Decellularized Grafts with Axially Aligned Channels for Peripheral Nerve Regeneration

Rukmani Sridharan^{1,2,3}, M.Sc. , Richard B. Reilly Ph.D.^{1,2,4} and Conor T. Buckley, Ph.D.^{1,2*}

¹ Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Ireland.

² School of Engineering, Trinity College Dublin, Ireland.

³ Tissue Engineering Research Group, Department of Anatomy, Royal College of Surgeons in Ireland, Dublin, Ireland

⁴ School of Medicine, Trinity College Dublin, Ireland

*Corresponding author: Conor T. Buckley

E-mail address: conor.buckley@tcd.ie

Address: Trinity Centre for Bioengineering

Trinity Biomedical Sciences Institute

Trinity College Dublin

Ireland

Telephone: +353-1-896-2061

Fax: +353-1-679-5554

Abbreviations:

CPD – Critical Point Drying

ECM – Extracellular matrix

FBS – Fetal Bovine Serum

H&E – Hematoxylin and Eosin

HYP – Hydroxyproline

MSC – Mesenchymal Stem Cell

NGC – Nerve Guidance Conduit

NGF – Nerve Growth Factor

PBS – Phosphate Buffered Saline

PCL – Poly Caprolactone

PDMS - Poly (dimethyl siloxane)

PFA - Para-formaldehyde

PGA – Poly Glycolic Acid

PNI – Peripheral nerve regeneration

PSR – Picrosirius Red

SEM – Scanning Electron Micrography

sGAG – Sulfated Glycosaminoglycans

Key words: peripheral nerve; decellularization; freeze-drying; porous; scaffold; axially aligned; channels

Abstract

At least 2 million people worldwide suffer annually from peripheral nerve injuries (PNI), with estimated costs of \$7 billion incurred due to paralysis alone. The current “gold” standard for treatment of PNI is the autograft, which poses disadvantages such as high fiscal cost, possible loss of sensation at donor site and the requirement of two surgeries. Allografts are viable alternatives; however, intensive immunosuppressive treatments are often necessary to prevent host rejection. For this reason, significant efforts have been made to remove cellular material from allografts. These decellularized nerve grafts perform better than other clinically available grafts but not as well as autografts; therefore, current research on these grafts includes the incorporation of additional components such as growth factors and cells to provide chemical guidance to regenerating axons. However, effective cellular and axonal penetration is not achieved due to the small pore size (5-10 μ m) of the decellularized grafts. The overall objective of this study was to induce axially aligned channels in decellularized nerve grafts to facilitate enhanced cell penetration. The specific aims of this study were to optimize a decellularization method to enhance cellular removal, to induce axially aligned pore formation in decellularized grafts through a novel unidirectional freeze drying method, to study the bulk mechanical properties of these modified decellularized grafts and to assess cell penetration into these grafts. To this end we modified an existing decellularization protocol to improve cellular removal while preserving matrix structure in rat sciatic nerve sections. Standard freeze drying and unidirectional freeze drying were employed to impart the necessary pore architecture, and our results suggest that unidirectional freezing is a pertinent modification to the freeze drying process to obtain axially aligned channels. These highly porous scaffolds obtained using unidirectional freeze-drying possessed similar tensile properties to native nerve tissue and exhibited enhanced cellular penetration after 14 days of culture when compared to non-freeze dried and standard freeze-dried scaffolds. The results of

this study not only highlight the importance of aligned pores of diameters $\sim 20\text{-}60\mu\text{m}$ on cellular infiltration, but also presents unidirectional freeze drying as a viable technique for producing this required architecture in decellularized nerves. To the best of our knowledge, this study represents the first attempt to manipulate the physical structure of decellularized nerves to enhance cell penetration which may serve as a basis for future peripheral nerve regenerative strategies using decellularized allografts.

1. Introduction

The current “gold” standard for treatment of peripheral nerve injuries (PNI), the autograft, poses several disadvantages such as high financial cost, morbidity at donor site and the requirement of two surgeries (Noble et al., 1998). Moreover, there is a frequent size mismatch of donor nerves and a low success rate of only 50%, with higher rates of failure for patients above 50 years and for defects longer than 7 cm (Lee and Wolfe, 2000). For the past two decades, several biomaterials have been developed to facilitate accelerated repair and better axon regeneration (Belkas et al., 2004; Biazar et al., 2010; Chang et al., 2007; Daly et al., 2012). Much progress has been made in identifying important characteristics necessary for success of a biomaterial as an autograft replacement and in understanding the interactions of growing axons within these biomaterials; however, the regenerative levels of the autograft remain unmatched and effective nerve regeneration is still an urgent clinical need (Gu et al., 2011; Rajaram et al., 2012).

