Repairing Peripheral Nerves: Is there a Role for Carbon Nanotubes?

Karen M. Oprych, Raymond L. D. Whitby, Sergey V. Mikhalovsky, Paul Tomlins, and Jimi Adu**

Peripheral nerve injury continues to be a major global health problem that can result in debilitating neurological deficits and neuropathic pain. Current state-of-the-art treatment involves reforming the damaged nerve pathway using a nerve autograft. Engineered nerve repair conduits can provide an alternative to the nerve autograft avoiding the inevitable tissue damage caused at the graft donor site. Commercially available nerve repair conduits are currently only considered suitable for repairing small nerve lesions; the design and performance of engineered conduits requires significant improvements to enable their use for repairing larger nerve defects.
Carbon nanotubes (CNTs) are an emerging novel material for biomedical applications currently being developed for a range of therapeutic technologies including scaffolds for engineering and interfacing with neurological tissues. CNTs possess a unique set of physicochemical properties that could be useful within nerve repair conduits. This progress report aims to evaluate and consolidate the current literature pertinent to CNTs as a biomaterial for supporting peripheral nerve regeneration. The report is presented in the context of the state-of-the-art in nerve repair conduit design; outlining how CNTs may enhance the performance of next generation peripheral nerve repair conduits.

1. Introduction

Peripheral nerve injury (PNI) often occurs during trauma involving crushing, laceration, ischemia, and stretching of the body and its limbs. Despite the robust pro-regenerative response of peripheral neurons to injury, functional recovery is often unsatisfactory.

PNI continues to be a major global health problem with significant negative impact on the quality of life of patients. Neurological deficits can range from temporary sensory and motor impairments, to permanent paralysis of entire limbs or organs, often accompanied by severe neuropathic pain. Transected nerves can be repaired by direct end-to-end suture, but when tensionless coaptation is not possible a nerve autograft must be used to bridge the gap between the two ends of the damaged nerve. Autografts protect, promote and guide axon regeneration from the proximal injured nerve into the distal nerve stump. Despite the success of this repair technique the inevitable damage caused at the graft donor site including loss of sensation, painful neuroma formation and scarring, makes this treatment far from ideal.

A variety of bioengineered nerve repair conduits (NRCs) have been developed to overcome the problems associated with nerve autografts. These conduits have proven successful for smaller often digital nerve lesions however, the nerve autograft is still the preferred gold standard treatment for larger nerve defects (>30 mm). Improving the performance of current NRCs to circumvent the significant side-effects of nerve autografts for large nerve lesion repair is consequently an intensely studied area of research that has inspired a variety of biomaterial approaches including nanomaterial-based strategies.

Recent advances in the field of carbon nanotubes (CNTs) have instigated a wealth of research into potential nano-biomaterial applications including supporting and promoting

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DOI: 10.1002/adhm.201500864

the regeneration of neural tissue. CNTs possess a unique set of physicochemical properties that could be useful within NRCs. This progress report will evaluate and consolidate the current literature pertinent to CNTs as a biomaterial for augmenting peripheral nerve repair (PNR) devices. After an introduction to peripheral nerve regeneration and existing repair conduits, we discuss how CNTs may enhance the performance of next generation NRCs.

2. Physiology of Peripheral Nerve Regeneration

After transection of a peripheral nerve, feedback mechanisms from the axon tip induce a set of morphological changes termed chromatolysis: swelling of the perikaryon, displacement of the nucleus towards the periphery and dissolution of Nissl bodies (**Figure 1**). If the injury is very close to the neuronal cell body such as in nerve root avulsion injuries, neuronal apoptosis often occurs. If the injury is more distal the expression of a plethora of pro-regeneration associated genes (RAGs) enhance neuronal survival and prepare the severed axon for regeneration.[1] The distal end of the axons, cut off from their nutritive and regulatory source, the perikaryon, degenerate via a cascade of events collectively termed Wallerian degeneration (WD).[2]

The proximal portion of the axon still attached to the perikaryon is sealed and begins to regenerate. At the tip of the axon, a highly motile structure called the growth cone forms and begins to advance into the lesion space. The growth cone guides the growing axon by sensing its microenvironment via the extension and retraction of filopodia and lamellipodia. Growth cone migration is governed via contact with extracellular matrix fibers, Schwann cell tracts and a complex array of diffuse and substrate bound molecules.[3]

Schwann cells are key participants in the complex processes of WD and axon regeneration in the peripheral nervous system.[4] Granular disintegration of the distal axon cytoskeleton induces the de-differentiation and proliferation of Schwann cells to an immature phenotype.[5] Along with recruited macrophages, Schwann cells clear the distal nerve stump of axonal debris, degraded myelin and growth inhibitory molecules leaving behind fascicles of empty basal lamina endoneurial tubes that once surrounded the nerve axons (Figure 1b).

Proliferating Schwann cells then assemble into longitudinal chains or 'regeneration tracts' known as the bands of Büngner that line the endoneurial tubes and extend into the lesion gap (Figure 1c). These regeneration tracts provide longitudinal guidance channels for directing axon sprouts from the regenerating proximal nerve stump. Gradients of diffusible trophic factors produced by Schwann cells and target organs attract growth cones towards and along the distal endoneurial tubes. Extracellular matrix (ECM) proteins such as fibronectin, laminin and tenascin produced by Schwann cells enhance the substratum for growth cone adherence and guidance.

The continual production of growth factors by for example, denervated target muscles and Schwann cells, is vital in sustaining axon growth during repair of larger nerve lesions. Axons regenerate at rates of approximately 1–4 mm per day.[6] Injuries that occur far from the target organ and more proximal to the neuronal cell body must regenerate over very long distances, taking many months to years. Unfortunately, over such long periods of time, denervated muscles atrophy, motor end plates destabilise and trophic factor expression is downregulated. If reinnervation of motor end plates does not ensue within 12 months of injury, functional outcomes are likely to be poor.[7,8] It is therefore vital that nerve repair occurs without delay to exploit this narrow window of optimum conditions for axon regeneration.

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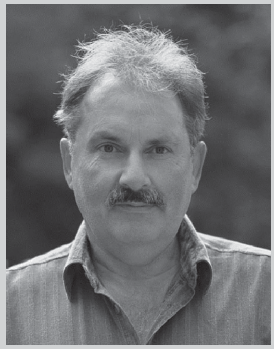

Paul Tomlins is a consultant specialising in biomaterials and tissue repair. Paul gained a PhD in physics from the University of Leeds and has spent most of his career working at the National Physical Laboratory in the UK and is active in the development of standards and good measurement practice in regenerative medicine.

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3. Evolution of NRC-design

The search for alternatives to the traditional gold standard nerve autograft began in the late 19th century with the introduction

