Heterogeneous scaffold designs for selective neural regeneration

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HETEROGENEOUS SCAFFOLD DESIGNS FOR SELECTIVE NEURAL REGENERATION

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DISSERTATION

to obtain
the degree of doctor at the University of Twente,
on the authority of the rector magnificus,
prof.dr. H. Brinksma,
on account of the decision of the graduation committee,
to be publicly defended
on April 24th, 2014 at 14:45

by

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Born on April 18th, 1980
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Chapter 1

General Introduction: Strategies to Create Biomimetic Microarchitectures for Peripheral Nerve Regeneration

Introduction

The use of tissue-engineered scaffolds to improve or control the regeneration of the peripheral nervous system (PNS) has been an area of extensive research for the past 30 years. The PNS has an intrinsic capacity to regenerate, such that minor damage can often be repaired spontaneously. However, if the damage is too extensive, with major disruption of the native extracellular matrix (ECM) structure, functional recovery becomes severely compromised or impossible[1]. The clinical 'golden standard' for repairing such PNS injury is the insertion of an autograft (a piece of the patients' own nerve) within the gap between the transected ends of a severed nerve. This provides a similar ECM environment conducive to regeneration, but also requires additional surgery to harvest a nerve segment from a 'less critical' region of the patient. To circumvent the use of an autograft, alternative tissue engineering strategies have been developed to improve regeneration to optimal levels typically observed in nominal cases. This has prompted the development of tissue engineered synthetic scaffolds in treating nerve injury.

The nervous system of the human body is traditionally separated into the central nervous system (CNS), comprised of the brain and spinal cord, and the PNS, comprised of a network of neurons that connect the CNS to the body. In most basic terms, the CNS is responsible for processing and integrating incoming information, decision making and, subsequently, initiating actions. Meanwhile the PNS is comprised of afferent neurons, which relay sensory information from the body back to the CNS (afferent signals), and efferent neurons, which transmit signals from the CNS to limbs and organs (efferent signals).

While not the focus of this work, CNS regeneration is recognized as a related area of research in the development of repair strategies: many recent reviews describe advances for the restoration of brain function after disease or trauma[2–6] as well as repair after spinal cord injury (SCI)[7–13]. SCI repair, in particular, has garnered much interest as an example of the CNS with a lower capacity for regeneration compared to the PNS. This is attributed primarily to the post-injury glial scar formation at the site of SCI, creating an inhibitory microenvironment of ECM components that physically and chemically prevent the successful regeneration of

CNS neurons. The complex array of biological signals involved still have to be fully explored and, as such, the therapeutic application of tissue engineering solutions has had limited success.

In contrast, the PNS exhibits a higher regenerative capacity and has been more amenable to therapeutic intervention. Scaffold structures placed between the gap of a transected nerve have been shown to enable recovery from otherwise debilitating PNS injury. To this end, the basic tube-like nerve guidance conduits (NGCs) are a clinical success as an alternative to the autograft. However, despite the encouraging results of NGCs, there are still limitations to the extent of successful recovery from PNS injury; the reader is directed to Daly *et al.*[14] and Gu *et al.*[15] for thorough reviews of NGCs. Whereas an NGC is essentially a hollow lumen, the ECM of both the natural PNS environment and the autograft exhibit a macroarchitecture and microenvironments conducive to guided neurite regeneration, which are far beyond the current available NGCs. Recent developments aim to produce a scaffold that recreates the native ECM, presenting appropriate chemical and physical cues in a spatiotemporally relevant manner to improve cell response.

Here, we describe tissue-engineering strategies for optimized PNS regeneration, focusing in particular on recent developments in the design and fabrication of physical cues and structural elements of interluminal guidance scaffolds. After establishing the parameters of an ideal synthetic ECM, current strategies are discussed according to two main categories: (i) non-patterned luminal fillers, supporting cell growth via intrinsic material properties, and (ii) patterned interluminal scaffolds, designed with a higher degree of complex organization. A brief summary of other promising design elements is briefly provided, such as the incorporation of diffusible growth factors or cell adhesion peptides, followed by a prospective on future design considerations and potential applications.

The Peripheral Nervous System

PNS Natural State

The axons of the PNS can be broadly classified as: efferent, transmitting signals from the brain to the body; and afferent, relaying signals from the body to the brain. In addition, the nervous system can be categorized according to general function. The efferent and afferent neurons of the autonomic nervous system govern homeostasis, as well as other functions over which we can exert no conscious or explicit control, whereas the somatic nervous system pertains to aspects of which we are aware, including sensory feedback, such as touch and heat, and volitional control of skeletal muscles.

Figure 1. Schematic view of the anatomy of the peripheral nerve (reproduced from 27).

In its natural state, the peripheral nerve is comprised of a multi-level organization of tissues, involving different ECM components and different cell types. The anatomy of a peripheral nerve is shown in Figure 1. Axons myelinated by Schwann cells (SCs) are grouped into fascicles supported by a collagenous *endoneurium*. Each fascicle is delineated by a *perineurium* sheath, a perineural epithelial cell layer serving as a blood-nerve barrier[16], and individual fascicles are bound into one nerve trunk by the *epineurium*[17] and the outermost *mesoneurium*[16]. The immediate endoneurial environment of these axons is comprised of both type I and type III collagen fibrils[18,19], as well as laminin molecules. These ECM components are

organized to surround the axons to form endoneurial tubes, characterized by a basal lamina of aligned collagen fibers produced by myelinating SCs[20,21]. Tube dimension correlates to the size of resident axons, ranging in diameter from 2 to 20 µm[22]. The interstitial spaces between endoneurial tubes are filled with hydroscopic glycosaminoglycans (GAGs) and also contain fibroblasts[23,24]. Macroscopically, endoneurial tubes are homogeneously interspersed within fascicles and the residing axons occupy approximately 50% of the overall cross-sectional area of the endoneurium[25,26].

Neural Regeneration

Peripheral nerve injuries (PNI) are a large-scale problem[14] and their functional recovery is still a clinical challenge[28], mainly because of the high complexity of the nervous system anatomy and physiology[29]. Damage to the nervous system, caused by mechanical, thermal, chemical, or ischemic factors, can impair various functions[30]. PNI most commonly results from blunt trauma (nerve crush) or from penetrating projectiles such as bullets or other objects, but it is also associated with fractures and fracture-dislocations[17].

A PNI results in the interruption of communication between nerve cell bodies and their targets[30].This results in Wallerian degeneration that is characterized by the disintegration of axoplasmic microtubules and neurofilaments[31]. Then, activated macrophages migrate into the degenerating nerve stumps and phagocytose the disintegrating nerve fibres and myelin. This is a critical event that increases the potential for nerve regeneration because myelin-associated glycoprotein present in PNS myelin inhibits peripheral axon regeneration[32]. Successful PNS regeneration occurs only after myelin is cleared and myelin-specific proteins are downregulated by SCs[33]. The SCs proliferate in response to myelin debris and macrophage-derived cytokines, and form longitudinal bands (bands of Büngner) as they divide and remain within the basal-lamina-lined endoneurial tubes[34]. These bands containing oriented arrays of SCs are believed to help in the process of regeneration[35].

In order to assess the progress and relative success of peripheral nerve repair, grading systems have been developed that correlate the cellular changes occurring after nerve injury to state and symptoms experienced by a patient. The most widely used assessment regimes are shown in Figure 2, developed by Seddon[36] and Sunderland[37].

Regeneration of severed axons requires that the parent cell bodies survive the initial trauma. Injuries closer to the spinal cord or brainstem are more likely to cause neuronal death and are therefore more complex than an injury at the distal periphery[17].

Figure 2. *System of classification of peripheral nerve injury (reproduced from* 38*).*

The structural and functional recovery of the PNS from injury has been shown to depend upon a variety of factors[39]. There are elements that influence the intrinsic ability of the neurons to regenerate, while external variables include the local environment at the site of injury[29]. Comparisons between the PNS and the CNS revealed that the CNS lacks several factors that are presented within the PNS. This includes the presence of SCs, which act to myelinate regenerating axons after

neuronal axotomy and synthesize growth-promoting molecules and growth factors[40] to provide nutrient support and chemoattractant guidance. As well, SCs reorganize within the endoneurial tubes to form the bands of Büngner to provide neurites with additional guidance[35].

A "regenerating unit" is formed by the sprouting of myelinated and unmyelinated fibers at the injury site where the axons are still intact[41]. This sprouting requires the elongation of the axon that is mediated by the axonal growth cone, an actin-based structure that extends into and senses the immediate environment in order to guide the axon to its target[31]. Growth cones are guided to their targets by a combination of contact-mediated (haptotactic) and diffusible (chemotactic) cues[42]. The axons that do not reach the target are withdrawn and result in reduced nerve function[30].

For nerve gaps less than 5 mm, spontaneous natural regeneration may occur. For nerve defects less than 4 cm, the application of simple hollow nerve guidance conduits (NGC) has been shown to enhance regeneration. Despite advances in peripheral nerve repair, the ideal solution to correct larger nerve defects has yet to be discovered and treatment remains dependent on the use of autografts or allografts[43].

Autografts and Allografts

Owing to the profound impact of PNS damage, many strategies have been developed and others are being investigated to facilitate axonal reinnervation and to direct axonal outgrowth[29]. Bridging systems are used in an attempt to provide guidance cues, similar to the bands of Büngner, that are able to offer an excellent support for orientated axon regeneration. When nerve endings are unable to be joined without tension, the crushed section of the nerve is cut, removed and replaced by a nerve taken from a less important site of the same patient, typically the sural nerve (Figure 3)[44]. Despite all the advances in the area, the use of nerve autografts still remains the clinical gold standard in bridging nerve injury gaps[30].

Nerve autografting has inherent flaws, but up to now no other conduit has proven to be superior. A second surgery is required in order to retrieve the donor tissue[17] and length and/or diameter of the autologous nerve may be insufficient for

effective reconstruction[45]. Another major shortcoming of this technique is the fact that endoneurial tubes can never be exactly re-approximated, resulting in a mismatch of regenerated axons. This leads to inappropriate and incomplete re-innervation, and subsequent poor recovery in function[46]. Finally, autograft use is currently limited to a critical nerve gap of approximately 5 cm in length. Beyond this distance, the use of an allograft is required due to the reasons mentioned above[14].

Figure 3. *Schematic representation of an autologous nerve graft. The alignment of the fascicles is critical for successful regeneration of peripheral nerves (reproduced from* 47*).*

An allograft is a decellularized nerve segment which has been harvested from a cadaver. Decellularization is a process that removes or destructs the cells present in a tissue, preventing the strong immunogenic response that implantation of the tissue would cause in the host while still preserving the structural elements (ECM components) from the donor. In this way, the 3D structure and composition of the native nerves is maintained[48]. Several studies have revealed that the additional removal of axonal growth-inhibitory molecules (e.g. chondroitin sulfate) from the basal lamina could potentially increase the regenerative efficacy of acellular allogeneic nerve grafts[49,50].

The resulting cell-free tissues can be used to prepare nerve grafts, an alternative to autologous nerve grafts, especially in the repair of non-critical peripheral nerve gaps with a small length and diameter[51–53]. Decellularized allografts using a

protocol involving detergents and chondroitinases have already been used in the clinic for the repair of facial nerve defects in humans[54]. Successful outcomes were also observed for hand nerve defects up to 3 cm in length[55]. However, cadaveric nerve allografts can be rapidly rejected unless the patient receives an adequate immunosuppression treatment that minimizes or prevents the rejection whilst permitting nerve regeneration[51].

Ideal Design of a Tissue Engineered Nerve Graft

Research has focused on the development of a synthetic alternative to auto- or allografting. This work has fallen under the purview of tissue engineering, an interdisciplinary field that applies the principles of engineering and life sciences. Through the development of biocompatible materials and advances in material processing and fabrication technologies, research in this rapidly growing field has produced polymeric structures and incorporated biomolecules to create bioactive scaffolds capable of restoring, maintaining or improving tissue function[56]. This provides a promising framework to pursue alternative strategies to autografts, providing innovative approaches to promote and guide peripheral nerve regeneration[15]. As this work has evolved, a number of ideal design requirements have emerged as guiding principles for continued nerve graft development.

In order to further improve synthetic nerve grafts, different issues are being studied, such as variations in material design and in the fulfilment of a number of criteria. An ideal graft should[14]:

a) direct axon growth from the proximal to the distal nerve stump;
b) obviate the infiltration of fibrous scar tissue, but allow diffusion of nutrients into the conduit and waste products to exit the conduit;
c) present adequate mechanical properties for structural support for the regeneration of nerve fibres;
d) create an optimal microenvironment for nerve regeneration;
e) exhibit low immune response[31,44,57,58].

While an autograft intrinsically satisfies all of these criteria, an ideal neural scaffold has to at least satisfy biological and physicochemical requirements such as

biocompatibility and biodegradability. These requirements should be fulfilled to eliminate the need of a secondary surgery, to prevent chronic inflammation, as well as the pain caused by nerve compression due to scaffold collapse[59,60]. A scaffold should also present an adequate permeability[61,62] and have suitable surface properties[59,63]. Ultimately, a synthetic nerve guide should exhibit performance that surpasses the autologous nerve graft in order to be considered clinically viable and relevant.

Figure 4. Graph of the probability of successful reinnervation with nerve guides in rats and mice. It is considerably reduced once an injury gap reaches a certain value. The critical gap length is the length where successful regeneration may occur 50% of the time (reproduced from 29*).*

Nerve Conduits

Today, the use of hollow NGCs is a clinically approved alternative to autograft repair[14]. These hollow tubes are used to bridge a nerve defect gap, with the proximal and distal ends of a transected nerve fixed into the respective ends of the tube. For small "subcritical" injury gaps (around 3–10 mm), NGCs are the current clinical approach and have shown successful results to the level of the autograft[17]. The use of hollow NGCs is currently limited to a critical nerve gap of approximately 4 cm[59]. Beyond this size, deficient levels of regeneration have been observed. This is usually attributed to an inadequate formation of ECM components during the initial stages of regeneration[63,64]. Without the formation of a fibrin cable, there is a limited migration of native SCs into the site of the lesion and consequently a reduction in the formation of glial bands of Büngner[64–66].

Despite these apparent shortcomings, the development of NGCs has been actively pursued due to advantages over the autograft. NGCs have shown improved alignment of nerve fascicles[31] and obviate the need of a second surgery[30]. Because they present a more controlled and enclosed environment[14], the penetration of fibrous scar tissue is minimized and, conversely, there is a local accumulation of factors produced by the nerve stumps that help the process of regeneration[34].

In the process of developing strategies, the critical gap lengths have been determined depending on the nerve and the animal model used in the study (Table 1). As shown in Figure 4, the gap length directly determines the successful regeneration (%N) of hollow silicone NGCs in rats and mice. Evaluation of studies performed with a defect size at or below the critical length must compare the quality and speed of the regenerative process with respect to an already robust response of the NGC. Defects larger than the critical size represent a more challenging condition, where any degree of regenerative growth is an improvement over the typical failure of an NGC. A tissue scaffold that promotes regeneration under these circumstances has potential to be clinically relevant and, thus, should also be assessed in comparison to an autologous graft to determine its relative performance.

Table 1. *Critical Size Defects*

Animal	Nerve	Defect size	Type of NGC	Reference
Human	Digital	3 cm	PGA tube	71
Monkey	Ulnar	3 cm	Pseudosynovial tube PGA tube	72,73
Rabbit	Peroneal	3 cm	Vein conduit	74
Rat	Sciatic	1 cm	Silicone conduit	75
Mouse	Sciatic	0.5 cm	Silicone conduit	29

A number of physical alterations to NGC fabrication have been performed in an attempt to improve regenerative capacity, including the enhancement of the porosity of the luminal wall[67] and the use of different materials, both natural and

synthetic[43,68,69]. For a complete review of NGC design and materials used in their fabrication, readers are directed to reviews by Gu *et al.*[15] and Nectow *et al.*[70].

Considerable efforts have been made in the development of NGCs with internal lumen architecture which recapitulates the hierarchical organization and biological function of the native ECM[64]. The introduction of such matrices within nerve guides increases the critical gap length that one can bridge to varying degrees, an important step in creating successful therapeutic strategies. This current review focuses on techniques used in the fabrication of structured interlumen fillers. A brief overview of previous luminal filler strategies provides design considerations, followed by an assessment of current approaches and promising methods that showed or may prove to show potential in creating an ideal, synthetic neuroregenerative scaffold.

Assessing Nerve Regeneration: a Discretionary Note
The reader's attention is brought to potential, sometimes subtle, discrepancies between studies that can make it difficult to draw comparison between different strategies for PNI repair. As mentioned in the previous section, the aspect of gap length is extremely important when evaluating the response of an NGC or scaffold. The most frequent animals models discussed in the following studies are mouse and rat, each with a critical gap length of approximately 5 mm and 10 mm, respectively. Studies that employ gap lengths above this critical distance are the more challenging scenario and are considered more clinically relevant. While studies involving defects at or below the critical size can be informative, the reader is cautioned about the interpretation and significance of resulting stemming from these studies. In addition, some studies did not include both a hollow NGC and an autograft controls to establish negative and positive baseline for neuroregenerative capacity. The authors reported the performance of these studies while explicitly stating the limitations of the experimental design.

Furthermore, methods to assess peripheral nerve regeneration are not uniformly applied, again impeding one's ability to make direct comparisons. The most common methods are outlined by Vleggeert-Lamkamp[76], where it is also stressed that a proper evaluation of PNI recovery requires a combination of methodologies.

Histological examination often reports the number of myelinated axons, the axon diameters, and the thickness of the myelin sheath enveloping the axon, where larger values for all indicate a strong neuroregenerative response. To examine the electrophysiological performance of regenerate nerves, measurements of the velocity and amplitude of evoked compound muscle action potentials (CMAPs) are performed. This provides a means of assessing the functional capacity of regenerated nerves and is a robust functional measure of PNI recovery. Additionally, return of motive function can be measured by assessing the walking gait of recovering animals, while sensory recovery can be determined via the response to noxious stimuli (i.e. the 'pinch test'). In discussing the reported results, authors confined general PNI recovery to pertain specifically to histological assessment. The assessment of electrophysiological function or motive function, if performed, is otherwise explicitly described.

Isotropic Hydrogel Interlumen Fillers

Hydrogels are attractive biomaterials for nerve regeneration mainly because of their physical properties that are similar to native tissues, allowing the creation of a 3D environment that better mimics the biological properties[77]. Hydrogels have the ability to swell in water and retain a significant fraction of water within their structure, mimicking the hydrated ECM microenvironment. The mechanical environment with which cells may interact can affect their viability and behaviour. Thus, the use of a hydrogel with specific mechanical and physical features, including stiffness, porosity, overall architecture, and degradability can affect the function or subsistence of a hydrogel in a tissue (Figure 5)[78].Neurite outgrowth through dense hydrogels require neurites to remodel the local hydrogel environment[79]. For this to occur efficiently, sufficient cell adhesion[80] and cleaving sites to facilitate matrix remodeling is required[81–83]. Both of these elements are not intrinsic to synthetic hydrogels and must be incorporated[84,85]. Natural hydrogels based on endogenous proteins often have the benefit of having both adhesion sites and can be remodeled [86]. Exogenous protein hydrogels or synthetic hydrogels do not necessarily meet these requirements and must be modified. Considering the low frequency of application in peripheral nerve injury studies, a full treatment of both exogenous and synthetic hydrogels is beyond the scope of this current discussion. Readers are directed to recent reviews[85,86].

Figure 5. *Sample parameters for selected hydrogel properties as a function of time (t). Compressive modulus (solid line) is a measure of the hydrogel strength and increases as the hydrogel polymerizes. During polymerization, cross-links form between monomers, increasing the cross-link density (dotted line). The number of cross-links declines as the bonds are hydrolysed or cleaved enzymatically during degradation. This also results in a decrease of compressive modulus. The time to degradation (t) is dependent on the chemical composition of the hydrogel and physical properties, such as the incorporation of pores. In most hydrogels, the compressive modulus remains constant during the time between polymerization and degradation (reproduced from* 78*).*

Early work towards recreating a suitable interlumen relied on bulk filling NGCs with hydrogels based on proteins naturally occurring within the PNS ECM. Fibrin is also present after neural injury and during subsequent regeneration[87] and has subsequently also been employed as luminal filling. Outlined below is the incorporation of these bulk materials in an isotropic, architecturally non-patterned form of hydrogels, fibers and sponges.

Fibrin

When an entubulation strategy is applied after PNI, a protein gel forms within the NGC from endogeneous proteins produced by the transected ends of the nerve[87]. Provided that the nerve gap is not beyond the critical length and that regeneration is sufficiently expedient, neurite regeneration occurs through this matrix. However, in the case that the gap is beyond the critical length, regeneration fails[88]. A major component of the ECM that fills the NGC at this initial stage is fibrin. Both neuronal and non-neuronal cells are known to invade and remodel this environment, depositing other constituent ECM proteins such as collagen and laminin[89,90].

As such, fibrin has been trialed as an interlumen material by prefilling the conduit with fibrin in lieu of endogenous filling. A series of earlier experiments by Williams *et al*. found that presenting the regenerative nerve with such a gel-filled lumen had generally positive effects compared to an empty tube[91]; this was performed for only a 10 mm gap defect in rat sciatic nerve, just at the critical gap length for this animal model. It was observed from this work that performance was dependent on the gelation and composition of the fibrin matrix, with innervation increasing with lower matrix density and increased longitudinal arrangement of polymerized fibrin fibers; these factors were later confirmed *in vitro*[92,93]. Under these experimental conditions, a low-density fibrin matrix with sufficiently ordered fibers proved to be superior to an empty NGC. However, when evaluated for a gap length of 13 mm, neural outgrowth in fibrin-filled NGCs was comparable to that of hollow nerve guides. Yet, performance was markedly worse compared to the standard autograft[94].

ECM Hydrogels

To further enhance the success of gel-based luminal fillers, different combinations of natural ECM proteins have been trialed. Initial reports by Madison *et al*. describe the use of laminin/collagen-based fillers – an early, non-commercial form of Matrigel[95]. This was first applied to optic nerve regeneration in rabbits[96] followed by a 5 mm PNI defect in mice. Initial results showed improved regeneration over a two week period compared to a hollow tube[97], though a follow up study revealed that long-term growth (6 weeks) was impeded[98].

Valentini *et al*. later confirmed this using different concentrations of either Matrigel and collagen-only gels for a 5 mm defect in the same animal model over a 12 week period[99]. The conclusion reached was that the degree of long term regeneration was compromised by the presence of dense bulk luminal fillers, attributed to the physical impediment posed by the hydrogel itself. This early work depicts a scenario of neurite regeneration across gap lengths below the critical defect size where a solid hydrogel filler promotes initial neurite outgrowth, but inevitably impedes regeneration compared to the more permissive environment of a hollow tube.

However, this differs once gap lengths above the critical defect size are examined. Additional work examined neurite regeneration over a 16 weeks period, comparing Matrigel and collagen fillers to hollow NGCs for a supracritical defect gap of 15 - 20 mm in rats[100]. It was found that all tubes without filler experienced no growth, whereas tubes with a gel lumen (either Matrigel or collagen) experienced regeneration. This was later corroborated by a study which examined the effects of sub- and supra critical gap lengths in mice (4 mm or 6 mm), comparing regenerative growth over a 5 week period with lumen fillers consisting of various hydrogel compositions and densities[101]. For subcritical lengths, the lowest collagen and laminin concentrations performed best, surpassing the standard fibrin gel fillers, hyaluronate gel (GAG), and even the hollow NGC. This confirmed that presenting an ECM-like environment is beneficial for neurite regeneration, provided it is adequately permissive to innervation and cellular invasion. Supracritical lengths were then examined, focusing on diluted gel compositions. In this context, the laminin-containing Matrigel proved to be slightly better than collagen, reciprocating *in vitro* findings[102,103]. Despite these observations of modulated neurite growth by natural ECM hydrogel fillers, a comparison revealed they were still outperformed by the 'gold standard' autograft[104,105].

Exogenous and Synthetic Hydrogels

A number of other potential hydrogel sources are available which are not based on endogenous proteins. These include exogenous gels, such as agarose, alginate or keratin, or synthetic hydrogels, such as those based on polyethylene glycol (PEG)[80,106–110]. Many of these materials are widely abundant, making them an attractive option compared to more scarce or expensive EMC proteins. Since these hydrogels do not exhibit cell adhesion proteins or other cell signaling motifs, they also represent a class of biocompatible materials whose properties can be systematically tuned and controllably modified with specific bioactive molecules to selectively stimulate cell behavior.

However, these hydrogels have yet to find widespread application as NGC luminal fillers for *in vivo* PNS injury studies. Those studies that have been performed support the conclusion that a less permissive environment presented by a bulk,

dense hydrogel filler is not conducive to peripheral regeneration. The performances of agarose or alginate fillers over supracritical gap lengths has been shown to be equivalent[111] or less[112], respectively, compared to a saline-filled NGC, requiring the incorporation of growth factors or additional adhesion proteins to improve regenerative performance. Even when applied, however, the performance of agarose fillers remains poor. This can be attributed to relatively poor cell adhesion compared to the native fibrin matrix, though outgrowth has also been shown to be inversely proportional to hydrogel density[113].

Keratin is another exogenous protein used for creating a hydrogel luminal filler, recently shown to be a promising candidate for subcritical PNI defect size in mice[108]. Long term evaluation shows equivalent functional recovery and increased neurite growth compared to both a saline-filled NGC and an autograft implant[110], though application to a supra-critical defect size is required to be able to fully assess the performance. Regardless, the availability of other promising hydrogel substrates, as well as the ability to design and specifically tailor synthetic materials for an intended application, holds great potential for the field of peripheral nerve repair and remains an exciting and developing area of research.

The Role of Hydrogel Fibril Alignment
In line with earlier observations of improved neurite growth resulting from longitudinal fibril formation in fibrin gels, aligned fibrils were induced within collagen gels and laminin gels (Figure 6) to investigate this effect for this class of ECM proteins[114,115]. Examining both sub- and supra- critical gap lengths (4 mm and 6 mm) in mice, aligned collagen was found to produce a notable improvement in the extent of PNI recovery over non-aligned collagen and saline-filled conduits. Over a gap length of 6 mm, magnetically aligned collagen and laminin hydrogels also resulted in improved functional recovery compared to non-aligned gels. However, it was noted that the extent of recovery, though an improvement over non-aligned gels and unfilled NGCs, was still relatively low. Regeneration was further impeded when hydrogels were more heavily crosslinked[114], further emphasizing the importance of a permissive environment for neurite growth.

The general consensus of existing literature suggests that the presence of a hydrogel filler is beneficial for nerve defects larger than the critical gap length, although the regenerative capacity is still lower that the standard autologous graft. There are also indications that the density and structural organization of the hydrogel fibrils directly influences regenerative performance. Lower density environments and those with aligned fibrils for structural guidance showed improved neurite extension, suggesting reducing the impediment of invading tissue and introducing topographical cues are important design considerations for 3D environments created to enhance PNI recovery.

Figure 6. Magnetically aligned hydrogels. Fibrin hydrogels, made with fluorescently labelled fibrinogen, were imaged with confocal laser scanning microscopy. (A) Isotropic fibrin gels showed no directional alignment, whereas (B) fibrin gels exposed to a 9.4 Tesla magnetic field were unidirectionally aligned. (C) As seen under bright field microscopy, neurite outgrowth from dorsal root ganglions (DRGs) was highly directional in magnetically aligned fibrin. Oriented neurite outgrowth from DRGs also occurred in (D) aligned collagen gels, compared to (E) isotropic collagen gels; in (D) and (E), neurites are stained green, SCs are stained red and co-localized neurites and SC appear yellow. (Adapted from 35,92).

Fibrous Interluminal Fillers

Alternative methods for providing internal structure within the lumen of an NGC have also been trialed, focused on providing a more permissive environment for neurite and cellular infiltration. Taking inspiration from *in vivo* observations of the PNS ECM and from previously mentioned work on aligned fibrils within

hydrogels, many groups have introduced longitudinal guidance structures within the lumen in the form of aligned fibers. These environments are typically more open and permissive compared to hydrogels, however the approaches outlined in this section simply introduced fibrous structure as a filler. No additional control is exacted over fiber placement or the resulting macrostructure, with modulation of neurite growth induced by the presence, but not the explicit organization, of the residing fibers.

Synthetic Fibers and Filaments

One of the first reports of aligned fibers within an NGC was in 1946 by Weiss and Taylor[116]. Using a variety of materials, parallel fibers approximately 30 mm in diameter were placed within a tube and held in place by coagulated blood (similar to fibrin gel). These were then sutured within a 20 mm defect in a cat animal model. Initial observations found that the fibers tended to cluster, obstructing cell invasion and growth; furthermore, the mechanical mismatch and micromotion of the stiffer materials also led to failed regeneration. To overcome these issues, softer fibers of cellulose or Nylon were used and surgically fixed to the nerve tissue to prevent relative motion. This resulted in successful regeneration and functional recovery, although it was found that in some cases fibers were clustered, with an eccentric displacement. This was accompanied by limited cell infiltration, indicating that regions of high fiber density still had the capacity to occlude regeneration. Some materials resulted in fibrotic tissue surrounded the fibers, within which no axons or SCs could be found.

Nevertheless, regeneration was well supported and exhibited improved aligned neurite growth compared to a hollow tube filled with blood alone. This initial attempt highlights the promise of this technique, but also the limitations in terms of appropriate material selection and maintenance of fiber organization.

The advent of new biomaterials and advances in biofabrication prompted a revisit to this approach, initially with the introduction of relatively large polyimide filaments (250 μm in diameter) within the NGC lumen[117]. This was originally motivated as a means of both directing cell infiltration and migration and for stabilizing endogenous fibrin matrix formation. Observations from the regeneration

of a subcritical 10 mm defect in a rat found that fast resorbing fiber types improved the degree of regeneration[118], attributed to macrophage activation from the degradation of the filaments[119]. The most promising among the materials used was catgut, which experienced both the maximal nerve regrowth and also the most degradation and macrophage response. Furthermore, the presence of interluminal filaments for a supracritical gap defect (15 mm in rat) resulted in observable regeneration compared to none for a saline-filled conduit. Interestingly, this more challenging regenerative environment also abolished any observable differences between filaments types: simply the introduction of such interluminal structure proved to be sufficient to promote regeneration[120].

Attempts to optimize filament packing density found that neurite growth was impeded when too many fibers were included[121]. Packing densities ranging from 1.7% to 24% were examined (respective open space available ranging from 98.3% to 76% of the original NGC area). Not only did more filaments occupy more space, but also large areas were taken up by *de novo* connective tissue formed around the large fibers to further occlude neurite growth. Of note, in this study catgut filaments displayed the poorest performance in this context, where a high filament packing density combined with the previously mentioned macrophage response resulted in the merging of newly formed connective tissue that was completely void of axon.

A later study employed smaller poly-L-lactide microfilaments (40 to 100 μm in diameter), again examining the role of packing density for different defect lengths[122]. This study confirmed that the presence of filaments was beneficial for sub- and supracritical gap lengths, compared to a saline-filled NGC. It was also found that a lower packing density of 3.8% was best for filaments of this scale; filaments were again encapsulated by macrophages and neurite growth appeared to be occluded by subsequent growth of connective tissue. In particular, increasing the number of filaments results in an increase in nerve cable diameter but a reduction in the number of actual myelinated axons. This underlines that such an increase in the number of larger scale filaments intensifies the macrophage response and produces a larger amount of consolidated fibrotic tissue, presenting a barrier to neural regeneration.

However, this work also highlighted the importance of filament organization, with some observed cases of the lowest packing density allowing filaments to rearrange and cluster. This was the attributed cause of failed regeneration in a few isolated cases. In a follow up study, filaments were stabilized within Matrigel, but this dense composite luminal filler produced inconclusive results[123].

Collagen Fibers and Filaments
Resorting to natural materials, collagen fibers of a similar diameter (100-150 μm) were also trialed by Itoh *et al.*[124], providing a comparison to collagen gel-filled NGC. The collagen filaments were prepared by mechanically elongating and air-drying columns of collagen gel. At a packing density of approximately 5% (95% open area), NGCs with collagen fibers were found to have successful regeneration compared to both saline and collagen filled conduits. The axons and SCs were found in the intercollagen filament spaces, with fibroblasts/macrophage in and around the collagen fibers; larger fibers or swelling was noted to be a concern for obstructing growth. A decrease in collagen fiber size (70 μm fibers) with an increase in packing density to 12.2 % also produced sufficient regeneration and functional recovery. This possibly suggests that the use of smaller filaments made of natural materials allows for a greater packing density and more effective PNS regeneration. However, the use of a subcritical 10 mm defect size in both studies precludes any form of comparison with previous studies. Yet, the reported bridging of an 80 mm gap in a dog model using collagen filament interluminal filler [125] suggests this approach is a worthwhile avenue of investigation.

