of using a structured scaffold to confer alignment on cells, we previously reported how self-alignment of Schwann cells could be achieved within a tethered collagen hydrogel [15].

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This method exploited the natural ability of cells and ECM to form anisotropic 3D structures in response to endogenous (cell-generated) tension [3]. However, in the fully hydrated state such cellular hydrogels require continuous tethering to maintain their anisotropic structure, limiting their potential for use in clinical repair. Here we report an effective solution to this problem in which cellular self-alignment within a tethered collagen gel can be stabilized using plastic compression. Plastic compression involves the rapid removal of fluid from collagen gels [16] and has been adopted as a means to form tissue-like constructs for a range of repair scenarios [17–21]. By using this approach to stabilise self-aligned cell-seeded collagen gels we have developed a powerful new method to engineer anisotropic cellular biomaterials. The technology avoids the limitations associated with seeding cells into preformed scaffolds, uses native collagen rather than synthetic materials, and achieves a robust stable structure without the use of chemical cross-linking agents. In the study reported here we have focused on incorporating Schwann cells within the aligned material in order to form engineered neural tissue (EngNT) constructs that could potentially be used in peripheral nerve repair. It is clear however that, with suitable alternative cells, a similar approach could be adopted for the fabrication of a wide range of tissues.

Here we describe the methodology used to generate sheets of EngNT, and the characterisation of the cellular and extracellular structure of the material. Furthermore, the ability of EngNT to support and guide neuronal growth both *in vitro* and *in vivo* has been demonstrated.

2. Materials and methods

2.1. Fabrication of Schwann cell EngNT

Schwann cells were aligned within tethered collagen gels in rectangular stainless steel moulds according to methods described previously [3,22], before stabilisation by plastic compression as shown in Fig. 1. Schwann cells were from the rat Schwann cell line SCL 4.1/F7 (Health Protection Agency, UK) and were maintained in culture medium (Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with penicillin and streptomycin (100U/ml and 100 mg/ml, respectively; Sigma) and 10% v/v foetal calf serum) in standard cell culture flasks. To prepare gels, 1 volume of 10× minimum essential medium (Sigma) was mixed with 8 volumes of type I rat tail collagen (2 mg/ml in 0.6% acetic acid; First Link, UK) and the mixture neutralised using sodium hydroxide before addition of 1 volume of Schwann cell suspension (final density 4×10^6 cells per ml of gel). One ml of this mixture was added to each mould at 4 °C and integrated with tethering mesh at opposite ends before setting at 37 °C for 10 min. Tethered gels were immersed in culture medium and incubated at 37 °C in a humidified incubator with 5% CO_2/95% air for 24 h to allow alignment to develop. Aligned cellular gels were stabilised by plastic compression, which has been described previously for unaligned cellular gels [3,16]. The plastic compression parameters used here were selected to ensure stabilisation was rapid, sufficient to retain cellular alignment in the absence of tethering, and caused minimal cell death. Aligned tethered gels were separated from the tethering mesh using a scalpel, placed on an absorbent paper pad and immediately compressed by loading the gel with 120 g for 1 min during which time fluid was absorbed by the paper pad underneath. The resulting sheets of EngNT were either transferred directly to 24-well plates for *in vitro* experiments, or rolled to form rods (approximately 200 μm diameter × 15 mm length), according to the spiral assembly protocol reported previously for unaligned plastic compressed gels [16] and maintained in culture medium for up to 24 h prior to *in vivo* experiments.

2.2. Scanning electron microscopy (SEM)

EngNT sheets were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 24 h at 4°C. These were post-fixed in 1% osmium tetroxide in phosphate buffer (PB), dehydrated through a graded series of acetone, infiltrated in liquid carbon dioxide in a critical point drying apparatus (Polaron, UK) before drying at the critical point of 31 °C. The dried samples were mounted on aluminium SEM stubs with double-sided carbon sticky tabs (Agar Scientific, UK), sputter coated with gold (Polaron sputter coater SC7640, UK) and examined in a Zeiss Supra 55 VP FEGSEM at 3 kV.

2.3. Transmission electron microscopy (TEM)

After excision and dissection of the middle of the repair constructs, samples were fixed in 4% paraformaldehyde in PBS for 24 h at 4 °C. These were post-fixed in 1% osmium tetroxide in PB, dehydrated through a graded series of acetone, flat-embedded in Epon epoxy resin and polymerized at 60 °C for 48 h. Semi-thin sections of 1 μm were cut using a glass knife on a UCT ultra microtome (Leica, UK), dried onto a poly-L-lysine coated microscope slides and stained with 1% toluidine blue with added 5% sodium borate. Ultrathin sections of 70 nm were cut with a diamond knife (Diatome, UK) and collected on copper slot grids with Pioloform/carbon support films. Sections were counter-stained with aqueous uranyl acetate and Reynolds' lead citrate before examination in a JEM 1400 TEM (JEOL, UK).