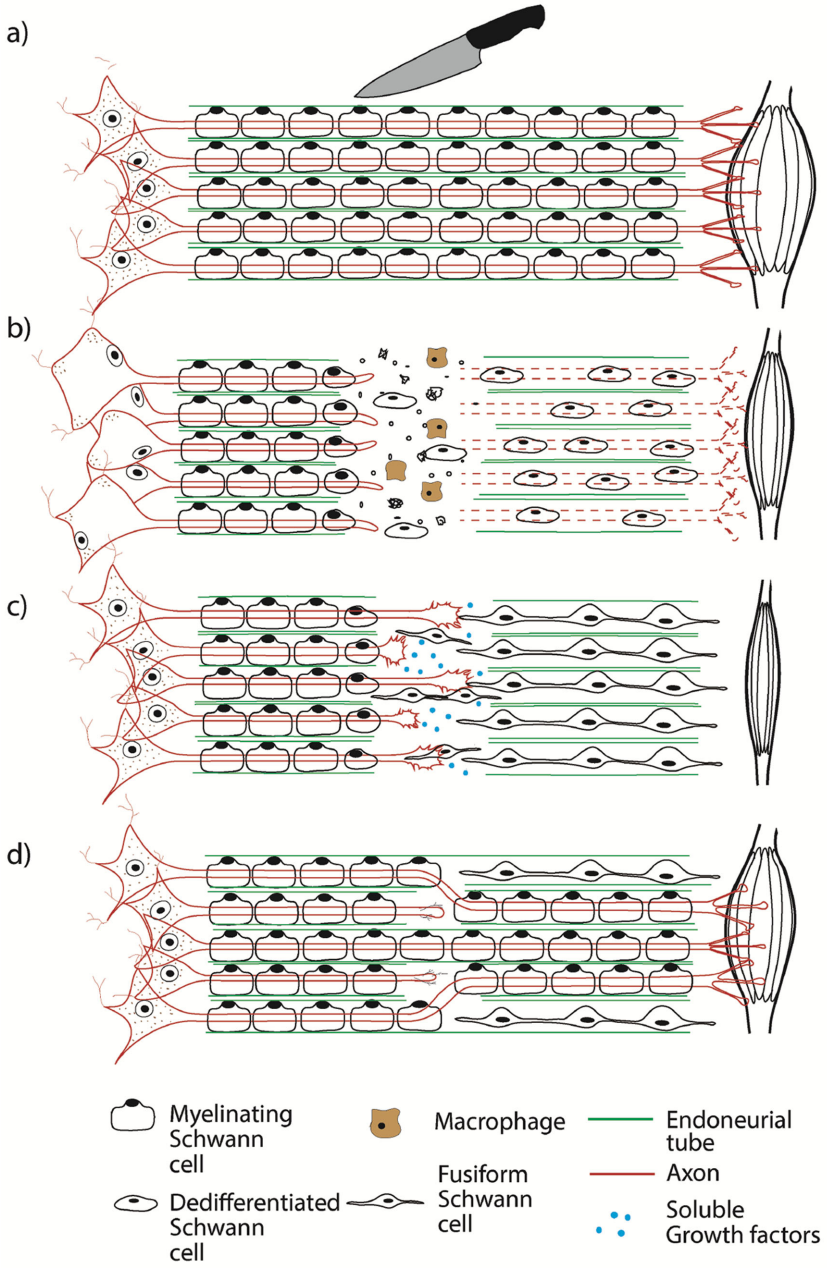

Figure 1. Wallerian degeneration and regeneration after neurotmesis in the peripheral nervous system. a) Peripheral nerve innervating skeletal muscle. Axons are myelinated by Schwann cells and surrounded by the basal lamina endoneurial tubes. b) After neurotmesis the proximal tip of the severed axons are sealed whilst the distal axon and myelin sheaths degenerate. Distally, Schwann cells dedifferentiate, proliferate and along with macrophages remove cellular debris and growth inhibiting molecules from the lesion and endoneurial tubes. Depending on the proximity of the lesion to the neuronal soma, chromatolytic changes may occur within the perikaryon. The denervated muscle begins to atrophy. c) The proximal axon sprouts and begins to regenerate into the lesion space. Axon regeneration is promoted by soluble growth factors produced by Schwann cells and target muscles. Schwann cells line the endoneurial tubes and form the bands of Büngner that guide axon growth. d) Axons regenerate along the endoneurial tubes to reinnervate the target muscle. Misalignment or aberrant growth of axons and a lack of specific topological axon guidance can result in regeneration into the wrong endoneurial tube and subsequent innervation of inappropriate targets.

of nerve allografts and the construction of the first NRC from decalcified bone, arteries and veins.[7] In the following decades, clinicians and scientists investigated the efficacy of conduits made from a wide range of biological and synthetic materials.[7,9] Findings from these studies began to identify the ideal design characteristics for NRCs (reviewed in de Ruiter et al. and Kehoe et al.). Thus, a bespoke NRC should provide structural support, but be flexible enough to allow ease of surgical insertion and normal anatomical movements; it should be permeable to allow angiogenesis and the diffusion of essential molecules, but prevent infiltration of scar tissue. Ideal NRCs should persist long enough for regeneration to occur, but also be biodegradable to minimise fibrosis, foreign body response and constriction of the nerve, as well as attenuating the long-term risks of infection. Advanced NRCs should be biocompatible to prevent inflammation and meet technical requirements for good manufacturing production, storage and handling.[10,11]

The design of current commercially available NRCs consists of a tube or wrap with a single hollow lumen into which the two ends of the damaged nerve are inserted. A recent survey identified eleven conduit devices that have secured Food and Drug Administration (FDA) approval including the commercially available Neurotube (polyglycolic acid), Neurolac (poly (L-lactic acid-co-caprolactone)); Neurogen (collagen type I); NeuroMatrix, Neuroflex (collagen type I) and Salubridge (polyvinyl alcohol hydrogel). The Neurotube and NeuroGen conduits have achieved the most clinical success and are often used to repair short digital nerve defects.[11] These conduits support functional recovery comparable to autografts however, for lesions greater than 3–4 cm their performance often falls short with clinicians still preferring the nerve autograph.[12]

Since 2007, the AxoGen Avance decellularised allograft has also been available for clinical use and has been transplanted into ca 5000 patients.[13] A recent clinical trial study of 136 nerve repairs supported by the AxoGen Avance allograft reported no host immune rejection and good functional recovery comparable to nerve autografts for lesions up to 31 mm.[14] The efficacy of nerve allografts and commercially available NRCs has been extensively reviewed in several recent articles.[7,11,15–17] With improvements in our understanding of nerve repair physiology it has become clear that a major limitation of commercial NRCs is the lack of a three-dimensional luminal matrix that can direct and promote the growth of advancing regenerating axons.

The successful repair of smaller nerve lesions using NRCs can be attributed to the formation of a fibrin matrix between

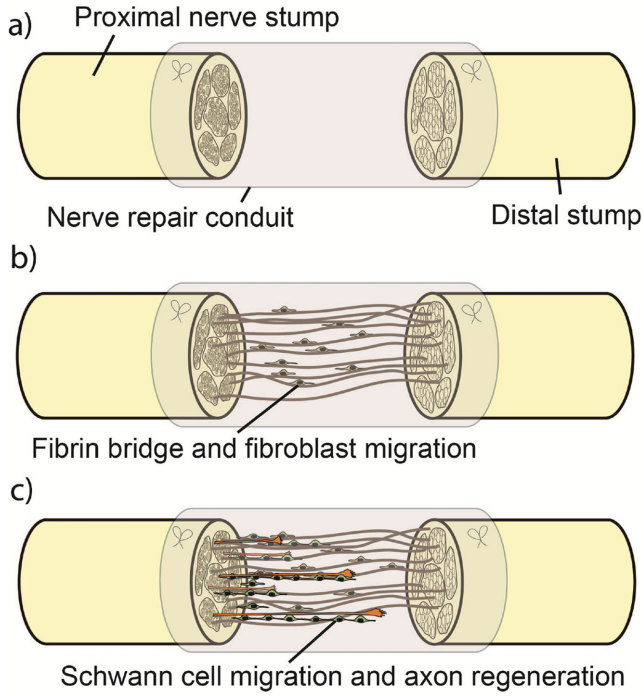

Figure 2. Regeneration of a non-critical length nerve defect (<1 cm in rodents) through a traditional hollow lumen NRC. a) The conduit fills with extracellular fluids b) A fibrin cable forms connecting the two nerve stumps and provides a bridge for cellular migration c) Schwann cells form the bands of Büngner and axon regeneration proceeds across the lesion. Reproduced with permission.[19] Copyright 1998, Elsevier.

the nerve stumps within the NRC lumen that facilitates the coordinated migration of fibroblasts, Schwann, endothelial and perineurial cells to optimise nerve repair (**Figure 2**). In larger lesions, greater than 3 cm for humans and 1 cm for rats, this matrix is either unable to form or is unstable hindering the migration of Schwann cells and the formation of the bands of Büngner. Without a guiding and supporting matrix, axon growth through larger lesions is significantly impaired often resulting in the formation of painful neuromas and poor functional recovery.[18,19] Recent advances in our understanding of the mechanisms of axon guidance and regeneration coupled with technological advances in biological and material engineering promise to bring a new generation of sophisticated, biomimetic NRCs with enhanced efficacy.

4. CNTs: Potential Role in Nerve Repair

CNTs are nanoscale tubes of carbon with a structure similar to a rolled up sheet of graphene with single or multiple concentric layers. Produced by a variety of techniques, most commonly by chemical vapor deposition involving the restructuring of carbon atoms using a metal catalyst, their diameters can range from 0.4–100 nm and lengths from a few nanometres up to 0.5 meter.[20]

With a unique set of physiochemical properties, CNTs have been dubbed the miracle material of the 21st century and promoted to have applications in numerous fields of science and technology.[21] CNTs are excellent electrical conductors and possess nanoscale topographical features. When normalised to account for differences in density, CNTs possess a strength that is over 50 times greater than steel wire. Extensive research into the surface chemistry of CNTs has provided methods to convert their very hydrophobic structure into one that is dispersible in aqueous solution and can be functionalised to impart useful properties for many applications (**Figure 3**).[22]

Several research groups have suggested the utilisation of CNTs in the field of neuroregeneration and PNR. However, the potential roles within NRCs have yet to be fully defined. Within NRCs, CNT scaffolds could be utilised to enhance axon growth rates and anisotropic organization via electrical stimulation and contact guidance cues, respectively. They are ideal for providing structural reinforcement to biodegradable conduit materials. Furthermore, surface functionalisation of CNTs could provide a route for the localised administration of growth promoting molecules and proteins.

4.1. Directing and Supporting Axonal Growth

4.1.1. The State-of-the-art

Structural and topological features formed by the ECM and cell surfaces have important roles in guiding axonal growth. This is particularly evident during the regeneration of nerve crush injuries in comparison to regeneration after nerve transection. After nerve crush lesions, when the connective tissue of the nerve is not breached, axons are able to follow the exact pathways of endoneurial tubes back to their original targets with 90% accuracy. The precision of regeneration after nerve transection injuries is significantly reduced due to loss of alignment of axons with their original endoneurial tubes.[23] The importance of including biomimetic directional guidance cues within the lumen of NRCs is now well recognised.