Even smaller collagen fibers derived from bovine skin were employed, this time without the protective encapsulation of a conduit[126]. About 2000 collagen fibers, each 20 μm in diameter, were assembled to form a free-standing fibrous graft with an approximate packing density of 10%. Placed in a 20 mm long PNS defect in a rat, this approach produced a similar degree of outgrowth compared to an autologous implant, though many macrophages were present to degrade collagen fibers and neurites within the autograft were larger and more mature. Furthermore, no functional recovery was noted for either implant, attributed to the insufficient time for complete recovery over this 8-week study. A follow up study with a 30

mm defect over 12 weeks reported functional recovery, where the use of 4000 bundled fibers showed a slightly better muscle actuation than 2000 fibers. Although the lack of a conduit boundary makes an accurate estimate of packing density difficult, regeneration into the less dense scaffold (2000 fibers) resulted in fewer, more mature axons. In comparison, more axons with smaller diameters were observed in the denser scaffold (4000 fibers). Again, this suggests the importance of presenting an adequately and finely tuned permissive environment in order to elicit an optimal regenerative response.

Electrospun Fibers

Another versatile method to produce continuous fibres with diameters within the micron to sub-micron range is electrospinning (ESP)[127]. This is an electrostatically driven method where nanofibres are formed from a charged liquid polymer solution or melt that is ejected through a capillary tube towards a grounded collecting target[128]. This versatile technique can form fibres out of a wide range of materials, including natural and synthetic polymers, composites and ceramics[127]. Varying parameters such as solution viscosity and concentration, voltage, and feed rate, can control the nanofibre size. Adjusting the grounded collector can control fiber orientation [128]. For further details about how to control nanofibres morphology and alignment, as well as how to control scaffold architecture, one may refer to the works of Xie et al.[129], Chew et al.[130] and Murugan et al.[131].

ESP fibers of collagen represent a seemingly ideal cell substrate, mimicking the natural ECM in terms of adhesion motifs and dimension[132,133]. However, synthetic fibers can also be created from specifically synthesized polymers or with incorporation of additives. This provides the possibility to design fibers for specific biomedical applications, exacting control over fiber dimensions, mechanical and degradation properties, and allowing for the incorporation of specific cell adhesion motifs[134] or small molecules for controlled release[127].

ESP fibers represent a versatile platform for presenting neurons with nanotopographical cues, the effects of which are treated thoroughly by Spivey et al.[135]. A number of in vitro studies have assessed the response of neurons and supportive glial cells to fiber diameter, orientation, density and possible

functionalization[15,128,136–144]. A summary of relevant studies reveals that directed neurite outgrowth and SC migration can be induced with aligned fibers over a range of 1-5 μm in diameter. Relevant to earlier sections, studies have also implicated fibre density as a determining factor of neurite extension and SC migration *in vitro*[145,146].

Despite the extensive number of studies investigating neurite growth *in vitro* on ESP fibers, the application of electrospun fibers within the context of repairing peripheral nerve injury has mainly been restricted to the development and fabrication of the outer nerve guide conduits[127]. This may be attributed to the difficulty in the handling of delicate fiber constructs. However, a few recent studies have explored the possibility of implementing aligned, electrospun fibers as interluminal constructs to promote regeneration. Koh *et al.*[64] employed an electrospun filler of poly-(lactic-co-glycolic) acid (PLGA) nanofiber yarn, created in a manner similar to previous reports[147]. PLGA fibers from 200 to 500 nm were bundled together into yarn-like segments of 25 ± 5 mm in diameter, after which approximately 360 yarn segments were inserted into an NGC; this represents a packing density of approximately 10%. Although observations over a 12-week in a 10 mm defect in a rat found higher density of axon growth in the autograft control, functional recovery of the lumen-filled guide was found to be superior.

The Bellamkonda lab has also applied ESP fibers as interluminal fillers, inserting multiple thin films of 400 to 600 nm poly(acrylonitrile-co-methylacrylate) (PAN-MA) fibers, aligned or random, into an NGC. The initial study employed 10 to 12 sheets within an NGC, representing an approximate packing density of <9%, and applied these constructs to a 17 mm nerve defect in rats[142]. The sheets of aligned fibers were found to effectively support neurite growth to a similar degree compared to the autologous control implant. However, fiber-filled conduit exhibited a segmented growth with respect to the cross section of the regenerated nerve cable compared to both the autograft and normal nerve, with distinct regions of neural cells juxtaposed to regions of non-neuronal cells; in contrast, the normal nerve and the autograft displayed evenly distributed neuronal cells. Growth through both implants resulted in neuromuscular junction formation and a return of function, although recovery was slightly higher in the autograft.

A follow-up study by Clements *et al.*[148] investigated a similar platform, but reduced the number of ESP films. Furthermore, fiber films were integrated into the conduit to hold them in place, allowing for a more precise strategy for film placement and exacting a degree of control over the macroscopic arrangement of fibers within the lumen. Two arrangement of ESP films were trialed: a single film inserted into the NGC to, bisecting the cross-sectional area of the lumen into 2 semicircles (Figure 6A); and 3 films inserted to form a Z pattern across the face of the lumen, separating it into 4 distinct luminal regions (Figure 6B). These fibrous interluminal films occupied less than 0.6% of the NGC cross sectional area. Results showed that the 3-film conduit exhibited a larger cross sectional area of the regeneration nerve cable, accounted for by the increased interluminal surface area provided. However, the single film conduit experienced more axonal growth, with better self-organization of the nerve cable tissue and improved functional recovery. This suggests that the arrangement of additional electrospun sheets contributed to fragmented ingrowth of the regeneration nerve, with the thin films acting as a barrier to spontaneous tissue organization and resulting in reduced regenerative capacity. Unfortunately, drawing conclusions about the relative performance of these conduits is not possible due to a lack of autologous graft controls performed.

Figure 6. A cross-section of a nerve conduit with a single ESP film inserted (A), providing additional surface area and topographical guidance. Arrangements were also created where 3 films were inserted (B), forming a Z-like pattern to create four distinct interluminal regions (reproduced from 148).

These findings highlight that a construct which presents a highly permissive environment for tissue infiltration and, as well, exhibits instructive topographical cues may still prove detrimental to nerve regeneration. This suggests that the design of an interlumen filler and its macroscopic architecture must either create an environment which does not inhibit tissue self-assembly or more closely mimics the endoneurium as with the autologous nerve graft.

Structurally Patterned Interlumens
The previous sections have outlined how simply providing biologically relevant bulk fillers as in the case of hydrogel fillers, or basic structural elements as with fiber fillers, act to improve regeneration when compared to a non-filled NGC; this was particularly evident when gap defects over a critical size were treated. These methods employed bulk fillers that were conducive to neural tissue infiltration but relied on the ability of this invading tissue to remodel the environment to accommodate neural regeneration. However, performance of these fillers failed to match that of the autologous nerve graft both in the return of function and in the quality of the regenerated tissue.

Many groups have pursued the creation of a more organized interluminal structure over the past 35 years, resulting in many attempts to design and fabricate a synthetic scaffold with optimized structural architecture capable of enhancing PNS regeneration. There is a pervading theme in literature that the presence of an interluminal structure has the capacity to hinder growth if the macroscopic architecture is not sufficiently permissive or does not match the structure/function relationship reflected in the autograft and native PNS ECM. As described previously, channel-like structures within the endoneurium guide axons innervation. Ranging in diameter from approximately 2 to 20 μm[22] and delineated by longitudinally aligned collagen fibers, these channels create anisotropic porosity within the ECM that makes approximately 50% of endoneurium cross-sectional area open for neurite ingrowth[25,26]. Many techniques have been developed to design and create an organized interlumen, intended to either increase available open area for ingrowth in a controlled manner or to achieve the ultimate goal of approximating the endoneurial structure. The design ethos embodied in many of

these fabrication approaches focuses on the creation of continuous channels, or interconnect anisotropic pores, which provide passage of neurite growth from the proximal to the distal stump of a transected nerve.

While some techniques have been evaluated *in vivo*, others are still in stages of development. Outlined below are various methods cited as viable means of creating patterned and structurally relevant 3D scaffolds towards constructing nerve guidance conduits with an unprecedented degree of complexity. Broadly speaking these techniques have been classified according to: (i) template molding; (ii) phase separation; (iii) sacrificial molding; (iv) self assembly; and (v) direct writing.

Template Molding

A traditional approach to producing polymer scaffold with designed structure is the molding of a polymer melt or polymer solution over a negative mold template, followed by mold extraction to produce a polymer cast. For the creation of an anisotropic luminal filler, the mold is typically in the form of an array of parallel cables or wires to subsequently create a scaffold with a controlled arrangement of parallel channels. An example of this approach was first reported by Hadlock *et al.*[149,150], but has since been reported by many others[151–155].

The size of the channels, typically ranging from 60 to 500 μm, is restricted by the mechanical stability of the molding wires used. The typical size of these structures precludes any parallels drawn between the native endoneurium, with these constructs often deemed multilumen conduits instead. These are conceived to provide more surface area for ingrowing tissue and to allow for ingrowth to be organized into fascicular-like subunits. An initial study used PLGA injected into a mold at lower pressure to form a multilumen conduit with five 500 μm luminal channel guides, which showed promising tissue growth over a 7 mm defect in a rat[149], although functional recovery was not assessed.

A thorough study by Yao *et al.*[153] evaluated similar multilumen constructs made from collagen, implanted in a 10 mm defect in a rat. Comprised of either 2, 4 or 7 channels, with diameters of 530 μm, 530 μm or 410 μm, respectively, it was found

that the fascicle-like growth induced by the 4 channel multilumen graft exhibited larger axons and increased myelination compared to the commercially available single lumen graft; this was also reflected in slightly higher compound muscle action potentials, though the difference in improved functional recovery was not statistically significant. However, the autograft implant was found to have consistently higher number of myelinated axons and markedly better degree of functional recovery. The relatively poor performance of the multilumen conduits results could stem from the only 20% to 30% open area they present, which poses a conspicuous barrier to neural tissue infiltration.

A recently reported approach combines the use of template cables to form orderly microchannels within an electrospun fibrous matrix, intended to create a highly biomimetic interluminal structure for PNS recovery[156]. By depositing aligned electrospun fibers onto a planar array of parallel cables (either suture material or fishing line), the resulting sheet is then rolled into a column and the template cables are removed to form an architecture of through-channels framed by aligned nano-scale polymer fibers. The microchannels so far produced are approximately 130 μm in diameter, on the same scale as previous molding attempts. However, the channels closely approximate the observed structure of the endoneurium, although not in size, and channel density is greater compared to other molding methods, providing approximately 50% available open area for nerve ingrowth. Although the mechanical instability of the resulting thin ESP fiber walls may prove detrimental, no *in vivo* studies have yet been performed with this construct to assess clinical performance. Regardless, these initial reports represent a novel and promising scaffold fabrication strategy with high potential for creating an effective biomimetic interluminal filler.

Freeze Drying/Phase Separation
The freezing of a hydrogel solution and subsequent water sublimation has been a long standing and well described method for creating a porous foam structure from a hydrogel solution[157,158]. If the rate of ice formation within a hydrogel is sufficiently slow, the solute material of the hydrogel will be pushed out of the way and excluded from the advancing ice crystal. Once freezing is complete, the hydrogel solute is confined to the boundary regions between opposing ice crystals.

Upon sublimation, the solute solidifies in place, to form a foam with a honeycomb-like structure. By controlling general parameters of the freezing process, pore diameters ranging from 95 to 150 μm are possible[159].

This has been used to create isotropic collagen sponges to be used as interluminal fillers for PNS repair[25,160,161]. Although the pores lack directionality, histological analysis of implanted scaffolds revealed that their scale, structure and interconnected nature was able to induce successful innervation. Collagen sponges also resulted in slightly higher action potential and conduction velocities compared to interluminal collagen fibers, although this difference was not statistically significant[25].

Additional work has shown that the creation of pores with a bias direction and improved interconnectivity formation is possible via anisotropic ice crystal formation within a hydrogel, achieved by inducing a thermal gradient during the freezing process[158,162–165]. A study by Hu *et al.*[166] provided a clear comparison between collagen/chitosan grafts with either isotropic or anisotropic pores, implanted in a 15 mm defect in a rat model. The anisotropic pores created in this study were 34 μm on average, closely approximating both the structure and scale of the endoneurium. Regeneration through anisotropic grafts was markedly better than that observed for grafts with isotropic pores, with larger, more myelinated axons and action potentials with higher amplitudes and faster conduction velocities. Furthermore, the anisotropic graft approached the performance of the control autologous graft; although values were consistently lower than those of the control, no statistical differences were found for both the histological and functional analysis.

Similarly positive results have been produced with pure collagen interlumen with 50 μm pores[167], though a direct comparison between studies is not possible because functional recovery was not assessed. The autograft control again had slightly larger axons and thicker myelination. Scaffolds prepared to degrade faster managed to approach these values to a statistically similar degree, implying that invading neural tissue must still remodel the environment in order to achieve the performance of the autograft. Although promising, these scaffolds do not surpass

the performance of the autograft. This top-down approach is limited in the extent to which it can tailor the microenvironment: the reported channel size is still slightly larger than endogenous PNS structures and no suggestion of inducing fiber alignment within channels has been reported. However, these findings support the idea that a scaffold with a preformed biomimetic structure is an effective means of achieving peripheral nerve repair.

Sacrificial Molding

An alternative method of molding polymer scaffolds relies on the use of a sacrificial molding material, forming an interlumen architecture by dissolving away the mold after polymer casting. Assuming a polymer solution is used for the casting process, this requires the use of a multisolvent system whereby the solvent for the scaffold material is a non-solvent for the mold material and *vice versa*. Such a system was first reported by Flynn *et al.*[168], used to create 200 to 400 µm channels within poly(2-hydroxyethyl methacrylate) (pHEMA) hydrogels by selectively leaching out embedded PCL fibers with acetone. This produced scaffolds with up to 40% open area available for growth. Despite such constructs supported cell growth *in vitro*[169], no follow up *in vivo* study has been reported for this particular scaffold.

Other solvent systems have also been employed, though primarily for the development of scaffolds for spinal cord repair[170–172]. A parallel array of 200 µm diameter channels was formed within agarose via sacrificial removal of Polystyrene fibers using tetrahydrofuran. This created a scaffold with a 44% open area, throughout which successful growth was observed. Although the dimensions for these studies are still considerably larger than the 2-20 µm observed in the endoneurium, they provide working examples of possible solvent/non-solvent system suitable for this approach.

A recent report by Scott *et al.*[173] describes the use of a similar non-solvent/solvent system to create a likewise parallel array of channels ranging from 15 to 150 µm within a fibrin gel. Using either PMMA or cellulose acetate fibers formed by melt spinning, these embedded fibers were later removed using acetone. This represents the creation of microchannel structures that most closely approximate the

dimensions observed in native endoneurial tissue. Initial *in vitro* testing using DRG explants revealed that all ranges of channel sizes promoted a promising degree of ingrowth, though *in vivo* performance remains to be validated.

An innovative solvent/non-solvent system has relied on the use of sugars (also deemed carbohydrate glass) as the soluble template[174–176]. Various methods have been employed to embed the sacrificial cables to create the final polymer matrix. Initial approaches drew fibers from a caramelized sugar bath to produce sacrificial cables ranging in diameter from 8 to 200 μm[174,175], after which templates fibers were encapsulated within the polymer matrix. This has been achieved by dip coating fibers into a non-solvent polymer solution, such as PLLA dissolved in chloroform[174]. The sugar template from these coated fibers can be leached with distilled water to form standalone tubules or assembled into a parallel array of coated fibers and undergo leaching; these assembly of tubules present an estimated open area of approximately 80%. The *in vitro* work performed thus far shows these scaffolds promote neurite extension and guide SCs infiltration. The final arrays were also reported to be irregular in shape and the mechanical stability of these constructs was also not explicitly stated.

Alternatively, fibers have also been coated with polymer microspheres, with these fibers later assembled into a parallel array[176]. Applying pressure to this assembly causes the microspheres to merge, creating a single polymer matrix with embedded fibers. After leaching in distilled water, scaffolds have been produced with up to 40% of open area available for ingrowth. Though no PNS application has yet been trialed, this matrix has produced promising results *in vivo* for spinal cord repair studies.

Miller *et al.* optimized the melting and viscosity of a sugar melt (carbohydrate glass) to enable the 3D printing of a sacrificial lattice template via rapid prototyping[176]. The template was then dip-coated to obtain a thin PDLGA layer, providing an improved barrier between the polymer solution and the sacrificial layer. This allowed for the template to be embedded within a hydrogel solution (photocrosslinked PEGDA, fibrin or Matrigel) while retarding template dissolution in the aqueous solution. After embedding, the whole construct was embedded in

medium to dissolve the template out the ends of the created lattice, leaving behind a hydrogel construct with intact channels. The thin polymer barrier is also cited to prevent the diffusion of template fiber solute into the surrounding hydrogel matrix during the leaching process, instead of restricting the flow of dissolved sugar through the channels. This allows for cells to be embedded within the hydrogel during the template encapsulation process, where high concentrations of solute are avoided within the matrix and cell viability is thus maintained.

Originally developed for the *in vitro* study of angiogenesis, this technology can create complex architecture of channels with diameters down to 150 μm. Although no neural applications have yet been reported and the channel size is of the same scale as previously mentioned approaches, the ability to directly and precisely create sacrificial templates in this manner presents many promising possibilities.

Self-assembly
Relying on a completely different mode of fabrication, the development of a hydrogel system in which aligned, parallel microchannels are spontaneously self-assembled represents a highly novel method of creating an anisotropic tissue scaffold[177,178]. This approach relies on the non-convective diffusion of ions into an ion-crosslinkable polymer solution, with alginate being an often-used biocompatible candidate. This simple and unique approach produces microchannels in the range of 10 to 77 μm and an open area from 5% to 30%; channel dimensions can be tuned by controlling the ions used in the crosslinking process and eventual additives to the polymer solution[178]. Until now, *in vivo* studies focused on spinal cord repair[177], although *in vitro* studies have shown ingrowth of sensory neuron and SC invasion. However, the current fabrication process is limited in terms of controlling microchannel distribution and density, producing scaffolds that would pose a considerable physical barrier to the regeneration of PNS tissue.

Direct Writing
The methods of scaffold fabrication described so far require the creation of a molding template or rely on the spontaneous, uncontrolled formation of microchannels. More direct approaches exist for the controlled formation of a

microstructural pattern within a 3D matrix, such as multi-photon ablation[179–181] or stereolithography[182,183]. These methods allow for the direct fabrication of designed structures, though each is accompanied by possible limitation. Using a multi-photon beam, sufficient energy for material ablation is imparted only to the focal point of the incident laser, with microchannel dimensions limited by the laser wavelength and the point spread function of the focused radiation energy. In addition, this approach is limited to optically transparent materials susceptible to ablation. *In vitro* experiments have clearly shown the utility and flexibility of two-photon ablation in creating microstructure within a 3D culture environment, coaxing neurite outgrowth to follow ablated microchannels as small as 5 μm. Though the large scale production necessary for the creating of an *in vivo* implant remains an obstacle, new high throughput techniques are in development to make this method of fabrication a promising avenue for providing detailed design and fabrication of a microchannel matrix[184].

Stereolithography is another method of direct writing, suitable for large-scale fabrication of NGCs with interluminal structures. This approach rapidly cures a photocrosslinkable polymer solution, layer by layer, to form complex 3D shapes. Arcaute *et al.*[182] and Pateman *et al.*[183] have recently shown the utility of this technology for creating NGCs internal architecture. Channel sizes reported were on order of 300 to 400 μm in diameter, although the minimum dimension of fabricated structures is primarily determined by the resolution of the translation stages used. This approach is also restricted by the material choice to those that are photopolymerizable, the pending regulatory approval of which may pose a barrier to final clinical application. Continued development may make this a viable possibility for fabricating future scaffold with relevant feature sizes.

Discussion

In order for a technological solution to be implemented within a clinical setting, its performance must not only be effective but also surpass the currently accepted treatment methods. Owing to an intrinsic ability to recover, the PNS often does not require intervention. Even in many cases where small defects occur, the simplest approach is shown to be the most effective. This is evidenced by hollow NGCs shown to facilitate levels of recovery that match an autograft when applied to nerve

defects below the critical gap length. The application of further tissue engineered solutions for these cases can even have a detrimental impact on recovery, such as for luminal hydrogel fillers described above. The introduction of interluminal fillers for more extreme injuries with longer gap defects does show a benefit over the basic hollow NGC. However, the various metrics and analysis used to determine performance makes it difficult to directly compare different strategies. To be able to resolve the true impact of these approaches, attempts have been made to mathematically extrapolate past experimental outcomes into a universal model[185] and recent calls to establish standardized methods of evaluation for future studies[76]. Regardless, the general consensus of literature addressed in this review indicates that homogenous fillers are inadequate for enhancing PNS regeneration and require the incorporation of biomimetic microarchitecture in order to approach the recovery level achieved by the autograft.

Surpassing the performance of autografts will likely require NGCs to combine the implementation of biomimetic luminal fillers with other design elements and strategies not covered in this current review. One cannot overlook the importance of the housing NGC conduit, an essential component to most scaffold strategies. The outer NGC provides a mechanically stable platform within which to realize an enhanced neuroregenerative scaffold and facilitate the final surgical application. Although relatively basic compared to some of the scaffolds described, not all NGCs are equal. Tuning the properties of the NGC can have a significant effect on its regenerative capacity, including aspects such as mechanical stiffness, porosity, and biodegradability. For a concise overview of pertinent NGC design considerations, the reader is directed to Jiang *et al.*[43].

The incorporation and delivery of diffusible guidance cues, in the form of growth factors or other small molecules, is another strategy known to greatly enhance neurite growth. During PNS regeneration, distal SCs generate gradients of growth factors that attract and guide neurite extension[186]. The emulation of these gradients within an NGC have been shown to positively influence regeneration, with many different means of incorporating diffusible cues[187]. Other aspects of scaffold design include the incorporation of cell adhesion motifs as a means of improving and controlling regeneration. Many of the approaches described above employ natural

ECM materials as a means of promoting cell adhesion and innervation. Yet, the ability to create and incorporate specifically tailored cell adhesive peptides presents another avenue by which neurite growth can be stimulated and controlled.

The combination of all of these elements culminates towards an increasingly complex guidance scaffold. Extrapolating trends outlined here, it can be expected that PNI recovery will continue to improve as scaffolds approach the level of structural and biochemical heterogeneity observed in native tissue. As scaffolds become more competent, developments and discoveries may find applications in other fields, such as providing treatment options for neuropathologies, enabling SCI repair, or facilitating neural interface development to improve neuroprosthetic control. Optimization of constructs as they become more multi-faceted will require precise and systematic control over composition and structural organization. The approaches highlighted here provide an overview of current technological solution, and provide a context for future biofabrication development.

Scope and Outline of Thesis

The summary above provides a context for scaffold fabrication strategies developed in this thesis where we focused on the generation of biomimetic guidance scaffolds capable of spatial segregation of regenerating neurites based on different neural subtypes. This physical separation is intended to facilitate the targeted stimulation and recording of specific neural subpopulations towards improving the selectivity of regenerative neural interfaces. To this end, the role of nanotopographical guidance cues was investigated (Chapter 2) using nanoimprinted substrates. Moving towards a 3D culturing environment, electrospinning (ESP) was employed to create a scaffold of aligned nanofibers (Chapter 3). The conjugation of different bioactive molecules to the fiber surface was implemented and the response of Schwann cells to the bioactive compounds was assessed. Complex nanofibrous scaffolds were then fabricated via a newly developed tandem electrospinning technique (Chapter 4), capable of depositing two fiber populations in an overlapping, but divergent and spatially defined pattern. A means of selectively functionalizing these different fiber populations was then implemented (Chapter 5), with biased neurite growth demonstrated on a tandem electrospun scaffold. A highly biomimetic three-dimensional (3D)

hydrogel scaffold to investigate neurite growth was finally developed (Chapter 6). Representing the closest synthetic approximation of an endoneurial tube so far, this is shown to be a viable platform for neurite outgrowth from explanted Dorsal Root Ganglia, which can be easily translated to an *in vivo* implant. A final summary describes how these new fabrication approaches advance the field of PNS scaffold development and bring closer the realization of a highly functional regenerative neural interface (Chapter 7).

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Chapter 2

Nanotopography induced contact guidance of F11 cell line during neuronal differentiation: a neuronal model cell line for tissue scaffold development

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Abstract

The F11 hybridoma, a Dorsal Root Ganglion-derived cell line, was used to investigate the response of nociceptive sensory neurons to nanotopographical guidance cues. This establishes this cell line as a model of peripheral sensory neuron growth for tissue scaffold design. Cells were seeded on substrates of Cyclic Olefin Co-polymer (COC) films imprinted via nano imprint lithography (NIL) with a grating pattern of nano-scale grooves and ridges. Different ridge widths were employed to alter focal adhesion formation, thereby changing cell/substrate interaction. Differentiation was stimulated with Forskolin in culture medium consisting of either 1% or 10% Fetal Bovine Serum (FBS). Per medium condition, similar neurite alignment was achieved over the 4 day period, with the 1% serum condition exhibiting longer, more aligned neurites. Immunostaining for focal adhesions found the 1% FBS condition to also have fewer, less developed focal adhesions. The robust response of the F11 to guidance cues further builds on the utility of this cell line as a sensory neuron model, representing a useful tool to explore the design of regenerative guidance tissue scaffolds.

Introduction

This work introduces the F11 neuronal cell line as a suitable tool for the development of tissue scaffolds specific for the regeneration of the peripheral nervous system (PNS). The response of neurite growth to nanotopographical guidance cues is analyzed, towards a more effective scaffold design.

Over the past few decades, many studies have investigated the use of engineered synthetic tissue scaffolds for *in vivo* neural tissue repair and regeneration. These examples, though functional, often perform poorly with respect to the "Golden Standard" of the autograph[1–3]. Thus far, scaffold design relies on intuition and accumulated knowledge, which has led to increasingly complex increasingly complex, multicomponent scaffolds which incorporate structural guidance cues, chemical cues, and supporting glial cells[4–8]. While these works show great improvements, the trial-and-error approach that is still employed intrinsically limits neural repair to what is achievable instead of what is desired.

Producing effective and efficient tissue engineered systems requires an intelligent design approach for tissue scaffolds, with reliance on tools and methods to further understand and manipulate the biological systems in question. As neural regenerative investigations continue, a complex picture is emerging of neural pathfinding and cellular response to the extracellular components within the body, including growth factors[9], ligand-binding extracellular matrix (ECM) proteins[10], and mechanical properties of the ECM[11].

Particularly relevant to scaffold design is the role of mechanical cues and their effect on cellular differentiation, migration and guidance. Cascades of intracellular signals which dictate cell behavior can be influenced by surface properties of the materials employed, the presence of extracellular proteins and peptides, nanotopographical structures, and even the mechanical properties of the underlying scaffold[12–16]. While this provides a wealth of possibilities for attaining highly functional tissue scaffolds, this also presents a great challenge to understand which extracellular signals are relevant and how to best implement them.

In particular, the influence of micro- and nanotopography on neurite outgrowth has been explored under various conditions, both *in vitro*[17,18] and *in vivo*[19,20]. Many neuronal cell types have been investigated, including primary central nervous system (CNS) neurons, PNS neurons and neuronal secondary cell lines. The ability to direct neurite growth has been shown to depend on both the dimensions of the underlying patterned substrate and, importantly, the cell type[21–24]. In particular, the work of Raijnek et *al.*[22] highlights the importance of using a representative neuronal model: *xenopus* spinal neurons are shown to grow parallel to microstructure compared to rat hippocampal neurons, which grow perpendicular.

Cells explore their surroundings by extending filopodia and lamellipodia from the lamella at the cell periphery, probing the surrounding environment. Mechanical cell guidance is mediated via the mechanisms of cell adhesion, notably the formation and maturation of focal adhesion (FA) complexes. The reader is directed to recent reviews on this subject by Geiger *et al.*[25] and Scales *et al.*[26]. Initial cell adhesion begins with integrin transmembrane receptors binding to extracellular proteins, forming nascent FAs and an initial linkage to actin fibres of the

cytoskeletan. The actin fibers apply force to integrins and those that resist will coalesce to form FA complexes and, later, mature FAs. FAs which do not provide sufficient adhesion are disassembled.

New protrusions then extend from regions with successful FA formation, biasing the net direction of cell motion in that direction. Therefore, ability to influence focal adhesion formation allows control of cell shape and guide neurite outgrowth.

Previous findings from our group have confirmed an association between the development of focal adhesions, the underlying nanograting patterned substrate, and the resulting PC12 cell polarity and neurite outgrowth direction[23,27]. The grating is a repeating ridge-groove nanopattern such that focal adhesion formation is restricted and, depending on the ridge dimension, the FA can only expand in the direction of ridge orientation. This induced polarization imparts direction upon the cell, establishing a physiological basis for the observed influence of topographical guidance cues.

In applying this examination to the case of peripheral neuron regeneration, this current article represents the first time the F11 cell line has been used to study cell response to nanopatterned substrates. The F11 cell line is a hybridoma of a rat dorsal root ganglion (DRG) cell and a mouse neuroblastoma[28]. In contrast to a typical heterogeneous DRG populations comprised of different sensory neuron subtypes, the F11 cell line represents a homogeneous population, exhibiting markers and receptors of nociceptive sensory neurons lineage[29,30] and producing an electrical response to nociceptive stimuli[31,32]. Previously used as a model for differentiation[33], cytoskeletal reorganization and plasticity[34], and for studies of gene regulation during PNS regeneration[35], this work establishes the F11 as a functional DRG model for providing insight into mechanical guidance during nerve regeneration.

The response of F11 cells to nanograting patterns of various dimensions (see Figure 1) and under different medium compositions is explored. We highlight the biomolecular response in terms of cytoskeletal organization and focal adhesion formation, capitalizing on the utility of this cell line to assess the influence of

nanotopographical cues towards an improved tissue scaffold for the peripheral nervous system.

Figure 1. The dimensions of the nanograting patterned culturing substrate. A groove width of 500 nm (A) and groove depth of 300 nm (B) was used for all experiments. Different ridge widths (C) were employed to investigate the role of restricting focal adhesion formation on neurite guidance.

Materials and Methods

Cell Culturing

F11 hybridoma cells (a kind gift from Dr. Ratto, CNR, Pisa, Italy) are a fusion of a mouse dorsal root ganglion and a rat neuroblastoma. They were grown in Dulbecco's Modified Eagle Medium (DMEM) with the addition of 10% fetal bovine serum (FBS), penicillin (10 units/ml), streptomycin (10 mg/ml) and l-glutamine (2 mM)[32] in an incubator at 37°C and 5% CO_2. Cells were grown in standard tissue-culture treated dishes and medium was refreshed every 2 days until cell growth was confluent (approximately every 4 days), at which point cells were dissociated using EDTA-trypsin (0.05%) and passaged 1:15. Experiments used cells from passages 10 to 14. All culturing materials were purchased from Invitrogen unless otherwise stated.

Substrate Manufacturing

The substrate material used was cyclic olefin copolymer (COC) from Ibidi, a thermoplastic polymer with excellent optical properties subjected to a proprietary tissue-culture treatment to enhance cell adhesion. Nanotopography was introduced to the substrates via nano imprint lithography (NIL), as described previously[23].

Briefly, imprinting molds were fabricated by spin coating a thin film of PMMA onto a p-doped silicon wafer. Electron beam lithography was used to create a

negative mask of the nanopattern grating in the PMMA layer and subsequent RIE etching was performed. The remaining PMMA was removed to reveal the relief nanopattern of grooves and ridges, with an approximate groove depth of 350 nm. 6 different nanograting patterns were investigated, each maintaining a constant groove width of 500 nm but with a different ridge width of either 500, 750, 1000, 1250, 1500, or 2000 nm. Patterns covered an area of 16 mm^2. The pattern substrates were glued to Wilco dishes using silicone glue (RS692-524, RS Components). As a control, flat COC culture dishes were used (μ-Dish$^{35mm, low}$, Ibidi). Dishes were sterilized with ethanol and rinsed twice with sterile PBS before cell seeding.

Cell Seeding and Differentiation

Cells were trypsinized, resuspended in culture medium and seeded on substrates with a density of approximately 6,000 cells/cm^2. The cell suspension was left for 3 hours at 37°C and 5% CO_2, allowing cells to attach. Medium was then removed and replaced with differentiation medium consisting of DMEM, penicillin (10 units/ml), streptomycin (10 mg/ml), l-glutamine (2 mM), and Forskolin (10 μM) (FSK) to stimulate differentiation. Two different serum concentrations were investigated: 1% FBS concentration, reflecting the established differentiation protocol for this cell line[33]; the standard growth medium concentration of 10% FBS, mimicking conditions of our previous work with PC12 cells[23]. Substrates were used first for the 10% FBS experiment, cleaned with EDTA-trypsin for 30 minutes at 37°C and 5% CO_2 followed by a 3x PBS wash. Substrates were then used for the 1% FBS experiment. Cells were maintained at 37°C and 5% CO_2 and differentiation medium was refreshed every 2 days.

Data Acquisition

Cell cultures were examined for cell body growth and neurite elongation 24, 48, 72 and 96 hrs after FSK stimulation. 8-bit gray scale images were recorded with a Nikon TI using a DIC and a 40x oil-immersion objective or a 20x objective, depending on the extent of neurite growth. Images for cell counting were acquired using a 10x objective, an epifluorescent source and a DAPI filter cube. Images were processed and measurements performed with the Fiji/Imagej image

processing software and associated plugins. Each experimental condition was repeated 3 times.