2.4. Assessment of EngNT in co-culture with neurons

All experimental procedures involving animals were conducted in accordance with the UK Animals (Scientific Procedures) Act (1986) and approved by the Open University animal ethics advisory group. Dissociated dorsal root ganglion (DRG) neurons were prepared from adult (200–300 g) Sprague Dawley rats. DRGs were incubated in collagenase (0.125%; Sigma) for 1.5 h at 37 °C then dissociated by trituration and washed twice with 20 ml of culture medium before being incubated for 18 h with cytosine arabinoside (0.01 mM) to deplete glia. The resulting cultures contained (>99%) neurons, which were seeded onto the surface of EngNT sheets, allowed to settle for 30 min, then constructs were immersed in culture medium at 37 °C in a humidified incubator with 5% CO_2/95% air. After 3 days the EngNT-neuron co-cultures were washed briefly in PBS and fixed in 4% paraformaldehyde at 4 °C for 24 h, then immunofluorescence staining was carried out as described previously for collagen gels [23,24], to detect βIII-tubulin positive neurons and S100 positive Schwann cells.

2.5. Surgical repair of rat sciatic nerve

Sprague Dawley rats were deeply anesthetised by inhalation of isoflurane (rats were from a colony that express a β-actin-green fluorescent protein reporter, though this marker was not used in the present study). The left sciatic nerve of each animal was exposed at mid-thigh and transected, then either a repair conduit or a nerve graft was positioned between the stumps to produce an inter-stump distance of 5 or 15 mm. Conduits or grafts were retained in place using three 10/0 epineurial sutures at each stump, then wounds were closed in layers and animals were allowed to recover for 4 or 8 weeks. Two experiments were conducted using the rat sciatic nerve model:

1. A 4-week experiment to assess two different ways to incorporate EngNT into a repair device ($n = 12$, 225–300 g rats) included three groups (4 rats in each) and used a 5 mm inter-stump distance with a NeuraWrap™ (Integra, US) sheath containing either (A) two EngNT rods, (B) two sheets of EngNT, and (C) empty conduit. NeuraWrap was cut to a length of 8 mm to allow a 1.5 mm overlap with each nerve stump, rods or sheets were positioned between the stumps, then the NeuraWrap was closed using 10/0 sutures along the seam. For group (B) the two EngNT sheets were used to line the central 5 mm section of the NeuraWrap prior to closure.
2. An 8-week experiment tested the ability of EngNT rods to support neuronal regeneration across a 15 mm inter-stump distance ($n = 15$, 250–500 g rats) and included three groups (5 rats in each). This used a NeuraWrap sheath (18 mm) for groups (A) two EngNT rods and (B) empty conduit, or a 15 mm nerve graft (C) taken from a littermate culled using CO_2 asphyxiation.

For each experiment, animals were culled following the recovery period using CO_2 asphyxiation and repaired nerves were excised under a dissecting microscope. For experiment 1, the proximal part of the repair device was embedded in OCT, frozen and sectioned transversely (10 μm sections) using a cryostat. For experiment 2, the middle of the repair device was removed and prepared for TEM, and cryostat sections were prepared as above from the proximal and distal parts of the device and the nerve stumps. The transverse sections that were used for analysis were from positions 1 mm into the proximal and distal stumps, or 1 mm into the proximal and distal parts of the repair site, measured from the end of the nerve stump in each case.

Sections were immunostained using mouse monoclonal anti-200 kDa neurofilament to detect axons (1:1000, Covance, Princeton, NJ) and visualised with DyLight 488 horse anti-mouse immunoglobulin secondary antibody (1:200, Vector Laboratories, Burlingame, CA). Primary antibody was incubated overnight at 4 °C and secondary antibody was incubated at room temperature for 45 min.

2.6. Microscopy and image analysis

Confocal microscopy (Leica SP5) was used in the assessment of Schwann cell alignment in EngNT and Schwann cell and neurite alignment and neurite growth in the EngNT-neuron co-cultures. Six equivalent fields were analysed per gel using a standardised sampling protocol. Images were captured using a ×40 oil immersion lens, z-stacks were 20 μm with a step size of 1 μm. Image analysis was conducted using Volocity™ software (Perkin Elmer, Waltham, MA) running automated 3D image analysis protocols to measure the angle of Schwann cell alignment and neurite alignment in each field. Neurite length per mm^2 was measured by tracing all