Progress in tissue engineering and three-dimensional (3D) scaffold fabrication techniques have enabled the production of biomimetic scaffolds with nano- and micro-features.[24] These scaffolds exploit the ability of sub-cellular physical features to control cell behavior and provide topological contact guidance cues to direct growth cone migration.[3,25] Among others, neurite outgrowth in vitro has been directed by nano-imprinted grooves and ridges (**Figure 4**a), electrospun fibers (Figure 4b), printed patterns of ECM proteins, aligned or printed hydrogels, aligned Schwann cells and CNT configurations including arrays, yarns, tubular scaffolds and ropes (Figure 4c–f).[26–29] The geometries of topographical features such as ridge height, width, inter-ridge distance, diameter of aligned fibers and width of patterned ECM proteins dramatically influence growth cone behavior.[25,30,31] Fine-tuning these geometries can enhance neurite alignment and outgrowth such as that seen on increasingly smaller diameter (500-5 µm) aligned polymer fibers.[32]

A wide variety of NRC luminal fillers have been investigated in vivo and often showed considerably enhanced performance compared to traditional hollow lumen NRCs. Experimental luminal fillers frequently consist of gels or sponges supplemented with ECM proteins and growth factors, supportive cells such as Schwann cells, or longitudinal contact guidance cues

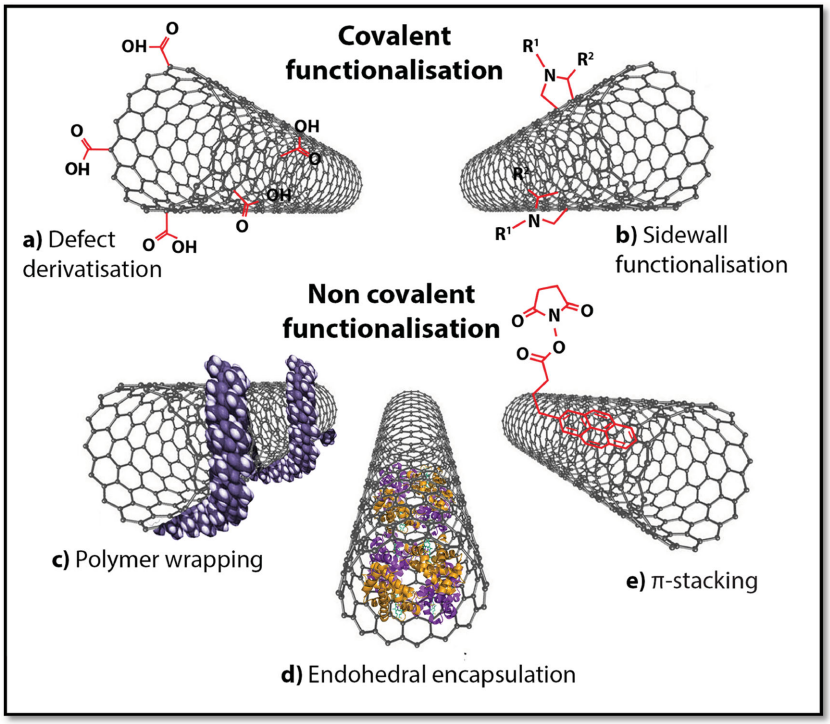

Figure 3. Examples of CNT surface modifications a) derivatization of sidewall defects formed via oxidation b) sidewall functionalization via 1,3-Dipolar addition of azomethine ylides c) polymer wrapping d) internalization of molecules within the CNT lumen e) π-stacking of aromatic molecules such as pyrene.

such as aligned textures, filaments, rods, and channels. These intra-luminal scaffolds and matrices increase the surface area for cellular migration, augment the availability and presentation of growth instructive molecules, and have been shown to improve the directional organization of axons towards the distal nerve stump.[33]

Aligned electrospun fibers have proven to be especially popular for enhancing anisotropic axonal growth.[34,35] Electrospun fibers of tuneable diameters can be generated from natural or synthetic materials. Synthetic polymers of poly(α-hydroxy esters) are commonly used owing to their FDA approved status, biocompatibility and adjustable degradation profiles.[36] A particularly impressive repair of an eighty millimetre lesion of the canine peroneal nerve was achieved using laminin coated collagen-electrospun fibers.[37]

Advances in a variety of techniques such as 3D-printing, self-assembling scaffolds and photolithography offer the ability to construct conduits with precise 3D micro-structures for enhancing organized axon growth.[24] For example, Owens et al. used bioprinting to generate nerve conduits made from multicellular cylinders whilst Georgiou et al. generated aligned Schwann cell conduits using tethered collagen gels. Both grafts showed potential for repairing rat peripheral nerve lesions.[29,38]

4.1.2. Application of CNTs

CNTs can be used to generate different types of 2D and 3D scaffolds with sub-cellular textures similar to ECM fibers. With the added benefits of exceptional strength, electrical conductivity and capabilities for surface functionalisation, CNT scaffolds could provide a superior material for directing and enhancing axonal growth within next generation NRCs.

Mattson et al. (2000) were the first to grow neurons on CNT substrates and suggested their use as a tool to investigate the mechanisms that govern neurite outgrowth at the nanometre scale.[39] Since then, interest in CNT substrates to support and control neuronal growth, to study neuronal network formation and for coating electrodes for interfacing neurological prosthesis has increased rapidly.

Although to date the application of CNT containing scaffolds for nerve repair in vivo has been limited, a variety of neuronal cell types have been grown on CNT substrates in vitro enabling assessment of their biocompatibility and growth promoting properties. Over 100 studies have reported that CNT scaffolds are capable of supporting neuronal survival, growth and maturation. Hippocampal, dorsal root ganglia (DRG), cortical, cerebellar neurons as well as the neuron-like PC-12, NG108, Neuro2a, and SH-SY5Y cell lines, have all been grown on different CNT substrate configurations.[40–46]

In addition to providing a permissive substrate for neuronal growth, patterned CNT substrates are also able to control the direction of neurite outgrowth.[47–49] Zhang et al. reported directed neurite outgrowth of the H19-7 hippocampal cell line by poly L-lysine (PLL) coated multi-walled carbon nanotube (MWCNT) arrays (Figure 4c). Alignment of neurites with the CNT patterns was dependent on the height of CNT arrays with taller arrays (10 µm) performing better than shorter (500 nm) arrays.[40] Human mesenchymal stem cells induced to undergo neuron-like differentiation, grew on patterned MWCNT sheets with cell bodies and neurite-like extensions orientated along the MWCNT in the sheets.[50] When compared to bare glass as a control, the MWNCT sheets significantly upregulated the expression of the ß1-integrin receptor, a cell adhesion protein.[50]

Patterned CNT substrates have provided a useful tool for controlling neuronal adhesion in studies of in vitro neuronal network formation. Controlled deposition of CNTs on quartz (SiO_2) substrates provided islands of adhesive growth surfaces allowing precise control over the formation of interconnected self-organising neural networks.[51,52] Scanning electron micrographs revealed that the entanglement of neurites and growth cone processes may be an important mechanical mechanism that enables neurons to adhere and migrate on CNT substrates.[45,53]

Collectively, these studies highlight the potential of CNTs as biomaterials in the repair of damaged neural tissue. Particularly promising for applications in NRCs are studies employing anisotropic 3D CNT scaffolds such as CNT yarns, ropes or aligned

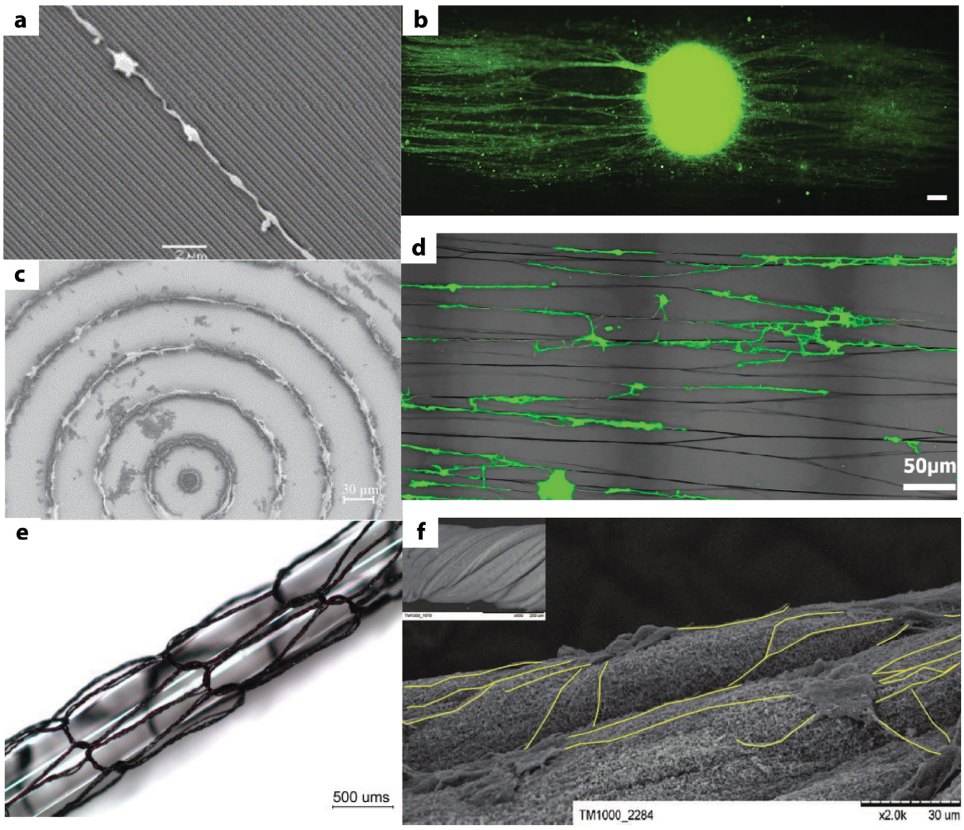

Figure 4. Neurite outgrowth directed by contact guidance cues on a) nano imprinted groves and ridges, b) electrospun fibers, c) MWCNT arrays, d) CNT yarns, and f) CNT ropes. a, c and f – are scanning electron micrographs. b and d are immunofluorescent micrographs. e) Knitted 9-ply CNT tubular scaffold. Reproduced with permission.[56] Copyright 2012, Johns Wiley and Sons. Reproduced with permission.[40] Copyright 2005, Elsevier. Reproduced with permission.[55] Copyright 2009, Elsevier. Reproduced with permission.[199] Copyright 2006, Elsevier. Reproduced with permission.[200] Copyright 2005, Elsevier. Reproduced with permission.[54] Copyright 2012, American Chemical Society.