Cell Morphology Measurements

Cell bodies were quantified using the LiveWire ImageJ plugin, developed by Daniel Lelis Baggio. Only cells that were distinguishable and exhibited measurable neurite outgrowth, as specified above, were considered. When possible, a minimum of 30 cell bodies was recorded per substrate, per day. In addition to cell body area, the average cell body diameter was also found only for Day 1, flat substrates, both medium conditions. This length has been traditionally used as the neurite length threshold, such that analysis only considered 'mature' neurites[36]. The average value (approx. 35 µm, in line with[37]) was used as a threshold for later neurite analysis.

Image sequences of neurites longer than a single image frame were merged into a single image with a 2D stitching plugin (developed by Stephan Preibisch). Neurite extensions were traced with the NeuronJ plugin (developed by Erik Meijerink) from the point of origin at the perimeter of the cell body to the tip of the neurite growth cone. At bifurcation points of neurites, a single neurite path was traced by selecting the thicker, longer branch of the extension (in that order). Only neurites that terminated in a free end or with growth cones cleanly abutting neighboring cells were considered. Neurites whose growth was obstructed or altered by other neurites or cell bodies were disregarded. A minimum of 40 individual neurons was recorded per substrate, per day.

Neurite length was measured in terms of actual length (the distance of the traced neurite path) and only neurites above the length threshold, described above, were included. Also investigated was the number of neurites per cell, examining whether nanogratings increase the incidence of uni/bi-polar neurons versus neurons with 3 or more neurite extensions.

Contact Guidance Measurements

The Feret (or Caliper) angle of the cell was used to describe the alignment of the cell body with respect to the underlying substrate orientation. To measure the

influence of patterned substrates on cell body morphology, the cell circularity was calculated (equal to 4*pi (area/perimeter^2)) as a number between 0 and 1 to indicate the degree of elongation of the cell body (lower values indicate more elongation).

Neurite alignment was measured by approximating the neurite as a straight line from the initiation point to end point and taking the relative angle of this line with respect to the underlying nanograting orientation.

For all measurements, the orientation of the grating was found by using a subsection of a reference image that was free of cells (only with the nanopatterned surface). The angle was extract using the Fourier Component Analysis of the Directionality plugin (by Jean Yves Tinevez). Flat substrates were given a 0 grating angle.

Cell Proliferation Assay
Flat substrates and patterns with 500 nm ridges and 2000 nm ridge were examined, representing the extremes of the patterns used. Substrates were produced as before, then punched out into 6 mm diameter discs to fit inside a 96 well plate (Nunc). Viton® polymer rings (Eriks b.v., The Netherlands), with an outer diameter of 6.5 mm and inner diameter of 5 mm, were sterilized in 70% ethanol and inserted into the well to hold substrates to the bottom. They also served to confine seeded cells to patterned substrate only. The wells were sterilized with ethanol, washed 3x with PBS, then filled with growth medium containing 10% FBS (200 ml per well).

Substrates were seeded at 7500 cells/cm^2 (minimum cell requirement for metabolic assay), in triplicate, and both the 10% and 1% serum conditions were investigated as before. Cells were cultured over a 4 day period, with a medium change on Day 2.

As a measure of metabolic activity, PrestoBlue (Invitrogen) assays were performed on Day 2 and Day 4, coinciding with medium changes. Medium was replaced with identical medium containing 10% PrestoBlue solution, 300 μl per well. Three blank wells were also filled for reference. Substrates incubated at 37°C and 5%

CO_2 for 2 hrs in the dark.

One hundred ml was transferred from sample wells to a white, solid-bottom 96 well plate (Nunc) and the fluorescence was measured, correlating to cell metabolic activity, using a Perkin Elmer Victor[3] 1420 Multilabel Counter (top-read; excitation filter: 560/10; emission filter: 590/10). A duplicate measure of the resulting solution was performed and averaged and corrected for background fluorescence. The average and standard deviation was calculated from the experimental triplicates. Per day and serum concentration, a one-way anova test was used to compare substrates. A paired T test was used to compare the same conditions at Day 2 and Day 4.

On Day 4, Hoechst 33342 (Invitrogen) nuclei stain was applied and cells were counted to determine the final number of cells. Substrates were washed 1 time with PBS after the PrestoBlue assay, followed by 200 µl of medium containing 2 mg/ml Hoechst solution. This was left to incubate for 25 minutes at 37°C and 5% CO_2, after which substrates were washed once with PBS and replaced with PBS for imaging. Using an 10x objective, 5 non-overlapping images were collected. Per sample, the average number of cells per cm^2 was calculated. The average and standard deviation was calculated from the experimental triplicates. Per serum concentration, a one-way ANOVA test was used to compare substrates. A T-test was used to compare identical substrates with different serum concentrations.

Immunocytochemistry - Nucleus, Actin, Focal Adhesion Staining
For fluorescence immunocytochemistry, F11 cells, FSK-stimulated on different nanogratings (flat COC, 1 and 2.5) for 24h or 96h, have been processed as previously reported[34]. Briefly, cells have been fixed for 15 min in 4% paraformaldehyde with 4% sucrose in PBS at room temperature, washed in PBS and then incubated with the primary antibody anti-vinculin (Sigma; mouse, monoclonal, dil. 1:100) and phalloidin-Alexa Fluor 647 (Invitrogen; 1:20) in GDB buffer (0.2% gelatin, 0.8M NaCl, 0.5% Triton X-100, 30mM phosphate buffer, pH 7.4), overnight at 4°C.

Samples have been then washed and incubated with secondary antibody conjugated to Alexa Fluor 488 (Invitrogen; anti-mouse, dil. 1:100) in GDB buffer, for 2h at room temperature. Samples were washed twice and mounted with medium Vecta containing DAPI (to visualize cell nuclei). Fluorescent samples were then examined with at Leica SP2 confocal microscope. Negative control experiments have been also performed, replacing the incubation with the primary antibody with the blocking solution; the staining was absent on these samples.

Statistical Analysis

For each metric considered, comparisons between each condition were usually made using the average of the means each condition repetition (repeated in triplicate). The exception was the number of neurites per cell, which used the average of the medians for further analysis. The means of each repeated experiment were assumed to be normally distributed about the true mean, with normality confirmed via a Shapiro-Wilks test (alpha = 0.01) available within the R statistical software. Data are shown with the standard error of the mean (mean±SEM, n = 3).

For analysis between conditions over the 4 day period, a repeated measured (mixed-model) ANOVA analysis was performed. When comparing different culture medium and substrates at a single time point, a regular two-way ANOVA analysis was used. When examining growth at a single time point for one culturing medium (1% or 10% FBS), a one-way ANOVA analysis was performed. Where applicable, all analysis was followed by a post-hoc Bonferroni test to further specify differences. A significance level of 0.05 was used, unless otherwise stated. This statistical analysis was performed using the Graphpad Prism software package (GraphPad Software, San Diego, CA, USA) and R statistical software in combination with the Deducer package (http://www.r-project.org/).

Results

Qualitative Assessment

Observations of F11 cell growth shows that cell bodies and neurites were generally well aligned on all the patterned substrates compared to the flat condition (Fig. 2).

Figure 2. *F11 hydridoma cultured on various substrates on Day 1. Flat substrates (a,d) exhibit notably less cell polarization compared to both the 500 nm ridge patterns (b,e) and the 2000 nm ridge patterns(c,f). Images a-c show cells grown in 1% FBS, while d-f show cells grown in 10% FBS. The arrow indicates the direction of the grating pattern; the scale bar indicates 35 μm.*

The 1% FBS condition permitted differentiation and extensive neurite outgrowth while limiting cell division. Sufficient cell-cell contact appeared to be required for survival, where seeding density below 5,000 cells/cm^2 led to poor cell viability. The 10% FBS condition was performed for comparison to culture conditions of previous PC12 experiments, as well as to improve cell viability in low density cultures. However, the 10% FBS condition experienced extensive cell division, reaching confluence and resulting in difficulty in achieving consistent measurements of non-occluded neurites and cells over the 4 day period.

Cell Morphology

Morphometric measurements of the cell body area revealed a notable change in cell body area over time between different culture medium: 1% FBS had a general increase while the measurable cells of the 10% FBS condition first increased and then decreased. However, per culture medium at identical time points, no significant difference in cell area was observed with respect to different culturing

substrates (including the flat control, Figure 3).

Figure 3. Cell body area measurements on different substrate patters over a 4 day period for (a) 10% and (b) 1% FBS. A comparison (c) of the average cell area (all substrate conditions combined) over the 4 day period is shown to have a significant change over the 4 day period (p < 0.0001) for both culturing conditions. (Mean±SEM, n=3, >8000 cells measured)

Both the 1% and 10% FBS conditions exhibit no significant difference in cell circularity on any substrate over the 4 day period, with an overall average of 0.75 (see Supplementary Data, Table S1 and S2). Examining the length of neurite outgrowth shown in Figure 4, one sees that there is no different between substrate conditions for either the flat or the patterned surface condition. However, there is a distinct increase in length over time, as expected. Comparing the Day 4 neurite lengths, the 1% FBS neurites are distinctly longer compared to the 10% FBS neurites ($p < 0.0001$, two-way ANOVA).

Examining the number of neurites per cell, we find that in the 1% FBS condition a more prevalent bipolar morphology is established (Figure 5). In comparison, the 10% FBS condition shows no temporal trend: for each time point, there is no difference between substrates (patterned or flat) for either culture condition.

Figure 4. *The average neurite length over a 4 day period on different substrates and different culturing conditions: (a) 10% FBS; (b) 1% FBS. No differences between conditions were noted within the same time point, though statistically significant differences over time were detected (p < 0.05 for 10% FBS; p < 0.0001 for 1% FBS). Inset shows the average neurite length over all conditions for the 4 day period. By Day 4, neurites are longer under the 1% serum compared to the 10% serum (P<0.0001). (mean±SEM, n=3,>15,000 neurites analyzed)*

Figure 5. *The graph above shows the average median of neurites per cell for both the (a) 10% FBS and (b) 1% FBS culturing conditions over a 4 day period on different substrates. Per time point, no significant difference is found between different substrates. , the 1% FBS condition is found to have a significant decrease in number of neurites per cell over time (p < 0.01, two-way ANOVA), with an emerging prevalence of bipolarity (±SEM, n= 3, >7000 total number of cells analyzed).*

Contact Guidance

Examining the alignment of the actual cell body to the underlying nanopattern (see Fig. 6), the 1% FBS shows a distinct difference between the patterned conditions and the flat control condition, at all time points. The 10% FBS condition shows a variable pattern of significant differences between pattern and the flat control condition. However, no significant differences between patterned substrates were observed for both culture conditions.

Figure 6. The Feret Angle measurements of the cell over a 4 day period on different substrates. Mean±SEM, n=3.. The asterisks (*) indicates a significant difference between substrates vs. flat control (p < 0.05). (Mean±SEM, n=3, >8000 cells measured)

Analysis also shows there is a significant change ($p < 0.05$) over time for the 10% and 1% FBS condition as the cell bodies become less aligned. Similar to the cell bodies, a two-way mixed model ANOVA analysis of neurite alignment shows that the nanopatterned substrates induce neurite alignment to their topographical signal, compared to the control flat substrate in both culture conditions(Fig. 7a-b).

Examining only the patterned substrates (flat controls not included), a trend can be observed of decreasing neurite alignment with increasing ridge width and in particular for 10% FBS condition. Since a mixed-model ANOVA analysis (per serum condition, all groups over the 4 day period) found no significant differences between patterned substrates for both the 1% and 10% FBS conditions, a one-way ANOVA analysis was applied to single time points (per serum condition, all groups on individual days) in order to increase statistical power. This confirmed the observable trend for the 10% FBS condition on Day 1 (Fig.7c), but further

concluded that no difference in neurite alignment exists with respect to nanopattern ridge width for the remaining time points.

Figure 7. *Shown above is average neurite alignment angle and SEM for both the (a) 10% FBS and (b) 1% FBS culturing conditions over a 4 day period on different substrates. a-b) * p < 0.05 flat vs. all other nanopatterns without the asterisk. Grouping all patterned substrates together, (c) shows the overall average and SEM of alignment angle for all combined substrates. No difference is observed between conditions on Day 1, but is apparent on Day 2 (p<0.01) and is maintained for the remaining days. Examining the 10% FBS on Day 1 in isolate (d), a one-way ANOVA test reveals the neurite alignment on 2000 nm ridge pattern is significantly less compared to patterns under the line marked with "‡" and the same on 1500 nm ridge pattern compared to patterns marked with a "†".(mean±SEM, n=3,>15,000 neurites analyzed)*

Based on the assumption that patterned substrates experience statistically similar degrees of alignment, the average neurite angle of all patterned substrates combined was plotted over time. This clearly shows that the alignment angle remains constant under the 1% FBS condition while the 10% FBS condition experiences decreasing alignment. A regular two-way ANOVA analysis comparing daily values of the combined neurite alignment shows a significant difference

Figure 8. *Histograms of neurite angle distributions for (a) 1% serum and (b) 10% serum, shown over a 4 day period for all substrates examined (bin size of 10 degrees).*

between 10% and 1% FBS is established on Day 2 and maintained for the remaining days (p<0.01; see Fig. 7d). This can clearly been seen in the angle histograms shown in Fig. 8, where the neurite angle distribution 1% serum

condition remains relatively unchanged over the 4 day period while the 10% serum condition shows a reduction in alignment.

Overall, the F11 hybridoma cell line adopt a polarized cell morphology when grown on nanopatterned surfaces compared to the control flat surface. The 1% FBS condition shows enhanced neurite outgrowth and consistently induced neurite alignment in response to the underlying nanopattern.

Cell Proliferation

Figure 9. The number of cells present after a 4 day period. Shown are values for cells on flat substrates and the two extreme of the nanograting patterns (500 nm and 2000 nm ridge width). Comparing 1% serum to the 10% serum, a student T-test found only the flat substrate was significantly different (*; $p < 0.05$). However, both the 500 nm and 2000 nm display patterns approaching significance ($p = 0.06$ and $p = 0.089$, respectively). The dotted line represents the initial cell seeding density (mean ± standard deviation, n=3).

The final cell count shows a general trend, with the 10% serum concentration resulting in a greater cell number over a 4 day period compared to the 1% serum condition (Fig. 9). Though not completely statistically significant, this implies that the higher serum concentration leads to increased cell division over this time period.

This is further supported by examining the metabolic acitivity at Day 2 and Day 4 for both serum conditions on all substrates (Fig. 10). Though the cell activity

remains relatively unchanged for the 1% serum concentration between Day 2 and Day 4, there is a significant increase in metabolic activity for the 10% serum concentration over the same time period.

Figure 10. *The metabolic activity of F11 cells is shown at Day 2 and Day 4 in 1% serum and 10% serum, on flat substrate and the two extreme of the nanograting patterns: 500 nm ridge width; and 2000 nm ridge width (mean ± standard deviation). Per time point, per serum concentration, no significant difference is found between substrates (p < 0.05). A significant difference is only found for the 10% serum condition between Day 2 and Day 4 (pair-wise T test, p < 0.05).*

Focal Adhesion Analysis

Because of the central role of focal adhesions in surface sensing[26], the organization of focal adhesions and actin fibers have been visualized by immunostaining. Figures 8 and 9 show focal adhesion formation for the extremes of nanopattern dimensions (flat, 500 nm ridge or 2000 nm ridge patterns) in both 1% FBS or 10% FBS conditions. The cells have been immunofluorescently labeled for the focal adhesion component vinculin (green) and actin (red). Vinculin is a protein found in maturing focal adhesions that is involved in the linkage of integrin adhesion molecules to the actin cytoskeleton. The locations where vinculin concentrates or strongly co-localizes with actin (yellow spots) represents where a focal adhesion has formed.

Figure 11. *Images of F11 cells grown for 1 day in 1% FBS on 500 nm ridge patterns (a-c), 2000 nm ridge patterns (d-f), or flat substrates (g-i). The cells were fixed and fluorescently labeled for actin (red;a,d,g) and vinculin (green;b,e,h). Also shown are merged images of actin and vinculin to reveal colocalization (c,f,i). The low intensity vinculin signal seen throughout the cell is cytoplasmic vinculin; focal adhesion-associated vinculin can be distinguished by a greater intensity and actin colocalization. Scale bar indicates 10 μm.*

As can been observed from the panels in Fig. 11 and 12, FAs tend to form at cell periphery, and in particular along neurites and within growth cones. A qualitative analysis reveals that FAs are more developed in the 10% FBS for both nanopatterns, where they are generally larger and more consolidated. Moreover, improved FA alignment can be observed on the narrower ridge pattern (500 nm) compared to the wider ridge size (2000 nm).

Figure 12. *Images of F11 cells grown for 1 day in 10% FBS on 500 nm ridge patterns (a-c), 2000 nm ridge patterns (d-f), or flat substrates (g-i). The cells were fixed and fluorescently labeled for actin (red;a,d,g) and vinculin (green;b,e,h). Shown are the actin and vinculin separately, then combined in an overlay to reveal colocalization(c,f,i). Scale bar is 10 μm.*

Discussion

In general, the response of the F11 cell line to nanotopography was robust under various conditions. Using 1% FBS proved more conducive to measurement and analysis, while the 10% FBS regime provided insight into focal adhesion formation and possible factors affecting guidance.

The present results show that F11 neuronal cells develop a polarized morphology compared to the flat control surface. Both cell bodies and neurites align to the nanopattern topography, in particular at a lower FBS concentration (1%).

Cell Morphology

Statistical analysis reveals that cell body areas and circularity are all roughly equal for all culturing substrates. The 1% serum condition shows an increase in cell area over time, indicating normal cell spreading. The 10% serum condition shows a

faster increase in cell area that decreases after Day 3, likely because of proliferation and reaching a semi-confluent state. This can be attributed to the fact that serum promotes cells to attach and proliferate, thus cells grow larger and then divide into smaller cells.

Looking at the neurite length, it seems that the 1% serum regime results in stronger neural differentiation as indicated by longer outgrowth compared to the 10% FBS. In general, the F11 is a suitable model for cell and neurite development and guidance, with extensive outgrowth on nanopatterned substrates that, at times, was in excess of hundreds of micrometers.

1% versus 10% Serum

There is a degradation in neurite alignment between the 10% and 1% FBS culturing regimes. Comparing FAs formed in 10% serum to those in 1% serum, they appear larger and more consolidated on both the smallest and the largest nanopatterns examined. The increased serum content may lead to increased deposition of cell-adhesion promoting proteins to the substrate surface[38] or lead to a serum-dependent increase in the production of endogenous extracellular protein[39] to further promote cell adhesion. For this reason, focal adhesion formation could be enhanced. In general, the influence of the underlying nanotopography becomes less significant as cell adhesion is increasingly facilitated[40].

Alternatively, serum could also modify focal adhesion dynamics as has been shown in other cell types. For example, Sprouty2 is an intracellular signaling protein present in F11s[35] which is known to modify focal adhesion formation and maturation[41]. Endogenous Sprouty2 (or Spry2) concentration has also been shown to increase with increased medium serum content in mouse cerebellar granule neurons grown *in vitro*[42]. Thus, serum content can directly affect cellular response to underlying topography by affecting FA formation, a cause suboptimal cell path finding[15]. Sprouty2 has even been shown to hinder DRG neurite outgrowth[43,44], indicating a possible reason for the differences in neurite growth observed in this DRG-derived cell line.

Neurite guidance in 1% serum and Focal Adhesion Formation

With respect to neurite guidance, the 1% serum conditions revealed that each pattern was able to induce alignment to an equal degree compared to the randomly oriented neurite growth observed on the flat control substrates. Qualitative assessment of focal adhesion formation on nanograting patterns reveals that the 1% serum condition has less consolidated FAs in response to all nanopatterns. This light adherence makes cells highly responsive to the patterned substrate, such that neurite alignment occurs to an equal degree on all patterns.

The ability to achieve equal neurite alignment via differently sized nanotopographical cues opens the possibility of exploring the influence of nanotopography on other aspects of nerve regeneration (speed, selectivity, etc.) towards optimized neuro-regenerative scaffolds.

Conclusion

The F11 cell line is a highly unique tool in the study of neural regeneration, previously used as an electrically active cell model, as a nociceptor model and for examining regenerative-associated gene (RAG) upregulatoin. This represents the first time this cell line has been used as a peripheral sensory neuron model for nanotopographical guidance. Under suitable culturing conditions this cell type is capable of extensive neurite outgrowth suitable for axon guidance studies and provides a robust guidance response.

This provides an interesting tool for investigating the role of nanotopography to adjust other neurite growth characteristics based on the induction of different focal adhesion sizes (and, thus, different traction forces) without sacrificing neurite guidance. Possibilities include: selective guidance of specific neural subtypes; fine tuning Schwann cell scaffold invasion preceding neurite regeneration; or allowing axons to regenerate at equal rates to address issues of reduced functional which normally occur because some axons naturally regenerate faster than others[45]. Within the scope of tissue scaffold design, the F11 proves to be a promising tool for future development.

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Chapter 2: Supplementary Data

Table S1. *The average circularity (and SEM) of cells grown on various substrates in 10% FBS over a 4 day period. (n=3, >2500 cells measured)*

Day	500 nm	750 nm	1000 nm	1250 nm	1500 nm	2000 nm	Flat
1	0.706	0.726	0.742	0.682	0.707	0.747	0.719
	(0.068)	(0.030)	(0.029)	(0.040)	(0.071)	(0.051)	(0.035)
2	0.756	0.720	0.688	0.684	0.762	0.741	0.726
	(0.013)	(0.029)	(0.038)	(0.018)	(0.014)	(0.025)	(0.025)
3	0.733	0.697	0.659	0.774	0.739	0.738	0.750
	(0.068)	(0.028)	(0.087)	(0.036)	(0.025)	(0.046)	(0.023)
4	0.764	0.748	0.792	0.780	0.774	0.792	0.773
	(0.028)	(0.036)	(0.008)	(0.021)	(0.032)	(0.012)	(0.005)

Table S2. *The average circularity of cells grown on various substrates in 1% FBS over a 4 day period. The SEM is show within brackets ().(n=3, >2500 cells measured)*

Day	500 nm	750 nm	1000 nm	1250 nm	1500 nm	2000 nm	Flat
1	0.735	0.763	0.761	0.754	0.774	0.798	0.765
	(0.032)	(0.060)	(0.053)	(0.049)	(0.025)	(0.035)	(0.047)
2	0.792	0.774	0.715	0.763	0.795	0.709	0.749
	(0.031)	(0.030)	(0.066)	(0.031)	(0.010)	(0.037)	(0.016)
3	0.798	0.770	0.744	0.725	0.828	0.794	0.798
	(0.038)	(0.037)	(0.053)	(0.023)	(0.029)	(0.042)	(0.004)
4	0.789	0.815	0.791	0.788	0.804	0.757	0.846
	(0.027)	(0.025)	(0.052)	(0.022)	(0.009)	(0.025)	(0.020)

Chapter 3

Peptide functionalized polyhydroxyalkanoate nanofibrous scaffolds enhance Schwann cell activity

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Abstract

Interactions between Schwann cells (SCs) and scaffolds are important for tissue development during nerve regeneration, because SCs physiologically assist in directing the growth of regenerating axons. In this study, we prepared electrospun scaffolds combining poly (3-hydroxybutyrate) (PHB) and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) functionalized with either collagen I, H-Gly-Arg-Gly-Asp-Ser-OH (GRGDS), H-Tyr-Ile-Gly-Ser-Arg-NH2 (YIGSR), or H-Arg-Asn-Ile-Ala-Glu-Ile-Ile-Lys-Asp-Ile-OH (p20) neuromimetic peptides to mimic naturally occurring ECM motifs for nerve regeneration. Cells cultured on fibrous mats presenting these biomolecules showed a significant increase in metabolic activity and proliferation while exhibiting unidirectional orientation along the orientation of the fibers. Real-time PCR showed cells cultured on peptide-modified scaffolds had a significantly higher neurotrophin expression compared to those on untreated nanofibers. Our study suggests that biofunctionalized aligned PHB/PHBV nanofibrous scaffolds may elicit essential cues for SCs activity and could serve as a potential scaffold for nerve regeneration.

Introduction

Schwann cells (SCs) are currently being investigated as a component of nerve repair strategies because of their known ability to support nerve regeneration in both the central nervous system (CNS) and the peripheral nervous system (PNS), where enhanced nerve repair has been reported following SCs transplantation[1]. *In vitro* and *in vivo* assessments have identified the use of bioengineered scaffolds seeded with SCs as a promising approach in synthetic nerve grafts to bridge nerve gaps[2,3]. The success of SCs in nerve regeneration is related to the production of cell adhesion molecules and neurotrophic factors, which mediate neurite attachment and growth[4,5].

Scaffolds for nerve regeneration require appropriate biocompatibility and biodegradability, good pliability for suture and mechanical integrity, and *in vivo* physiological loading during axon regeneration across large nerve defects. Scaffolds should also provide contact guidance for cell migration and axon outgrowth along the gap defect to support nerve functional regeneration[6].

Recently, electrospun nanofibrous scaffolds served as suitable environments for cell attachment and proliferation thanks to similar physical dimensions and cues compared to natural extracellular matrix (ECM)[7]. Poly[(R)-3-hydroxybutyrate] (PHB) and poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyvalerate] (PHBV) are two extensively studied natural derived poly(hydroxyalkanoates) (PHAs) due to their good biocompatibility and mechanical properties[8]. In recent years, promising studies aimed at the engineering of tissues such as bone[9], cartilage[10], skin[11]and nerve[3]have used PHB or PHBV substrates as a scaffold.

Normally, cell affinity towards polymers is poor as a result of their low hydrophilicity and lack of surface cell recognition sites[12]. Therefore, surface treatment of polymeric scaffolds is necessary to improve their bioactivity to achieve functional tissue regeneration. While various studies improved the hydrophilicity and surface properties of PHAs scaffolds using techniques such as composite electrospinning[13], plasma treatment[14,15], photografting[16] and alkaline hydrolysis[15,17], very few studies have been reported in the literature on the surface functionalization of PHAs electrospun fibrous scaffolds with peptides for neural tissue engineering applications. The majority of employed biomolecules have been so far ECM proteins, on the basis that cells in native tissues are surrounded by and attach to this network of fibril proteins. Meng *et al.* fabricated nanofibrous scaffolds using a blended solution of PHBV/collagen, shown to accelerate adhesion and growth of NIH-3T3 cells[13]. Alternatively, collagen can be

immobilized on the surface of PHBV scaffolds, as reported by Tesema *et al*. and Baek *et al*.[18,19], to improve their osteoblasts compatibility. Surface functionalization of PHBV-chitosan scaffolds grafted with hyaluronic acid (HA) has also been demonstrated by Hu *et al.*, showing that antibacterial properties were maintained while protein adsorption was effectively reduced [20]. Within the context of nerve repair, Armstrong *et al*. and Novikova *et al.* showed that coating of PHB nerve conduits with ECM molecules such as laminin,and fibronectin enhanced SCs activity to release neurite promoting factors[3,21], highlighting the potential of adding ECM biomolecules to bioengineered nerve conduits in order to improve nerve regeneration. However, the complexity and source of ECM proteins can cause issues with consistency and control over cell response as well as final clinical translation[22]. Synthetically-produced peptides represent a viable alternative, as shown by Wang *et al.*[23] with the introduction of RGD peptides on PHBV films; although not for a neural regeneration application, the viability of fibroblast-like NIH 3T3 cells was shown to improve.

Building on our previous work developing fibrous PHB/PHBV electrospun scaffolds with optimal physical properties and fiber alignment for improved SC activity, we compare here for the first time the biological activity of electrospun PHB/PHBV fibrous scaffolds which have been functionalized with relevant peptides: GRGDS, and the two laminin derived neuromimetic peptide sequences YIGSR and p20. These biomolecules were chosen based on their biological functionalities as cues present in the basal ECM and known to be involved in cell-cell and cell-ECM communications. Also shown is the immobilization of collagen type I for further comparison, following our earlier observations that SC adhesion and proliferation was enhanced when collagen was blended into the PHB/PHBV fibers[24].

Methods

Materials

PHB with M_w of 437'000, PHBV with 5% wt poly (3-hydroxyvalerate) and M_w of 150'000, chloroform, N, N-dimethyl formamide (DMF), 2-(N-morpholino) ethanesulfonic acid (MES), sodium hydroxide (NaOH), N-Hydroxysulfosuccinimide sodium salt (sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) were acquired from Sigma Aldrich (USA). Purchased reagents for cell culture were as follows: fetal bovine serum (FBS) from Hyclone (USA), Dulbecco Modified Eagle Medium (DMEM), phosphate buffered saline (PBS), penicillin/streptomycin and trypsin-EDTA from Gibco BRL (USA). Acid soluble collagen type I powder of bovine origin was a generous gift from Kensey Nash Corporation (USA). Peptides for biofunctionalization consisted in H-Gly-Arg-Gly-Asp-Ser-OH (GRGDS; Mw: 490.47 g/m), H-Tyr-lle-Gly-Ser-Arg-NH2 (YIGSR; Mw: 594.67 g/m), and H-Arg-Asn-lle-Ala-Glu-lle-lle-Lys-Asp-lle-OH (RNIAEIIKDI (p20; Mw: 1184.40 g/m) peptides were purchased from Bachem (Switzerland).

Electrospinning of aligned PHB/PHBV nanofibers

PHB/PHBV (1:1) solutions were prepared by dissolving the polymers in a chloroform (90) / DMF (10) solvent mixture at a concentration of 6% wt. Using a syringe pump (KDS 100, KD Scientific), the solution was fed at a rate of 1.5 ml/min through a 10 cc syringe with a 23 G needle placed 15 cm from a rotating mandrel collector with a speed of 5000 rpm. A high-voltage power supply was used to apply a voltage of 16 kV DC to produce nanofibers. Temperature and humidity were monitored during the process and ranged between 24-26 ºC and 37-42%, respectively.

Figure 1. Schematic representation of covalent attachment of biomolecules on the surface of PHB/PHBV nanofibrous scaffolds. Electrospun fibers are first subjected to alkaline hydrolysis, after which fibers were treated with a solution of EDAC and NHS. Fibers were then functionalized with collagen or peptide sequences, where the amine-reactive NHS groups on the fiber surface covalently bind biomolecules in solution.

Covalent attachment of biomolecules on PHB/PHBV nanofibers

For biofunctionalization, PHB/PHBV nanofibrous mats were firstly treated with NaOH (1N) for 80 min at room temperature, washed and dried at 37 °C overnight to obtain hydrolysed scaffolds. Afterwards, the hydrolysed scaffolds were washed in MES buffer solution (0.1 M, pH 5.0) for 30 min at room temperature to be subsequently activated with 5 mg/ml EDAC and 2.5 mg/ml sulfo-NHS in MES buffer solution for 90 min at room temperature. Biofunctionalizaton with collagen (5 mg/ml PBS) and peptides (0.2 mg/ml PBS) solutions was performed for 24 hr at room temperature. Scaffolds were further rinsed with PBS and dried at 37 °C overnight (Figure 1).

Characterization of nanofibrous scaffolds

Scanning electron microscopy (SEM)

The morphology of electrospun fibers was observed using SEM (XL 30 ESEM-FEG, Philips). Fiber diameters were calculated from SEM micrographs by measuring 100 fibers using Manual Microstructure Distance Measurement software (NahaminPardazan Asia Co., Iran).

Monitoring of biofunctionalization method on scaffolds

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) of collagen immobilized and bulk PHB/PHBV nanofibrous scaffolds were performed over a range of 400–4000 cm^{-1} at a resolution of 2 cm^{-1} using a Nicolet spectrometer system.

The XPS spectra of GRGDS immobilized and bulk PHB/PHBV nanofibrous scaffolds were also obtained on VGEscalab 2201-XL Base System (Thermo VG Scientific, UK) with a take-off angle of 90∘. Briefly, survey scan spectra were taken and the ratio of C 1s, N 1s and O 1s of samples were indicated.

Surface wettability measurements

Water contact angle (θ) of each scaffold was determined on a contact angle goniometer (Dataphysics OCA-20) by at least five independent measurements, while deionised water was used as a probe liquid.

Pretreatment of scaffolds before cell culture

Electrospun discs with a diameter of 15 mm and thickness of about 300 µm were soaked in 70% ethanol for 2 hr, washed twice with PBS, transferred to a 24 well non-treated plate and incubated overnight in basic cell culture medium. Rubber O-rings (Eriks BV, The Netherlands) were used to secure the scaffolds in place and prevent them from floating.

Quantification of protein immobilization

The amount of surface protein was evaluated using a BCA protein assay (Thermo Scientific). Scaffolds were washed for 24 hours in PBS at 4 $^{\circ}$C followed by another 1 hour wash in 1 % w/v SDS in TRIS buffer at pH 8.5 (Sigma Aldrich, USA) to hydrolyze NHS groups and remove any residual absorbed proteins. Afterwards, scaffolds were rinsed twice in 1% SDS/TRIS solution, followed by a 2 times wash in PBS. Finally, scaffolds were immersed in the BCA solution (with appropriate dilution to simulate the addition of a protein solution) and mixed at 1000 rpm for 30 min at 37 $^{\circ}$C and 60 $^{\circ}$C for collagen and peptide immobilized scaffolds, respectively. To resolve protein concentration, BCA standard curves of each protein in solution were generated using a dilution series. All scaffold conditions were prepared in duplicate and measured in triplicate. Data analysis was performed using R and Deducer Package (Ian Fellows).