Characteristics of an ideal nerve guidance conduit (NGC) include biocompatibility, biodegradability, semi-permeability, the existence of longitudinal channels to promote axon regeneration, the presence of necessary guidance cues from extracellular matrix (ECM)-like components in addition to long term storage and ease of handling/suturing (Deumens et al., 2010; Kehoe et al., 2012; Spivey et al., 2012). Although many natural (e.g. collagen) (Kemp

et al., 2009; Li et al., 1991) and synthetic (Polycaprolactone (PCL), chitosan, polyglycolic acid (PGA)) (Jansen et al., 2004; Lee et al., 2012; Meek and Jansen, 2009; Yao et al., 2009) conduits have been developed in an attempt to replace the requirement for autografts, functional recovery that matches autograft levels is seldom observed (Kehoe et al., 2012). Several studies have reported that decellularized allografts have consistently performed better than other commercially available grafts, which is most likely due to these grafts possessing the necessary architecture and guidance cues from nerve ECM components to promote axon regeneration (Karabekmez et al., 2009; Kehoe et al., 2012; Whitlock et al., 2009). A majority of protocols that decellularize nerve grafts are based on those developed by Sondell et al. (Sondell et al., 1998) and Hudson et al. (Hudson et al., 2004b) that predominantly use chemical decellularization agents. It has been shown for other tissues that enzymatic treatment with nucleases (Crapo et al., 2011; Mangold et al., 2012; Yang et al., 2010) enhances removal of immunogenic components from decellularized nerves. Hence, our first aim was to test the effects of an optimized decellularization process using chemical and enzymatic reagents on the biochemical components and nerve architecture of rat sciatic nerve sections.

While decellularized grafts perform better than other commercially available grafts, the clinical requirement is for a graft that can perform better or equal to that of an autograft to enhance regenerative levels and reduce the time taken for complete functional recovery (Spivey et al., 2012). To fulfill this requirement, additional factors have been incorporated into decellularized grafts such as cells (eg. Schwann cells and Mesenchymal stem cells (MSC)) (Wang et al., 2008; Wang et al., 2012; Zhang et al., 2010), growth factors (Hagg et al., 1991; Li et al., 2012) and gels (Zhao et al., 2012). However, current decellularized grafts are limited due to the size of the basal laminal tubes (5-10 μ m), which are smaller than the typical diameter of cells (10-15 μ m). Moreover, while small endoneurial tubes are optimal for

protecting fully grown axons, they are less ideal for regenerating axons. It has also been previously shown in a rat model that motor grafts lead to higher nerve fiber count and better recovery than sensory grafts in part due to the larger diameter of endoneurial tubes in motor nerves (Moradzadeh et al., 2008).

Previous studies using different biomaterials such as alginate, chitosan and collagen have investigated the effects of subcellular topography on axon regeneration and shown that medium sized pores (20-60 μm) along the longitudinal direction were optimal for maximum axon penetration while allowing for minimum axon misdirection (Bozkurt et al., 2009; Pawar et al., 2011). Whether such large uniform channels can be formed in decellularized nerves has not been demonstrated to date. With these questions in mind, the overall objective of this study was to modify the physical structure of decellularized rat nerves by introducing large axially aligned channels to assist in enhanced cellular penetration. We hypothesized that unidirectional freezing (which has been established as a method to produce aligned pore structures in synthetic polymers and collagen (Bozkurt et al., 2009; Hu et al., 2009)) can induce the formation of such channels. Specifically, the aims of this work were to first optimize a decellularization method to enhance cellular removal, to induce the formation of axially aligned channels in these decellularized grafts using unidirectional freeze-drying, to characterize the formation of channels and bulk mechanical properties and finally to assess cell penetration into these grafts and compare them to decellularized grafts without channels. To the best of our knowledge this present work represents the first attempt to manipulate the physical structure of decellularized nerves containing large, axially aligned and open channels.

2. Materials & Methods

All reagents were purchased from Sigma Aldrich, Wicklow, Ireland unless specifically mentioned otherwise.