electrospun CNT composite fibers. Fan et al. developed super-aligned poly D-lysine (PDL) coated MWCNT yarns that provided excellent directional guidance to regenerating rat hippocampal neurites with minimal neurite branching (Figure 4d).[54] A similar study reported pristine MWCNT yarns that supported the migration of fibroblasts, Schwann cells and directed neurite outgrowth from murine DRG explants along the longitudinal orientation of the yarn.[46] MWCNTs yarns can also be knitted together in a variety of configurations to form novel scaffolds, for example, Edwards et al. knitted a 9-ply tubular scaffold (Figure 4e).[55] CNT yarns can also be wound into large rope-like structures capable of directing neurite outgrowth. Neural stem cells (NSCs) grown on a 1-mm CNT rope differentiated in a similar manner to control NSCs on tissue culture polystyrene (TCP). The NSCs survived for up to three weeks and neurite extension favoured the direction of the CNT fibers spiralling around the rope structure (Figure 4f).[56]

CNTs can also be incorporated into electrospun polymer fibers either by blending with the polymer solution during the electrospinning process so that the CNTs are embedded within the fibers, or by coating the surface of already spun fibers. Kabiri et al. generated poly(L-lactic acid) (PLLA) electrospun fibers blended with either single-walled carbon nanotubes (SWCNTs) or MWCNTs. Poor adherence of mouse embryonic stem cells to both the MWCNT and SWCNT-PLLA fibers was attributed to their surface hydrophobicity. After oxygen plasma treatment, CNT-PLLA fibers promoted neural differentiation and cell growth was aligned along the orientation of the fibers. Cell attachment was comparable to that on PLLA only fibers.[57]

Whilst data from these preliminary studies are promising, conflicting reports of reduced neuronal viability and growth on CNTs raises uncertainty as to the suitability of CNT scaffolds for enhancing axon growth in NRCs.[43,58–63] For example, Hu et al. compared the growth of postnatal hippocampal neurons on nonfunctionalised MWCNTs with that on polyethylenimine (PEI) coated onto glass coverslips as the control substrate.[64] They reported that neurite growth parameters including the number of neurites per neuron, the total neurite length per neuron and neurite branching were substantially reduced on the as-prepared (nonfunctionalised) MWCNT substrates compared to growth on PEI. However, no significant differences in the average length of individual neurites extended on PEI compared to those on the as-prepared MWCNTs were observed. This suggests that the as-prepared MWCNT substrates reduced the initiation and branching of neurites resulting in a reduction in total neurite area, but once initiated, the length of neurites compared to control was unaffected.

To explore the relationship between MWCNT surface charge and neurite outgrowth physiology, Hu et al. compared the growth of hippocampal neurons on MWCNTs modified

with functional groups bearing differing charges including poly m-aminobenzene sulfonic acid (zwitterionic), ethylenediamine (+ve), and carboxylic acid (-ve). Intriguingly, the positively charge MWCNT substrate enhanced the number of growth cones generated, neurite branching and neurite length compared to the negatively charged and zwitterionic MWCNT substrates, but was still substantially inferior to growth on PEI coated coverslips.[64]

Hu et al. subsequently compared neuronal growth on PEI-coated glass coverslips with that on either unmodified MWCNTs or SWCNTs covalently functionalised with positively charged PEI to assess whether PEI functionalization could rescue the reduced neurite growth observed on unmodified CNTs.[65] All neurite growth parameters measured including average neurite length were significantly reduced on unmodified MWCNTs compared to PEI-coated glass coverslips as previously observed. Neurite growth on PEI-SWCNTs was superior to that on unmodified MWCNTs with an increase in neurite branching and slight improvements in neurite length however, the number of growth cones and neurites per neuron were not different from unmodified MWCNTs, and neurite growth on PEI-SWCNTs was still inferior to PEI-coated coverslips.

In a recent similar study, embryonic hippocampal neurons were grown on a set of MWCNTs (MWCNT-A_0-A_5) chemically functionalised covalently or non-covalently to assess the effect of functionalisation on neuronal growth.[66] MWCNT-A_1-A_5 were all prepared from raw MWCNT-A_0 that was initially coated onto silicon wafer. MWCNT-A_1-A_3 were non-covalently functionalised with trimethyl-(2-oxo-2-pyren-1-yl-ethyl)-ammonium (positively charged), 1-pyrenebutyric acid (negatively charged) and pyrene–PEG_{5000}, respectively. After oxygen plasma-treatment, MWCNT-A_4 became functionalised with carboxyl, carbonyl and hydroxide groups while MWCNT-A_5 was additionally covalently functionalised with PEG_{5000}. PDL served as control in this study.

Compared to PDL coated on glass coverslips, all of the functionalised-MWCNT substrates had substantially fewer cells per unit area, except for MWCNT-A_4 that supported similar cell densities as the control. Neuronal growth differed dramatically on the different functionalised-MWCNTs with neurons on MWCNT-A_4 possessing similar length and number of neurites to the PDL control whilst neurons grown on MWCNT-A_1-A_3 possessed shorter and fewer neurites. Neurites were often absent on MWCNT-A_5. After eight days in vitro (DIV), neurons on MWCNTs -A_0 – A_3 aggregated to form clusters and many neurites were organized into fascicles, whilst on MWCNT-A_4 growth of neurons and neurites remained dispersed, similar to the PDL control substrate. This suggests that MWCNT substrates A_0-A_3 promoted greater cell-cell interactions over cell-substrate interactions. Conversely, cell-substrate interactions appeared to be favored on MWCNT-A_4. Clearly, neuronal adhesion and neurite growth on functionalised-CNT substrates can be highly variable and greatly influenced by surface coating and functionalisation state.

For applications in PNR it is not sufficient that a scaffold simply permits axonal growth. Importantly, the rate of axonal growth should also be maximised to minimise lesion repair time and improve the probability of functional recovery. CNT scaffolds must therefore enable rapid and efficient axon elongation comparable or superior to axon growth through the gold standard autologous nerve graft. When assessing the suitability of functionalised CNT scaffolds in vitro, careful consideration must be given to control substrates for comparison. Glass coverslips and TCP coated with polyamines such as PDL, PLL, PEI and poly ornithine have been extensively used as control substrates when neurite outgrowth has been assessed on CNTs.[43,46,47,64,66] Polyamines are commonly used for coating culture surfaces to enhance cellular adhesion through interactions with negatively charged groups on cell surfaces.[67] These coatings have the capacity to support neuronal adhesion, neurite outgrowth and maturation however, for evaluating the ability of a biomaterial to support rapid and extensive neurite outgrowth for applications in PNR, a superior benchmark should be sought such as extracellular matrix proteins with known neurite outgrowth promoting properties.

Laminin is a heterotrimeric glycoprotein that, along with collagen type IV and fibronectin constitutes a major component of the basement membrane. Laminin is pivotal to the process of embryonic neuronal wiring, defining migratory and axonal pathways.[68] It is present in all tissues that support axon growth in the regenerating adult nervous system such as the olfactory pathway; blocking its function via anti-sera significantly impairs regeneration.[69–71]

Laminin is consistently reported to be a potent stimulator of neurite outgrowth and when directly compared with polyamine coated substrates, growth of neurons on laminin is significantly enhanced, especially for peripheral neurons.[68,72–77] It is often blended or coated onto biomaterials developed for nerve regeneration and consistently boosts biomaterial performance.[32,78,79] To evaluate the relative efficacy of anisotropic CNT scaffolds and similar nanomaterials in supporting the guidance and regeneration of peripheral axons, it is essential that scaffold performance should at the very least be compared to well-characterised and physiologically relevant substrates such as laminin.

Compared to laminin, neurite outgrowth on CNT substrates is poor.[58] However, functionalising CNTs with laminin can support substantially greater neurite outgrowth compared to laminin-coated TCP or other laminin coated biomaterial controls.[80,81] CNT/chitosan blended electrospun fibers provided a poor substrate for the adhesion of PC-12 cells compared to chitosan fibers and laminin coated TCP. However, after oxygen plasma-treatment and coating with laminin, PC-12 adhesion onto CNT containing fibers increased 20-fold and was reportedly almost double that on laminin-TCP or chitosan substrates.[81] Neurite projections followed the orientation of the fibers regardless of fiber diameter that ranged from 54–284 μm.

Electrospun poly (L-lactic acid-co-caprolactone) (PLCL) fibers coated with water soluble MWCNTs, PLL and laminin, directed neurite outgrowth from rat DRG neurons along the longitudinal axis of the fibers, significantly enhancing neurite length compared to PLL/laminin coated PLCL fibers without CNTs. These MWCNT coated fibers also enhanced the expression of focal adhesion kinase (FAK) in nerve growth factor (NGF) differentiated PC-12 cells. FAK is a key cytoplasmic enzyme that mediates the signal transduction pathways of the integrin family of laminin cell surface receptors.[80] Collectively, these studies show that with the appropriate surface treatment CNTs

can significantly improve neuronal attachment and neurite outgrowth over laminin as a control reference substrate.