Cell Culture

Schwann cell line RT4-D6P2T (ATCC, USA) was cultured in high glucose DMEM, and supplemented with 10% FBS and 1% Penicillin/Streptomycin. Cells were seeded at a density of about 50'000 cells/well on sterilized scaffold in 50 µl basic medium and incubated for 30 min to allow cell attachment and topped up to 1 ml with culture medium.

Cell-scaffold Studies

Cell Morphology Study

For morphology investigations, the cells were fixed in 4% formalin for 30 min and dehydrated in a series of increasing ethanol concentrations (70, 80, 90, 96, and 100%), 30 min in each concentration, before being dried using a critical point dryer (Balzers CPD-030). Finally, the samples were sputter-coated with gold (Cressington) for SEM observation.

Immunofluorescent staining

Cells were washed with PBS, fixed with 4% (v/v) paraformaldehyde (Sigma) in PBS for 30 min and washed again with PBS. Afterwards, blocking was performed with 10% (wt/v) BSA in PBS for 1 hr. Primary antibodies against p75 low affinity NGF receptor (p75LNGFR, 1:500, Abcam; ab6172) were applied in dilute buffer consisting of 10% (wt/v) BSA in PBS overnight at 4°C. Cells were then washed and the secondary antibody, goat anti-mouse IgG conjugated-fluorescein isothiocyanate (FITC, 1:50), was applied for 45 min at 37°C. Cells were counterstained with DAPI for 10 min and observed under fluorescence microscope. For negative controls, the primary antibody was excluded.

To quantify the p75 positive staining, we used the Sauvola binerization method. Firstly, we converted original true color immunostaining images (0:255 values) of cultured SCs on each scaffold to binary images (0:1 values). Then, we applied digital image processing algorithms to these double precision (n=10). After creation of a disk morphological structuring element with the specified radius and applying the Sauvola binarization method, the area of white zones in each binary image was measured as a quantitative value of p75 positive zones.

Cell Metabolism Study (Presto Blue Assay)

The metabolic activity of cells were monitored after 1, 3, 7 and 14 days (n=3) by Presto blue assay according to the manufacturer's protocol. Briefly, cells were incubated with medium containing 10% (v/v) Presto blue solution (Biosource, USA). After the designated time periods, aliquots were pipetted into a 96-well plate and the absorbance of the content of each well was measured at 590 nm using a Multilabel plate reader (Victor3, Perkin Elmer, USA).

Proteinase-K Digestion and Cell Proliferation Study (DNA Assay)

After 1 and 14 days of cell culture, scaffolds were washed in PBS and frozen at -80 $^{\circ}C$ until further processing. Subsequently, they were digested at 56 $^{\circ}C$ (> 16 hr) in a Tris-EDTA buffered solution containing 1 mg/ml Proteinase-K, 18.5 µg/ml pepstatin A, and 1 µg/ml iodoacetamide (Sigma-Aldrich). DNA quantification assay was performed with a CyQuant dye kit according to the manufacturer description (Molecular Probes, Eugene, USA), using above spectrofluorometer, at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

ELISA Assay of NGF Secretion (NGF assay)

To quantify the concentration of nerve growth factor (NGF) in cell cultured supernatant, commercially available ELISA kits were used according to the

manufacturer's instruction (Promega). The plates were read at 450 nm and analysed using a Lightcycler II (Roche Diagnostics GmbH, Germany). Secretions of NGF were measured at 3, 6, 12 and 14 days of culture (n =3).

RNA extraction and quantitative real-time RT-PCR

For time reverse transcription polymerase chain reaction (real-time PCR) analysis, total RNA was isolated using a combination of TRIzol® with a NucleoSpin®RNA II isolation kit (Bioké). Briefly, after 7 days of cell culture, scaffolds (n=3) were washed with PBS once and 1ml of TRIzol (Invitrogen) was added to the samples. The samples were then stored at -80 °C for RNA isolation. After chloroform addition and phase separation by centrifugation, the aqueous phase containing the RNA was collected, mixed with an equal volume of 75% ethanol and loaded onto an RNA binding column. RNA was collected in RNAse-free water. 750 nanograms of RNA were used for first strand cDNA synthesis using iScript (Bio-Rad) and 1 µl of undiluted cDNA was used for subsequent analysis. RT-PCR was performed on an iQ5 real time PCR machine (Bio-Rad) using SYBR Green supermix (Bio-Rad). Expression of neural marker genes (Table S2) was normalised to β-Actin levels and fold inductions were calculated using the comparative ΔCT method.

Statistical analysis

All data presented are expressed as mean ± standard deviation (SD). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. A value of $p < 0.05$ was considered statistically significant.

Results

Characterization of fibrous Scaffolds

Electrospinning of PHB(50) /PHBV(50) solutions resulted in a scaffold composed of uniform, uniaxially oriented nanofibers of an average diameter of 925±156 nm (Figure 2). More specifically, the distribution of the fibers had two peaks with average diameters of 577.5 ± 80.4 nm and 1074 ± 123.3 nm, respectively. Our previous studies showed that approximately 60 % of the fibers had a diameter of 1074 nm, thus confirming a rather narrow fiber distribution[24]. Apart from common peaks of the PHAs backbone, ATR-FTIR spectra of both scaffolds exhibited additional absorption peaks for collagen immobilized PHB/PHBV fibers at 1640 cm^{-1} associated with the C=O stretching of amide I and at 1560cm^{-1} associated with the N-H bending of amide II (Figure S1). These peaks confirmed the successful immobilization of the collagen on the surface of the scaffolds.

XPS analysis of unmodified and GRGDS immobilized fibers showed an additional N1s peak in the survey spectra of GRGDS modified sample at 401.92 eV (Figure S2), which clearly suggests that the GRGDS is chemically conjugated on the surface of the fibers. Table S1 also lists the intensities (i.e., the measured peaks divided by the corresponding sensitivity factors and normalized to 100% total intensity) of the elements on the two different surfaces.

Figure 2. SEM micrograph (A) and fiber diameter distribution (B) of PHB/PHBV nanofibres. SEM images of SCs on (C) untreated, (D) alkaline hydrolysed, (E) collagen, (F) GRGDS, (G) p20 and (H) YIGSR immobilized scaffolds 1 day after cell culture. Scales bars: (A) 10 µm; (C-H) 50 µm.

BCA analysis revealed that there is a greater amount of collagen bound compared to the peptide sequences, in accordance with the relatively larger size of whole

collagen fibrils and their initial concentration in solution (Figure S3). Immobilized peptide concentration of GRGDS, YIGSR and p20 decreases by weight was in agreement with the relative reduction of molar concentration of the initial peptide solutions. A similar comparison with the collagen solution was not possible because of the unknown molecular weight.

Figure 3. *Immunostaining identification of SCs by p75LNGFR and counterstained by DAPI, after 7 days on electrospun scaffolds which were untreated, hydrolised, or functionalized with respective biomolecules. Tissue culture plastic well plate served as a negative control. Cell adhesion and p75LNGFR expression is shown for all scaffolds, with little or no expression observed for the negative control condition. Scale bars show 100 μm and 10 μm for low and high magnifications, respectively.*

Wettability measurements showed that the water contact angle values were 107.4 ± 5.0° for aligned PHB/PHBV nanofibers and reached zero after alkaline hydrolysis, resulting in 100% wettability by the water droplet.

Morphological studies of SCs

After 1 day from seeding, cells oriented themselves along the direction of fiber alignments and clustered around the aligned fibers in a longitudinal fashion (Figure 2). Cells seemed to spread more on laminin derived peptide functionalized scaffolds than on other meshes. Immunocytochemistry indicated that cultured SCs on biofunctionalized scaffolds were positive for p75LNGFR, thus confirming normal cell functionality (Figure 3). Figure 4 shows outputs of digital image processing analysis to quantify amounts of p75 positive zones in each immunostaining image. Biofunctionalized scaffolds supported a stronger expression of p75LNGFR, with collagen, GRGDS, and YIGSR functionalized fibers significantly better than p20, thus suggesting enhanced SCs differentiation compared to non-functionalized and alkaline hydrolyzed scaffolds.

Figure 4. (A) Original immunostaining image of SCs on untreated PHB(50)/PHBV(50) nanofibrous scaffold, (B) average percentage of p75LNGFR positive zones on different scaffolds calculated by image processing algorithms . Asterisks represent statistically significant difference at $p < 0.05$.

Figure 5. *Metabolic activity of SCs on collagen and RGD functionalized scaffolds (A) and YIGSR and neuromimetic functionalized scaffold (B). SC proliferation (C and D) on unmodified and modified scaffolds. Peptide-functionalized scaffolds are shown to have consistently increased metabolic activity and proliferation compared to collagen-functionalized and non-functionalized scaffolds. Asterisks represent statistically significant difference at p < 0.05.*

Metabolic activity and proliferation of SCs on fibrous scaffolds

Metabolic activity of cells on all scaffolds increased during the 14-day period (Figure 5A and 5B). While in the first 7 days of cell culture, SCs metabolic activity increased just on GRGDS immobilized fibers, after two weeks of cultivation such an increase was significant on all functionalized nanofibers compared to those observed on the unmodified and alkaline hydrolysed samples (p < 0.05). The DNA quantification results also showed increased proliferation of SCs on different scaffolds from day 1 to day 14 (Figures 5C and 5D). On day 14, SCs proliferation on all functionalized nanofibers increased significantly compared to unmodified

and alkaline hydrolysed scaffolds, while on day 1 SCs proliferation was found to be higher ($p < 0.05$) just on GRGDS immobilized nanofibrous scaffolds, in agreement with metabolic activity results. These findings indicated a better effect of the applied biomolecules on SCs metabolic activity, likely resulting in higher proliferation rate compared to unmodified or alkaline hydrolysed scaffolds.

Figure 6. *Real-time PCR of neurotrophin gene expression in SCs grown on scaffolds functionalized with GRGDS or collagen, compared to untreated or hydrolysed scaffolds at Day 7 (A). Showing fold induction with respect to an endogenous reference of β-Actin, GRGDS was shown to result in increased expression of BDNF, CNTF and NGF over the other scaffolds. However, looking at NGF secretion over a 2 week period shows a major difference on Day 6 between biofunctionalized and non-functionalized scaffolds (C), with no observable differences between collagen and GRGDS. Neurotrophin gene expression on peptide functionalized scaffolds is also shown to similarly upregulate BDNF, CNTF and NGF compared to non-functionalized scaffolds (B). Subsequent NGF secretion shows marked differences for only YIGSR-functionalizaed scaffolds on Day 3 and Day 12. Asterisks represent statistically significant difference at $p < 0.05$.*

Gene expressions and NGF released from cultured SCs on nanofibrous scaffolds

The gene expression of different SCs markers was analysed using quantitative RT-PCR and obtained results are presented in Figure 6. No statistically significant difference was observed on SCs cultured on unmodified and biofunctionalized scaffolds for PMP22. The presence of GRGDS and both laminin derived peptides in the scaffolds tended to significantly upregulate GDNF, BDNF, CNTF and NGF-F expression in comparison to untreated scaffolds indicating that applied peptides might have accelerated SCs differentiation ($p < 0.05$).

Furthermore, no significant difference between GDNF and PMP22 expressions of SCs on GRGDS, YIGSR and p20 groups was observed, while BDNF and CNTF expressions were significantly higher on both laminin derived peptide modified scaffolds compared to GRGDS ($p < 0.05$). Also the difference between BDNF and CNTF expression of GRGDS immobilized sample was not significant. Significantly higher expression of NGF-F on YIGSR immobilized scaffold was also found than those of p20 and GRGDS ones ($p < 0.05$).

Figures 6C and 6D show concentrations of NGF released from cultured SCs on different scaffolds. Biofunctionalization with collagen and GRGDS did not have any positive effect on NGF secretion of the cultured SCs, except at day 6 (Figure 6C). After 3 and 12 days of cell culture, NGF concentration of YIGSR immobilized scaffolds were significantly higher than that of untreated nanofibers ($p < 0.05$) (Figure 6D). In general, NGF assay results of SCs on YIGSR modified nanofibers confirmed NGF-F expression obtained by real-time PCR.

Discussion

Characterization of unmodified and modified nanofibrous scaffolds

Electrospinning is an established method for fabricating nanofibrous scaffolds to mimic ECM-like properties such as nanoscale dimensions, high porosity, and large surface area for tissue engineering applications. This method produces non-woven meshes containing fibers ranging in diameter from tens of microns to tens of nanometers with different orientation, which makes electrospinning more appealing than other submicron fiber fabrication[7,25]. In this study, we used a high speed mandrel to collect uniaxially aligned PHB/PHBV nanofibers. SEM results showed homogenous and continuous structure of electrospun fibers with a high degree of orientation (Figure 2A). Similarly, Matthews *et al*. reported that electrospun collagen fibers maintained random orientation at rotational speeds less than 500 rpm and significant fiber alignment when the speed of the mandrel was increased to 4500 rpm[26].

Surface properties of scaffolds are also extremely important, as they affect the biological interactions of the substrate with cells and tailor the responses across the interface with the host tissue[27]. Normally, the relatively poor hydrophilicity of PHB and PHBV affects their biocompatibility for application in tissue engineering. To improve the hydrophilicity and surface bioactivity of PHB/PHBV scaffolds, different types of physicochemical and post-processing surface modification techniques such as hydrolysis with lipases and NaOH[28], low pressure plasma treatment[14] and ion implantation[29], have been attempted. Immobilization of specific proteins or peptide sequences on the scaffold surface that mimic natural components is a promising method to obtain surface properties especially tailored for tissue engineering. When proteins are considered for functionalizing an artificial substrate, natural choices are ECM molecules such as laminin,

fibronectin, and collagen, which activate integrin receptors[12]. However, these ECM molecules often present cells with multiple types of integrin binding sites and other cell signalling motifs and, therefore, limit the degree of specificity over the cell response that can be elicited[22]. An alternative is biofunctionalizing a surface with peptide sequences based on moieties found in ECM proteins, employing peptides that bind specific membrane-bound receptors to promote cellular adhesion, differentiation, and other intracellular signalling cascades via defined cell/substrate interactions[5].

In this study, we used collagen I and the integrin binding GRGDS, YIGSR and p20 peptides to functionalize the surface of aligned PHB/PHBV nanofibers. Covalent modification was performed by presenting reactive carboxyl groups (-COOH) through alkaline hydrolysis. ATR-FTIR showed that after immobilization with collagen the extra absorption peaks of amide I and amide II appeared in the spectra of functionalized nanofibrous scaffolds (Figure S1). Similar results have been reported by Mattanaave *et al*. during immobilization of collagen onto surface of PCL nanofibers[30]. Because of a low concentration of peptides used for functionalization, we used XPS as a more accurate detection method to determine the presence of GRGDS peptide on the surface of PHB/PHBV nanofibrous scaffolds as an evidence for effective functionalization.

From Figure S2, it can be seen that unmodified PHB/PHBV fibers fail to show any N1s peaks, while we can clearly attribute the N1s peak to the chemically immobilized GRGDS on the surface of the nanofibers, thus confirming successful peptide biofunctionalization. Similar N1s peak around 400 eV was also observed by Wang *et al*. after immobilization of RGD peptides on the surface of PHBV films[23]. Furthermore, immunostaining analysis against anti-collagen I showed that functionalized coating was stable after 22 month from biofunctionalization in a

sealed container at room temperature (data not shown), thus suggesting that our procedure was stable in time.

In vitro studies of SCs with unmodified and modified nanofibrous scaffolds

As SCs play an important role in axonal regeneration and function recovery of neurons, they should effectively attach, spread, and differentiate on scaffolds. SEM images of SCs obtained on day 1 of cell culture on scaffolds showed that SCs cultured on the aligned webs oriented along the fibers; thus, the aligned fibrous scaffolds could exhibit SCs columns, also known as band of Büngner (Figure 2C to 2H). It has well been documented that fiber orientation influences cell adhesion and growth, while it also modulates elongated cellular patterns that match cell morphology found in native tissues[6,31,32]. For PNS and spinal cord, previous studies demonstrated that aligned structures are capable of guiding neurite extension through a lesion site for axonal regeneration within the nerve[33,34]. This finding is confirmed by similar results obtained by other researchers who reported that oriented arrangement of SCs on aligned electrospun fibrous mats might positively influence axonal regeneration[6313536 6, 31, 35, 36]. Immunostaining analysis against p75LNGFR showed that SCs can adhere, migrate, and form elongated cellular processes on the surface of all scaffolds. Although it has been previously shown that neat PHB and PHBV scaffolds are non-toxic to SCs[37], the current study revealed a higher degree of spreading and a more intense p75LNGFR staining when PHB/PHBV nanofibers were functionalized with biomolecules (Figure 4). In this way, the positive response of the p75LNGFR observed in biofunctionalized scaffolds indicate improved reactivity that may further stimulate *in vivo* regeneration.

Furthermore, we have also shown that biofunctionalization of PHB/PHBV scaffolds increased metabolic activity and proliferation of SCs compared to

unmodified scaffold (Figure 5). Previous studies proved that presence of RGD peptides on the surface of scaffolds can enhance proliferation of different cell types such as fibroblasts and SCs[23,36,38]. In addition, it is well known that laminin contains bioactive neurite binding sites for neural cell attachment and differentiation, which enhance neurite extensions[39]. From our results, the presence of biological cues enhanced SCs proliferation and metabolic activity compared to other groups (Figure 5). Thus, it appears that these peptides activated signalling pathways with an influence on cell cycle.

The improved SCs activity on peptide-modified scaffolds could be first the result of more hydrophilicity, which increases serum proteins adsorption. Secondly and most important, the presence of biomolecules on the surface of fibers might have allowed SCs to preferentially attach to the proteins of their native environment providing a surface bioactive scaffold. Previous studies showed that cell activity is extremely sensitive to surface chemical groups of scaffolds and varies with individual functional groups more significantly than with general surface properties like hydrophilicity[40]. Wang *et al.* believed that protonation in the culture medium helps the adhesion of cells that carry negative charges on the membrane surface. N-containing groups, which resulted from peptide introduction to the surface of scaffolds, may be positively charged at physiological pH and caused protonation of cell culture medium[23]. Ren *et al.* also showed that amino groups (NH_2) are very effective on nerve stem cell proliferation and migration[41]. Hence, it may be concluded that the presence of amino groups on the surface of biofunctionalized scaffolds may account for improved SCs.

Another crucial issue regarding the application of biofunctionalized PHB/PHBV nanofibrous scaffolds in neural tissue engineering is whether the presence of biomolecules altered SCs phenotype. After cultivation for 7 days, the expressions

of neural genes associated to differentiation were detected. Also considered was the upregulated gene expression of GDNF, BDNF, NGF, and CNTF, four well characterized neurotrophins known to stimulate growth of different types of neurons. These neurotrophic factors, therefore, may promote neurite sprouting from the transplantation site when translated into an *in vivo* model[42]. PMP22, as the primary component of myelin protein, is known to be involved in formation and controlling myelin thickness and stability[43].

In the present study, the comparison of the untreated and biofunctionalized scaffolds obviously indicated that SCs gene expressions in the unmodified group were down-regulated (Figures 6A and 6B), which agrees well with Feng *et al*. findings for avidin treated scaffolds compared to untreated ones[44]. Previously, Ren *et al*. have also reported that, compared to –COOH surfaces, $-NH_2$ seemed to promote neuronal stem cell differentiation[41]. PMP22 was not altered by the presence of biologically active motifs. Chew *et al*. reported similar results indicating that aligned electrospun PCL scaffolds seeded with SCs did not change PMP22 expression[45]. Further *in vivo* investigations are required to determine the effect of scaffold alignment and laminin motifs introduction on myelination since the result of the PMP22 gene expression was not conclusive *in vitro*.

The significant up-regulation of these neural genes indicates differentiation and maturation of SCs, which could benefit axonal regeneration and provide a more favourable microenvironment for peripheral nerve. Previous studies demonstrated that complementing exogenous neurotrophin or up-regulated neurotrophic gene expression can promote axonal regeneration[44,46] There is considerable evidence indicating that various neurotrophins such as NGF, BDNF, CNTF and GDNF, play an important role in nerve regeneration both *in vitro* and *in vivo*[47]. Cai *et al*. showed that the neutrophins released from SCs may enhance the regeneration of

not only neural tissues, but also non-neural tissues, such as ligament, bone and periodontal membrane[48]. Additionally, the up-regulation of SCs gene expression was significantly higher when fibers were functionalized with GRGDS compared to collagen type I and laminin derived peptides compared to all the other scaffolds.

To further analyse cell differentiation, we also investigated NGF secretion of cultured SCs. NGF has been widely explored among neurotrophic factors and shown to play a prominent role in experimental diabetic neuropathy[49]. ELISA assay results showed a significant increase in NGF released by SCs cultured on YIGSR immobilized scaffolds on days 3 and 12 compared to p20 and alkaline hydrolysed scaffolds (Figure 6D). Neither did GRGDS nor collagen containing scaffolds show any greater NGF secretion of SCs compared to untreated scaffolds except day 6 (Figure 6C). This might be explained by an early state of SCs differentiation triggered by the presence of the biomolecules, which results in upregulation of neural genes without a correspondent increase in NGF secretion. As exogenous NGF is rapidly disintegrated[50], we could also hypothesize that degradation of NGF during culturing times may reduce its secretion. Further studies should aim at evaluating protein secretion after a longer culturing period.

On the basis of the data obtained, we believe that the use of peptide functionalized PHB/PHBV fibrous substrates enhances the response of SCs *in vitro*. Designed scaffolds not only enhanced SCs metabolic activity and proliferation, but also maintained neural gene expression, which in the case of laminin derived peptides was also correlated to increased neurotrophic factors secretion. Therefore, laminin derived functionalized nanofibrous scaffolds can be pivotal in functional peripheral nerve regeneration.

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Chapter 3: Supplementary Material

Table S1 .*Quantities of C, O, and N Calculated from the XPS Survey Spectra of unmodified and GRGDS immobilized PHB/PHBV nanofibers*

Fiber type	C	N	O
PHB/PHBVnanofibers	71.18	0.13	28.69
GRGDSimmobilizedPHB/PHBVnanofibers	69.87	1.31	28.82

Table S2. *Designed primers of genes for real-time PCR*

Gene	Primer Sequence	Length
β-Actin	F:5'-CACCCGCGAGTACAACCTTC-3' R:5'-CCCTATCCCACCATCACACC-3'	207
CNTF	F:5'-TTTGCGAGAGCAAACACCTCT-3' R:5'-TGCTAGCCAGATAGAACGGCTAC-3'	67
GDNF	F:5'-GGCGACGGGACTCTAGAATGA-3' R:5'-GTCAGGATAATCTTCGGGCATATTG-3'	194
BDNF	F:5'-GCCA TTCA TTCAGGCTTCCA-3' R:5'-GCCA TTCA TTCAGGCTTCCA-3'	93
NGF-F	F:5'-TGATCGGCGTACAGGCAGA-3' R:5'- GAGGGCTGTGTCAAGGGAAT -3'	107
PMP22	F:5'-TGTACCACATCCGCCTTGG-3' R:5'-GAGCTGGCAGAAGAACAGGAAC-3'	138

Figure S1. *ATR-FTIR of untreated PHB/PHBV and collagen immobilized nanofibers.*

Figure S2. *XPS spectra of (A) unmodified and (B) GRGDS immobilized PHB/PHBV nanofibers.*

Figure S3. *Microgram quantities (A) and relative molar concentrations (B) of immobilized biomolecules per scaffold. Measured immobilised amounts were 26.6 ± 4.5 μg, 8.5 ± 2.9 μg (408 μM), 8.7 ± 0.9 μg (336 μM) and 3.3 ± 0.5 μg (169 μM), for collagen, GRGDS, YIGSR and p20 biomolecules, respectively.*

Chapter 4

Tandem electrospinning for heterogeneous nanofiber patterns

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Abstract

Smart nanofibrillar constructs can be a promising technological solution for many emerging and established fields, facilitating the potential impact of nano-scale strategies to address relevant technological challenges. As a fabrication technique, electrospinning (ESP) is relatively well-known, accessible, economic, and fast, though until now has shown limitation over control and design of the fibrillar constructs which can be produced. Here, we introduce "Tandem Electrospinning" (T-ESP), a novel technique able to create increasingly complex patterns of fibrous polymeric constructs on a micro and nano-scale. Modifying a standard ESP configuration results in a focusing electric field that is able to spatially define the deposition pattern of multiple polymer jets simultaneously. Additional spatially defined heterogeneity is achieved by tuning polymer solution properties to obtain a gradient of fiber alignment. Heterogeneous fibrous meshes are created with either random, aligned, or a divergent fiber patterns. This approach holds potential for many fields, with application examples shown for Tissue Engineering and Separation Technologies. The technique outlined here provides a rapid, versatile, and accessible method for polymeric nanofabrication of patterned heterogeneous fibrous constructs. Polymer properties are also shown to dictate fiber alignment, providing further insight into the mechanisms involved in the electrospinning fabrication process.

Significance Statement

Despite early promises of nanotechnology, a number of barriers exist in terms of fabrication costs and reliability for both the research and industrial setting. This has hampered the full utilization of nanofabrication techniques for driving innovation and technological advances. Electrospinning technology is often cited as an accessible and simple technology for nano-scale fabrication of polymeric fiber constructs. However, the application of this approach is still limited by the low degree of control and complexity achievable. Here we outline a novel and versatile electrospinning method to fabricate smart nanofibrillar constructs with unprecedented control over fiber spatial deposition. This provides a facile route to

creating more complex nanofiber patterns. Additionally, mechanisms are explored to provide insight for further refining this developing technology.

Introduction

Recent years have seen a renewed interest in electrospinning (ESP) for micro- and nano-scale fabrication, driven by new applications in the fields of environment and security, such as filtration or particle sensing, biomedical technologies, in form of tissue scaffolds or drug release platforms, and in the energy sector, with potential for improving both solar and fuel cell technologies[1–6]. The promise of creating micro- and nano-sized fibers in a simple, relatively inexpensive and high-throughput manner has spurred development of ESP fabrication techniques, leading to the production of fibrous meshes with high surface area and tunable porosity.

Conventional ESP results in a chaotic fibrous mesh, narrowing the application of this form of ESP to areas such as textile and filtration industries. Strategies have been developed to produce fiber constructs useful for other applications, but these approaches still exhibit limitations in terms of spatially defined fiber deposition and orientation, and typically produce simple polymer constructs consisting of a single fiber type. ESP across a planar target gap electrode or patterned target electrodes produces simple parallel fibers or cross-hatched fiber patterns, respectively, with limited spatial definition and restricted to single fiber composition or layer-by-layer fiber assemblies[1,7–11]. In general, deposition and spatial control of multiple polymer fibers is hampered by the mutual repulsion of multiple charged jets[12–14]. Attempts to create multimodal fiber constructs have relied on the chaotic fluctuations of the 'whipping' polymer jets to achieve overlap between fiber populations, intrinsically precluding any form of fiber orientation. ESP onto a rotating mandrel also produces simple parallel fibers, with increased complexity possible by simultaneously electrospinning different polymers onto a rotating mandrel[15]. However, more elaborate, spatially defined multimodal patterns are not possible. The most detailed fiber patterns have been created by near-field electrospinning (NFES) or melt electrospinning, which are less accessible and unable to simultaneously deposit multiple fiber types within a confined region[16,17].

Here, we describe the development of T-ESP techniques, which allow for simultaneous electrospinning of up to three polymer fibers. Using modifications to a traditional ESP setup, we can achieve a high degree of control of planar ESP patterning. Control over multiple jets is achieved by manipulating the electrostatic field, resulting in the creation of ESP multi-material fibrous constructs. This allows ESP fiber patterns of intermingled, heterogeneous fiber populations in random, parallel or divergent ("Y-shaped") orientations. Finally, we show how this facile and economic fabrication method can be used to mimic branched structures found in biological systems and present an example of a divergent tissue scaffold supporting spatially defined differences in neurite outgrowth.

Results and Discussion

Electrode configuration focuses multiple polymer jets for controlled overlapping fiber deposition. Traditional ESP process can be described as an electrostatic extrusion of a charged polymer solution from a supply needle towards a grounded collector. During this process, a polymer jet is formed which is initially straight but then enters an unstable whipping phase. This chaotic motion further extrudes the polymer jet and accelerates solvent evaporation to form solid polymer fibers[18]. To achieve more control over fiber deposition, this study manipulated the electrostatic field by using a grounded target comprised of millimeter-wide electrodes with a centimeter gap (Figure 1). The electric field across the gap is known to exert forces on the fibers during the whipping phase (Figure 1A), leading to fiber alignment across the gap[19]. However, the use of millimeter wide electrodes in this configuration (Figure 1C, 1A Inset: "Collector") also achieved a focusing effect of the electric field in the orthogonal plane (Figure 1D) to further direct the evolution of the whipping phase.

Figure 1. The Tandem ESP Apparatus. (A) A depiction showing an upper parallel plate to which 2 needles are mounted and high voltage is applied and a base plate with a gap electrode arrangement (detail shown in Inset: "Collector"). A 10 mm gap was made between two 2 mm wide electrodes. Shown is the placement of a 14 mm glass coverslip on top of the two electrodes, used to collect fibers patterned across the gap. ESP with single needles were centered over the coverslip. Tandem and triple ESP needles were arranged along the X axis (see B for orientation). Also shown is a representation of two simultaneous jets (red and green) as well as an image of the polymer jets of two LW polymer solutions (Inset: "Tandem Jets"; scale bar: 5 cm); dark arrows indicate the stable jet phase and a white arrow indicates the onset of the whipping phase, where the jet becomes too small to be easily visible. (B) The gap electrode configuration was modeled to show the electric field generation in both the X and Y plane. (C) The electric field in the Y plane of the gap electrode configuration shows the divergent electric field (inset) which causes fibers to align across the gap. (D) The same electric field in the orthogonal X plane clearly shows the focusing electric field this electrode arrangement creates.

ESP of one polymer solution with this arrangement led to the spatially confined deposition of aligned fibers across this gap (Figure 2A). Yet, this novel electrode architecture produced an electric field able to confine the simultaneous deposition of two ESP jets to produced aligned parallel deposition of different fiber populations (Figure 2B). To the best of our knowledge, this is the first report of concurrent and controlled deposition of different populations of aligned fibers with a well-defined region of overlap (Figure 2C).

Figure 2. Aligned Tandem ESP constructs. (A) Aligned ESP of a single jet of LW polymer solution, confined to the approximate width of the electrodes (scale bar: 2 mm). Fibers have a diameter of 0.756 ± 0.160 μm and are well aligned, with a coherence measurement of 0.780 ± 0.059 (1 is perfectly aligned, 0 is isotropic; see Figure S1 for additional information). (B) The tandem ESP deposition pattern of two identical LW polymer solutions with Macrolex® Red G and Green dyes added, respectively (scale bar: 2 mm). This particular polymer solution produces consistent electrospun fibers (green: 0.653±0.143 μm; red: 0.637±0.136 μm) and achieves a high degree of alignment when using a gap target electrode configuration (green: 0.734±0.115; red:0.817±0.097). (C) A magnified view of the overlapping regions between the two fiber populations is shown, clearly depicting the defined yellow gradient transition from red to green (scale bar: 2 mm).

Despite initial mutual repulsion of the two jets during the stable jet phase (Figure 1A Inset: "Tandem Jets"), the focused electric field provided sufficient force upon the fibers during the whipping phase to cause colocalized deposition. Fluorescence microscopy (Figure 2C) revealed that the two regions were overlapping with no distinct boundary region between fiber populations. The fibers showed a relatively homogenous diameter and were well aligned, with no statistically significant differences between populations.

Polymer properties dictate alignment for gap electrode ESP. The degree of fiber alignment achieved was found to be dependent on the polymer used. This provided the possibility of further control over fiber deposition and degree of multimodal complexity achievable. The polymer used in the experiment above (designated LW) is of a family of segmented block copolymers compromised of

poly(ethylene oxide terephthalate) (PEOT) and poly(butylene terephthalate) (PBT), synthesized using PEOT and PBT segments of specific lengths added in known proportions to produce a randomly distributed block copolymer. Originally devised as materials for tissue scaffold fabrication and controlled drug delivery systems, the polymer properties are tuned per application by controlling the length of the initial PEOT segments and varying proportions of PEOT to PBT[20–22]. A polymer with initial PEOT segments of 300 Da and a PEOT/PBT of 55/45 has the designation of 300PEOT55PBT45.

The subset of polymers used in this study was selected for having similar molecular weights of approximately 50 kDa but having different compositions and distinct mechanical properties. A distinguishing characteristic of the different polymers selected was the entanglement molecular weight (M_e); this is a measure of the chain length between physical entanglement points of polymer chains in a bulk polymer and has an impact on the viscoelastic properties of the polymer[23]. M_e, along with viscosity and solution conductivity, has been cited as an important parameter in describing the polymer solutions used for ESP[24–26]. The three polymers selected were: 300PEOT55PBT45 (M_e: 250 Da) and designated in this study as LW (low M_e); 300PEOT70PBT30 (M_e: 450 Da), referred to as IW (intermediate M_e); and 1000PEOT70PBT30 (M_e: 710 Da), called here HW (high M_e)[27]. These were prepared as polymer solutions in solvent blends of chloroform and hexafluoroisopropanol (HFIP; see Supplementary Material, Table S1 and S2 for a details on polymers and solutions used).