2.1 Study design

The study was divided into three sections. First, to assess the performance of combining enzyme and chemical treatments, the following groups were analyzed; Native, Buffer only, Enzyme only, Chemical only and the combination of enzymes, chemicals and a sterilization reagent termed the Decell group. These groups help identify the exact reagents facilitating cellular removal. Second, to identify the effect of unidirectional freezing (UFD), we analyzed four groups; Native, Decell, FD (nerves frozen parallel to freezing shelf), UFD (nerves frozen perpendicular to the freezing shelf using unidirectional freezing method) (Fig1). Finally, to verify higher cell penetration in UFD nerve segments, comparisons were made with Decell and FD groups.

2.2 Nerve isolation and decellularization process

Ethical approval was obtained from the institutional ethics committee at Trinity College Dublin. Hanover Wistar rats, aged 3-4 months old, were euthanized using carbon dioxide and/or cervical dislocation. Sciatic nerves were isolated, cleared of fat and blood vessels and preserved in phosphate buffered saline (PBS, pH 7.2) at 4°C (for a maximum of 24 hours) with 0.002% sodium azide until further use. Nerve segments were decellularized using a modified protocol adapted from Sondell et al. (Sondell et al., 1998). Briefly, nerve segments were washed in de-ionized water for 7 hours at room temperature. Segments were then treated overnight at room temperature with 3% Triton X-100 in de-ionized water, followed by a 24 hour agitation with 2% deoxycholic acid in 50 mM Trizma base buffer at pH 7. This treatment was then repeated with the two detergents, followed by an overnight incubation in DNase and RNase (both 5units/ml, Sigma-Aldrich, Ireland) in 10mM magnesium chloride ($MgCl_2$) buffer at 37°C. Decellularized nerves were then sterilized in 0.1% peracetic acid for 1 hour at room temperature and finally stored in PBS (pH 7.2) at 4°C until further use.

2.3 Biochemical analysis for DNA, proteoglycan and collagen content

Biochemical analysis was performed to analyze DNA, proteoglycan and collagen content. Samples were digested with papain (125 µg/ml) in 0.1 M sodium acetate, 5 mM l-cysteine HCl, 0.05 M EDTA, pH 6.0 at 60 °C and 10 r.p.m. for 36 hours. DNA content was quantified using the Hoechst Bisbenzimidazole 33258 dye assay, using a calf thymus DNA standard. Proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycans (sGAG) using the dimethylmethylene blue dye binding assay (Blyscan, Biocolor Ltd., UK), with a chondroitin sulphate standard. Total collagen content was determined by measuring the hydroxyproline content. Briefly, samples were mixed with 38% hydrochloric acid and incubated at 110 °C for 18 h to allow hydrolysis to occur. Thereafter samples were dried in a fume hood overnight and the sediments suspended in ultra-pure water. Chloramine-T and 4-(dimethylamino)benzaldehyde were added and hydroxyproline content was quantified with a trans-4-hydroxy-L-proline (Fluka analytical) standard using a Synergy™ HT (BioTek Instruments Inc.) multi-detection microplate reader at a wavelength of 570 nm. Each biochemical constituent was normalized to the tissue dry weight.

2.4 Histological characterisation

Samples for histology were fixed in 4% Paraformaldehyde (PFA) for 2 hours followed by several washes in PBS for 24 hours. Fixed samples were dehydrated in a graded series of ethanol, embedded in paraffin wax, sectioned to 8µm, and affixed to microscope slides. Sections were stained with Hematoxylin and Eosin (H&E) to visualize cells and Picrosirius red (PSR) to visualize collagen distribution and structure.

2.5 Freeze drying to impart porosity and axially aligned channelled structure

To impart porosity and pore structure into decellularized nerves, segments were placed either parallel (FD) or perpendicular (UFD) to the freeze-dryer plate (Fig 1, step 2). For unidirectional freezing, the samples were placed perpendicular to the base and insulated using Poly (dimethyl siloxane) (PDMS) molds to allow longitudinal ice crystal formation (Zhang et al., 2005). Briefly, samples were placed on a stainless steel base in the freeze dryer (Labconco TriadTM, Kansas City, MO USA) and frozen at a constant cooling rate of 1°C/min to a final temperature of -30°C. After a 1 hour hold period, a vacuum cycle was initiated and allowed to stabilize to a final pressure of 200mTorr. Next, a constant heating rate of 1°C/min was employed to reach a final temperature of -10°C for 24 hours until primary drying was complete. Finally, secondary drying was carried out for 5 hours at 20°C at the same pressure. The orientation of samples (as shown in step 2, Fig 1) in the freeze dryer determines the type of pore formation, with longitudinal channels only being formed when samples were sufficiently insulated on the sides and placed perpendicular to the conducting base (Step 2, Fig 1). Freeze-dried samples were stored in airtight containers at room temperature until further use.