In vivo evidence of the efficacy of CNT coated fibers in promoting nerve regeneration as luminal fillers in NRCs comes from the recent study of Ahn et al.[82] They covalently linked aminated CNTs onto the surface of aligned phosphate glass fibers (PGFs). Their in vitro studies showed that after coating with PDL and laminin, the CNT-PGFs supported the attachment and growth of mouse DRG neurons. The growth of these neurons on the CNT-PGFs was comparable to that on laminin. Both PGFs and CNT-PGFs appeared to align neurites along the longitudinal axis of the fibers. Interestingly, DRG neurites were significantly longer on the CNT-PGFs compared to PGFs alone.

Bundles of these fibers were then wrapped within an aligned electrospun nanofiber mat, inserted into a porous poly (L/D-lactic acid) nerve repair conduit and used to repair a 10 mm rat sciatic nerve lesion. After 16 weeks, the CNT-PGF conduits significantly enhanced both the number of regenerating SMI32 positive axons in the distal nerve stump and functional recovery of reinnervated gastrocnemius muscle. However, the performance of the CNT-PGF conduit was substantially inferior to autologous nerve grafts. Despite the reduced efficacy compared to autografts, attributed to the suboptimal interspacing between the PGFs within the NRC; this study demonstrates the feasibility and biocompatibility of CNT tethered biomaterials for NRCs.

4.2. CNTs for Mechanical Reinforcement of Biodegradable Conduits

4.2.1. The State-of-the-Art

It is clear that NRCs composed of biodegradable materials provide improved long-term efficacy over non-degradable materials. Although nerve regeneration can proceed through non-biodegradable materials such as silicon, these tubes result in constriction of the nerve, foreign body reaction, extrusion and require a second surgery for removal.[83] These limitations have resulted in a move away from non-biodegradable materials. However, utilising biodegradable materials presents another set of obstacles: over time the mechanical properties of biodegradable materials inevitably deteriorate and so, the fine-tuning of conduit degradation times is of the upmost importance to prevent fibrosis and nerve constriction whilst retaining sufficient mechanical strength until regeneration is complete. Achieving an appropriate degradation profile for the repair of larger nerve defects is a particularly difficult challenge.

Naturally occurring biodegradable materials whilst offering excellent biocompatibility, often suffer from batch to batch variability, require extensive purification, degrade rapidly in vivo and often have poor mechanical properties.[9] Synthetic biodegradable materials can provide superior mechanical properties over natural materials however, the degradation of some polymers such as polyglycolic acid (PGA) results in the release of acidic, cytotoxic by-products reducing their biocompatibility. The strength of natural biomaterials can be significantly improved by chemical modifications such as cross-linking as well as blending or reinforcing with synthetic polymers or fibers.[84] Innovative fabrication methods can also significantly improve structural properties. For example, chitosan conduit moulds cast around a tube of braided chitosan yarns possessed a tensile strength of 3.69 MPa, 9 times greater than chitosan conduits without braided yarns.[85] Although cross-linking enhances the stiffness of hydrogels, excessive cross-linking can result in reduced cell migration and proliferation.

4.2.2. Application of CNTs

CNTs possess outstanding mechanical properties such as elastic modulus of 0.2–1 TPa and strength 200–900 MPa, whilst being lightweight and flexible.[86–88] Hence, CNTs are already utilised in a variety of products such as car bumpers, tennis rackets and Kevlar textiles to improve elasticity and strength of the host material.[89] Interest in using CNTs to improve the mechanical properties of biomedical materials has increased significantly in the last decade with a variety of studies describing the incorporation of CNTs with biodegradable polymers, gels and ECM proteins.[42,84,90–94] Although few studies focus on the production of CNT-composites specifically for NRCs, many studies combine CNTs with natural materials and synthetic polymers commonly used to fabricate NRCs.

4.2.3. Natural Biomaterials

Collagen type I is an essential mechanical and structural component of the ECM and is often used for creating tissue-engineered scaffolds including NRCs. During collagen extraction and purification natural cross-links between collagen fibrils are lost thus, scaffolds produced from purified collagen lack the robust fiber structure and mechanical stability of collagen in situ in the body. Combining collagen scaffolds with CNTs increases the static tensile modulus, compressive strength and stiffness of the constructs.[87,95] CNTs enhance the mechanical properties of alginate, hyaluronic acid and gelatine hydrogels.[86,92,96]

Pure unmodified SWCNTs were incorporated into 3D alginate scaffolds using a freeform multi-nozzle deposition system. SWCNT at 1 wt% reinforcement increased the tensile strength of alginate struts from 436 to 542 kPa. Cell attachment and proliferation were also improved and attributed to an increase in surface roughness on SWCNT containing scaffolds.[86] Functionalised-SWCNTs incorporated into hyaluronic acid hydrogels (0.06 wt% SWCNTs) enhanced the viscoelastic properties of the gels reported as a 4-fold increase in storage modulus, without significantly affecting their swelling capacity or shear thinning behavior.[96] Gelatin methacrylate coated-CNTs incorporated into hydrogels produced 3D scaffolds that were biocompatible and possessed enhanced mechanical properties. The compressive modulus of the gel was enhanced from 10 to approximately 30 kPa whilst still preserving the scaffold porous structure. The addition of CNTs also appeared to slow the degradation of the scaffolds with an inverse relationship between CNT content and the degradation rate reported. CNTs have also been used to strengthen fibers electrospun from chitosan and agarose.[81,97,98] Huang et al. observed that the addition of CNTs to laminin

coated chitosan fibers significantly improved the poor mechanical strength of plain chitosan fibers making it feasible for their application in nerve repair.[81]

4.2.4. Synthetic Materials

Biodegradable aliphatic polyesters are classic synthetic biomaterials used for a wide range of tissue engineering and surgical products including NRCs. The addition of 1–3 wt% unmodified or functionalised MWCNTs significantly enhanced the compressive strength and modulus of poly(lactide-co-glycolide) (PLGA) scaffolds.[99] The MWCNTs were chemically functionalised with hydroxyl or carboxylic acid groups. Scanning electron microscopy (SEM) analysis showed these PLGA-MWCNT composites supported growth of osteoblast precursors however, after 21 DIV, proliferation on the functionalised MWCNT composites was significantly reduced compared to PLGA alone and unmodified MWCNT-PLGA composites. Analysis of the in vivo biocompatibility of the composites after subcutaneous implantation in adult rats showed initially a minimal inflammatory response similar to the PLGA control, but after four weeks this increased to moderate or even severe responses. Inflammation varied between the different functionalised MWCNTs. The formation of a fibrous capsule around the implants was observed without systemic or neurological adverse side-effects.

Polycaprolactone (PCL) modified with as little as 0.5 wt% MWCNTs exhibited significant improvements in tensile and compressive modulus and enhanced the proliferation and differentiation of rat bone marrow-derived stromal cells.[100] Inclusion of 1 or 3% MWCNTs in PCL hollow fibers increased the tensile modulus of the composite fibers by 20 and 30%, respectively, compared to pure PCL fibers. An increase in MWCNT concentration produced an increase in material stiffness that resulted in a lower elongation to break for composites containing 3% MWCNTs.[61] A similar study reported that as the concentration of MWCNTs in a polylactide – caprolactone (PLC) composite increased from 2 to 5 wt%, dispersion of MWCNTs changed from very uniform to agglomerated resulting in greater porosity of the composite and significantly reduced tensile strength, perhaps due to poor interfacial bonding and poor load transfer at 5 wt% CNT. PLC composites with 2 wt% CNT exhibited a 100% increase in elastic modulus, a 160% increase in tensile strength and an increase in osteoblast viability similar to the data reported by Yildirim et al. in 2008. The observed improvements in osteoblast viability were attributed to the increased surface roughness of the CNT-PLC composites.[101]

Incorporation of 0.2 wt% 4-tert-butylphenylene functionalised-SWCNTs resulted in extraordinary improvements to the mechanical properties of the injectable degradable polymer poly(propylene fumarate) for tissue engineering of load bearing bones.[102] MWCNTs at 1–3 wt% in poly(2-hydroxyethylmethacryate), (pHEMA) composites supported growth of neuroblastoma cells similar to controls (TCP and unmodified pHEMA) whilst 6 wt% MWCNTs in pHEMA resulted in almost complete cell death by the seventh DIV suggesting that a fine balance must be struck between enhancing material mechanical properties whilst retaining biocompatibility.[103]

4.2.5. CNT-composite NRCs

Preliminary in vivo studies incorporating CNTs into the wall of experimental NRCs have also been undertaken. NRCs made from electrospun collagen/PCL fibers containing 0.1% carboxyl-functionalised MWCNTs showed improved hydrophilicity and substantially slower degradation rates compared to collagen/PCL alone. However, the mechanical strength of the conduit was not significantly improved. This was ascribed to insufficient dispersion of CNTs within the composite matrix.[104] Despite the lack of improved mechanical properties, MWCNT-collagen/PCL NRCs supported nerve regeneration in a rat sciatic nerve injury model and were found to support Schwann cell growth in vitro. Electrophysiological and histological assessment showed that the composite conduits performed better then silicon, but were inferior to nerve autografts. Acute infections, inflammation or rejection were not observed however, the internal surface of the conduit was coated with multi-nucleated giant cells indicating a potential foreign body response.