Despite similar mean molecular weights and a 20% w/v polymer concentration, the LW, IW and HW polymers solutions demonstrated different viscosities of 120, 125 and 388 cP which produced fiber diameters of 0.86±0.28 0.74±0.34, and 1.03±0.35 mm respectively. Viscosity of a polymer solution is dependent on polymer chain entanglements, or overlap, with a higher polymer concentration or molecular weight increasing the availability of polymer chains to entangle in solution and leading to an observable increase in solution viscosity; this is also dependent on the solubility of the polymer, as evidenced here by the higher viscosity of the HW solution[28]. Viscosity is often correlated to either changes in fiber size or quality,

exemplified in the current study by the higher viscosity HW solution which produced larger fibers[24,29,30].

Unexpectedly, image analysis of fiber orientation found statistically significant decrease ($p < 0.01$) in alignment with increasing values of M_e. Using a score from 0 to 1 (random to aligned), alignments of 0.773 ± 0.089, 0.641 ± 0.102, and 0.33 ± 0.077 were measured for LW, IW, and HW fibers, respectively. To account for differences in viscosity, the concentration of the HW polymer solution was adjusted to achieve a similar viscosity of 122 cP (referred to as HW_{mv}, 'matched viscosity'; see Table S2). However, the resulting fiber alignment of 0.222 ± 0.0698 indicated that viscosity, thus entanglement in the initial polymer solution, was not an effective predictor of fiber alignment. Instead, M_e as a bulk polymer property appeared to be the predominant factor. Until now, M_e has been used to describe the viscoelasticity of the polymer solution as it relates to the formation of 'beads-on-a string' fiber morphology formed in the initial stages of the polymer jet[24]. This finding suggested a potential role of M_e on fiber morphology after they have returned to a bulk polymer state later in the whipping phase.

M_e affects whipping phase evolution and fiber alignment. The difference between whipping phases of the different polymers is clearly observed from the macroscopic patterns of fiber deposition, with the size and shape of the region of deposition dependent on how the whipping phase develops. The highly entangled LW polymer deposits fibers along the length of the electrodes, indicating a smooth, elongated progression of the whipping phase. In comparison, the HW polymer has fewer fibers along the length of the electrodes and a wider, more circular deposition in the electrode gap, suggesting a more abrupt, arrested evolution of the whipping fiber (Supplementary Material Figure S1). It is suggested here that M_e influences both fiber formation during the whipping phase and the overall evolution of the whipping phase, accounting for many of the observed differences between the polymers fibers.

In addition to the initial perturbations (bending) preceding the onset of the whipping instability, it has been previously reported that the fiber can also experience secondary and tertiary bending during the whipping phase. The

consequence is that fibers are not necessarily smooth and straight, but can have a wavy, corrugated appearance[18]. Considering also the strain of the solidifying fiber during the whipping phase and the correlation between degree of entanglement and plastic deformation, it is proposed here that a fiber with a higher entanglement molecular weight (M_e) experiences more plastic deformation during the whipping phase[31,32]. In turn, this increased drawing results in a curled, corrugated fiber, likely caused by non-axisymmetric residual stress[33]. In comparison, a fiber of a highly entangled polymer would have increased elasticity, resisting deformation, and would incur less residual stress to produce a straight fiber morphology. It should also be remarked that the long working distance used (20 cm) excludes the curled fibers to be the result of buckling[34].

We suggest that the electric field imposes orientation along the length of the fibers, such that a general 'global' scale alignment is achieved and fiber orientation is not entirely random. However, the resulting microscopic alignment of the fiber also depends on how corrugated the fiber has become. As observed, higher M_e polymers would produce fibers with increasing disorder, although not entirely random. Since strain and disentanglement can also be affected by differences in molecular weight, use of these particular polymers proved essential in identifying the role of M_e[35].

These additional perturbations may also disrupt the whipping profile development, explaining the observed differences in the elongated deposition of fibers. An elongated whipping profile may also increase the degree of microscopic fiber alignment of the LW polymer fibers by improving global fiber alignment and effectively stretching out fiber perturbations.

Heterogeneous fiber patterns can be tuned by adjusting polymer solution properties. T-ESP was extended to produce heterogeneous fiber populations, using the LW and the HW polymers as far extremes offered by this family of polymers in terms of both fiber size and fiber alignment. Heterogeneous T-ESP produced the expected regions of aligned LW fibers (0.699±0.104), less aligned HW fibers (0.401±0.06), and an overlapping region between the two populations. Unexpectedly, this also resulted in a fiber deposition pattern with an extreme

spatial bias, with the HW fibers now shifted to the edge of the deposition region (Figure 3A).

Figure 3. *Heterogeneous Tandem ESP Scaffolds. (A) Tandem ESP fibers of LW (green) and HW (red) produced a biased pattern of fiber deposition, though an overlapping gradient region between the two fiber populations was still maintained (inset). (B) The LW fibers had a diameter of 0.723 ± 0.261 μm and were well aligned (0.699 ± 0.061). (C) HW fibers were significantly larger in diameter (1.61 ± 0.345 μm, p<0.01), and were less aligned (0.397 ± 0.076, p<0.01). (D) Tandem electrospun fibers of LW polymer solution and HW^+ solution produced a centered pattern of fiber deposition with an overlapping gradient region between the two fibers also evident (inset). (E) LW had fiber diameters of 0.859 ± 0.311μm and were relatively aligned (0.551 ± 0.106). (F) HW^+ fibers were smaller in size, though still significantly larger than LW fibers (0.865 ± 0.164 μm, p>0.01), and maintained the same approximate alignment as the original HW fibers (0.397 ± 0.076, p<0.01). (Scale bars: A,D 2 mm; Inset 500 μm)*

Aiming to understand the sources of this bias, a comparison was made between the fluidic jet profiles with two identical LW solutions versus two different polymer solutions (Figure 1A Inset: "Tandem Jets" and Figure S4F, respectively). This revealed that T-ESP with identical polymer solutions produced stable jets of equal length while heterogeneous T-ESP exhibited different lengths, with the LW solution initiating the chaotic whipping phase at an earlier stage compared to the HW solution. This presented the possibility that the whipping phase of the LW jet electrostatically deflected the HW polymer jet to create the observed spatial bias.

To investigate this premise, the HW solution was replaced with the previously described HW$_{mv}$ solution (See Supplementary Table S2 for details). According to Hohman *et al.*[36], the onset of whipping occurs once the polymer jet reaches a critical radius, with less viscous solutions reaching this stage earlier in the fluidic jet evolution. Despite the HW$_{mv}$ solution now initiating the whipping phase earlier compared to the LW solution, a spatially biased fiber pattern was still produced with LW still dominant on the target substrate (Supplementary Figure S5). Looking at the later stages of jet evolution showed that LW fibers experienced a final pull towards the target area (Supplementary Figure S5K). From this it was surmised that an LW fiber experienced a larger force during the final stages prior to deposition. This was attributed to the elongated whipping profile having more exposure to the electrostatic field and experiencing more electrostatic attraction.

To increase the effective force experienced by the HW fibers, the surface charge of the fibers was increased by adding 0.05 mg/ml of salt (NaCl) to the polymer solution[26]. As evidence of increased surface charge, this should also hasten the onset of the chaotic whipping phase[37]. NaCl was first added to the original HW solution (referred to hereafter as HW$^+$), resulting in an increased conductivity (Supplementary Table S2). The increased surface charge of the HW$^+$ fibers was evident from the whipping phase now initiating at approximately the same height as the LW jet (Supplementary Figure S6F). The end result was a more centered distribution of fibers (Figure 3E). The HW$^+$ fibers were smaller, consistent with a previous report on the effect of salt[38], and there was no change in alignment between the salt and salt-free condition.

Adding salt to the HW$_{mv}$ solution (hereafter HW$_{mv}$$^+$), evidence of increased surface charge was again observed by a much shorter stable jet length (approximately 1.5 cm) and a more centered distribution of tandem fiber deposition with a defined region of overlap (Supplementary Figure S7). The resulting HW$_{mv}$$^+$ fibers were now equal in diameter (0.774±0.287 μm) to the tandem-spun LW fibers (0.859±0.311 μm, p<0.01) but maintained a reduced degree of alignment (0.247±0.0586). In summary, tuning polymer solutions used for heterogeneous T-

ESP makes possible fiber constructs with both distinct fiber alignments and tailored distribution patterns.

Complex T-ESP fiber patterning. Once the optimization of T-ESP was established, the strategies for multi-jet patterned spinning were further extended. For example, moving from two to three jets resulted in the simultaneous overlapping deposition of three populations of aligned fibers (Figure 4A). The target electrode arrangement was further altered to achieve a divergent pattern of deposition (Figure 4C; Supplementary Figure S9 for electrode details). Figure 4B shows two fiber populations which have both deposited onto a 'shared' bottom electrode in an aligned, overlapping manner (Figure 4D). These fibers then diverge and separate, with the left polymer fibers (red) oriented towards the left upper electrode and the right polymer fibers (green) directed towards the right electrode. This creates a unique fiber pattern with a degree of complexity not yet seen for ESP.

Figure 4. Triple ESP and Divergent Tandem ESP. (A) An example of focused aligned electrospinning with 3 separate LW solutions simultaneously (scale bar: 2 mm). A different dye was added to each solution (see Methods section). The blue autofluorescence of LW was enhanced by adding Pyrene, though the residual blue autofluorescence of the other fiber populations modified the typical green and red dye colors to mint and pink, respectively. (B) The result of tandem ESP on an 'epsilon' gap electrode arrangement (shown in C), producing a region of overlapping fibers (scale bars, B: 2mm; D: 500 µm) which then diverge. Further details of the needle and target collector arrangements for these patterns are described in Supplementary Material Figure S8. Fiber diameter is 0.788 ± 0.279 µm, consistent with previous LW ESP fibers.

T-ESP applications. This new fabrication approach has an immediate application in the field of tissue engineering and regenerative medicine. The design of fibrous

scaffolds follows a common biomimetic principle, with the intent of achieving an appropriate cell response by imitating the natural fibrous extracellular matrix found in the body. Current ESP scaffold can be tailored to elicit specific cell behavior, but fibers are typically modified homogeneously. However, this does not reflect the heterogeneous cell populations found *in vivo*. The possibility to create tissue scaffolds with purposely-designed heterogeneity may enhance scaffold effectiveness, exacting spatial control of cell response imparted by the properties of respective fiber types. Furthermore, bifurcated branching structures are ubiquitous throughout the body, including, but not limited to, nerves, vasculature, pulmonary and breast tissue[39–42]. The ability to make fibrous scaffolds as described in this work provides a promising tool for studying these types of biological systems as well as for the development of tissue engineering solutions.

To show the utility of T-ESP fiber tissue scaffolds, we developed a heterogeneous divergent scaffold of LW fibers and an LW-collagen blended fibers (Figure 5A) to explore neurite outgrowth of an explant dorsal root ganglion (DRG). An earlier study showed modulated neurite growth on ESP scaffolds of PCL/collagen blended fibers compared to scaffolds of PCL-only fibers[43]. We were able to replicate these earlier findings on our heterogeneous scaffold, with longer and more consolidated neurite extension on LW-collagen fibers compared to LW fibres (Figure 5C). Neurites also followed the fiber orientation, creating a divergent growth pattern and lending itself to quantification via radially segment Scholl analysis to provide quantification of differential outgrowth (Figure 5D-E). This presents interesting possibilities for neural tissue engineering, such as the spatially selective promotion of different neural subpopulations along divergently oriented fibers, or a general tissue engineering approach for sorting of a heterogeneous cell population.

Figure 5. *Spatially modulated neurite outgrowth on a divergent heterogeneous Tandem ESP scaffold. (A) A divergent heterogeneous T-ESP pattern comprised of LW-collagen fibers diverging to the left half labeled 'Coll' (0.235±0.048 mm diameter; faintly autofluorescent green) and LW fibers diverging to the right half labeled 'LW' (with MacrolexGreen dye, visible as bright green; scale bar 500 μm). (B) Sensory neurite outgrowth from a rat Dorsal Root Ganglion placed at the junction of a divergent tandem ESP. Neurites were stained for b3-tubulin, shown in red (scale bar 500 μm). (C) A merged image shows neurite alignment along the direction of the divergent fibers and reveals a differential pattern of growth, with the collagen-containing fibers promoting longer, more consolidated growth while the LW fibers promote more shorter, dispersed neurites (scale bar 500 μm). (D) A visual representation the segmented Scholl analysis performed to measure neurite outgrowth. The image was divided into 10° angular sections, indicated by red lines for the LW-collagen half and blue for the LW half. Each sector was divided radially into 250 mm segments indicated by the different colored bands. This created a 2D polar bin map with which the number of neurites per bin were then counted according to standard Scholl Analysis. (E) A rose plot of the cumulative number of neurites per bin. Each radial distance is indicated by the color and the size of each colored segment represents the neurite count within that distance. This analysis clearly shows that the LW-collagen fibers promote longer growth consolidated over fewer sectors compared to the LW fibers, which show dispersed growth over a broader range of sectors.*

A gradient of fiber alignment could also spatially modulate cell morphology and, thus, creating a gradient of cell function. Figure S10 shows the modulation of

Schwann cell morphology on a T-ESP scaffold of aligned LW fibers and less aligned HW_{mv}^{+} fibers, where such differences in Schwann cell morphology are known to modulate their production of neurotrophic factors[44].

The application of this technology is not limited to the field of Tissue Engineering. Initial trials have also employed tandem scaffolds to separate mixed solutions on the basis of fiber affinity (Supplementary Material S11), showing promise in the field of Separation Technologies. This fabrication technique can used to prepare heterogeneous catalytic or filtration substrates or high surface area nanofibrous cathode/anode electrode configurations able to sequester specific analytes.

Conclusions

Until now, the ease and promising characteristics of ESP has found limited applicability due to limitations in the ability to control and customize the resulting fiber meshes. This work presents the highly accessible T-ESP approach, providing the versatility to create an array of micro- and nano-scale fibrous constructs with complex, ordered patterns. At the same time, this work provides general considerations for optimal polymer selection as well as furthering the understanding of the ESP process. Though a promising tool for tissue engineering applications, this approach holds potential for other fields. The methodology presented here further extends the growing arsenal of ESP solutions to create increasingly complex polymer fiber constructs in a simple manner to address the growing demands of new material and engineering challenges.

Materials and Methods

Polymer Solution Preparation and Characterization

All polymers were dissolved in associated solvent solutions overnight (See Supplementary Table S1 for complete solution details) and appropriate dyes were added at 0.01% w/v. NaCl (Sigma Aldrich GmbH, Germany) was added to HW and HW_{mv} polymer solutions at final concentration of 0.05 mg/ml. Conductivity was measured using a custom gold parallel plate apparatus at 20 ^{o}C (see Supplementary Material S12 for full description) and viscosity was measured using a Brookfield DV-E with an s21 mandrel at 30 ^{o}C and 100 rpm.

Electrospinning for T-ESP Development

A custom ESP setup was used with an environmental chamber (25°C, 30% humidity) and a large upper parallel plate (30 cm by 20 cm). ESP was performed at 25 KV, a working distance of 20 cm, a flow rate of 1 ml/hr and for an ESP interval of 1 minute. For tandem ESP and triple ESP, needles were mounted along the Y axis of the collector target (Figure 1B) at an inter-needle separation of 1 cm and 5 cm, respectively. A 14 mm coverslip (Menzel-Glaser) was the target substrate used to collect deposited fibers, placed on the collector electrodes of either a parallel or epsilon arrangement (see Supplementary Figure S8).

Electrospinning T-ESP Scaffolds for Specific Applications

Cell scaffolds were prepared using the epsilon electrode configuration shown in S9 and fibers were deposited on flexible mesh rings (outer diameter 15 mm, inner diameter 12 mm) in lieu of coverslips for improved handling. A working distance of 20 cm and a voltage of 20 KV was used for an ESP time of 30 seconds. To ensure jet stability, the LW solution had a flow rate of 1 ml/hr and a flow rate of 0.5 ml/hr for the LW-collagen solution. Parallel T-ESP scaffolds for liquid phase separation were prepared with the standard gap electrode configuration using an LW solution (1.0 ml/hr) and a 10% PVA solution (0.5 ml/hr) on glass coverslips. Scaffold were immersed in methanol with 5% paraformaldehyde for 24 hrs to crosslink the PVA fibers and then air dried for 24 hrs before use.

Analysis of ESP Process

Images of whipping were captured by a Luminix DMC G3 and fiber pattern images were stitched together using a Nikon Eclipse Ti with an automated stage at 10x magnification. Fibers were gold sputter-coated and examined with a XL 30 ESEM-FEG (Phillips) operating at 10 kV. A minimum of 100 fibers were measured per population and minimum of 5 images per fiber population were evaluated for fiber orientation using the OrientationJ plugin[45], providing a coherence value between 0 (isotropic) and 1 (perfectly anisotropic). Fiber orientation was also evaluated by creating an FFT image of SEM images[46].

Cell Culture

Dorsal root ranglia (DRGs) were explanted from 2 day old rat pups (Wistar Unilever: HsdCpb:WA). All procedures followed national and European laws and guidelines and were approved by the local ethical committee. Briefly, rats were sacrificed by cervical dislocation under general anaesthesia (4% Isoflurane) and then decapitated. Individual ganglia were removed from the spinal column and nerve roots were stripped under aseptic conditions with the aid of a stereomicroscope. DRGs were cut in half to expose interior neurites and placed at the divergent junction of the tandem ESP scaffold. DRGs were cultures in NeuralBasal A- medium with B27 supplement, 0.5 mM L-glutamine and pen/strep added (Gibco/Invitrogen) and 10 ng/ml NGF (Sigma Aldrich). Cultures were maintained for 5 days, with medium refreshed after Day 1 and Day 3. Cells were fixed with ice cold 2% paraformaldehyde (PFA) for 15 minutes at $4^{o}C$, then permeabilized for 10 minutes with 0.1% TritonX-100. Cultures were blocked for 1 hour in 5% Normal Goat Serum and 1% BSA in a TRIS buffered solution (TBS), followed by a 16 hr incubation with a mouse anti-b3-tubulin primary antibody (1:1000,Abcam), a triple wash in 1% BSA TBS solution and a 16 hr incubation with an Alexa 546 anti-mouse secondary antibody (1:1000, Invitrogen) with 1% Normal Goat serum. Scaffolds were then washed and mounted with Mowiol 4-88 with 2.5% DABCO.

Additional methodology information is available in Supplementary Material section.

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Chapter 4: Supplementary Information Appendix

Experimental Section

Polymer solution preparation and characterization
All PEOT/PBT polymers were dissolved over night in solvent solutions of chloroform ($CHCl_3$) and hexafluoroisopropanol (HFIP) in the ratios listed in Table S1. Macrolex® Fluorescent Yellow 10GN and Fluorescent Red G hydrophobic dyes (Lanxess NV, Belgium) were added at 0.01% w/v to assist in visualization. A 10mg/ml NaCl (Sigma Aldrich, Germany) suspension in HFIP was added to HW-based polymer solutions to final concentration of 0.05 mg/ml.

Conductivity was measured using a custom gold parallel plate apparatus. Applied voltage of approximately 0.707 V_{rms} was applied by a function generator(5400A Generator, Krohn-hite) at a frequency of 1 kHz. The voltage across the 'sense' resistor (R_{sense}), connected in series with the probe, was used to determine the current through the circuit (Figure S9); R_{sense} was adjusted to match the voltage drop across the probe. The voltage was measured using a Tenma 72-7725 MultiMeter. The conductivity probe was calibrated with a solution of 1 mol/L NaCl in MilliQ water with a known conductivity of 85 mS/cm. Viscosity was measured using a Brookfield DV-E with an s21 mandrel at 30 ^{o}C and 100 rpm.

For additional electrospun patterns (SI Appendix, Figure S8), 5% w/v solutions of Polyethylene Oxide (PEO, 900,000 MW, Sigma Aldrich) were prepared in demineralized water. To distinguish different PEO fiber populations, solutions were prepared with Fluorescein (green, 0.01% w/v, Sigma Aldrich), Rhodamine (red,0.1% w/v,) and 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, blue, 0.01% w/v, Sigma Aldrich). The PEOT/PBT-collagen blend was prepared by first preparing a 20% w/v LW solution and an 8% w/v collagen in HFIP. These were then mixed in a 1:1 ratio for a final solution of 10% w/v LW and 4% w/v collagen. A 10% solution of polyvinyl alcohol (PVA, 98% anhydrous, Sigma Aldrich) was prepared in demineralized H_2O. A mixed drop of corn oil with hydrophobic MacrolexGreen (20 mg/ml) and water with hydrophilic Rhodamine B (20 mg/ml)

is placed on the central region of the scaffold. A time series was captured using a non-inverted Nikon E600 and a triple band pass filter (DAPI/Green/Red).

Electrospinning

Electrospinning was performed in an environmental chamber, with a maintained temperature of 25 °C and a relative humidity from 22% to 27%. Polymer solutions were loaded into 5 ml syringes (BD Biosciences) and connected via Teflon tubing to a stainless steel needle (0.8 mm outer diameter, 0.5 mm inner diameter). Needles were mounted to the centre of a large upper parallel plate (30 cm by 20 cm) and centered over the collector target at a working distance of 20 cm. A voltage of 25 KV was applied to the upper parallel plate and needles (Gamma HV Research). For tandem spinning, needles were mounted along the Y axis of the collector target (Figure 1B) at an inter-needle separation of 1 cm; needles were symmetrically offset with respect to the X axis. Triple spinning used an inter-needle separation of 5 cm along the Y axis, with one needle centered at the YX axis intersection. Using a syringe pump (KDS-100-CE, KD Scientific), one per syringe, solutions were electrospun at flow rate of 1 ml/hr, unless otherwise stated. A 14 mm coverslip (Menzel-Glaser) was the target substrate used to collect deposited fibers, placed on the collector electrodes of either a parallel or epsilon arrangement (see SI Appendix, Figure S8).

Whipping and Fiber Analysis

Images of whipping were captured by a Luminix DMC G3 with bar LED illumination. Fiber patterns were recorded with a Nikon Eclipse Ti with an automated stage at 10x magnification in brightfield and with an epifluorescent lamp with filters for FITC, Rhodamine, DAPI and brightfield. Images were stitched using Nikon Elements software. Fibers were gold sputter-coated with a Polaron E5600 sputter-coater and examined with a XL 30 ESEM-FEG (Phillips) operating at 10 kV. Fiber diameter was determined by measuring a minimum of 100 fibers, taken from a minimum of 5 images at random locations with a 1000x magnification. A minimum of 5 images per fiber population were evaluated for fiber orientation using the OrientationJ plugin(1), providing a coherence value between 0 (isotropic) and 1 (perfectly anisotropic). Fiber orientation was also evaluated by creating an FFT image of SEM images (2). Images of whipping

fibers, fiber patterns and fibers were processed with Fiji image processing software (3). Figures were prepared with Scientifig plugin (4).

Statistics

All statistics were performed with R statistical software (http://www.R-project.org/) and graphs were created using the Deducer plugin (by Ian Fellows, http://www.jstatsoft.org/v49/i08/). When comparing between tandem ESP fiber populations, a Student's T test was used. When comparing whole populations of the same fiber family, a one way ANOVA was use followed by a *post hoc* Holm method. A significance level of $p < 0.01$ was adopted.

Figure S1. *Fiber alignment of LW, IW and HW polymer solutions when electrospun individually (solo ESP versus tandem ESP). (A) plots fiber alignment, based on the Coherence metric provided by the OrientationJ plugin for ImageJ/Fiji (1). (B-D) show representative SEM images of the LW, IW and HW fibers. (E) shows LW, with MacrolexGreen dye, having an elongated deposition pattern along the electrodes, which becomes more apparent when applying a color thresholded to highly green pixels. In contrast, G shows a less elongated, more circular deposition of HW polymer fibers, with a similar threshold filter making this more clear in (H). (scalebar: E, G 1 cm).*

Figure S2. *(A,B) SEM analysis of aligned ESP fibers of a single jet of LW polymer solution show they are well formed and aligned. Alignment is corroborated by indications of alignment in an FFT image of the SE micrograph (C) and an image coherence measurement of 0.780 ± 0.059 (1 is perfectly aligned, 0 is isotropic). Fibers had a diameter of 0.756 ± 0.160 μm, with a size distribution shown in (D). The whipping profile of a single jet is also shown in (E, scalebar: 10 cm), with a close up in (F, scalebar: 5 cm) showing a stable jet length (thin dark line) of approximately 3.5 cm.*

Figure S3. *SEM images of tandem ESP of two LW solutions on a 14 mm coverslip (A,D) show parallel depositions of aligned fibers of the two fibre populations with similar degrees of fiber alignment (0.734±0.115 and 0.817±0.097, FFT of SEM images in (B) and (E)). Both fiber populations were of similar diameters (0.653±0.143 μm; 0.143±0.136 μm) and had similar fiber diameter distributions (C, F). Whipping jet profiles (scale bars: G 5 cm; H 10 cm) shows equal stable jet lengths and initiation of whipping at the same point.*

Figure S4. *Tandem ESP of LW and HW polymer solutions produce LW fibers with a coherence of 0.699 ± 0.061, indicating fair alignment (FTT shown in (A), and a diameter of 0.723 ± 0.261 μm, distribution shown in (C). In comparison, the HW fibers were less aligned (0.397 ± 0.076, FFT (B), and had a diameter of 1.61 ± 0.345 μm (histogram shown in (D)). The whipping jet profiles (E, scale bar: 10 cm; F, scale bar: 5 cm) reveal that the HW solution initiates whipping later compare to the LW solution. Distinct ridges of accumulated parallel fibers are noted at the transition between the two fiber types (G, H), a possible consequence of electrostatic interactions between the two fiber populations.*

Figure S5. *Tandem ESP of LW and HW$_{mv}$, which produces a biased deposition of fibers (A, scale bar: 2 mm) as well as a region of overlap (C, scale bar: 500 μm). Looking at the stable jet phases of the tandem ESP (E, scale bar: 10 cm; F, scale bar: 5 cm), the lower viscous HW$_{mv}$ had a shorter stable jet length (right) compared to the LW jet (left) An image of the lower whipping phase (K), processed with Fiji to increase contrast (imaged was processed with functions: smooth, CLAHE local contrast enhancement, background substraction) to make the small fibers visible, shows the LW jet is deflected by HW$_{mv}$ whipping but experiences a final 'pull' of the LW fibers (left jet, white arrow) towards the electrode area (indicated by the black arrow, center bottom). In comparison, the right HW$_{mv}$ jet shows no such redirection. Based on this observation, the assumption was made that the biased deposition was the result of greater electrostatic force upon the LW fibers at the final stages of deposition. The transition region again produces accumulated bundles of fibers, forming striated like structures (L). LW fibers are of similar diameter to previous LW fibers (0.647±0.223 μm) and share a similar distribution (I). As well, alignment is also similar (0.804±0.049, (G)). The fibers of the less viscous HW$_{mv}$ solution produce a smaller fiber diameter (0.790 ± 0.402 μm) than the previous HW solution and have a slightly improved alignment, though not statistically significant (0.432 ± 0.076).*

Figure S6. *Tandem ESP of LW and HW⁺ polymer solutions produces LW fibers with an alignment of 0.633±0.061 (corresponding FFT image (A)) and a diameter of 0.630 ± 0.242 μm, with the diameter distribution shown in (C)). The HW⁺ fibers have a smaller diameter compared to the original HW solution (0.865±0.164 μm) and a narrower diameter distribution (D), though alignment is roughly equal (0.397±0.076 (corresponding FFT image (B)). The whipping profiles shown in (E) and (F) show that the LW jet (left) and the HW⁺ jet have similar stable jet lengths (scale bars of 10 cm and 5 cm, respectively). As before, the transition region exhibits striated patterns of highly aligned fiber bundles (G-I).*

Figure S7. *Tandem ESP of LW and HW$_{mv}^+$ solutions produced a centered fiber distribution (A, scale bar: 2 mm), this time with a region of overlap (C, scale bar 500 μm) which did not result in a striated transition region (K,L). The jet profiles show that the HW$_{mv}^+$ stable jet (left) is now much shorter than the LW stable jet (right) (E, scale bar: 10 cm; F, scale bar: 5 cm). LW fibers had a diameter of 0.859 ± 0.311 μm, larger than previous LW fibers and with a wider distribution (I), and have comparatively less alignment (0.551 ± 0.106, SEM image (B) and corresponding FFT image (G)). HW$_{mv}^+$ fibers have a diameter of 0.774 ± 0.287 μm (diameter distribution shown in (J)), equal to the tandem LW fibers produce, and exhibit a low degree of alignment of 0.247 ± 0.059, corroborated by SEM (D) and FFT analysis(H).*

Figure S8. *Additional examples of patterned spinning with a 5% PEO solution (900,000 MW). (A) Using the parallel electrode arrangment, a similar pattern can be achieved with this polymer solution. (B) A divergent pattern can also be achieved, though completely separation of polymer types was less robust and subject to fine adjustment in needle alignment. Note the presence of green fibers diverging in the same direction as red fibers. (C) As well, a triple spin is possible with PEO. The central PEO fibers, with fluorescein, were adjusted to false white to improve visualization. (scalebars: 4 mm)*

Figure S9. *(A) The electrode arrangement used for parallel tandem ESP, with a gap width of 10 mm and electrode width of 2 mm. A Teflon mount was used to center a 14 mm coverslip (Menzel-Glaser) between the electrodes with a 2 mm of overlapping coverlsip over each electrode. The Teflon mount raised the cover slip 2 mm above the electrodes to ensure no direct contact. Tandem ESP with two jets used an inter-needle separation of 1 cm, centered about the coverslip along the X axis. Triple ESP used an inter-needle separation of 5 cm to ensure jet stability. (B) The divergent patterns were created using an epsilon-type gap electrode triple electrode arrangement shown. The base electrode repositioned 6 mm under the glass coverslip while two additional electrodes where positioned at the cover slip periphery at an angle of 45° with respect to the coverslip center. As before, an inter-needle separation of 1 cm, centered about the coverslip along the X axis. The coverslip was again placed on top of a Teflon mount to maintain position and ensure no direct contact with electrodes.*

Figure S10. *Schwann cells on a heterogeneous LW/HW Tandem ESP scaffold. Rat Schwann cells cultured on a tandem ESP scaffold of CA (strongly cell adherent) and NA (less cell adherent) polymer fibres. (A) Cells on CA show clear cell adhesion and elongation in the direction of fiber orientation, while (B) shows that cells adhere poorly to NA fibres and retain a rounded, less elongated cell morphology. Also notable is the wave-like morphology of the NA fibres, the result of swelling because of the high hydrophilicity of this polymer (scale bar: 20 μm).*

Figure S11. *Oil/Water separation on a tandem ESP scaffold of LW (hydrophobic) and PVA (hydrophilic) fibers. (A) A merged image showing the location of LW (green) and PVA (black) fibers. A mixed drop of corn oil with hydrophobic MacrolexGreen (20 mg/ml; shown as white) and water with hydrophilic Rhodamine B (20 mg/ml) is placed on the central region of the scaffold and imaged after (B) 30 seconds, (C) 2 minutes and (D) 12 minutes. Observed is migration and separation of the two phases in accordance with the underlying fibers.*

Figure S12. *A depiction of the circuit used to measure the conductivity of polymer solutions. By measuring the V_{sense} across the known R_{sense}, i_{sense} could be calculated. The voltage across the probe (V_{probe}) was calculated by substracting V_{sense} from V_{source} By applying Ohm's Law to i_{sense} and V_{probe}, the resistance of the polymer solution is determined. Conductivity was calculated using the empirically derived constant of the probe using a dilution series of Glycerol with NaCl concentrations and compared against published values of conductivity.(5)*

Table S1. *Polymer Properties[a]*

Polymer	Mw (kDa)[a]	Me (Da)[a]	Ha (MPa)[a,b]	PEO segment[c]		PBT segment[c]	
				Tg (^{o}C)	Tm (^{o}C)	Tg (^{o}C)	Tm (^{o}C)
300PEOT55 PBT45	50.67	250	97.15	-23	-[d]	26	145
300PEOT70 PBT30	48.45	450	42.7	-23	-	-	114
1000PEOT7 0PPBT30	57.43	710	29.6	-50	6	-	149

[a] *Data taken from Moroni et al., 2005(6)*
[b] *Ha is the equilibrium modulus*
[c] *Data take from Deschamps et al., 2001(7)*
[d] *not observed*

Table S2. *Polymer Solution Properties*

ID	Polymer[a]	Conc. % w/v	Solvent[b]	NaCl[c]	Viscosity[d]	mS/cm (20°C)
LW	300PEOT55 PBT45	20	CHCl$_3$(70)/ HFIP (30)		120 cP	1.3295
IW	300PEOT70 PBT30	20	CHCl$_3$(70)/ HFIP (30)		125 cP	1.1906
HW	1000PEOT7 0PPBT30	20	CHCl$_3$(70)/ HFIP (30)		388 cP	1.5589
HW$^+$	1000PEOT7 0PPBT30	20	CHCl$_3$(70)/ HFIP (30)	Yes	372.5 cP	9.2822
HW$_{mv}$	1000PEOT7 0PPBT30	15	CHCl$_3$(80)/ HFIP (20)		122 cP	1.3577
HW$_{mv}$$^+$	1000PEOT7 0PPBT30	15	CHCl$_3$(80)/ HFIP (20)	Yes	135.5 cP	6.6394

[a]*PEOT/PBT polymers used in this study are available under the name PolyActive® (PolyVation b.v., Groningen, Netherlands). They have a generalized nomenclature of aPEOTbPBTc: a refers to the molecular weight, in kDa; b refers the percentage of PEOT; and c refers to the percentage of PBT.*
[b]*The solvent blends were made of chloroform (CHCl3) and hexafluoroisopropanol (HFIP) by volume percentage (i.e. 70% CHCl$_3$ and 30% HFIP: CHCl3(70)/HFIP (30)). The HW$_{mv}$ solutions required a CHCl$_3$(80)/HFIP(20) blend to ensure polymer solubility, with a slight change in solvent volatility.*
[c]*NaCl was added for a concentration of 0.05 mg/ml.*
[d]*measured at (30°C, 100 rpm)*
[e]Conductivity

Table S3. *Summary of 'solo' ESP Fiber Diameters*

Description	Polymer Solution	Diameter±std. dev. (mm)	n*
Solo ESP	LW	0.863±0.281	331
Solo ESP	IW	0.738±0.335	387
Solo ESP	HW	1.03±0.358	354

Total number of fibers measured.