2.6 Scanning Electron Microscopy (SEM) assessment and pore size analysis

Samples were fixed in 4% Paraformaldehyde (PFA) for 2 hours, washed in PBS for 24 hours and snap-frozen by immersion in liquid nitrogen. Fixed samples were dehydrated through successive graded ethanol baths (10 to 100%) followed by critical point drying (CPD) with CO₂. Samples were mounted in carbon cement and sputter coated with an approximate 10nm thick gold film, and examined by SEM (Tescan Mira FEG-SEM XMU, Libušina, Czech Republic) using a lens detector with a 5kV acceleration voltage at calibrated magnifications.

The diameter of pores (>250 pores for each experimental group) from transverse sections of nerve segments were analysed using ImageJ™.

2.7 Assessment of biomechanical tensile properties

Uniaxial tensile tests were performed using a Zwick tensile testing machine (Zwick Z005, Roell, Germany). Freeze dried samples were rehydrated in PBS for at least 24 hours before testing. Whole nerve segments (1.5-2cm in length) were mounted on custom grips fitted with sandpaper to prevent slippage and preconditioned using a 10% pre-strain and a 2% superimposed cyclical loading profile for 10 cycles followed by testing to failure at a rate of 2% strain/s using a 50N load cell. Video tracking was used to verify that samples did not fail at the grips. A minimum of 3 samples were tested for each group. The length of the samples was taken as the separation of the grips before the start of the test and the area was calculated by averaging 10 separate measurements of the diameter. Samples were excluded from analysis if the video tracking indicated failure at grips.

2.8 In vitro cell culture and characterization

To characterize *in vitro* cell penetration, a rat adrenal pheochromocytoma cell line (PC12) that is induced by Nerve Growth Factor (NGF) into a neuronal phenotype was utilized. PC12 cells (passage 11-18) were maintained in 175 cm² tissue culture flasks coated with 0.01% type I Bovine collagen (Gibco Biosciences, Ireland). High glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated horse serum (HIS), 5% Fetal Bovine Serum (FBS), with penicillin (100U/ml)-streptomycin (100µg/ml) (all GIBCO, Invitrogen, Ireland) and amphotericin B (0.25µg/mL; Sigma-Aldrich, Ireland). Cells were cultured in an incubator at 37°C and 5% CO₂, and media was changed every two days.

Freeze dried decellularized scaffolds were seeded on one side with 2.5µl of cell suspension at a concentration of 120 million cells/ml in 24 well plates (BD Biosciences, Ireland). After 30 minutes, another 2.5µl of cell suspension was added to the same side of the decellularized scaffold. Finally after 1 hour, 1 ml of media was added to the wells and placed in an incubator at 37°C and 5% CO₂. After 24 hours, fresh media containing 100ng/ml Nerve Growth Factor (NGF) was added to each well. This was considered to be Day 0, and seeded scaffolds were cultured for 1, 7, and 14 days to analyze viability and penetration behavior at different time points.

2.9 Quantification of cell number and depth penetration

ImageJ (Ver 1.46r) was used to quantify the number of cells per histological image for cell number analysis using the cell counter plugin, which was normalized per scaffold area (mm²). For penetration depth analysis, the penetration depth was calculated as the maximum distance from the cell-seeded edge of scaffold where cells were identified (µm).

2.10 Statistical analysis

Statistical analysis was performed using GraphPad Prismv4.0 software with 3-4 samples analyzed for each experimental group. One way ANOVA was used for analysis of variance with Tukey's post-tests to compare between groups. Significance was accepted at a level of $p < 0.05$. Numerical and graphical results are displayed as mean $\pm$ standard deviation.

3. Results

3.1 Improved decellularization using combined enzymatic and chemical treatment

DNA content of native nerve tissue was found to be 1288 ± 138.90 ng/mg with buffer treated samples having similar levels of DNA (1205 ± 361.9 ng/mg) (Fig 2A). Both enzyme and

chemical treatments led to a reduction of DNA content by half, with values of 761 ± 150.8 ng/mg and 668 ± 124.8 ng/mg respectively. Decell group exhibited the highest reduction (fivefold) in DNA content (257 ± 36.9 ng/mg).