In a similar study using NRCs fabricated from silk fibroin containing carboxylated SWCNTs, these NRCs were observed to be biocompatible with U373 cells in vitro and supported regeneration of the sciatic nerve in a rat injury model.[105] This study did not include an autograft or non-CNT containing silk fibroin conduit control making evaluation of efficacy difficult. In addition, no information was provided on the influence of the CNT content on mechanical properties of the conduits.

CNTs can clearly enhance the mechanical performance of biomaterials, particularly the tensile strength however, optimal integration of CNTs within the composite matrix is vital to ensure transfer of mechanical loads and superior mechanical properties are attained.[89,106,107] The poor aqueous solubility of CNTs can significantly hinder their homogenous dispersion in aqueous solution leading to structural flaws in the resulting materials where aggregates of CNTs have formed. Surface functionalisation can significantly enhance interactions between CNTs and the material matrix improving their integration.[87] For example, collagen gels with 5 wt% albumin-adsorbed on SWCNTs possessed larger collagen fibrils and increased compressive modulus than gels with 5 wt% unmodified SWCNTs. Adsorption of albumin to SWCNTs clearly improved their dispersion in collagen imparting improved mechanical properties.[95]

CNT reinforcement also appears to slow down the degradation of a surrounding biodegradable matrix which could be applied to NRCs for larger defects with longer repair times.[92] Further studies are required to characterise and refine the biocompatibility and degradation profiles of CNT-composite NRCs. Although these limited number of in vivo studies indicate that NRCs containing CNTs are able to repair injuries in animal models, whether these conduits offer superior performance to equivalent NRCs without CNTs and nerve autografts remains to be proven in well-controlled studies.

4.3. CNTs for Electrical Stimulation in NRCs

4.3.1. The State-of-the-Art

Although not yet widely adopted in the clinic, studies have shown that electrical stimulation via percutaneous or nerve

cuff electrodes can enhance regeneration of injured peripheral nerves in animal models and in humans.[108,109] Designed to provoke firing of action potentials at physiological rates, electrical stimulation via these techniques can accelerate end organ reinnervation and functional recovery by enhancing the number of axons that regenerate, by reducing the latency between injury and initiation of axon growth and by enhancing the maturation of regenerating fibers.[110,111] However, the speed of axon growth through the distal nerve once regeneration is initiated does not appear to be affected.[112] The molecular mechanisms by which this type of electrical stimulation promotes nerve regeneration are not fully understood. Studies have shown an enhanced growth state and expression of RAGs such as GAP-43, neurotrophic growth factors and their receptors are important.[113–115] Carefully designed protocols are required to prevent excessive electrical stimulation which can impair nerve cable formation and functional recovery.[110]

An alternative method of electrical stimulation makes use of weak electrical fields and currents, normally below the threshold for producing an action potential. Endogenous bioelectric fields regulate many cellular activities including proliferation, migration, and differentiation.[116,117] Since the early 1900s, it has been known that weak exogenous electrical stimulation in vitro can affect neuronal growth.[113,118] Reviewed by Ghasemi-Mobarakeh et al., different forms of stimulation have been investigated for their ability to control neurite growth including steady direct current (DC) fields, magnetic fields, pulsed electromagnetic fields and DC passed through conductive growth scaffolds.[118,119]

These studies have shown that electric fields can dramatically affect the direction of neurite growth, increase neurite initiation, neurite length, neurite branching and the speed of neurite growth of primary neurons in vitro.[116,120–122] Several studies investigating the underlying mechanism have implicated the induction of an asymmetric distribution of charged receptors on the surface of growth cones resulting in an asymmetric response to external growth cues and hence, growth cone turning.[116] The efficacy of DC electrical field stimulation for treating spinal cord injury has been investigated in vivo in guinea pigs, dogs and humans in a phase I clinical trial.[123–125] Results have been promising with return of a variety of functions such as muscle reflexes and minor improvements in sensation detection in humans.

Electrical stimulation can also exert effects on neurite outgrowth when applied to neurons through conductive growth substrates. The use of conductive materials in NRCs would enable precise control over the intensity and spatial distribution of electrical stimulus delivered directly to regenerating axons. As well as direct effects, electrical stimulation can enhance the ability of supporting cells to promote axon regeneration. Olfactory ensheathing cells (OECs) and Schwann cells cultured on polypyrrole (PPy) composite substrates with electrical stimulation upregulated expression of neuronal growth factors including brain-derived neurotrophic factor (BDNF), NGF, vascular endothelial growth factor and neuronal cell adhesion molecules such as N-CAM. Concomitantly, the production of the growth inhibitory protein, NOGO-A in OECs was reduced compared to unstimulated controls.[126,127]

Polyaniline (PANI), PPy, poly(3, 4-ethylenedioxythiophene) (PEDOT) and poly(thio-phene) (PT) are the most widely utilised conducting polymers for applications in tissue engineering and brain machine interfacing.[119,128] Piezoelectric materials such as poly(vinylidene fluoride) generate transient surface charges under material deformation which can also enhance process outgrowth in vitro.[129] To impart conductivity, these polymers must be modified through a process known as doping resulting in a transfer of charge from dopant molecules to the π-conjugated polymer backbone.[119]

These conductive polymers are generally rigid, hard to manipulate and non-biodegradable however, by blending with biodegradable materials such as chitosan, flexible and useful biomaterials for nerve regeneration can be created.[126,130] Blending with natural polymers such as collagen or the use of biologically active dopants such as hyaluronic acid can improve their biocompatibility and impart biological functionality.[119] Furthermore, blending with electrospinnable polymers permits the production of conductive nanofibers. These can be used to form either NRC walls or aligned structures within the lumen. A major disadvantage of this method is a reduction in conductive properties of the resulting blended fibers compared to the pure polymer. However, innovative fabrication techniques can overcome this such as core-shell techniques or by simply coating the degradable electrospun fibers with nanometre-thick layers of conducting polymer.[131]

PPy has been the most intensely investigated conducting polymer for neuroregenerative applications and shows biocompatibility with neurons and Schwann cells in vitro and in vivo.[132] Electrical stimulation of neurons on pure PPy and PPy composites resulted in significantly longer and more numerous neurites then unstimulated controls.[131,133–135] Enhanced deposition of proteins such as fibronectin onto PPy during electrical stimulation has been shown to contribute to the growth promoting affects.[136] PPy NRCs have also been used to repair peripheral nerves in vivo with a recent study by Xu et al. showing efficacy as good as the gold standard nerve autograft for the repair of 10 mm sciatic nerve lesion in rats, although it is not clear if electrical stimulation was applied in this study.[135,136] Despite the widely reported positive effects of in vitro electrical stimulation on neurite growth, there are limited reports of the successful application of conductive NRCs and electrical stimulation to enhance peripheral nerve regeneration in vivo. This technique has also yet to be trialled successfully in the clinic however, development of novel biodegradable and biocompatible conducting materials for NRCs continues to be an area of research focus.[137]

4.3.2. Application of CNTs

CNTs are excellent conductors of electricity withstanding current densities exceeding 10^9 A cm^{-2}.[138] SWCNTs can exhibit either a semi-conducting or metallic behavior whilst MWCNTs are metallic. CNTs are a very attractive material for electrically interfacing with neurons on account of their unique combination of superior electrical conductivity, exceptional strength, chemical inertness, high specific surface area and flexibility. Recently reviewed by Bareket-Keren et al., a wealth of research has been conducted into the suitability of CNTs for stimulating and recording neuronal activity. Wang et al. were the first to

report the repeated stimulation of primary neurons in vitro with a PEG-functionalised CNT pillar array electrode.[139] Early studies focused on the stimulation of neuron-like cells from the NG108 cell line, primary rat peripheral and hippocampal neurons through CNT films.[43,140,141] Patch clamping of NG108 cells grown on CNT films showed normal electrophysiological features and electrical excitation upon application of current through the CNT substrate.[140] Stimulation through SWCNTs reliably evoked postsynaptic responses in hippocampal neurons and with clear potential for electrical coupling with neurons, the development of CNT-based electrodes and conductive CNT-composite materials rapidly followed.[141]

4.3.3. CNT Electrodes in the Repair of Central Neurons

Novel electrodes incorporating CNTs could enable a new generation of high performance neural prostheses and enhance the efficacy of therapeutic treatments such as deep-brain stimulation. Traditional metal electrodes often suffer from low signal to noise ratio, low spatial resolution due to large electrode dimensions, inadequate biocompatibility and induction of inflammatory responses due to a lack of compliance.[128] Smaller electrodes would improve biocompatibility and spatial resolution. However, these often suffer from high impedance and insufficient charge injection. To overcome these significant problems, electrodes that can handle larger currents and charge densities with high specific surface area are required. Close physical contact or coupling of electrodes to neurons is also important to ensure efficacy of recording, stimulation and to reduce signal-to-noise ratios however, the metallic surface of traditional electrodes does not facilitate cell-material interactions.

The mechanism of charge injection can greatly affect long-term performance and biocompatibility of electrodes. Faradaic charge injection exhibited by traditional metal electrodes including gold, platinum and titanium can result in electrochemical products leading to tissue damage and electrode degradation. Conversely, capacitive charge injection is not associated with electrochemical hazards and is therefore more desirable for neuronal interfacing.