Table S4. *Summary of Tandem ESP Fiber Diameters*

Description	Polymer Solution	Diameter±std. dev. (mm)	n*
Tandem ESP	HW_{mv}	0.790±0.402	115
	LW	0.647±0.223	149
Tandem ESP	HW_{mv}^{+}	0.774±0.287	113
	LW	0.859±0.311	107
Tandem ESP	HW	1.607±0.345	113
	LW	0.723±0.261	130
Tandem ESP	HW^{+}	0.865±0.164	117
	LW	0.630±0.242	112
Tandem ESP	LW	0.653±0.143	108
	LW	0.637±0.136	118

Total number of fibers measured.

Table S5. *Summary of Solo ESP fiber alignments*

Description	Polymer Solution	Coherence±std. dev.*	n**
Gap Electrode	LW	0.773±0.089	24
Gap Electrode	IW	0.641±0.102	22
Gap Electrode	HW	0.339±0.077	20
Random ESP	LW	0.088±0.055	7

Coherence as a metric for orientation of objects in an image, as measured by the OrientationJ plugin(1). 1 is perfect alignment while 0 is complete anisotropy.
**Total number of images analyzed.*

Table S6. *Summary of Tandem ESP fiber alignments*

Description	Polymer Solution	Coherence±std. dev.*	n**
Tandem ESP	HW_{mv}	0.432±0.076	6
	LW	0.804±0.049	7
Tandem ESP	HW_{mv}^{+}	0.247±0.059	6
	LW	0.551±0.106	6
Tandem ESP	HW	0.401±0.060	7
	LW	0.699±0.104	6
Tandem ESP	HW^{+}	0.397±0.076	7
	LW	0.633±0.061	6
Tandem ESP	LW	0.734±0.115	6
	LW	0.817±0.097	6

*Coherence as a metric for orientation of objects in an image, as measured by the OrientationJ plugin(1). 1 is perfect alignment while 0 is complete anisotropy.
**Total number of images analyzed.

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Chapter 5

A one-step biofunctionalization strategy of electrospun scaffolds enables spatially selective presentation of biological cues

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Abstract

To recapitulate the heterogeneous complexity of tissues in our body with synthetic mimics of the extracellular matrix (ECM), it is important to develop methods that can easily allow the selective functionalization of defined spatial domains. Here, we introduce a facile method to functionalize microfibrillar meshes with different reactive groups able to bind biological moieties in a one-step reaction. The resulting scaffolds proved to selectively support a differential neuite growth after being seeded with dorsal root ganglia. Considering the general principles behind the method developed, this is a promising strategy to realize enhanced biomimicry of native ECM for different regenerative medicine applications.

Introduction

The intrinsic heterogeneity of the body poses a great challenge when attempting to restore or replace lost functions. An emerging perspective in both biological sciences and tissue scaffold design is the influence of the microenvironment on cellular and tissue function[1]. While the traditional 2D culturing environment fails to replicate the complex and multifaceted 3D environment of the extracellular matrix (ECM), many traditional biofabrication strategies also do not capture the nuanced heterogeneous complexity of native cellular environments in a controlled manner. This limits the possibility of inducing relevant cell responses, preventing the adequate study of more complex biological systems and posing a barrier to further tissue scaffold development. In response, 3D culturing environments have been developed that exhibit controlled patterns of biologically relevant cues, including the immobilization of cell adhesion molecules in heterogeneous 3D patterns[2] and the formation of complex 3D structures[3,4].

Of the various scaffold fabrication strategies available, electrospinning (ESP) is often cited as a promising method of recreating the fibrous structure of the natural extracellular matrix (ECM)[5]. ESP scaffolds have proven to be particularly promising for neural tissue engineering applications[6]. By changing various parameters, this process can form fibers from tens of nanometers to tens of micrometer and can produce both randomly or oriented patterns of fibers. However, these fibers must often be modified to present cell adhesion moeities or other cues in order to better approximate the native ECM[7]. These are typically

affixed to the fiber surface by the physical adsorption of proteins, chemical conjugation of biomolecules, or by incorporating ECM proteins, such as collagen, into to the polymer solution. While physical adsorption is relatively unstable, a blended fiber can require a large amount of protein mixed into the organic solvent of the polymer solution. This mixing potentially denatures the protein and renders it non-functional; as a result, this approach is expensive and potentially ineffective. The bioconjugation approach typically requires the surface of ESP fibers to be modified with reactive groups; fibers are then exposed to a solution of biomolecules, which chemically bind to the reactive groups they encounter. This method remains an efficient and effective means of fiber functionalization. However, this approach homogenously functionalizes the entire scaffold structure and cannot be used to impart a spatially defined pattern onto the fibrous construct.

I) Blend solution
(PA + functionalized PEG)

II) Electrospinning

III) PA fibers with
functional groups

Figure 1. Preparation of the functionalized ESP fibers. The blended ESP polymer solution is first prepared (I), with the addition of short polymer chains (red), homofunctionalized with reactive species (blue crosses), to the bulk polymer solution. This solution is then used in the electrospinning process (II), where the resulting fibers exhibit reactive groups on the fiber surface (III).

Spatially selective patterns can also be achieved by photolithographic means, though this can lead to inefficient conjugation and polymer degradation[8–11]. Furthermore, conjugation only occurs on surfaces that are efficiently exposed to radiation, limiting this application to only the superficial 'visible' regions of 3D fibrous scaffolds. Here, we present an approach to create 'pre-functionalized' ESP fibers, whereby poly(ethylene glycol) (PEG) chains homofunctionalized with

reactive groups are introduced to the polymer solution of a chosen biomaterial *a priori* the ESP process (Figure 1). Three different reactive groups are characterized: (i) non-selective succinimidyl valerate (SVA, an *N*-Hydroxysuccinimide variant) for amine conjugation; (ii) a more selective Thiol-Maleimide conjugation approach; and (iii) a bio-orthogonal Alkyne-Azide 'click chemistry' strategy. The use of small chain PEG as a carrier results in the presentation of functional groups on the fiber surface without additional processing required, representing a simple method for achieving functionalized fibers.

When the different fibers are fabricated into a single construct, this provides a powerful method to selectively functionalize specific subsets of fibers within a scaffold. Combined with patterned ESP techniques, this creates a spatially defined pattern of functionalized fibers that ensures the entire fiber surface is activated. We verified the ability to create spatially defined functionalization within a 3D fibrous architecture and examine neurite growth on scaffolds functionalized with the peptide sequence H-Gly-Arg-Gly-Asp-Ser-OH (GRGDS) or the laminin-derived sequence H-Arg-Asn-lle-Ala-Glu-lle-lle-Lys-Asp-lle-OH (RNIAEIIKDI, p20)[12]. Homogeneously functionalized scaffolds successfully support neurite growth, with no difference in growth observed between peptides. However, a scaffold functionalized with both peptides in a spatially defined manner is shown to produce spatially modulated neurite growth, underscoring the value of employing complex ECM-mimicking environments to further elucidate cell behavior.

Materials and Methods

Polymer solutions preparations
300PEOT55PBT45, known commercially as PolyActive™ 30055/45 (PA), was provided by PolyVation B.V. (Groningen, The Netherlands). The chemical composition is represented by the notation aPEOTbPBTc, where a is the molecular weight, in g mol^{-1}, of the starting PEG segments used in the polymerization process, whilst b and c are the weight ratio between PEOT and PBT blocks, respectively. PA was used as main polymer, prepared as a 20% w/v solution dissolved overnight in a mixture of chloroform (CHCl$_3$) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFiP) at a volume ratio of 7:3. The pre-functionalized

agent was added 4 hours before ESP under magnetic stirring at either a 5% or 2% w/v concentration. The three different prefunctionalization agents trialed in this study were PEG-bis(SVA), PEG-bis(Alkyne) and PEG-bis(Thiol) 5000 MW, provided by Laysan Bio (Alabama, USA). The same solutions were prepared for thin film formation time-of-flight secondary ion mass spectrometry (TOFT-SIMS).

Electrospinning

Electrospinning was performed with a customized environmental chamber, maintained at a temperature of 25 $^{\circ}$C and 30% humidity. The polymer solution was loaded into a syringe, mounted into a syringe pump and connected to a spinneret needle via Teflon tubing to supply solution at a flow rate of 1 ml/hr. The spinneret was mounted to a parallel plate, all of which was held at 20 KV and positioned 20 cm above the grounded collector. The collector was a gap electrode arrangement, with 2 mm wide electrodes and a 1 cm gap, producing aligned fibers across the gap. ESP fibers were collected onto mesh support rings, 15 mm outer diameter and 12 mm inner diameter, placed across the gap. ESP was performed for 1 minute to achieve a sufficient fiber density. Heterogeneous scaffolds were produced via the Tandem ESP (T-ESP) technique. Briefly, the same electrode setup was employed but the single spinneret was replaced with two needles supplying different solutions mounted 1 cm apart.

ToF SIMS

Separate samples were prepared on ~0.5 cm^2 segments of silicon wafer coated via evaporation of fluoroctatrichlorosilane (FOTS, Sigma Aldrich) in an enclosed chamber. Thin film control samples of polymer were prepared by spin coating (2000 rpm, 1 minute) the polymer solution on to wafer segments. Silicon wafer segments were also prepared with ESP fibers by placing them between the gap of the target electrode. The same spinning parameters were employed as before.

ToF-SIMS was performed on a TOF.SIMS5 instrument (ION-TOF GmbH, Münster, Germany) at the Institute of Functional Interfaces, Karlsruhe Institute of Technology (KIT). The spectrometer was equipped with a Bi cluster primary ion source and a reflectron type time-of-flight analyzer. UHV base pressure was < 5x10^{-9} mbar. For high mass resolution the Bi source was operated in the "high

current bunched" mode providing short Bi_1^+ or Bi_3^+ primary ion pulses at 25 keV energy and a lateral resolution of approximately 4 μm. The short pulse length of 1.1 to 1.3 ns allowed for high mass resolution. The primary ion beam was rastered across a 500×500μm field of view on the sample, and 128×128 data points were recorded. Primary ion doses were kept below 10^{11} ions/cm^2 (static SIMS limit). If charge compensation was necessary an electron flood gun providing electrons of 21 eV was applied and the secondary ion reflectron tuned accordingly. Spectra were calibrated on the omnipresent C^-, C_2^-, C_3^-, or on the C^+, CH^+, CH_2^+, and CH_3^+ peaks. Based on these datasets the chemical assignments for characteristic fragments were determined.

For high lateral resolution imaging the primary ion source was operated in "burst alignment" mode. Here, only nominal mass resolution was obtained but the lateral resolution of the instrument is in the range of 150nm. Therefore, peaks like S^- and HS^- were used for imaging since both peaks do not show other signals at the same nominal mass (M7z).

<u>Fluorescent Label Conjugation</u>
Reactivity and availability of functional groups were confirmed by the conjugation of fluorescent dyes with complimentary binding motifs. SVA was conjugated to FITC-labeled BSA (Sigma) at 10 mg/ml in a PBS solution at pH 7.2. The Thiol functional group was conjugated with a Dylight 488 Maleimide probe (Invitrogen), which was applied at a concentration of 10 mg/ml in PBS. The click chemistry Alkyne functional group was conjugated with azido-labeled Megastokes 673 dye (Sigma) at a concentration of 10 mg/ml in PBS with 25 mM ascorbic acid and 2.5 mM copper (II) sulfate. Dye solutions were applied over night. A T-ESP scaffold was simultaneously conjugated with a solution containing both the maleimide and azide probes (same concentrations). Substrates were washed 3 times with a tris buffered solution (TBS), pH 8, followed by a1 hour wash with a TRIS-based EDTA solution, pH 9, and then demineralized water (dH$_2$O) three times. To facilitate spatial analysis of observed neurite growth, T-ESP scaffolds conjugated the azide-Megastokes dye to the alkyne fibers as described above, but with a 50 mg/ml dye concentration incubated for 2 days.

Peptide preparation

Both the click chemistry and the thiol strategies require functionalization of the biomolecule of interest with the complimentary binding partner. Peptide solutions of H-Gly-Arg-Gly-Asp-Ser-OH (GRGDS) and H-Arg-Asn-Ile-Ala-Glu-Ile-Ile-Lys-Asp-Ile-OH (RNIAEIIKDI; p20)(Bachem) were prepared at 5 mg/ml in PBS. These were activated with a maleimide and an azide, respectively, via amine-reactive linker molecules of NHS-peg4-Azide (Jena Bioscience) and sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC, Thermo Scientific). Maleimide and azide linkers molecules were first dissolved in DMSO and then added to peptide solutions for a final linker concentration of 20 mM and 50 mM, respectively, representing a 5 times molar excess with respect to peptide concentration. Solutions were maintained at room temperature for 4 hours. The SVA conjugating strategies did not require any pre-preparation of the proteins to be applied.

Scaffold Preparation

Glass cover slips (14 mm in diameter) were placed in a 24 well plate, followed by the ESP fibers collected on mesh rings; the glass cover slip provided a necessary mechanical support for subsequent removal and imaging of the eletrospun mesh. A Viton rubber ring (Eriks B.V., The Netherlands) was inserted into the well to hold the scaffold in place. These constructs were placed inverted with respect to their fabrication orientation, such that the first deposited fibers were facing upwards; the mesh ring provides a physical barrier between the ESP fibers and the Viton ring, improving handling during subsequent processing of cell culture samples. For homogenously functionalized scaffolds, 150 ml of peptide solution was applied at a concentration of 0.5 mg/ml in PBS. Heterogeneous scaffolds were prepared using mixed peptide solutions at a concentration of 0.5 mg/ml of each peptide. Solutions involving click chemistry also had an additional 25 mM of L-ascorbic acid and a 2.5 mM Cu_2SO_4 (Sigma Aldrich). Solutions were left over night to allow for conjugation. Similar to the fluorescent label conjugation, substrates were washed 3 times with a tris buffered solution (TBS), pH 8, followed by a1 hour wash with a TRIS-based ethylenediaminetetraacetic acid (EDTA) buffer (Klinipath B.V., The Netherlands), pH 9, and then demineralized water three times.

Cell Culture

Scaffolds were sterilized with 70% ethanol for 30 minutes, which was later left to evaporate. Scaffolds were washed 3 times with sterile PBS, followed by a one time wash in Neuralbasal® culture medium (Invitrogen) supplemented with B27 supplement, 0.5 mM l-glutamine, 10 U/ml of penicillin/streptomycin (all from Invitrogen) and 10 ng/ml of NGF (Sigma Aldrich). Culture plates were left to warm with 150 ml of culture medium per well in an incubator at 37 ^{o}C and 5% CO_2. Dorsal root ganglia (DRGs) were extracted from 2-day-old post-natal Sprague-Dawley rat pups. All procedures followed national and European laws and guidelines and were approved by the local ethical committee. Briefly, rats were sacrificed by cervical dislocation under general anaesthesia (4% Isoflurane) and then decapitated. Individual ganglia were removed from the spinal column and nerve roots were stripped under aseptic conditions with the aid of a stereomicroscope. Connective tissue was removed and the DRGs were cut to expose the enclosed cells. One DRG was placed in the center of each ESP fiber scaffold; particular care was taken to ensure the DRG was placed in the overlapping region of the T-ESP scaffolds. Cultures were maintained for 5 days, with medium refreshed every other day.

Immunohistochemistry

Cells were first cooled to 4 °C, followed by the addition of an equal volume of ice cold 4% w/v paraformaldehyde (PFA) in PBS, added to each well for a final concentration of 2% PFA. Fixation proceeded for 20 minutes, after which samples were washed with Tris Buffered Solution (TBS). Samples were permeabilized with 0.1% Triton-X for 15 minutes, washed 2 times with TBS and blocked with 5% normal goat serum for 1 hour. This was followed by a 16 hour incubation at 4 ^{o}C in a 1:1000 dilution of BIII-tubulin (Sigma) raised in mouse and 1:500 S100 (sigma) raised in rabbit in TBS with 1% normal goat serum. Samples were then washed 3 times in TBS with 1% normal goat serum, Secondary antibody of anti-mouse Alexa 488 or Alexa 546 raised in goat (Invitrogen) and anti-rabbit Atto 647N raised in goat (Sigma) were applied at a dilution 1:500 for 12 hrs at 4 ^{o}C. Samples were then washed 3 times with TBS containing 1% BSA and 0.1% w/v sodium azide (Sigma Aldrich). After staining, the alkyne-containing fibers of the T-ESP scaffolds were selectively labeled as outlined in the *Fluorescent Label conjugation*

section above. Samples were washed again with TBS, then removed from the well and mounted on coverslip using 4-88 mowiol mounting fluid with 2.5% w/v 1,4-diazabicyclo[2.2.2]octane (DABCO, Sigma Aldrich) for microscopy imaging.

Microscopy and Image Analysis

Fluorophore conjugation to fibers was imaged using a Nikon TI confocal microscope, eliminating diffractive autofluorescence. Per fluorophore, laser power was adjusted using the conjugated scaffolds as a reference. The remaining scaffolds were imaged using the same power settings to determine the degree of selective conjugation. Cell labels were imaged using a BD Pathway 435 with a 4x objective. Neurites were imaged via the Alexa 488 or Alexa 546 labels, using the ex: 482/35-em: 536/40 or ex: 543/22-em: 593/40 filter sets respectively(ex: excitation; em: emission). Schwann cells were visualized with the Atto 647N fluorophore using an ex: 628/40-em: 692/40 filter set. The use of Megastoke 678 dye made possible the visualization of alkyne fibers by using an ex: 543/22-em: 692/40 filter set. Neurite outgrowth was assessed by spatially measuring the number of pixels in radially segmented concentric bands around a DRG. Images shown were processed using the Contrast Limited Adaptive Histogram Equalization (CLAHE) filter within the ImageJ/Fiji software package, with stitched mosaic created with the Grid Stitch plug-in [13].

Statistical Analysis

All statistics were performed with R statistical software (http://www.R-project.org/) and graphs were created using the Deducer plugin (by Ian Fellows, http://www.jstatsoft.org/v49/i08/). Comparisons of fiber diameters and fiber alignments were performed with a one way ANOVA was use followed by a *post hoc* Holm method, with significance level of $p < 0.01$ and a minimum of 100 fibers measured per fiber type.

Results

Initial ESP trials incorporated functionalized PEG additives at a concentration of 5% w/v. However, the quality of the fiber deposition across the gap electrode was inconsistent. An apparent phase separation of the blended polymer solution was also observed over a 12 hour period, suggesting poor solubility of the PEG additive.

Reducing the additive concentration to 2% produced a stable polymer solution and an improved ESP fiber desposition (Figure 2). ALK and SH fibers produced fibers of 0.88±0.23 μm and 0.71±0.19 μm in diameter, with a statistically significant difference between the two populations. The SVA fibers were much larger in comparsion, with a diameter of 1.56±0.47 μm. Image analysis of fiber alignments provides a coherence metric between 0 (random) and 1 (aligned). ALK and SH fibers were found to have statistically similar degrees of alignment (0.42±0.06 and 0.35±0.07, respectively), while the SVA fibers exhibited a higher degree of alignment (0.59±0.08).

TOF-SIMS analysis provides a method to identify elemental composition of the first few nanometer of a surface, providing an effective means of assessing functional group availability on the pre-functionalized ESP fibers. Both the 2% SVA and 2% SH fibers clearly exhibited available functional groups, shown in Figure 3. Unfortunately, ALK fibers were not possible to be assessed because the chemical signature of the incorporated alkyne groups was not sufficiently distinct to be discerned from the bulk PA polymer.

To ascertain whether chemical groups on the fiber surface were available for conjugation, solutions of fluorescent probes with complimentary conjugation molecules were employed; these included BSA-FITC for SVA conjugation, Dylight-488 with maleimide for SH conjugation and Megastokes 678 with an azide for alkyne (ALK) conjugation. Available groups on the fiber surface were reactive (Figure 4). The SVA fibers were shown to strongly retain the BSA-FITC protein (Figure 4A) compared to the ALK and SH fibers (Figure 4D,G), although a degree of non-specific binding could still be observed. A high degree of selective conjugation was noted for the smaller molecular probes used for the ALK fibers

(Figure 4F), with no observable cross-reactivity with either the SVA fibers (Figure 4C) or SH fibers (Figure 4I). Similarly, the maleimide fluorescent probe showed a clear preference for the SH fibers (Figure 4H) compared to no observed reactivity with the SVA or ALK fibers (Figure 4 B,E).

Figure 2. *ESP fiber size and alignment. Diameters are shown for PA fibers with 2% of ALK, SH and SVA conjugation agents (A). All three diameters were found to be statistically different from each other, according to a one-way ANOVA with a post-hoc Holm-corrected pairwise comparison (p< 0.01; n=100, fibers per sample). The degree of fiber alignment was also determined (B) using coherence as a metric, where 0 is random and 1 is perfectly aligned. Similar analysis found that fiber alignments of the ALK and SH fibers were similar, while the SVA fibers were significantly more aligned (p<0.01, n =6, images per sample). scalebar: 50 μm.*

Tandem ESP was also employed to create a heterogeneous scaffold of two aligned fiber populations positioned next to each other with defined overlapping region. A tandem scaffold of ALK and SH fibers was exposed to a solution of maleimide-Dylight 488 and azido-MegaStokes 673, verifying that spatially selective conjugation of different fibers types is possible with such a system.

Figure 3. Scanning results from ToF-SIMS analysis. Shown are the signals associated with $C_4H_4NO_2$ (A), the elemental signature of SVA, and an overlay (B) of SVA (red) with the PA signal (green). Colocalization of both signals appear as yellow. Similarly, a signal from the S- or SH groups is shown (C), along with a corresponding overlay (D) of SH (blue) and PA (green). Regions of colocalization appear as light blue/mint. No distinctive signal pertaining to the ALK additive could be observed.

To validate these conjugation strategies, neurite outgrowth was evaluated on functionalized scaffolds. The SH and ALK fibers were selected for *in vitro* evaluation, owing to similarities of fiber diameter and alignment as well as high conjugation selectivity and similar use of activated proteins. SH fibers and ALK fibers were functionalized with the GRGDS and p20 peptides, respectively.

Neurite outgrowth on these substrates was well aligned in the direction of fiber orientation (Figure 5A,C), with no distinct differences in the length or distribution of outgrowth on fibers functionalized with either the GRGDS or p20 peptide. The migration of Schwann cells from the explanted tissue was also observed on both fiber substrates (Figure 5B,D). Fibers functionalized with GRGDS exhibited a higher density of Schwann cells surrounding the explanted DRG.

Figure 4. *Fluorescent probe conjugation on different fiber types. The SVA additive fibers showed strong binding to BSA-FITC (A), where BSA is a representative large globular protein with available amine groups. No signal was observed when the maleimide dye (B) or azide dye (C) were appied to SVA fibers. ALK and SH fibers both experienced residual adhesion of the BSA (D, G). Both of these fibers were shown to have highly specific binding to azide-MegaStokes 673 (MS673) and Maleimide-Dylight 488 (Maleimide-488) (H, F). A T-ESP scaffold of ALK and SH fibres within a mesh frame (autofluorescent green boundary) showed selective conjugation of azide-MegaStokes 673 (red) and maleimide-Dylight 488 (green), respectively. Scale bar: A-I = 250 μm; J = 1 mm.*

Figure 5. *Representative images of neurite outgrowth and Schwann cell migration from explanted DRGs. Neurites outgrowth on the p20 fibers (A) or GRGDS fibers (C) was equivalent. P20- and GRGDS-functionlized fibers also stimulated Schwann cell migration from the DRG explant (B,D, respectively). Scale bar: 500 μm.*

Figure 6. Representative outgrowth on Tandem ESP (A). The cumulative pixel intensity of neurite outgrowth (B) provided a comparison of the density of neurite growth, with the GRGDS fibers exhibiting higher density outgrowth. Fibers with p20 functionalization were visualized (C, in white) versus the region of RGD-functionalized fibers (C, black region), with the DRG placed at the nexus of the two fiber populations. Also observed was Schwann cell migration from the explanted DRG (D). Scale bar: A,C,D = 500 μm.

These peptides were also applied to tandem ESP scaffolds, creating spatially defined regions of aligned fiber with specific biomolecule functionalization. Under these conditions, a preferential growth on GRGDS-modified fibers was revealed, despite the presence of the neurite-specific p20 peptide. This is despite an apparent uniform distribution of migrating Schwann cells.

Discussion

The goal of this study was to develop ESP fibers that could be functionalized with biomolecules without the need of post-modification by wet chemistry or photolithography. While representing a simple strategy for fiber bioconjugation, this was pursued to create fibers with orthogonal conjugation chemistries to enable the selective functionalization of specific subpopulations of fibers within a heterogeneous ESP scaffold. We were able to verify the presence of reactive species on ESP fibers and confirm the selectivity of the different chemistries employed. Applying this approach to the assembly of spatially defined heterogeneous fiber scaffolds, created via the T-ESP technique, a fibrous scaffold with spatially defined, orthogonal conjugation chemistries was created. As an

example, preliminary results showed that neurite growth on such scaffolds differed compared to homogeneous scaffold and exhibited spatially modulated behavior.

The ability to conjugate biomolecules to 3D culturing platforms in a spatially defined manner represents a promising tool to study and dictate cell response. While studies of cells on 2D substrates with biomolecule patterns have produced interesting results[14,15], planar substrates limit the applicability of these outcomes. Many of the relevant signals imparted to a cell are lost or altered when moving between a native 3D ECM and a 2D substrate. However, the creation of analogous biomolecular patterns within a 3D scaffold via spatial selectivity represents a biofabrication challenge, with emerging strategies employing two-photon activation of reactive molecules within optically-permissive hydrogel environments[2,16,17].

Outlined in this current study is a method to spatially define bioactive moieties within a 3D fibrous scaffold, providing cells with a combination of nanotopography and a pattern of biomolecular cues. Functionalization of ESP fibers traditionally relies on wet chemistry to bestow reactive groups on the fiber surface, a method that does not permit spatially defined bioconjugation. Recently, alternative approaches to functionalize ESP fibers have been explored whereby the polymer solution was modified to include functionalized reactive groups before ESP. These approaches included conjugation chemistries similar to those employed in the current study[18–20]. However, all methods required the custom synthesis of active carrier polymer chains. In contrast, the method described in the current study provides a simple means of attaining pre-functionalized ESP fibers through the use of commercially available modified PEG chains as a carrier. ToF-SIMS analysis revealed the availability of reactive groups on the fiber surface, confirmed by the successful conjugation of fluorescent probes. This approach is compatible with many polymer preparations currently used in ESP and creates fibers nearly identical in composition while varying surface reactivity by simply modifying the used additive.

Neurite growth from an explanted rat DRG was evaluated on scaffolds conjugated with either p20 or GRGDS peptides, with no observable differences in the degree

of outgrowth were observed. To showcase the utility of this approach, a heterogeneous scaffold comprised of two overlapping populations of aligned fibers was created. One fiber type incorporated the ALK additive and was subsequently functionalized with p20 using click chemistry, while the other contained the SH additive and functionalized with the GRGDS adhesion motifs via thiol-maleimide chemistry. An explanted DRG was then placed on the overlapping region between these two fiber groups. Despite the equal performance of these two peptides on homogenously conjugated scaffolds, biased neurite growth was observed on the RGD-functionalized fiber population.

RGD is a well known cell adhesion peptide sequence, present in many ECM proteins such as fibronectin, vitronectin, collagen and laminin[21]. This adhesion molecule is known to interact with cells through a well characterized family of integrins, with clear evidence of positive influence on neurite outgrowth[22]. In contrast, p20 is a lesser known sequence found specifically on the gamma 1 chain of laminin. Identified by Liesi *et al.* as promoting neurite outgrowth[12], it is now known to bind to the non-integrin cell membrane prion protein (PrPc). This receptor is considered a modulator of cell behaviour, maintaining the destabilization of β1 integrin- related focal adhesion formation and causing localized release of Ca^{2+} ions, resulting in increased cell motility and neurogenesis[23].

The DRG explant model used in this study also includes the complex interaction between neurons and supporting glial Schwann cells, also present in DRG explants and highly active during peripheral nerve regeneration. After injury, Schwann cells typically precede the regenerative front *in vivo*. They change phenotype to produce ECM molecules and diffusible growth factors, encouraging and guiding neurite growth[24]. Recent work in our lab has shown that Schwann cells exhibit a differential response to both the p20 and RGD peptides. While both of these peptides promoted increased metabolic and proliferative activity in comparison to non-functionalized fibers, the RGD sequence was observed to also upregulate NGF production[25].

Within the current study, the observed bias in neurite growth is thought to be the result of a localized release of NGF by Schwann cells in response to RGD-conjugated fibers. For scaffolds that are homogeneously functionalized with either the NGF-promoting RGD or neurite promoting p20, evidence of such a localized release of NGF is lost and resulting growth is similarly homogenous. For tandem-spun scaffolds, the spatially defined distribution of different peptides likely produced a local concentration gradient. This attracted the initial outgrowth of neurites in a biased manner, with a preference for the RGD sector. This is despite virtually no difference in the distribution of Schwann cells in and around the DRG, attributed to both RGD and p20 as effective substrates for this glia subtype[25].

When this approach is implemented within a heterogeneous scaffold, it presents a promising approach to investigate the complexity of a heterogeneous growth environment. These findings exemplify the importance of examining heterogeneity for both natural and designed ECM constructs. Though traditional homogeneous environments will continue to provide knowledge, these substrates overlook the influence of ECM components on the spatial-temporal regulation of cell behaviour. Though these preliminary results are still far from achieving decisively selective cell response, the methodology proposed here provides the first step towards mimicking the complexity of the ECM heterogeneity.

Conclusion

We introduced three facile methods for creating functionalized fibers and present the concept of combining these strategies to create an ordered, heterogeneous tissue scaffold. The use of such scaffolds reveals the synergistic influence of different peptides on the function of glia and neurite growth, revealing how such multimodal fabrication strategies might assist in both understanding the complexities of biological systems and providing tissue engineering solutions.

Acknowledgements

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Chapter 6

A Biomimetic 3D Hybrid Culturing Platform For Studying Peripheral Nerve Regeneration

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Abstract

The development of an effective tissue scaffold for the repair of peripheral nerve injury remains a challenge, with the need for scaffolds to become increasingly biomimetic and multi-faceted for improved neural regeneration. Here, we present the development of a synthetic 3D *in vitro* culturing system that faithfully recreates the fibrillar physical cues of the peripheral nervous system extracellular matrix (ECM). This hybrid hydrogel/nanofiber construct has been designed for studying nerve regeneration and optimizing tissue-engineered scaffolds, and represents an accurate synthetic mimic of the ECM in terms of composition, structure and scale. Hydrogel devices containing micro and nano fibers were fabricated, with the possibility to tune nanofibrillar orientation and size, and selectively leach the microfibrillar network, thus originating microchannels for axon growth. The device was tested with dorsal root ganglia and showed to support axonal elongation through the microchannels, which is enhanced in presence of neural morphogenetic signals.