In terms of sulphated glycosaminoglycan (sGAG) content expressed in micrograms (μg) of sGAG per mg of sample; Native, Buffer and Enzyme groups retained 21.5 ± 5.9 , 23 ± 5.3 and 19.2 ± 5.1 $\mu\text{g}/\text{mg}$ of sGAG respectively (Fig 2B), indicating that buffer and enzyme treatments do not significantly alter sGAG levels. However, Chemical and Decell groups exhibited a six fold reduction in sGAG content (3.49 ± 1.42 $\mu\text{g}/\text{mg}$ and 1.79 ± 1.17 $\mu\text{g}/\text{mg}$ respectively). Hydroxyproline (HYP) content is directly proportional to collagen content. HYP content of native nerve was found to be 42.4 ± 8.6 $\mu\text{g}/\text{mg}$ (Fig 2C). Buffer, Enzyme and Chemical groups exhibit similar HYP levels (45.3 ± 14.2 , 32 ± 6.6 and 36.6 ± 11.6 $\mu\text{g}/\text{mg}$ respectively). However, Decell group had a reduced HYP content of 19.7 ± 9.6 $\mu\text{g}/\text{mg}$, which was 50% lower than that of native tissue. Additionally, the decellularization process was found to induce a translucent nature to the physical appearance of treated nerve segments (Fig 2D).

Enhanced removal of cellular content by Decell treatment was confirmed through histological analysis (Fig 3). H&E and PSR staining of the native tissue (Fig 3A,B) demonstrates the presence of a tight network of parallel collagen fibers with the presence of cells throughout (H&E- ECM stains pink, cells stain blue; PSR – Collagen fibrils stain red). Buffer and Enzyme groups had minor ECM disruptions with cells still present in both groups (Fig 3C-F). Chemical treatment showed a high degree of cellular removal but also resulted in further disruption of ECM (Fig 3G, H). On combining the enzyme and chemical treatments (i.e. Decell group), there is no further disruption of the collagen matrix in comparison to Chemical group (Fig 3I, J). It was also observed that the Decell group contained the lowest number of cells (visual observation from Fig 3). In all treatment groups, the overall structure of the axially aligned collagen fibers was maintained.

3.2 Unidirectional freezing induces axially aligned channels in decellularized nerves

Transverse sections of Native, Decell, FD and UFD groups (Fig4A-D) revealed that native nerves contained circular endoneurial tubes; these became less circular in treated groups. While the pores were found to be of similar size for Native, Decell and FD groups, the pores in the UFD group were approximately ten times larger (Fig4D). Additionally, voids were introduced between the periphery and lumen of the Decell and FD groups (denoted by red arrows), which was not observed in the UFD group. Histological analysis of longitudinal sections suggested that native nerve exhibited an inherent wave-like collagen network (Fig 4E) which became less pronounced once decellularized (Fig 4F). In the FD group, elliptical pores were observed; whereas in the UFD group, open-ended, continuous pores were induced along the longitudinal direction (Fig 4H,I). Pore size analysis (Fig 4J) confirmed these observations. Average pore sizes of Native, Decell and FD groups were $5.42 \pm 1.83 \mu\text{m}$, $4.22 \pm 1.42 \mu\text{m}$ and $5.77 \pm 1.86 \mu\text{m}$ respectively, and not significantly different to each other. However, the UFD group had an average pore size of $40.3 \pm 23.8 \mu\text{m}$, and was significantly greater compared to the other groups ($p < 0.0001$). In addition, on comparing the frequency of occurrence of pore size - Native, Decell and FD groups exhibited an average pore size of approximately $5 \mu\text{m}$; while the UFD group contained a broader range with a majority of pores between $20\text{--}60 \mu\text{m}$ in diameter (Fig 4K).

3.3 Tensile properties of peripheral nerves are modified on decellularization and freeze drying

Biomechanical tensile properties were assessed using a 10% pre-strain and a 2% superimposed cyclical loading profile for 10 cycles followed by testing to failure at a rate of 2% strain/s as illustrated in Fig 5A. Upon decellularization, the diameter of samples

decreased from 1.49 ± 0.07 mm for Native to 0.84 ± 0.07 mm for Decell group (Fig5B). However, on undergoing unidirectional freezing (UFD), the diameter of the sample is somewhat restored to native nerve values (1.24 ± 0.15 mm) (Fig5B). The young's modulus of Decell nerve segments (46.03 ± 15.65 MPa) and FD segments (46.97 ± 2.984 MPa) were found to be approximately twofold higher compared to Native samples (21.23 ± 5.36 MPa) ($p < 0.05$). However, UFD nerve segments exhibited no significant difference compared to Native samples (Fig 5C). A similar trend was observed for ultimate stress (Fig 5D). Ultimate strain analysis revealed that while Native and Decell groups failed at strains of 42.4 ± 4.1 % and 43.2 ± 8.7 % respectively, both freeze dried groups (FD and UFD) failed at higher strains of 63.9 ± 9.3 % and 66.7 ± 9.1 % respectively (Fig 5E).