By coating the surface of traditional electrodes with CNTs or CNT composites such as PPy or PEDOT, superior electrochemical properties can be achieved.[142–144] The extremely large surface area and electrical conductivity of CNTs enables the production of small electrodes whilst retaining high charge injection and low impedance.[139,142] The nanotextured surface of CNTs can enhance neural adhesion and neurite anchorage improving electrode-neuronal coupling and signal-to-noise ratio.[45,145] Due to their capacitive properties, CNTs can also safely deliver required charges without electrochemical hazards, ideal for neural stimulation.

CNT-based electrodes have now been utilised in a variety of configurations to effectively stimulate neurons in vitro and in vivo.[142,145] During long-term implantation, mismatch between the mechanical properties of traditional rigid and non-compliant electrodes and soft nervous tissue leads to tissue damage, adverse inflammation and reduction in electrode efficacy. Electrode arrays of pure CNTs can be generated on flexible films for compliant implantable neuro-prosthetics.[146] CNTs can also be wet-spun to produce small, soft and flexible CNT fiber microelectrodes with diameters of 12–43 μm for effective long-term in vivo neuronal recording and stimulation.[147] Vitale et al. implanted CNT microfiber electrodes chronically into Parkinsonian rodents and found these to be as effective as larger platinum electrodes for alleviating methamphetamine-induced rotation via deep brain stimulation. After 6 weeks, histological tissue analysis showed that the animals with CNT microfiber electrodes exhibited substantially reduced inflammation and gliosis compared to platinum electrodes. However, the zone of neurodegeneration was greater around the CNT electrodes compared to platinum controls, attributed to the implantation technique.

The body of research to date, on CNT-based electrodes, suggests they are capable of safe and efficient electrical stimulation of the nervous system. These properties can also be exploited for the development of electrically active biomaterials for applications in regenerative medicine, including PNR conduits.

4.3.4. Conductive CNT-biomaterial Composites

CNTs have been utilised to enhance the electrical properties of a variety of synthetic and natural materials, some of which could be developed for the electrical stimulation of regenerating nerves within NRCs. Several research groups have demonstrated that dispersion of CNTs in hydrogels such as collagen can enhance the electrical properties of the gels whilst retaining biological properties and supporting the viability of neuronal cells.[59,95,148,149] McDonald et al. reported that the electrical conductivity of collagen-SWCNTs gels increased with rising CNT content (0.8–4 wt%). These gels possessed electrical conductivity similar to native human tissue and supported good viability and morphology of human dermal fibroblasts cells at 3 DIV however, after 7 DIV, viability was reduced to 67% in the gels with the highest CNT content (4 wt%).[149] Subsequent studies by the same authors have shown that by ensuring good dispersion and alignment of CNTs within hydrogels, superior electrical percolation can be achieved. Alignment of CNTs, collagen fibrils and seeded cells can be achieved by applying an anisotropic mechanical strain to the hydrogels.[150]

In a rare recent study assessing the efficacy of electrical stimulation of nerve growth on conductive CNT composite gels, NGF-differentiated PC-12 cells were electrically stimulated at 0.1 V for 6 h on a MWCNT-collagen composite.[59] Results showed that the inclusion of MWCNTs improved conductivity of the matrix. When the matrix contained 5 wt% MWCNTs, neurite length was slightly enhanced over non-stimulated CNT-collagen controls, but was not improved above non-stimulated collagen only controls. Electrical stimulation has also been applied to SHSY5Y neuroblastoma cells seeded onto a pHEMA – MWCNT hydrogel composite developed for fabricating NRCs. The addition of 6 wt% MWCNTs to the pHEMA composite improved its conductivity by 11.4-fold. Electrical stimulation enhanced SHSY5Y viability however, neurite outgrowth was not assessed.

As previously discussed, polymer fibers can be used to fabricate NRCs and intraluminal anisotropic scaffolds. Coating or

incorporating CNTs into polymer fibers can greatly enhance their electrical conductivity.[151] Tethering of carboxylated-CNTs to the surface of phosphate glass fibers enhanced fiber conductivity from 10^{-13} to 10^{-5} through to 10^{-6} S cm^{-1}.[82] Huang et al. generated CNT/chitosan electrospun fibers and films (5:1 w/v) that possessed electrical conductivity of 1.13×10^{-2} S cm^{-1} compared to chitosan only (5.7×10^{-3} S cm^{-1}).[81] The addition of CNTs to PLLA electrospun fibers increased the conductivity of MWCNT-PLLA and SWCNT-PLLA composites from 10^{-8} and 10^{-9} mS cm^{-1} to 5 and 6 mS cm^{-1}, respectively.[57]

Electrical stimulation through CNT ropes and CNT composite fibers can enhance the differentiation of stem cells towards a neural phenotype. PLGA electrospun fibers were vacuum impregnated with SWCNTs and utilised as a substrate to enhance the differentiation of induced pluripotent derived NSCs. A regimen of 10 min, 30 μA DC electrical stimulation markedly increased neuronal differentiation after 14 DIV compared to unstimulated controls.[152] Electrical stimulation (5 mV, 0.5 mA, 25 ms) of NSCs grown on CNT ropes also enhanced neuronal differentiation and the speed of neurite outgrowth.[56]

Despite the number of studies describing the incorporation of CNTs into biomaterials with enhanced electrical conductivity, surprisingly few have reported the effect of electrical stimulation via these materials on neurite and axonal outgrowth rates. Neurite outgrowth on pure CNT scaffolds subjected to electrical stimulation has also been poorly reported. Electrical stimulation of regenerating peripheral nerves through NRCs incorporating CNTs has yet to be attempted.

4.4. CNTs as Neural Delivery Vehicles

4.4.1. The State-of-the-Art

Neurotrophic growth factors such as glial cell line-derived neurotrophic growth factor (GDNF) and the neurotrophins, NGF, BDNF and neurotrophin-3 are important secreted proteins that support survival and regeneration of peripheral neurons after injury.[153] Unfortunately, the production of these growth factors declines post-injury and so, for the repair of larger nerve lesions and those far from target organs, an exogenous supply of growth factors is required to sustain robust axonal growth.

The therapeutic application of exogenous growth factors has great potential for enhancing PNR. However, there is a need to develop delivery methods to maximize the efficacy of these treatments. Previous studies have shown that uncontrolled release of growth factors from NRCs can result in aberrant axonal growth and trapping of axons where growth factors become concentrated. Excessively high concentrations of growth factors can also inhibit Schwann cell maturation preventing normal myelination of regenerated axons.[154] To improve performance, methods of controlled release to sustain long-term therapeutic action, to prevent the "sweet shop" phenomena and to orchestrate unidirectional axonal growth are required.

A variety of different delivery methods have been investigated with the most common involving encapsulation of growth factors within biodegradable scaffolds and matrices such as gels, sponges, electrospun fibers and polymer rods.[155,156] These techniques often rely on the release of growth factors by passive diffusion as the supporting matrix degrades. Further control can be achieved by including methods of sequestering growth factors within the encapsulating matrix. One such method exploits the heparin-binding affinity of growth factors for retention on heparin cross-linked within a fibrin matrix.[157] Using this technique growth factor release is controlled by cellular activity enabling delivery to be concurrent with the progression of cell migration and regeneration through the conduit. This method significantly improves nerve regeneration in vivo over diffusion-based delivery techniques.[158,159]

An assortment of engineered nanoparticles is currently in development with potential to revolutionize the delivery of therapeutic agents. Reviewed in detail by GhoshMitra et al. and Shi et al., these nanocarriers or nanovectors can provide tissue or cell specific delivery of precise doses of therapeutics through the use of targeting ligands. In the future they could enable control of drug delivery via external stimuli such as temperature, electric or magnetic fields.[160–162]

The delivery of growth factors via engineered nanoparticles could provide a mechanism for directing axonal outgrowth from regenerating nerves towards desired targets. For example, magnetic hematite nanotubes functionalised with NGF stimulated neurite outgrowth from PC-12 cells in vitro, with growth cones observed extending towards the nanotubes.[163] Using carefully designed magnetic fields, movement of the magnetic nanotubes could be controlled to direct neurite growth.

As an alternative to direct delivery of growth factors, genetic manipulation via viral vector mediated gene transfer can be utilised to induce growth factor expression either by injection directly into injured nerves or in therapeutic cells ex vivo for subsequent transplantation to the site of injury.[154,164,165] Genetic manipulation using viral vectors to enhance PNR has predominantly focused on inducing expression of growth factors. However, these vectors also present additional opportunities to manipulate numerous potential therapeutic targets such as: prolonging the expression of intrinsic RAGs within neurons; preventing atrophy of denervated muscles, and enhancing expression of pro-regenerative proteins in Schwann cells.[166] Developments in viral vector technology have enabled greater control over the temporal and spatial expression of targeted genes.[166] Expression can be targeted to specific components of the peripheral nerve such as Schwann cells, fibroblasts and neurons themselves.[167] Adeno-associated viral (AAV) vectors offer improved efficiency of gene transfer and longevity of expression. An AAV-vector based gene therapy, Glybera, has recently been approved by the European Medicines Agency demonstrating the clinical applicability of this delivery technique.