Introduction

A long-standing pursuit in regeneration of the peripheral nervous system (PNS) has been the development of an effective replacement for autologous nerve grafts, which are still considered the 'gold standard' in nerve repair[1,2]. The PNS performs the critical task of rapidly communicating between the central nervous system (CNS) and the body. Fortunately, the PNS has an intrinsic capacity to regenerate and can repair minor neural damage without assistance[2]. However, regeneration fails if nerve damage is too extensive or a transected nerve is unable to be re-joined without tension. Defects shorter than 10 mm can be effectively bridged by nerve conduits, facilitating nerve repair by promoting the local accumulation of endogenous extracellular matrix (ECM) and growth factors[3]. The standard approach for longer defects is to bridge the gap with an autograft donor nerve segment, sacrificed from a 'less critical' site within the body[4]. Nerve damage more than 5 cm exceeds the length of available autografts, requiring a longer cadaver allograft and the indefinite use of immunosuppressant to prevent implant rejection[5]. In both cases, there is a risk of limited regenerative capacity because of

the possible size mismatch of donor tissue[6,7]. To avoid sacrificing healthy nerve tissue, and a second surgery site or issues of implant rejection, an intense area of research is the development of an instructive scaffold as a viable alternative to these approaches.

A suitable replacement has proven elusive, prompting increasingly multifaceted scaffold designs in order to reflect the organization and complexity of peripheral nervous tissue[8,9]. The main functional components of the PNS are axons, continuous cellular extensions that transmit action potentials both to and from the CNS to tissues of the body. Axons range from <1 µm up to 20 µm in diameter, but are found in organized bundles to form nerves with a diameter up to a millimeter or more[2]. Within the nerve, axons are embedded within the endoneurium, an ECM organized into tube-like structures that house individual axons and supporting Schwann cells[10]. These endoneurial tubes typically range in diameter from 5 to 20 mm[11], formed by 25 to 60 nm diameter fibers of type I and type III collagen aligned along the length of the axon[12]. Also present within the endoneurial tube are laminins, specific ECM proteins known to exhibit preferential neurite adhesion[13]. The remaining interstitial spaces of the endoneurium are filled with additional hydroscopic proteoglycans[14].

The ability of the PNS to repair itself is partly attributed to the guidance structures provided by the 'endoneurial tubes'[2]. Subsequently, scaffolds have been designed with structural guidance elements in order to similarly facilitate nerve repair. Nerve conduits with microchannels of 400 mm to 500 mm in diameter have shown enhanced regeneration compared to hollow conduits, but cannot match the performance of autografts[15]. Scaffolds have been fabricated from collagen in the form of foams with interconnected pores that form channel-like structures between 25 and 50 µm in diameter, better approximating the endoneurium in both scale and composition[16]. These scaffolds fair better, approaching the performance of autografts. However, none have surpassed autograft performance to fully justify a change from a well-established procedure. To achieve a sufficient substitute, further scaffold development is required.

In lieu of *in vivo* studies for continued scaffold optimization, the use of three-dimensional (3D) *in vitro* culturing environments has proven invaluable to explore biological systems[17] and to optimize elements of increasingly complex scaffold designs[18,19]. Three-dimensional culture systems overcome limitations of 2D environments to facilitate the understanding of complex biological processes; the first *in vitro* observation of axon myelination by Schwann cells was facilitated by culturing neural tissue with a natural ECM hydrogel[20]. Such *in vitro* scaffolds include aligned nanofiber meshes, used as an *in vitro* substitute for the fibrillar ECM of the endoneurium. A recent report highlights that aligned electrospun (ESP) fibers direct neurite extension, but that the 3D organization of these topographical elements is also an important factor[21]. Improved neurite growth was observed on 760 nm diameter fibers compared to 290 nm, although the smaller fibers better approximate the size of native collagen fibrils[22]. The authors stressed that the packing density of the fibers could be the determining factor for the observed cell response; the smaller fibers exhibited higher fiber density, suspected to limit cell infiltration and limiting the extent of the 3D environment presented. Nanofiber meshes were also shown to direct neurite growth *in vitro* and *in vivo*[23], but *in vivo* response was found to also be dependent on the macrostructural arrangement of nanofibers[24]. These results reflect recent opinions that nanofiber scaffolds are more similar to roughed 2D surface and that scaffold design should respect the 3D organization of structural elements[25].

Hydrogels are an alternative 3D *in vitro* culture environment and can even be anisotropic in nature, formed with aligned fibrils that are able to direct growth[26]. However, these constructs often do not have additional microchannel architecture and instead require cells to remodel the hydrogel matrix to make space for neurite growth[27]. This is reflected by the *in vivo* study of anisotropic hydrogels in PNS regeneration, where results showed that neurite growth was enhanced but still considered insufficient[28].

Outlined in this study is a novel hybrid 3D construct comprised of both hydrogel and nanofiber elements, designed to approximate the 3D endoneurial structure. This culturing platform consists of oriented microchannels on the scale of endoneurial tubes, interspersed within a compliant hydrogel to provide

microarchitecture accommodating to neurite growth, without requiring the hydrogel matrix to be remodeled. Similar to the endoneurium, aligned nanofibers are incorporated into the wall of the channels to provide additional nanotopographical cues. In addition to characterizing this hybrid construct, *in vitro* assessment of primary neurite growth through these synthetic endoneurial structures validated this as a promising culturing platform.

As scaffolds begin to increase in complexity, optimization becomes increasingly difficult. Here, we describe the development of a unique 3D culturing platform that incorporates both micropatterned architecture and nanofiber topography in a controlled manner. The modular assembly of this platform also creates a highly flexible system that can be tailored to reflect the subtle interplay of components within native ECM. This is ideal for scaffold optimization or controlled study of biological systems, providing an accessible, economic tool for making informed design decisions and advancing current understanding of neural growth. Furthermore, this approach employs fabrication methods that can be directly translated to a scaffold suitable for implantation.

Methods And Materials

<u>Device Assembly</u>

Preparation of Polymer Solutions for Electrospinning
300PEOT55PBT45 (300/55/45), a PEOT/PBT block copolymer within the PolyActive™ (PA) family, was kindly provided by PolyVation B.V. (Groningen, The Netherlands). The chemical composition is represented by the notation aPEOTbPBTc, where a is the molecular weight, in g mol^{-1}, of the starting PEG segments used in the polymerization process, whilst b and c mean the weight ratio between PEOT and PBT blocks, respectively. PA fibers were fabricated from a 20% w/v solution dissolved in a mixture of chloroform ($CHCl_3$) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFiP) at a ratio of 7:3 v/v. Blended fibers were prepared from a PA-Collagen solution which was a 1:1 blend of a 20% w/v PA solution, prepared in HFIP, and an 8% w/v bovine collagen type I (a generous gift from Kensey Nash Corporation (USA, Catalogue number 20003–04)), also in HFiP.

This produced a final solution with 4% w/v of collagen and 10% w/v of PA in pure HFIP. The sacrificial template fibers were fabricated from PL(DL)A 50/50 (Mw=40000, IsoTis S.A., The Netherlands), dissolved in a mixture of dicloromethane and HFiP (4:1), Concentrations of 50% w/v and 75%w/v were prepared. All solutions were prepared and stirred overnight at room temperature before use.

Electrospinning

ESP fibers were prepared with a custom apparatus with temperature and humidity control, maintained between 24–25 ºC and 28–32 %, respectively (Figure 1). Each polymer solution was loaded into syringes and then mounted on a syringe pump (KDS 100, KD Scientific) to control flow rate. Teflon tubing connected the syringe to a stainless steel spinneret needle with a 0.8 mm outer diameter and a 0.5 mm inner diameter. The spinneret was mounted to an upper parallel plate. Electrical potential was controlled during the spinning process by a high voltage generator (Gamma High Voltage Research Inc., FL, USA). Aligned fibers were achieved by employing a gap electrode grounded target, consisting of two aluminium electrodes 2 mm wide with a gap of approximately 15 mm (Figure 1, inset). A Teflon® mount was used to support a ring-like mesh support frame (17 mm OD, 12 mm ID) across the electrode gap. Fibers were collected onto the support frame, with both the fibres and the frame later embedded with the final hydrogel construct. PA fibers were electrospun at a flow rate of 1mL/h, a voltage of 18 to 20 kV and a 20 cm air gap between needle and the collector. For PA-collagen fibers, we used a flow rate of 0.2 ml/h at a voltage of 15 kV with an air gap of 15 cm. PLA fibers were electrospun at flow rates of 1, 5 and 10 mL/h for the 50% w/v solution, and 5 ml/hr for the 75% solution. Voltages ranged from 15 to 20 kV and an air gap between 20 cm and 25 cm.

PCL Plug Fabrication

Sacrificial plugs were used to form the seeding wells within this *in vitro* hydrogel device. PCL (Capa 6500, Perstorp, UK) plugs were manufactured using a Bioplotter device (Envisiontec GmbH, Germany). The polymer was inserted in a stainless steel syringe and heated to T = 150 °C. When the molten phase was achieved, fibers and support frame were positioned 1 mm from the syringe and the

molten polymer was extruded via manual pressure until the molten polymer contacted the fibers. Iteratively raising the syringe $\cong 0.2$cm, followed by extrusion and a polymer cooling period, a $\cong 1$ cm high solid column of PCL was cut from the syringe, creating well templates merged with the underlying aligned fiber matrix (Figure 1). Two wells per scaffold were created in this manner.

Hydrogel Embedding and Leaching

The hydrogel pre-polymer solution was prepared by dissolving PEGDA (Mw=5000, Laysan Bio) in PBS (Gibco, Invitrogen) at room temperature, for a final concentration of 10% w/v. A 10% w/v of Irgacure 2959 (BASF) solution, prepared in a 70:30 solution of ethanol and demineralized water, was added to the hydrogel prepolymer solution for a final photoinitiator concentration of 0.1% w/v. A custom aluminium mold of cylindrical wells (17 mm in diameter and 10 mm deep) was used. A 15 mm coverslip was sealed to the bottom of the mold well and 100 µl of PEGDA solution formed an even layer on the well bottom. Under constant N_2 flow, this layer was partially crosslinked in a crosslinking cabinet (Ultralum, USA) by placing the mold 10 cm from the 365 nm UV lamps for 2.5 minutes at an average power of 10 mW/cm^2. The support frames, with ESP fibers and PCL plugs, were then placed in the well and held down by a cylindrical insert (17 mm OD, 15 mm ID, 2 cm height). The well was then filled with a 300 ml of hydrogel and crosslinked for an additional 10 minutes as previously described. The PLA/PCL template was then dissolved by placing the construct in acetone (Sigma Aldrich) held at 50 ^{o}C. The acetone was refreshed every hour for the first 2 hours, then maintained for 16 hours (over night). This was followed by the same sequence in 100% ethanol at 37 ^{o}C, followed by the same sequence in sterile demineralized water at 37 ^{o}C.

Device Characterization

Fiber SEM

The morphology of ESP fibres above mentioned was observed using scanning electron microscopy (SEM) (XL 30 ESEM-FEG, Philips). Before the observation, the scaffolds were mounted on aluminium stubs and coated with gold using a sputter coater (Cressington SputterCoater 108auto). Fibre diameters were measured

from the SEM photographs by measuring 150 fibres per condition using the ImageJ image analysis software (National Institutes of Health, USA).

Hydrogel SEM

Structural characterization of the surface and bulk of the hydrogel devices was performed using SEM. Initially, the devices were cut with a sharp blade in places of interest and processed through a dehydration sequence of tert-Butanol (TBA, Sigma Aldrich). This involved dilution series of 50%, 70%, 100%, 100%, 100% TBA/H_2O for 12 hours, each maintained at 37 ^{o}C. The samples were kept in TBA and quickly frozen in liquid nitrogen. Samples were then quickly placed under vacuum at 4 ^{o}C and the TBA was sublimated overnight. The samples were later gold-sputtered and imaged.

Hydrogel Swelling

The hydrogels were initially weighed after crosslinking. Then, they were immersed in demineralized water and allowed to swell for one week. After, the gel was removed from the demineralized water, weighed, and dried under vacuum over night at room temperature to desorb all water. The final gels were then weighed with samples prepared in triplicates. The swelling ratio (Q) was calculated using the mass of the equilibrium swollen hydrogel ($M_{swollen}$) and the mass of the dried hydrogel (M_{dry}) by the following formula:

$$Q = 1 + \frac{\rho p_p}{\rho_s} \cdot \frac{(M_{swollen} - M_{dry})}{M_{dry}}$$

with ρ_p the polymer density and ρ_s the density of the solvent (PBS).

Microchannel Diffusion Assessment

To verify that the device had open channels connected to the wells, diffusion tests using fluorescent beads were performed. Diffusion studies were conducted with FluoSpheres® Carboxylate-Modified Microspheres (Invitrogen) with 100 and 200 nm diameters. Twenty *μL* of solution were added to one of the wells of the device and the diffusion was recorded using an epifluorescent microscope (EVOS® FL Digital Fluorescence Microscope). Confocal microscopy (Nikon A1 Confocal Microscope) was used for 3D reconstruction.

Primary Cell Culture

DRG Isolation
Dorsal root ganglia (DRGs) were isolated from postnatal rat pups (Wistar Unilever: HsdCpb:WA) between the ages of 2 and 8 days. All procedures followed national and European laws and guidelines and were approved by the local ethical committee. Briefly, rats were sacrificed by cervical dislocation under general anaesthesia (4% Isoflurane) and then decapitated. Individual ganglia were removed from the spinal column and nerve roots were stripped under aseptic conditions with the aid of a stereomicroscope.

Device Seeding and Culture Maintenance
Hydrogel devices were incubated in sterile phosphate-buffered solution (PBS; Invitrogen) for two hours, followed by placement in a 24 well plate of one device per well with each well filled with PBS to avoid trapping air bubbles under the hydrogel. The PBS was removed and hydrogels were held within the well using Viton® O-rings (Eriks BV, The Netherlands). Devices were then incubated in 200 µl of laminin (15 µg/ml; Sigma Aldrich) and poly-l-lysine (0.2 µg/ml; Sigma Aldrich) in PBS for 2 hours at 4 °C. Each device was then washed 1x with PBS and 2x with Neuralbasal® A- medium (Invitrogen), with the last wash applied for 1 hr. This was replaced with 200 µl of complete Neuralbasal® A- medium, supplemented with 0.5 mM of L-glutamine, 1x B27 supplement, and 10 U/ml of penicillin/streptomycin (all from Invitrogen). Medium was additionally augmented with either 50 ng/ml of glial-derived growth factor (GDNF; Sigma Aldrich) or 10 ng/ml of neurotrophic growth factor (NGF; Sigma Aldrich). Each scaffold was seeded with 1 DRG in one of the wells and cultures were maintained for 5 to 8 days, with medium changed every 2 days.

Immunohistochemistry and Imaging
Medium was aspirated from cell culture samples, cells were washed twice with a wash buffer [tris-buffered solution (TBS; Sigma Aldrich) + 1% w/v bovine serum albumin (BSA; Sigma Aldrich) and 3.4% w/v (100mM, 34 mg/mL) sucrose(Sigma Aldrich)] and fixed for 1 hr with a 4% w/v paraformaldehyde solution (Sigma

Aldrich) at 4 °C. After 3 times washing with the wash buffer, cells were permeabilized with 0.2% v/v Triton-X 100 (Sigma Aldrich) in wash buffer for 30 min. Next, they were washed again 2 times with the wash buffer and incubated with 5% v/v goat serum (Sigma Aldrich) for 1hr. Primary antibodies β-Tubullin III (1:1000, anti-Mouse, Sigma Aldrich) and S100 (1:500, anti-Rabbit , Sigma Aldrich) were diluted in wash buffer with 2% v/v goat serum and incubated for 24 hours at 4 °C. Samples were washed 3 times with wash buffer + 2% goat serum, then incubated with goat anti-Rabbit IgG Alexa 488 conjugated (Invitrogen) and goat anti-Mouse IgG Alexa 594 conjugated (Invitrogen) diluted in wash buffer (1:500) for 16 hours at RT, in the dark. GDNF-sensitive neurons were stained by first blocking endogenous biotin and avidin (VectorLabs), 30 minutes for each step, followed by incubation with Isolectin-B_4(IB4)-biotin (5 µg/ml, Invitrogen) for 24 hours at 4 °C in TBS. After a triple TBS wash, samples were incubated for 16 hours with streptavidin-488 (Jackson Immunoresearch, USA). To visualize the cell nucleus, cells were incubated for 20 minutes in 0.7 µg/ml 4',6-diamidino-2-phenylindole (DAPI; SigmaAldrich) as counterstaining, followed by a 2x wash in wash buffer. Samples were imaged with a confocal microscope (Nikon A1 Confocal Microscope).

Statistical Analysis

All statistics were performed with R statistical software (http://www.R-project.org/) and graphs were created using the Deducer plugin (by Ian Fellows, http://www.jstatsoft.org/v49/i08/). When comparing between template fiber diameter and final microchannel diameter, a Student's T test was used ($p<0.05$). Comparison of fiber diameters was performed with a one way ANOVA followed by a *post hoc* Holm method, with significance level of $p < 0.01$ and a minimum of 20 fibers measured.

Results

Device Characterization

Figure 1. *Outline of the fabrication strategy. The fiber network to be embedded was formed by sequential deposition of aligned fibers (A), achieving fiber alignment via a gap electrode collector (A, Inset). Fibers were deposited onto a mesh frame (grey) (B), creating a template layer of soluble fibers (green) between two insoluble layers of nanofibers (orange) (C). The fiber network was then heated for 1 hour at 65 ^{o}C (D), allowing for the insoluble fibers to merge on the periphery of the template fibers. PCL plugs (white) were then deposited onto the fiber network (E), forming the well templates for cell seeding. Using a mold with a cylindrical cavity (F), a thin hydrogel layer (blue) was first partially cross-linked (G). After placing the fiber network within the mold (H), held in place by an additional insert (I), a second hydrogel layer was formed (blue)(J). This created a hydrogel construct that enveloped the template mold and merged with the thin underlying layer (K). The construct was then place in heated acetone to dissolve the template (L), leaving the final hydrogel construct with microchannels lined with aligned nanofibrous topography (L, Inset).*

The electrospinning of PLA in combination with a gap electrode collector configuration, shown in Figure 1, allowed for the deposition of aligned fibers onto a supporting mesh frame, creating an ordered microchannel template that could be robustly handled throughout the fabrication process. Altering the electrospinning parameters or the properties of the PLA solution produced a defined range of fiber sizes (Figure 2A) with average diameters of 2.5 ± 0.40 μm, 6.0 ± 0.84 μm, or 15.7 ± 3.6 μm. This extended a degree of flexibility over the size of the microchannel template created. It should be noted that the fiber density of the more viscous 75% w/v PLA solution was inconsistent because of fibers localizing on one electrode or the other instead of depositing across the electrode gap. Based on a consistent density of fiber deposition and diameter most closely approximating the dimensions of an endoneurial tube[11], the median fibers (50% PLA, 5 ml/hr) were selected for use as an appropriate channel template.

Fiber alignment was measured by assessing SEM image coherence, a method that produces a metric between 0 (completely random) and 1 (perfectly aligned)[29]. Analysis found a coherence measurement of 0.77 (Figure S1), indicating a good degree of alignment for relatively straight microchannel formation.

The multimaterial strategy shown in Figure 1 takes advantage of the different material properties of PCL and PLA in order to make a template with interconnected macro-scale features (PCL well plugs) and micron-scale features (PLA fibers). The melting temperature of PCL is approximately 60 $^{\circ}$C, much lower than the 160 $^{\circ}$C melting point of PLA. This allowed for the PCL macrostructure to infiltrate and fuse with the microchannel template while maintaining the integrity of the PLA fibers (Figure S2). Importantly, acetone is a known solvent for both materials, completing this sacrificial template approach.

To test the effectiveness of this PCL/PLA combination to create the desired structures, templates were embedded without the presence of the insoluble nanofibers. Evaluation of the hydrogel found a swelling ratio of 19 ± 5 %. Such a limited degree of swelling permits faithful preservation of template dimensions, with an original 6 μm fiber resulting in a 7.14 μm channel. After encapsulation within the hydrogel and subsequent acetone leaching the template structures, a

disc-like hydrogel construct was produced with two wells (Figure 1G). A microchannel-like structure was observable by bright field microscopy (Figure 2B, 2C). Applying a solution of fluorescent microspheres to a well validated the accessibility of the formed microchannels via the wells (Figure 2D, 2F). Occurring within minutes, the flow of fluorescent beads clearly delineated the channels connecting the two wells.

The PLA fibers had a tendency to make contact with one another, existing at times as a single fiber or as a bundle of fibers along the length between the two well locations. Consequently, microchannels were found to have a variable, branched architecture (Figure 2C, 2D), mimicking the bifurcating appearance often found in biological systems[30,31]. Confocal analysis and 3D rendering of microchannels with fluorescent beads (Figure 3) showed that the structures retained a generally cylindrical morphology, mirroring the original PLA fibers. The intermittent clustering of PLA fiber bundles translated to a wide range of channel diameters, depending on the density of fiber deposition. A careful selection of channels assumed to be formed from single fibers found the average channel diameter to be approximately 6.6 ± 0.78 μm (n=8); a Student T-test found no statistically significant difference with the estimated value of channel size of 7.14 μm.

Nanofiber topography was integrated into resulting microchannels by first creating a sequentially ESP fiber network with aligned nanofibers above and below the PLA template. Both pure PA and PA-collagen fibers were used to fabricate these triple layer constructs (Figure S3). Heating the PLA fibers within their glass transition temperature allowed nanofibers, already in close contact with PLA fiber surface, to be drawn into the periphery of the template (Figure 4A). A series of optimizations (Figure S4) found that 1 hr at 65 °C achieved adequate nanofiber integration while maintaining the round morphology of PLA fibers (Figure 4B).

***Figure 2.** Microchannel characterization. Electrospinning of PLA fibers under different conditions (A). Three distinct diameter populations (p<0.01; n>20), confirmed by a one-way ANOVA analysis and a Holm-corrected pairwise test, were produced. This provided templates to form microchannels of different sizes. The diameter distribution of the PLA fibers was selected for the microchannel template (B), with an average diameter of 6.04 ± 0.85 µm. The resulting microchannels could be observed through bright field microscopy (C, D) and microchannel/well connectivity was confirmed by the diffusion of fluorescent microspheres (E, F). This also revealed arborized microchannels, the result of bundling of the template fibers (scale bars: C, F 500 µm; D, E 50 µm).*

***Figure 3.** A 3D rendering (A) of suspension of fluorescent nanospheres as they flow from the seeding well to fill microchannels formed within a hydrogel, acquired via confocal microscopy (B). Microchannels morphology (C) retains the similar rounded, cylindrical shape of the template fibers (scale bar: B, 400 µm).*

SEM analysis of a fully prepared hydrogel construct was initially impeded by insoluble fibers that occluded the presence of any microchannel architecture (Figure S5). Reducing the density of nanofibers within the hydrogel construct, the presence of both microchannels and nanofibers on their periphery could be confirmed (Figure 4D). After fabricating a completed hydrogel device

incorporating both microchannels and nanofibers, microchannel connectivity was again confirmed by the application of fluorescent microspheres in solution (Figure 4E-F).

Figure 4. *Optimization of insoluble fiber/template assembly and verification of fiber/channel integration. The triple layer after 65 °C showed merging of the soluble fibers on the periphery of the microchannel template fibers (A), while maintaining the template morphology (B). SEM analysis of a cross-section of an assembled hydrogel construct showed the formation of a row of microchannels, delineated by a dotted line (C), with integrated nanofibers (C – solid square outline, expanded in D). Connectivity of microchannels after nanofiber integration (D) was shown via the effective diffusion of fluorescent microspheres from t=0 (E) to t=30 min (F). (scale bars: A-C 20 μm; D 2.5 μm; E-G 500 μm).*

Explanted DRGs from 2 day-old pups were placed within hydrogel devices and allowed to adhere. This required a reduced volume of medium to be applied to the hydrogel. Initial trials found that DRG adhesion in devices incorporating PA fibers was insufficient, with DRGs either failing to adhere or not supporting neurite extension. A blended fiber of PA and collagen proved to be sufficient to induce DRGs outgrowth, indicating that adhesion and neurite extension required the presence of an appropriate nanofiber scaffold. However, DRG adhesion remained a challenge given the buoyant propensity of this tissue explant; sufficient adhesion was at times achieved only after 2 days, at which time neurite extension was permitted to occur.

These hydrogel devices were able to maintain neurite extension in GDNF-supplemented medium (Figure 5). The observation of nuclei within the well area

indicated the migration of glial cells from the explanted DRG, reaffirming the nanofiber scaffold as conducive to cell adhesion and migration. However, only neurites were observed to infiltrate the hydrogel along the nanofiber-lined microchannels, exhibiting highly aligned and elongated growth. The absence of any visible nuclei within the hydrogel region revealed that microchannel formation faithfully mirrored the fiber template, with no separation occurring between the hydrogel layers, and presented the only means of passage through the hydrogel. Microchannels of this size supported only the infiltration by neurites.

Figure 5. DRG growth within a hydrogel construct over an 8 day period in GDNF-supplemented medium. A bright field image (A) shows an explanted DRG (far right) within a seeding well (double arrows indicate well boundary). Aligned nanofibers could be observed running from the well into the hydrogel. GDNF-sensitive neurons are shown in green (B), extending from the DRG into the hydrogel. Nuclei of migrating cells are visualized by staining with DAPI (indicated in blue) (C), confirming the selective growth of neurites through microchannels formed within the hydrogel. (scale bar: 500 μm).

DRGs from post-natal 8 day-old pups were also shown to support neurite growth through the hydrogel constructs (Figure S6). Neurites were observed to cross the hydrogel barrier between wells, traverse the second well and begin to infiltrate the hydrogel on the far end of the second well; the fiber scaffold and template used spanned the entire length of the hydrogel, permitting such growth to occur. This highlights the instructive role of nanotopographical guidance structures and the permissive environment to neurite growth created by the microchannel structures.

Neurite achieved a maximal growth rate of approximately 0.9 mm/day. Growth was maintained for up to 8 days, allowing for refreshing of medium.

Discussion

The study outlines the development of a 3D hydrogel culturing environment that incorporates aligned microchannel architecture to enhance and direct cell infiltration as well as aligned nanofibers in and around the microchannels to provide cell adhesion and topographical guidance. Though the use of a leachable template to create microchannels has been described previously[32,33], the current study combines the use of a sacrificial template with insoluble guidance fibers within a highly compliant hydroscopic environment. This work represents, to the best of our knowledge, a 3D scaffold with the most complete degree of anisotropic complexity with respect to the composition of physical elements typically found in the natural ECM of the PNS.

Material and Fabrication Considerations

PEGDA (5000 MW) was selected as a biocompatible, but biologically inert hydrogel, such that any observed cell behavior could be attributed to the combination of the nanofiber scaffold and microchannels. The limited degree of swelling experienced by this hydrogel also allowed microchannels to maintain the approximate size of their template fibers and also limited the mismatch in swelling between the hydrogel and the encapsulated nanofibers. However, this templating approach can be extended to investigate constructs made from other hydrogel materials, such as fibrin[33], agarose[34], pHEMA[35], or collagen.

In addition, the use of electrospinning to create both the template and insoluble fibers makes this an incredibly flexible approach, permitting the use of different materials to create fibers of different dimensions, alignment and density[36,37]. This allows for a systematic assembly of different possible synthetic ECM configurations, providing a facile and economic means for scaffold optimization. This versatility is reflected in the substitution of the nanofiber template material from PA to a PA-collagen blend. Although PA is known to have cell adherent and biocompatibility properties[38], it was found that a blended fiber of PA and collagen better promoted cell adhesion and neurite growth, similar to previous reports[39].

Pure ESP collagen fibers were trialed but found to be too fragile to withstand all the fabrication steps. The additional application of laminin culminated in a synthetic scaffold that closely approximates the native ECM composition of the endoneurium.

PLA template fibers ranging from 1 to 15 μm were obtained, providing a means of producing microchannels over a range of diameters. However, it was observed that the 75% w/v polymer solution inconsistently deposited fibers across the collector electrode gap. Although it was possible to produce large template fiber diameters, it is thought that the increase in polymer solution viscosity caused reduced whipping of the polymer jet and limited deposition across the electrode gap. An alternative is the use of a rotating mandrel collector, achieving aligned fibers without the necessity of an extensive whipping phase. Previous reports have shown that fiber diameters up to 10 μm are possible when using a rotating mandrel as a collector[40].

It should be noted that this approach requires the careful selection of polymers with complimentary solvent/nonsolvent properties. Both the template and guidance fiber materials are insoluble in water, maintaining the fibrous structures while being embedded within the hydrogel. Once crosslinked, PEGDA proved to be insoluble in acetone, even at elevated temperatures. Also the PA nanofibers were insoluble in acetone, permitting the selective removal of the PCL and PLA structures. Similarly, the melting temperature (T_m) and the glass transition temperature (T_g) are critical material selection criteria for this fabrication process. The combination of PLA template fibers, having a T_g of approximately 60 ^{o}C, with the insoluble PA nanofiber, having a melting temperature of 145 ^{o}C[41], allows for the superficial merger of the insoluble scaffold along the periphery of the template fibers. Also, the selection of PCL (T_m = 60 ^{o}C) as the seeding well template allowed for both fibers to remain intact while being enveloped, avoiding hydrogel encapsulation. This ensures cells seeded with the formed wells to have access to both the nanofiber scaffold and microchannels.

In vitro Validation: Observations of Neurite Growth

The *in vitro* culturing platform was validated by the successful innervation of the hydrogel architecture, with neurites directed by the guiding fibers and growth confined to the microchannels. These initial results also showed neurite growth rates up to 0.9 mm/day, not accounting for growth delays due to the initial adhesion period of the DRG explant. This is in stark contrast to 0.13 mm/day for GDNF-induced outgrowth from DRGs embedded within a collagen matrix[42] and more closely approximates reported *in vivo* growth rates of >1.5 mm/day[43]. This hybrid scaffold holds the potential to represent a vast improvement over previous 3D culturing environments, with a representative scaffold architecture that produces rapid and direct neurite growth on a time scale approaching that observed *in vivo*.

Cells in 3D Culture

After an injury where an axon is severed, the proximal axon forms a growth cone, a unique structure that guides regeneration[2] by projecting lamellae and filopodia that sense haptotactic and chemotatic cues via integrins and growth factor receptors. Ideal neural regeneration occurs along the original endoneurial tube, which provides a clear path to the original target tissue[43]. Successful regeneration is impeded when this guiding architecture of the ECM is disrupted.

To understand the elements that are critical to nerve regeneration, 2D culturing environments have successfully deconvolved the complex *in vivo* setting of the PNS and established the growth cone response to ECM proteins, chemical gradients, and surface topographies[44]. Neurite guidance is ultimately dictated by the growth cones adhesion to a surface through the formation of focal adhesion complexes, through which the growth cone respond to adhesion proteins, topography and substrate stiffness[45–47]. Diffusible cues also modulate focal adhesion formation[48], reducing strategies for neural regeneration down to the successful modulation of FA formation. However, recent reviews highlighted an intractable link between form and function of the natural 3D ECM and the cells which it hosts[49–51]. In particular, changes occur in focal adhesions formation[52] and in their distribution around the cell membrane when in a 3D environment, leading

to changes in cell morphology compared to that observed on 2D substrates; such morphological changes also occur to growth cones[53].

Hydrogels are often selected to study cell behavior in 3D and can be designed to mimic many characteristics of the ECM, including variable substrate stiffness and the presentation of ECM binding motifs[17]. Curiously, hydrogels employed as 2D substrates show improved growth on moderately stiff gels, attributed to the focal adhesions of the growth cone able to exert increased traction force on the underlying hydrogel. In comparison, softer gels are considered optimal for embedded neurites in a 3D environment[53,54]. Neurite growth in a 3D culture typically requires the impinging neurite to actively create space by remodeling the local environment via enzymatic cleavage[27,55]. A reduction in the density of hydrogel reduces the extent of remodeling required and improves outgrowth[18]. However, this remodeling is metabolically expensive, slowing or inhibiting cell invasion[51], and is in contrast to the unobstructed path provided by the microarchitecture of the endoneurium. Furthermore, reducing hydrogel density to promote neurite extension also reduces stiffness below optimal levels for unobstructed neurite growth. This compromise is a direct consequence of an artificial 3D culture environment that lacks the anisotropic structure of the natural ECM.

Biomimicry in 3D Culturing Systems
Anisotropic 3D hydrogels do exist, directing neurites growth via gradients in stiffness[56], diffusible chemical cues[57] and adhesion molecules[19,58,59]. While these represent promising approaches, they fail to approximate the observed structural elements of the endoneurium. Microchannels have been created within a hydrogel using various microfabrication techniques[60,61], though these are typically an order of magnitude larger than PNS structures[62] or require highly specialized equipment[63].

The endoneurial tube also includes aligned collagen fibers, thought to provide structural support and topographical guidance for neurite growth; directed neurite growth on aligned ESP fibers supports this assertion[21,39]. The focal adhesion formation observed in 3D is also replicated on aligned 1D structures of the scale

producible by ESP fibers[64], suggesting such scaffolds are a suitable biomimetic 3D scaffold. In fact, recent work shows that oriented fibers within a 3D microenvironment have a greater impact on focal adhesion maturation than the bulk stiffness[65]. However, ESP scaffolds of dense fibers can prevent cell infiltration[66], emulating instead a roughened 2D[25]. Alignment of protein fibrils has been induced within collagen and fibrin hydrogels by the application of a magnetic field during gelation and successfully directed growth of embedded neurons[26,67]. However, this approach still requires remodeling of the hydrogel environment. Embedding ESP fibers within a hydrogel environment also produces similar hydrogels with fibrous anisotropy[25], though this approach also requires infiltrating cells to actively remodel their environment.