3.4 Freeze dried decellularized nerve segments support cell growth and enhanced cell penetration

Fig 6(A-I) represent H&E staining of Decell, FD and UFD groups on Day 1 (top), Day 7 (middle) and Day 14 (bottom) respectively. On day 1, few cells are present in all three groups, although it appears that more cells are present in the UFD group when compared to Decell or FD groups (Fig 6A-C). By day 7, cells appear to have proliferated in all three groups and while Decell and FD groups contained cells in the periphery, the UFD group contained cells in the lumen. Similarly, by day 14, it is clear that while cells remain confined to the periphery in Decell and FD groups (Fig 6G,H), the lumen of the UFD group was populated with migrating and proliferating cells and their morphology appears significantly different from other groups (Inset, Fig 6I). Cells appear rounded on day 1 and flattened by day 7 and 14, suggesting that they have adhered to the underlying substrate. A higher proportion of cells were observed in the UFD group in comparison to the FD and Decell groups. This was confirmed by semi-quantitative analysis of Cell Number/mm² of scaffolds

on Day 1 and Day 14 (Fig6J). On Day 1, Decell and FD groups had 106.8 ± 93.4 cells/mm² and 323.9 ± 161.7 cells/mm² respectively, while the UFD group had at least 5 times greater number of cells, with 1275.9 ± 1069.5 cells/mm². On Day 14, both FD and UFD groups showed statistically significant differences in cell number compared to the Decell group, although there was no significant difference between the FD and UFD groups. Similarly, on quantifying penetration depth of cells into scaffolds at day 1 and day 14 (Fig6K), it was observed that UFD group exhibited a significantly higher penetration depth ($50.0 \pm 18.5 \mu\text{m}$) compared to Decell ($16.8 \pm 6.4 \mu\text{m}$) and FD ($27.1 \pm 3.1 \mu\text{m}$) groups. On Day 14, the Decell and FD groups had similar penetration depths ($33.2 \pm 2.8 \mu\text{m}$ and $36.9 \pm 2.4 \mu\text{m}$ respectively) whereas the UFD group had 5 times higher penetration depth at $172.3 \pm 108.9 \mu\text{m}$. However, statistical significance was not achieved between the groups on Day 14 due to the small sample size (n=3) and variability in the UFD group. However, it is clear from the qualitative images and the semi-quantitative representations that a clear trend exists towards UFD exhibiting larger number of cells and higher penetration depths compared to Decell and FD groups.

4. Discussion

Limitations of the autograft for treating PNI have motivated the development of alternative biomaterial and tissue engineered replacements (Biazar et al., 2010; Daly et al., 2012; de Ruiter et al., 2009; Evans, 2001; Nectow et al., 2012; Schmidt and Leach, 2003). Recent reviews discuss the importance of subcellular topography for proper axon infiltration and extension (Spivey et al., 2012), and the necessity of certain ECM components such as collagen and laminin for sustained axon re-growth (Chen and Strickland, 2003; Gao et al., 2013). While many tissue engineering approaches use natural biomaterials such as collagen (Bozkurt et al., 2009) and chitosan (Hsu et al., 2013) to synthesize conduits, decellularized

allografts may offer a more appropriate substrate and architecture by presenting the diverse ECM components and necessary topography that are present in a peripheral nerve.

In addition to presenting natural nerve ECM, decellularized allografts also provide a guided substratum for promoting axon growth, as the lack of cells and myelin sheath creates available space for the axons to develop through the graft. However, previous studies report that the diameter of endoneurial tubes remains unchanged after decellularization (Hudson et al., 2004a; Sondell et al., 1998). These studies also report that such allografts do not perform better than the autograft. While it is important to demonstrate that decellularization does not affect structural parameters, we hypothesized that the small size of endoneurial tubes does not permit efficient penetration of cells. This is supported by studies on several substrates (PDMS (Goldner et al., 2006; Schmalenberg and Uhrich, 2005), chitosan (Hu et al., 2009), collagen (Bozkurt et al., 2009)) that report a pore size of 20-60 μ m to be optimal for maximum axon penetration with minimum axon misdirection. Furthermore, a direct comparison between decellularized sensory and motor nerves resulted in better regeneration and higher nerve fiber count through motor nerves, primarily due to the larger size of endoneurial tubes in motor compared to sensory nerves (Moradzadeh et al., 2008).

In this study, we first modified a previously existing decellularization method that has been employed by many groups for nerve decellularization. A fivefold reduction in DNA content and eradication of sGAG content (which has been implicated in stunting axon regeneration (Groves et al., 2005; Hattori et al., 2008; Neubauer et al., 2007)) was observed. We have conclusively shown through biochemical and histological assays that the combination of enzyme and chemical treatments perform better than either stand-alone treatment. While the DNA content of Decell group is still higher than industrial standards (Crapo et al., 2011), we believe further optimization of the protocol will reduce the immunogenic DNA content. Furthermore, although a 50% reduction in collagen content was observed in Decell group

compared to Native group, we verified through histology that this did not affect the appearance, density and continuity of collagen nerve fibers. Unpublished data confirms that the reduction in collagen content occurs as a result of peracetic acid treatment; hence we are currently exploring other sterilization methods such as gamma irradiation. It should be noted that the objective of the study was not to optimize a decellularization method for commercial use. It is merely to decellularized nerves with the objective of inducing axially aligned channels of required diameter in such decellularized nerves through unidirectional freezing. The unidirectional freeze-drying method employed in this work verified the formation of open-ended channels of ideal diameter (20-60 μ m) that extended the entire length of nerve segments. It is interesting to note that in the FD group that did not undergo unidirectional freezing, elliptical pores of approximately 100 μ m in diameter are formed in the longitudinal direction, parallel to the collagen fiber network. However, in the context of axonal penetration, as these pores are discontinuous, they may act as a barrier to axon growth. We therefore propose that unidirectional freezing is a crucial precursor to freeze-drying to induce the formation of continuous axially aligned channels.

Interestingly, the decellularization process had an effect on the overall mechanical properties, resulting in stiffness values twice that of native tissue. This is possibly due to the wave-like characteristic of collagen fibers normally observed in native nerve (which leads to an overall banded appearance called bands of fontana) which is lost upon decellularization. Also, as observed in Fig5B, the diameter of samples is considerably lower upon decellularization, which is consistent with differences reported for other soft tissues (eg. arterial tissues) (Sheridan et al., 2013). Previous reports of biomechanical testing of decellularized nerve tissue have not observed such pronounced differences in stiffness values (Ma et al., 2011; Zhao et al., 2012), although a trend towards increased stiffness does exist post decellularization treatment. Surprisingly, while the FD group does not show any changes

in stiffness compared to Decell group, the UFD group exhibited stiffness values similar to native nerve. In addition, it was observed that due to the unidirectional freezing process, the diameter of decellularized nerves was restored to values similar to native nerve. This is possibly due to the introduction of axially aligned channels in the UFD group, which provides additional space for the samples to swell when rehydrated. This could in part explain the similarity in stiffness values found for native tissue segments.

As the UFD group contained larger open channels compared to other groups, we anticipated higher cell penetration into this group. This was confirmed through in vitro cell experiments. UFD and FD nerve segments contained significantly more cells than the Decell group on day 14; and although UFD group had 10 times more cells than the FD group, statistical significance could not be achieved due to variability in the data. However, it is clear from the qualitative images and the semi-quantitative analysis that the UFD group contained higher number of cells at all timepoints analyzed, compared to FD and Decell groups. A clear trend is also observed in penetration depth of scaffolds, with the UFD group exhibiting higher penetration depths on both Day 1 and Day 14. This is in agreement with previous studies that reported increased cell penetration in nerves with larger pores (Pawar et al., 2011). Whether the pore structure could be further manipulated or altered by modifying the freezing process or regime warrants further investigation. Although several studies have utilized liquid nitrogen to quench samples (Bozkurt et al., 2007; Bozkurt et al., 2009; Hu et al., 2009), we found that a shelf temperature of -30°C was sufficient to produce the required pore size of 20-60 μm . A limitation of this work is the choice of PC12 cells used in this study. Although they help determine effects in terms of cellular viability and cell penetration, the use of Dorsal Root Ganglion (DRG) cultures would help provide further information regarding axon penetration and misdirection.