The culturing platform outlined in this study is the first report of a synthetic 3D environment that guides cell growth by a combination of corralling microstructure and incorporated ESP nanotopography. The controlled flexibility of this approach lends itself to the investigation of a multivariant 3D environment in defined manner. In addition to modifying the nanofibrous scaffold or microchannel architecture, the relatively inert hydrogel currently employed can also be substituted for a degradable material to allow for cell-driven remodeling within this highly ordered system. The fabrication approach outlined can also be employed to create a nerve graft, similar to Jeffries *et al.*[68,69], making it possible to faithfully translate an optimized *in vitro* scaffold to an *in vivo* environment.

This platform can also be extended to explore other cell behavior, such as cell migration within a 3D tubular environment and vascularization of fine capillaries. The two-well system permits various experimental designs, including growth factor release from one well to another or co-cultures connected via microchannel structures. The system can be expanded to multiples wells arranged in 3D, with the physical flexibility of the template fibers enabling interconnected porosity in a wide range of paths and orientations; this could ultimately lead to the assembly of 3D organ-on-a-chip devices.

Conclusions

We have developed and validated a unique hybrid scaffold that incorporates many of the architectural elements observed within natural ECM and is able to guide extensive neurite growth. The ability to create a hydroscopic environment that amalgamates aligned fibers with directed microchannel porosity in a controlled manner realizes a scaffold design with a new degree of complexity. This provides a versatile tool for the study of biological processes, which are relevant for neural as well as other highly ordered tissues.

Acknowledgements

The authors would like to thank Dr. Carlos Mota and Erwin Zant for invaluable input and insight during the development of this platform, without which this would not have been possible, and Dr. Fred de Winter for advising on the neural growth assays and immunohistochemistry.

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Chapter 6: Supplementary Material

Figure S1. *An example image of assessment of fiber alignment. The original image of fibers produced with 50% w/v PLA at a flow rate of 5 ml/hr (A) is processed with the OrientationJ plugin (B), available for ImageJ. This assessed the degree of alignment of features within the image, producing a value of coherence between 0 (random) and 1 (aligned). (Scalebar: 50 µm)*

Figure S2. *PCL plug integration with the fiber network. The PCL plug was deposited on the fiber network via a bioplotting device (A). The molten plug was able to adhere to the fiber mesh surface (B), penetrating through the fiber mesh (C). (Scalebar: 1 mm)*

Figure S3. *The diameters of both types of insoluble fiber diameter are shown as average values (A) and distributions (B). Pure PA fibers had an average diameter of 0.863±0.281 μm, while blended PA-collagen fibers were a 0.218±0.057 μm. These fibers were used to create either triple layer fiber constructs of either Collagen/PLA/Collagen (C, D) or PA/PLA/PA (E, F).*

Figure S4. *Optimization of the merging of PA and PLA fibers by the baking. Before the application of heat, the two fiber types are distinct (A, B). After the application of 60 °C for one hour (C, D), slight softening of the PLA fibers can be observed but the degree of merging is limted. Baking for either 70 °C (E, F) or 80 °C (G, H) for one hour increases the degree of merging between the fiber types, however the PLA fibers lose their rounded morphology.*

Figure S5. *Cross sectional view of a hydrogel device with the typical density of insoluble fibers (A). At this high density, the presence of microchannel structures is obscured by the insoluble fibers (B). (Scalebar: A, 100 μm; B, 20 μm)*

Figure S6. *(A) Neurite outgrowth of DRGs explanted from post-natal Day 8 rat pups, grown in NGF over an 8 day period. (B) neurite outgrowth of DRGs explanted from post-natal Day 2 rat pups, grown in NGF over a 5 day period. (Scalebar: 500 μm)*

Chapter 7

General Discussion

General Discussion

The motivation of the work described in this thesis was the development of a new class of regenerative peripheral nervous system (PNS) interface that incorporates a tissue guidance scaffold in order to address issues of long-term stability and selectivity. The proposed scaffold is conceived to deconvolve the nerve, physically segregating specific neural subpopulations to improve selectivity and provide more available area for establishing neuroelectric contact. To this end, this work focused on the development of fabrication techniques, towards the creation of a 3D tissue scaffold capable of selectively directing the regeneration of neural subtypes (Figure 1).

This work falls within the emerging field of neural engineering, which seeks to apply engineering design principles to understand the complexity of neural system, and to address neuropathologies and other deficiencies. An active area of development is the creation of a direct interface between an electronic device and the PNS, intended for neuroprosthetic devices that return lost function[1,2]. An ideal PNS interface must be stable and capable of selectively recording or stimulating specific axons or axonal subpopulations, facilitating the natural control of prosthetic devices and permitting the return of appropriate sensory feedback. Of the many possible strategies, pilot studies have shown that regenerative electrodes form a mechanically stable interface that provides a promising degree of selective contact with a finite, albeit undefined, subset of the neural population[3–5].

However, this approach relies on the successful and uncontrolled regeneration of neural tissue through the interface. This is hindered by the small amount of open area made available for regeneration by current interface designs and by the material required for these devices; long term studies have shown that the non-compliant materials required to realize these electrodes also act to compress the ingrown tissue as axons mature and enlarge, leading to axonopathy[4]. The implementation of a compliant guidance scaffold proposed in this thesis serves to artificially expand the nerve trunk while simultaneously separating different neural subtypes. This addresses the issues which plague current regenerative interfaces and further improves the degree of interfacing selectivity.

Figure 1. *Example of a regenerative neural interface. An outer 'Y' shaped nerve conduit (white, with cross-section cut out) with the left horizontal lumen housing a severed nerve is depicted. The nerve is comprised of a heterogeneous mixture of motor axons (blue) and sensory axons (red). Upon innervation of the 3D scaffold, the different neural subtypes are induced to selectively regenerate towards specified electrode sites: motor axons to the upper electrode and sensory axons to the lower electrode. The presence of microspheres for the controlled release of diffusible growth factors for the attraction of respective neural subtypes is also depicted.*

PNS regeneration is driven by the pathfinding process of the growth cone, which dictates the direction of neurite growth by integrating a myriad of chemical and physical signals. Neural subtypes exist within the PNS with specific functional classifications and also exhibit selective sensitivity to a subset of growth factors and extracellular (ECM) adhesion molecules[6–8]. During neural development, these different types of axons extend into the human body in response to a highly orchestrated spatiotemporal expression of diffusible growth factors and ECM molecules and are successfully guided to selectively innervate associated tissue types[9,10]. Although direct parallels cannot be drawn between developmental processes and PNS regeneration[11], evidence suggests that a degree of selective response persists into adulthood and plays a role in the PNS response to injury[12].

The 3D scaffold shown in Figure 1 was conceptualized to harness this selective capacity. Applying a biomimetic approach, a major design objective is to mimic the ECM of the PNS in order to emulate the intrinsic ability of the PNS to

regenerate. However, attaining the desired growth pattern from such a highly heterogeneous cell population requires the controlled assembly of various scaffold components. This highlighted a so far unaddressed niche of suitable culturing platforms for such scaffold optimization and a lack of fabrication techniques to realize such a 3D scaffold. The related field of peripheral nerve repair and development of synthetic nerve grafts provided a starting point (Chapter 1), with many scaffold designs also driven by a biomimetic imperative to replicate the structure/function relationship of native neural tissue[13,14]. A recurring theme found in the current state-of-the-art is that the simple implementation of structural elements observed *in vivo* without faithfully reproducing the associated macrostructural 3D organization leads to limited performance.

An understanding of the role of structural guidance on neurite growth is first required in order to effectively implement these physical cues into functional 3D constructs. Topographical cues are known to be key mediators of directed neurite growth, although the dearth of studies on the subject has produced conflicting reports[15–17]. Investigations into the biophysical component of cell response have shown that topology acts to geometrically constrain the formation of focal adhesions, the mechanism by which cells adhere to the surface. This has also been shown to occur in growth cones (Chapter 2), whereby the modulation of focal adhesions by the underlying topology was shown to induce directed neurite extension. Neurites presented with aligned grating with ridges ranging in size from 0.5 to 2.5 mm were found to achieve similarly aligned neurites, indicating that guidance topography within this size range results in robust direction of neurite extension. This is perhaps surprising considering that the collagen fibers that provide similar structural guidance within the neural ECM are considerably smaller, ranging in size from 20 to 100 nm. Previous reports exploring the topographical guidance of electrospun fibers have also shown aligned neurite growth on structures of a similarly large size[17,18]. One proposed explanation for this discrepancy is that the 3D organization of topographical cues may play an equally important role in neurite guidance. This calls into question the relevance of 2D patterned substrates for studying such mechanisms.

Despite the limited impact of feature size, Chapter 2 does report shorter neurite extension and larger focal adhesion formation when cells are grown in the presence of high serum concentrations. These results emphasize the complexity of manipulating focal adhesion formation. While focal adhesions are required for adequate cell adhesion, the disassembly of focal adhesions is equally important in order for neurites to appropriately respond to topographical stimuli and to achieve full extension. The stability and maturation of adhesions are known to be dependent on ECM availability and can be modulated by growth factors, both of which are present in serum. While this study provides a baseline for the dimensions required for topographical guidance, it also underscores multi-faceted considerations when mediating cell growth by regulating cell-surface interaction. This knowledge is ultimately important as a basis to design and implement functional criteria when translating to 3D interfaces.

Electrospinning has been extensively reported as a suitable fabrication technology to produce 3D scaffolds of polymer fibers of a diameter suitable to achieve topographical guidance[19]. This was confirmed in chapter 3, where the aligned nanofibers were shown to induce elongation of Schwann cells. Beyond providing structural guidance, it was shown that the bioactivity induced by these fibrous scaffolds can be tailored by the conjugation of specific biomolecules to the polymer surface. The surface functionalization with the cell adhesion peptide sequences H-Gly-Arg-Gly-Asp-Ser-OH (GRGDS) and H-Tyr-lle-Gly-Ser-Arg-NH2 (YIGSR) was able to modulate Schwann cell gene expression and protein release, resulting in increased neurotrophic growth factor (NGF) and glia-derived growth factor (GDNF) production. GRGDS and YIGSR are peptide sequences found within the laminin protein and are known to promote neurite extension[20–22]. Both peptides also bind to integrins receptors, the transmembrane proteins that form the bridge between the ECM and focal adhesion complexes.

Fibers were also functionalized with the laminin-derived H-Arg-Asn-lle-Ala-Glu-lle-lle-Lys-Asp-lle-OH (RNIAEIIKDI, p20) peptide. In contrast, while the p20 peptide is also known to support neurite growth, it is not a direct adhesion motif[23]. Instead, this peptide enhances neurite growth by modulating focal adhesion turnover; the lack of direct involvement in cell adhesion and subsequent

downstream intracellular signaling may account for the lack of growth factor expression in response to this peptide. This work exemplifies the utility and complexity of modulating cell behavior via ECM proteins, representing a small subset of the multitude of direct and indirect signaling pathways involved in controlling cell fate. In order to elicit the distinct cell responses required for selective neural regeneration, a combination of structural and biomolecular cues represents a promising strategy. However, to achieve a pattern of physically segregated cell growth requires a greater degree of spatial control over fiber scaffold organization and subsequent fiber bioactivation than what is offered by conventional electrospinning and functionalization methods.

The need to create fibrous constructs with a higher degree of organizational complexity prompted the development of a new approach that we called tandem electrospinning (T-ESP, Chapter 4). This makes possible the simultaneous deposition of two or more distinct populations of oriented fibers in an overlapping straight or diverging pattern. The resulting 3D fibrous scaffold incorporates ordered and oriented heterogeneity, and allows cell population to be simultaneously presented with two fibrous guidance stimuli. DRG outgrowth on a heterogeneous scaffold comprised of synthetic versus synthetic/collagen blended fibers confirmed the possibility to spatially modulate diverging neurite growth, with the presence of collagen shown to promote more consolidated and elongated neurite extension. Acknowledging the robust neurite alignment observed in Chapter 2 and the ability of cells to adapt to surrounding environments, this may also present a platform to optimize conditions for guidance by observing the outcome of two competing guidance stimuli. Beyond this proof of principle, the inclusion of different bioactive molecules within the polymer solution to impart both structural and bioactive heterogeneity is impractical and potentially ineffective. The concentration of proteins required in order for a sufficient amount to be present on the fiber surface after ESP fabrication limits this possibility to a subset of abundant and cheap ECM proteins. Furthermore, the use of organic solvents in the preparation of most ESP polymers solutions poses a risk that the added protein becomes denatured, potentially losing or diminishing its function[24].

An alternative strategy was developed in Chapter 5, whereby fiber populations were imparted with orthogonal bioconjugation chemistry via the addition of a short chain, homofunctionalized PEG to the ESP polymer solution. In this manner, it becomes possible to produce ESP fibers with surface-bound reactive groups without the need for additional activation. The two most promising candidates were either click chemistry or maleimide-thiol crosslinking, noted for the mild reaction conditions and selectivity. Combining this approach with tandem ESP, heterogeneous scaffolds were produced with oriented fiber populations exhibiting spatially defined selective functionalization. This novel scaffold construct permitted the evaluation of neurite response of an explanted DRG when simultaneously presented with two fiber populations selectively functionalized with GRGDS and p20 peptides, respectively. Evidence of spatially modulated neurite growth was observed on the T-ESP scaffold compared to homofunctionalized fibers, with the GRGDS functionalized fibers promoting more neurite growth compared to those with p20.

Observations in the course of the T-ESP development found that a polymer with a high degree of chain entanglement is required in order to achieve robust fiber orientation. In the semi-amorphous polymer employed in this study, the degree of chain entanglement depends on polymer chain length and the proportion of crystalline and amorphous regions of the polymer. The addition of a small polymer chain to a bulk polymer has the potential to act as a plasticizer, interposing within the polymer network to reducing the effective degree of entanglements[25]. Evidence of this can be observed in the reduced degree of fiber alignment of functionalized fibers produced in Chapter 5. Although in this case the degree of fiber orientation was sufficient to direct neurite growth, the ESP fibers incorporating functionalized short chain PEG molecules exhibited reduced fiber alignment compared to fibers without the additive. To achieve both robust fiber orientation and incorporated selective functionalization capacity, the custom synthesis of a polymer would be required which is designed to have a high degree of chain entanglement and incorporates reactive chemical groups for conjugation. A promising modular approach to polymer synthesis has recently been describe by Lin et al.[26], providing a biocompatible polymer capable of producing electrospun fibers exhibiting a variety of conjugation chemistries. Provided the molecular weight of entanglement

can also be tuned, this provides an avenue for further refinement of ESP scaffold fabrication.

Despite the potential of ESP scaffolds as an *in vitro* platform for heterogeneous scaffold development, the resulting scaffolds in their current form are not yet optimal for *in vivo* implementation. In addition to their potential structural fragility, *in vivo* studies have shown that the direct implementation of fibrous scaffolds results in reduced regeneration compared to the 'golden standard' autograft[27,28]. Artificial scaffolds have only managed to approach the performance of autografts by emulating the microchannel architecture observed in native PNS tissue. Because of issues related to surface tension and mechanical stability, an electrospun scaffold has difficulty maintaining porosity[29] or 3D microstructural architecture[30]. Therefore, in chapter 6 we have developed a hybrid hydrogel/fiber construct to overcome these issue. By embedding a soluble template in combination with insoluble nanofibres, stable microchannels can be formed within a compliant hydrogel and with aligned nanofibers affixed to the microchannel wall. This approach maintains the microarchitectural arrangement of aligned nanofibers in 3D that closely replicates the PNS endoneurium in terms of structure, constituent components, and scale.

Future Perspective

The development of 3D cell culture environments has found promising applications as a means of studying cell response in a more representative, native-like environment[31,32]. The study of cell behavior has relied on the use of controlled culturing environments in order to isolate variables and establish cause and affect relationships. However, there is an emerging consensus that traditional 2D culture environments may induce misleading cell response and that more representative 3D environments are needed to accurately observe and understand cell behavior. The development of these 3D constructs also facilitates the ability to control cell behavior and steers the design and fabrication of future tissue scaffolds. Each of the fabrication techniques developed in this thesis present promising tools to assess and develop 3D scaffolds for neural regenerative or other biomedical applications. It is the ultimate synthesis of these techniques into a single, powerful 3D culturing

platform that holds the most potential for broader applications within the field of tissue engineering.

Already validated in Chapter 6 as a tool for studying DRG outgrowth *in vitro*, the hybrid hydrogel/fiber construct itself provides a highly adaptable 3D culturing system. Beyond the planar microchannel/fiber architectures already described, the physical flexibility of the fiber template permits creating various orientations and 3D arrangements of fiber-lined microchannels within the 3D hydrogel environment; the possibility to stack fiber templates to form layers of distinct microarchitectures within the hydrogel further expands the geometric complexities that can be achieved. Considering also the many permutations of alternative materials for both the hydrogel and nanofiber components, many avenues for scaffold design can be envisioned, for example in the vascular and lympathic systems.

The hybrid hydrogel/fiber construct fabrication is also amenable to the implementation of techniques described in Chapter 4 and 5, whereby divergent patterns of selectively functionalized fibers are incorporated within the hydrogel. This makes possible the creation of highly complex 3D scaffolds for cell growth comprised of novel microstructural and fibrous guidance with spatially defined patterns of biomolecular haptotactic cues. In this way, an organized multifactorial 3D culturing environment can be realized to have incrementally increasing complexity in a designed and controlled fashion. This permits the presentation of a multitude of signaling cues to cells in order to control cell behavior, ideal for scaffold optimization and for the study of complex biological processes within a 3D context. Although an *in vitro* culturing environment cannot fully capture the complexity of the *in vivo* setting, the multifaceted construct described here more closely approximates this reality and could prove invaluable for creating lab-based assays that are more relevant to the final clinical application. Furthermore, these fabrication approaches can be scaled up to realize larger tissue scaffolds, preserving the optimized design features to increase the chances of translating observed *in vitro* performance to an *in vivo* setting.

Returning to the intended application of a 3D scaffold for a highly selective regenerative neural interface, the fabrication techniques outlined in this body of work provide a means of creating a structural mimic of the PNS ECM in order to enhance successful neural ingrowth while the method of scaffold assembly is also amenable to the incorporation of microelectrodes. At the same time, the scaffold also has the capacity of exacting spatial control over various structural and biomolecular elements known to be involved in the selective promotion of neural subtypes, bringing within reach the objective of physically segregating these neural subpopulations in order to form a highly selective, bidirectional neural interface.

As mentioned previously, one downfall of earlier regenerative electrodes was the compressive forces exerted on the axons once they began to mature and enlarge in diameter. While the compliant nature of the hydrogel may be sufficient to avoid the compressive forces that induce axonopathy, the use of a biodegradable hydrogel may further assist in avoiding this pitfall. On the other hand, the use of such a scaffold as a neural interface requires the stable anchoring of electrode components and the establishing of long-term contact between innervating tissue and resident electrodes. In light of this, it may be advantageous to limit the degree to which tissue can actively remodel the scaffold. At the very least, substituting nanofibers for a non-degradable polymer may be sufficient to retain the original scaffold architecture while allowing cells a certain degree of freedom to remodel the remaining hydrogel component.

One strategy for neurite guidance that has yet to be implemented in this 3D culturing platform is the use of diffusible chemo-attractants. This is well established to have an effect on nerve regenerative capacity, with *in vivo* observations clearly showing that regeneration is enhanced by the diffusible growth factors released by supporting Schwann cells after injury[12]. Artificially created gradients of diffusible growth factors have also been reported to selectively stimulated growth of specific neural subtypes[33]. A gradient of growth factors within the hybrid hydrogel construct would be straightforward to implement, requiring a source to maintain controlled release of the biomolecules in question. Such a source could take the form of degradable microspheres laden with growth factors or Schwann cells, which have been shown in Chapter 3 to upregulate

particular growth factor expression in the presence of specific ECM-derived peptides.

While techniques have been established to create growth factor gradients, there was a lack of fabrication strategies capable of creating the biomimetic arrangement of structural and topographical features to guide neurite growth. As such, the fulfilled objective of this work was to develop scaffold fabrication techniques that were able to fill this niche. Using diffusible biomolecular cues in combination with these complex scaffolds creates a 3D culturing environment which exhibits microarchitecture, nanofibrous topography, spatially defined haptotactic signaling and soluble growth factors, making an almost complete mimic of the *in vivo* setting. Such tissue scaffolds that better recapitulate the ordered heterogeneity of the *in vivo* situation will aid in understanding and controlling of complex biological systems and hasten the realization of effective tissue engineering solutions.

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Summary

Over the past 5 decades, there has been a drive to apply technology to enhance neural regeneration in order to improve patient recovery after disease or injury. This has evolved into the field of Neural Engineering, with the aim to understand, control and exploit the development and function of neural tissue. To improve peripheral nervous system (PNS) recovery, various tissue scaffolds have been developed; the most pivotal and promising are covered in Chapter 1. In the pursuit of exacting control over neural growth patterns, the use of topographical cues has been identified as a promising strategy to guide growth cones and direct neural growth. Chapter 2 explores this possibility by using nanoimprinted 2D culture environments to determine the role of topographical feature size.

To better approach the native cellular environment and to facilitate translation from the in vitro to the in vivo setting, 3D culturing environments have recently been employed to explore cell behaviour. Chapter 3 describes the exploration of topographical cues in a more 3D environment using electrospun (ESP) fibers to influence the behaviour of Schwann cells. Also explored in this chapter was the influence of biofunctionalization on cell behaviour, with results indicating that specific substrate-bound biomolecules contributed to the functional intricacy of the PNS. To recreate this complexity, Chapter 4 develops a new electrospinning technique (Tandem Electrospinning; T-ESP) capable of creating spatially defined, heterogeneous patterns of aligned fibers while Chapter 5 validates a method of selectively functionalizing different populations of fibers. Selective biofunctionalization of T-ESP fibers was shown to produce spatially modulated neurite outgrowth.

Chapter 6 outlines the creation of a hydrogel construct mimicking the PNS extracellular matrix, with microchannel architecture and incorporated nanofiber topography lining the channel walls. Shown to induce neurite alignment, this presents the possibility of creating defined patterns of neurite growth in a 3D context. Chapter 7 discusses the developed biofabrication techniques as a means to create a scaffold capable of selective neural regeneration, emphasizing the potential impact in developing a highly selective regenerative neural interface.

Samenvatting

In de afgelopen 5 decennia is er een streven geweest naar de toepassing van technologie ter bevordering van neurale regeneratie, dit om het herstel te versnellen bij patiënten na ziekte of herstel. Hieruit is een nieuw vakgebied ontstaan, genaamd 'Neural Engineering'. Dit vakgebied heeft als doel de ontwikkeling en werking van het zenuwstelsel te begrijpen, te beheersen en te gebruiken. Verschillende scaffolds zijn ontwikkeld om te zorgen voor een beter herstel van het perifere zenuwstelsel (PZS). In hoofdstuk 1 worden de voornaamste en meest veelbelovende scaffolds behandeld. In de literatuur komt onder andere naar voren dat het gebruik van topografische signalen een veelbelovende strategie is om meer controle te krijgen over neurale groeipatronen. In hoofdstuk 2 wordt deze mogelijkheid onderzocht door met behulp van nanoimprinted 2D-kweekomgevingen de rol van de afmetingen van nanostructuren te bepalen.

Sinds kort worden steeds vaker 3D-kweekomgevingen gebruikt om het celgedrag te bestuderen. Deze omgevingen blijken de natuurlijke cellulaire omgeving beter te benaderen waardoor het gemakkelijker is een vertaalslag te maken van de *in vitro* setting naar de *in vivo* setting . Hoofdstuk 3 beschrijft het onderzoek naar het gebruik van topografische signalen in een 3D-omgeving. Hierbij worden dunne vezels, gemaakt met een techniek genaamd electrospinning, gebruikt om het gedrag van Schwann cellen te beïnvloeden. In dit hoofdstuk is tevens het effect van biofunctionalisatie op het gedrag van deze cellen onderzocht. De resultaten laten zien dat specifieke substraatgebonden biomoleculen een bijdrage leveren aan de functionele complexiteit van het PZS. Om deze complexiteit beter te benaderen, hebben wij in hoofdstuk 4 een nieuwe electrospinning techniek (Tandem Electrospinning; T-ESP) ontwikkeld waarmee het mogelijk is om ruimtelijk geordende, heterogene patronen van uitgelijnde vezels te creëren. Hoofdstuk 5 valideert een methode om selectief verschillende groepen vezels te functionaliseren met specifieke biomoleculen. Deze methode maakt het mogelijk om in een complexere omgeving, zoals die is ontwikkeld in hoofdstuk 4, het effect van deze biomoleculen te testen. De resultaten laten zien dat het selectief functionaliseren van T-ESP vezels het mogelijk maakt de uitgroei van neurieten te induceren met controle over de groeirichting.

Hoofdstuk 6 beschrijft de ontwikkeling van een hydrogel construct met als doel de extracellulaire matrix van het PZS na te bootsen. Het ontwerp bestaat uit microkanalen in de gel met nanofibers langs de wanden van deze microkanalen. Deze kleine kanalen met nanofibers zorgen ervoor dat de neurieten gaan uitlijnen, waardoor het mogelijk is om gedefinieerde patronen van neuriet-uitgroei in een 3D omgeving te maken. In hoofdstuk 7 wordt een discussie gevoerd over de biofabricagetechnieken die in dit proefschrift zijn ontwikkeld en over de potentie om met deze technieken een selectieve neurale interface te creëren.

Acknowledgements

When I first moved to Italy, Maria Laura took me in and helped me get on my feet. Both you and Jack made me feel welcome and I will always remember your kindness and enthusiasm… and my first taste of ′Nduja. And the famed San Sepolcro Support Group: Alberto, Gerson, and Kat, thank you for being my surrogate family.

To Silvia, who kept me sane through the first year in Italy both personally and professionally, thank you for being a point of stability and calm in a pretty confused period. To my NEST comrades, Marco T., Sandro, Emanuela, Ilaria, Paolo and Marco C., it was a pleasure to learn, work and play with the best. I wish you the best in your careers and I hope our paths will cross in the future.

To awesome days of beach volleyball and Arno Vivo with my Pisano expat possé: you got me through it all without having to actually learn Italian! Bernard, thanks for the intense (and honest) volleyball critiques. Victoria, you're a goof but I love you anyway. Anything more I try to say would sound lame and you would mock me for it. Esteban, thanks for being my baby seal punching bag. You didn't always deserve it. Pepita, full of love, trees and rocks over statues and churches any day! And Leonard the Swede, thanks for at least trying to convince me to take things a little less seriously.

Returning to Enschede was made easier by those who welcomed me back. Joyce, you were a constant throughout and, though you escaped just as I made it back, I always look forward to catching up over some tea or coffee. Ana B., the first TR connection I had and a kindred engineer with a similar perspective, I always felt a little less crazy after having a chat. And the Pathmos girls: Fede, thank you for introducing the humanities perspective in all the things I do; Aimee, for the many shared Duvels; and Lucie, for your persevering friendship and slightly aggressive tendencies – may we always walk quietly on the stairs. And as well to Suzanne, Lise and Rin, Des and Clare, Chris and Nicole, Didem and Semih and Peter and Connie, thank you for being up for a chat, a drink, a barbeque or dinner. And an accolade to the support and eternal mockery of the veterans: Dom, Denbot, Markus, Yana and Jon. And Victor.

And my time here would definitely have been less exciting were it not for my second home. Bjorn, thank you for providing a roof over my head, a well stocked beer selection and many hours of conversation and commiseration: I think we solved the world's problems a few times, but unfortunately we cannot remember. And Ana R., the Boss, thank you for the balance and for taking my teasing so well.

My extended stay here would of course be difficult without some ice hockey to abate the homesickness. The distraction provided by the Slapping Studs and the Lions helped me survive the long, cold, wet winters. To Jurjen, Bjorn, Johan van Es, Erik, Johan, Anne, Tones Capones, Yvo, Hans, Jeroen, Bauer, Mark, Henk, and all the others with whom I've had the pleasure of playing, I look forward to beating you on the ice.

It has been amazing to get to know so many people with such diverse backgrounds, something truly unique to the ever-expansive network of MIRA and MESA[+]. 277 was where I made my second home and it would not have been the same without Bade, Sebastien, Shahriar, Marc and all the second floor riffraff. The technical staff, Anouk, Tom, Jacqueline, Ed and Marcel, have been essential in keeping things running smoothly. Bettie and Gerco, thank you for all the headless wonders and the flow hood discussions. Audrey, thanks for looking out for me.

And to my many TR/DBE/BSS/MTP/IMS/NBP/Matriomics colleagues, which embody the stereotypes and archetypes of the research lifestyle, thank you for your friendship, help, inspiration and constant entertainment. From the Italian connection to the continually evolving Portuguese mafia, the Spanish Inquisition, the office of beautiful people… and Erik, the Canucks, the Chinese Army, the poorly outnumbered natives, and everyone else in between, without you this would have been a tough slog.

And of course my army of Master's students, who's tireless (and youthful) toil pushed me to keep up. Sebastian, Andreia, André and Rita, I thank you for your dedication, hard work and for your patience while I learned alongside you. I wish you all the luck for the future.

Mirko, my very first Post Doc, I learned a lot. Sometimes even some scientific things. Thanks for being there when I needed it most. And Max and Hugo, the Post Doctoral influences of my UT existence. Thanks for the many beers and runs, respectively, and for the friendship, advice, guidance and the impartial, amused perspectives on the travails of a struggling PhD.

Roman, your continued enthusiasm and insight is always valued and I have enjoyed our a technical brainstorming sessions, dropping acronyms at an impressive rate.

Silvestro, thank you for providing me with the opportunity to pursue my goals and facilitating as much as possible the realization of this project, despite all of the difficulties. The completion of this thesis would not have been possible without the effort of both Richard and Lorenzo. Thank you both for welcoming me back to UT and for initiating the co-PhD agreement, enabling me to pursue my project. Your support and guidance up until the last moment has been critical and very much appreciated. And especially without your tight deadlines, endless reading and editing… and reading… and editing, this thesis would not exist.

To Mijke, I cannot say enough. The endless understanding, patience… and entertainment, hanging out in the lab until late, on the weekends, until the job was finished. Climbing, running, talking…. and talking. Your support and the support of your family has been amazing, with family dinners and lactose-free food drops keeping me well fed and in touch. My 'partner in crime', this would not have been possible, nor as much fun, without you and I look forward to more nerdy fun.

And I would like to acknowledge the patience and understanding of my friends at home who only see me once a year and still remember my name! I would also like to thank my extended family living in the Netherlands for providing me with a sense of security while living in this strange land. To my sisters, Rosanne and Marsha, and to Dave, Rob, Tadao, and Grace, you've kept me grounded and helped me to understand the importance of family.

And finally, I would like to thank my parents for making all of this possible. For raising me with the confidence to pursue my passion, the perseverance to overcome the obstacles and for providing me with infinite support regardless of whether they agreed with my choices.

List of Publications

E. Masaeli, **P. A. Wieringa**, M. Morshed, M. H. Nasr-Esfahani, S. Sadri, C. A. van Blitterswijk, and L. Moroni, "Peptide functionalized polyhydroxyalkanoate nanofibrous scaffolds enhance Schwann cells activity," *Nanomedicine Nanotechnology, Biol. Med.*, *In final revison*, 2014.

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Biography

Born in Owen Sound, Canada on April 18th, 1980, Paul was raised in the village of Markdale. In 1999, he began studies at the University of British Columbia, Vancouver, Canada where he enrolled in the Integrated Engineering Program, through which he received training in the principles of the main engineering disciplines (Mechanical, Electrical, Materials, etc.) with a focus on combining these expertise to finding engineering solutions. During this time, he held an NSERC USRA summer internship in the Molecular Mechatronics Lab under Dr. John Madden and Dr. Joseph Yan where he focused on the development and evaluation of synthetic muscle.

After graduating with a Bachelors of Applied Science (B.A.Sc.) in 2004, he travelled to Europe by sailboat to begin a Master's study at the University of Twente (UT) in 2006. He received his M.Sc in 2008 with dissertation entitled "Evaluation Of Neural Ingrowth Into A Microchannel Network: Towards A Regenerative Neural Interface", under the supervision of Dr. Wim Rutten in the Biomedical Signals and Systems (BSS) group at UT. This was followed by a year as a researcher under Dr. Rutten to continue the topics explored during his Master's thesis.

In 2010, Paul began his doctoral studies at Scuola Superiore Sant'Anna (SSSA), Pisa, Italy, under the supervision of Dr. Silvestro Micera and in collaboration with Dr. Marco Cecchini of the Scuola Normale Superiore/NEST laboratory, Pisa, Italy. In the fall of 2011, Paul returned to the UT for his 'Period Abroad' to continue his PhD work on the development of a regenerative neural interface. Working under Dr. Lorenzo Moroni and Dr. Roman Truckenmüller of the Tissue Regeneration Department, he applied their expertise in the field of tissue scaffold design to the creation of 3D neural guidance scaffolds. At the suggestion of Dr. Richard van Wezel of the BSS group, this later evolved into an official co-PhD between the UT and SSSA, the results of which are contained in this book.