Taken together, this proof-of-concept study identifies the importance of pore size of decellularized nerves on cellular penetration. The novelty of this work is the finding that unidirectional freeze drying is a beneficial precursor in the process to impart large pore structures of the required diameter, which in turn leads to higher cellular penetration. The incorporation of axially aligned channels in decellularized nerve segments may hence serve as a basis for future peripheral nerve regenerative strategies using decellularized allografts.

5. Conclusions

To the best of our knowledge, this study represents the first attempt to manipulate and physically enlarge the pore size of decellularized nerves to enhance cellular penetration. This study emphasizes the beneficial effects of unidirectional freezing as a precursor to the freeze-drying process for the introduction of axially aligned channels in peripheral nerves. One of the main advantages of this approach is its low processing time, which can be implemented with existing freeze-drying methodologies already in use for decellularized commercial products. We expect that continued studies with modified decellularized allografts will advance our understanding of nerve regeneration and will aid in the development of novel strategies to replace the autograft.

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Figures Legends

Fig 1: Overview of experimental design and process to create axially aligned porous structure in decellularized nerve segments. **STEP 1:** involves decellularization of fresh nerve segments to form a Decell nerve. **STEP 2:** Decell nerves are then subjected to a freeze-drying process by placing segments either parallel (FD) or perpendicular to the cooling shelf thereby facilitating unidirectional freeze drying (UFD) to impart porosity and control pore orientation. **STEP 3:** In vitro experiments were performed on Decell, FD and UFD samples to evaluate the effect of pore formation and orientation on cell penetration and growth.

Fig 2: Effect of decellularization treatments on the biochemical composition of nerve segments. (A) DNA content (ng/mg) (B) Glycosaminoglycan (GAG) content (μg/mg) (C) Hydroxyproline (HYP) content (μg/mg) (D) Physical appearance of Native (top) and Decell (bottom) groups. *,! indicates significance compared to all other groups ($p < 0.05$). # indicates significance compared to Native and Buffer groups ($p < 0.05$).

Fig 3: Histological evaluation of decellularization treatments compared to native nerve tissue. H&E stain for cell nuclei (Left) and PSR stain for collagen (Right). **(A, B)** Native

nerve (C, D) Buffer group (E, F) Enzyme group (G, H) Chemical group (I, J) Decell group.

Arrow heads indicate cells. Scale bar = 100 μm .

Fig 4: Pore size assessment and analysis. (A-D) SEM of cross section and (E-H) longitudinal histological sections of nerve segments (A, E) Native, (B, F) Decell, (C, G) FD and (D, H) UFD. Scale bar = 100 μm . (I) longitudinal section showing continuous channels in UFD nerve segments. Scale bar = 500 μm . (J) mean pore diameter (μm) of four groups (Native, Decell, FD and UFD) and (K) frequency distribution of pore sizes (μm). * indicates significance compared to all other groups ($p < 0.0001$). Red arrows highlight the void spaces introduced between periphery and lumen of Decell and FD scaffold. Number of pores analysed > 250 for all groups.

Fig 5: Assessment of biomechanical tensile properties (A) Illustration of tensile testing regime of 10 cyclical pre-loading strains followed by testing to failure (B) Cross sectional diameter of different groups (mm) (C) Young's modulus (MPa) (D) Ultimate stress (MPa) (E) Ultimate strain (%). * indicates significance compared to Native and UFD groups ($p < 0.05$). ! indicates significance compared to Decell and FD groups ($p < 0.05$). & indicates significant difference compared to Native and Decell groups ($p < 0.05$).

Fig 6: Cell number and penetration depth assessment of Decell, FD and UFD nerve segments post seeding with PC12 cells. H&E stain: Pink-ECM, Blue – Cells; **Top**– Day 1, **Middle**–Day 7, **Bottom**–Day 14. Decell, FD and UFD scaffolds on Day 1 (**A, B, C**) Day 7 (**D, E, F**) and Day 14 (**G, H, I**). Black arrows point to cells in lumen. Scale bar - 100μm. Scale bar inset - 20μm. Red arrows highlight cells in the peripheral region. (**J**) Quantification of Cell Number/mm². * indicates significant difference of Decell group to FD and UFD groups at Day 14 (p<0.05). (**K**) Penetration Depth (μm) from edge of scaffold where cells were seeded. * indicates significant difference between UFD and Decell groups (p<0.05).

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