4.4.2. Application of CNTs

CNTs are highly amenable to development as drug delivery vehicles owing to an exceptionally large specific surface area and ability to load with therapeutic molecules through a variety of different methods. Biomolecules can be attached to CNTs via a wide range of permanent or cleavable, covalent or non-covalent surface modifications, or by encapsulation of biomolecules within the nanotube internal cavity (Figure 3).[168] CNTs can be modified with single or multiple molecules enabling multiple functionalities. These can include tracking agents such as

radioactive particles, magnetic particles, fluorescent probes, or targeting agents such as antibodies and other ligand binding molecules.[169,170] The high aspect ratio of CNTs confers needle-like properties enabling them to penetrate cell membranes for direct translocation into cells.[171] CNTs can also be internalised via receptor-mediated and energy-dependent endocytosis.[172]

Research is currently underway to develop CNTs as delivery vehicles for a range of applications. Reviewed extensively by Wong et al. in 2013 these include delivery of anti-cancer drugs such as topoisomerase inhibitors and non-cancer related drugs such as anti-microbials, anti-inflammatories, anti-hypertensives among many others.[173,174] Varying degrees of success have been reported with CNT-based delivery systems. The majority of in vitro studies show CNT-biomolecule conjugates enhance cellular uptake and can reduce toxicity compared to the respective free drugs. The number of in vivo studies is limited, but nonetheless also demonstrate improved therapeutic efficacy and reduced toxicity in mice.[175] However, demonstration of their efficacy compared to other nanoparticulate delivery systems has not been undertaken and some studies have reported poor control over drug release.[176]

CNTs can be internalised by neurons, astrocytes and microglia, and by altering surface functionalization, uptake by neurons is preferentially enhanced.[177,178] As an alternative to viral vectors, CNTs have potential for therapeutic gene and short interfering RNA delivery. Encouraging results have already been achieved for enhancing neuroprotection after stroke by caspase-3 silencing however, applications in PNR have so far received little attention.[174] Preliminary in vitro studies have shown CNTs can be used to deliver and to enhance the therapeutic effects of neural growth factors in peripheral neurons. MWCNTs covalently functionalised with NGF and BDNF promoted neurite outgrowth of cultured embryonic chick DRG neurons similar to soluble NGF and BDNF.[179] Further investigation revealed water soluble CNTs amplified the neurite outgrowth promoting effects of soluble NGF via activation of the ERK signalling pathway and upregulation of neurotrophin receptors TrkA and $p75^{NTR}$.[180,181]

MWCNTs have also been conjugated to the neurite outgrowth promoting peptide sequences L-arginine-glycine-L-aspartic acid (RGD) and isoleucine-lysine-valine-alanine-valine (IKVAV).[182] As well as neurite outgrowth, these peptide components of fibronectin and laminin, promote cell adhesion, migration and proliferation by interaction with integrin receptors.[183] The MWCNT-peptide conjugates were found to be biocompatible when dispersed in culture media with no reported deleterious effects on the viability, morphology or function of dissociated rat hippocampal neurons. The conjugates were also non-immunogenic after intraperitoneal injection into albino (BALB/c) mice. Further experiments to assess the ability of these CNT-conjugates to enhance neurite outgrowth in an anisotropic scaffold would be very interesting. Collectively, these studies suggest that there may be significant potential for CNT-based delivery systems within NRCs however, the conjugation of CNTs with therapeutic biomolecules for enhancing nervous system regeneration has been limited and more studies investigating the neurite outgrowth promoting properties of CNTs conjugated to axon guidance molecules are essential.

Many studies have primarily focused on developing biocompatible CNTs with simple surface modifications often consisting of carboxylated, aminated, PEGylated or polyamine conjugated CNTs. Reviewed recently by Hwang et al., these modifications have however, produced some interesting effects and show potential for PNR.[184] Exposing cultures of hippocampal neurons to water soluble PEG-functionalised SWCNTs reduced the number of neurites whilst simultaneously increasing their length.[185] Subsequent studies suggested that neurite outgrowth was increased due to blockade of endocytosis and changes in calcium dynamics by the PEG-SWCNTs.[186,187] Treatment of spinal cord transected rats with PEG-SWCNTs increased the number of nerve fibers regenerating into the lesion, significantly reduced lesion volume and moderately improved behavioral recovery without causing toxicity or inducing gliosis. Despite these encouraging studies concerns about CNT toxicity and long-term affects after internalisation prevail. Using CNTs to deliver biomolecules to the surface of cells only without internalisation of the nanotube could reduce toxicity. This could be achieved within NRCs by utilising long CNT yarns or ropes. In this configuration CNTs could facilitate the spatially controlled delivery of axon guidance molecules for directing growth cone migration through NRCs as discussed in section 4.1.

5. Issues of Biocompatibility and Future Perspectives

Several issues currently impede the development of CNT-based biomaterials. Variability in CNT manufacture and surface modification can lead to batches possessing different quantities of impurities, morphological, structural and chemical characteristics, all of which significantly impact on toxicity, pharmacokinetics and functionality. In the future, producing a consistent product will be vital for clinical licensing. Reviewed recently by Zhao et al., the assessment of CNT toxicity has been particularly challenging.[188] Over the past 15 years an array of in vivo and in vitro studies have provided conflicting and highly variable accounts of toxicity and inflammation, often attributed to differences in experimental methodology, CNT properties such as solubility and confounding metal catalyst or amorphous carbon impurities in samples. CNTs must be purified to remove these toxic contaminants shown to produce reactive oxygen species and induce oxidative stress.[189] Surface functionalisation to improve CNT solubility and dispersal also reduces toxicity.[190]

Of crucial importance, several research groups have investigated the long-term in vivo fate of CNTs, again with variable results. The morphological characteristics and surface functionalisation of CNTs can significantly affect pharmacokinetics. Intravenously administered pristine SWCNTs, tracked by isotope ratio mass spectroscopy, showed high uptake in the lungs, liver, and spleen without apparent excretion within 28 days however; surface modification such as PEG-functionalisation increased the circulation time in blood, reduced retention within reticuloendothelial systems and accelerated excretion.[191–193]

CNT length also determines clearance rates with longer CNTs persisting as aggregates whilst shorter nanotubes are

more easily phagocytosed and cleared through different excretory pathways.[189] An in depth discussion of the toxicity of CNTs is beyond the scope of this review however, it is clear that each type of functionalised CNT engineered for biomedical applications will exhibit unique properties and require individual toxicological assessment appropriate to the mode of administration.

The slow progress of PNR will require CNT containing NRCs to exhibit long-term biocompatibility with minimal inflammation or fibrosis throughout regeneration, occurring over months to years. CNTs could be incorporated into NRCs in a variety of ways that will affect their long-term biocompatibility and persistence. They may be short and dispersed within a biodegradable gel or polymer matrix, or used to coat surfaces. On degradation of the matrix, CNTs will become available for cellular uptake and may cause unintended effects. Whether they persist within the nerve or are removed and degraded by phagocytic cells is not yet clear.

CNTs can be biodegraded within neutrophils and to a lesser extent within macrophages however, this is highly influenced by the number of CNT walls i.e., single or multi-walled, and the extent of surface functionalization.[194–196] Gradual internalisation and accumulation of short CNTs into peripheral neurons over years and decades, as well as translocation to the spinal cord could lead to deleterious effects and should not be overlooked.

Long CNT ropes and yarns are not biodegradable and will therefore be retained within the nerve tissue indefinitely. This may prevent unwanted effects associated with cellular CNT internalisation. However, to what extent CNTs will elicit a problematic foreign body response is unclear. A two-year histological study investigating the biopersistence of oxidised MWCNTs after subcutaneous implantation showed that large CNT agglomerates (>5 μm) remained within the intercellular space, became surrounded by granulation tissue consisting of fibroblasts and foreign body giant cells, whilst smaller aggregates (<5 mm) were taken up by macrophages, internalised into lysosomes, but only partially degraded. No severe inflammatory changes such as necrosis or carcinogenesis were reported. After 2 years a thin layer of fibrous connective tissue surrounded the MWCNTs. The authors concluded that the MWCNTs were biocompatible and suitable for use in medical devices.[197] Other studies have also reported minimal inflammatory response to CNT implantation.[147] These studies are promising for the long-term biocompatibility of CNT containing implants however, the formation of granulation tissue within the nerve repair pathway is of concern and could lead to disorganized axon growth and impair functional recovery.

In vitro studies have also indicated CNTs can affect neuronal calcium dynamics, inhibit endocytosis, act as potassium channel blockers, increase neuronal excitability and provide electrical shortcuts, all of which may have implications for normal propagation of action potentials.[65,185–187,198] How the presence of CNTs affects normal peripheral nerve function requires detailed in vivo investigations.

6. Summary and Conclusions

The AxoGen Avance decellurized human nerve allograft appears to be the most promising currently available alternative to the gold standard nerve graft. Multicenter clinical studies have shown Avance allografts are superior to standard collagen conduits and perform similar to autografts in lesions up to 5 cm however, further clinical data is needed on their performance for repairing larger lesions (>3 cm).[14] These grafts provide real nerve architecture, extracellular matrix and longitudinal topological guidance through basal lamina endoneurial tubes. They are available in a range of diameters and in lengths up to 7 cm. These grafts could also be combined with adjunctive therapies such as growth factor supplementation, gene therapy and nerve-cuff electrical stimulation. However, the following important factors may limit the widespread use of this product. As with any processed human donor tissue, the grafts cannot be guaranteed to be free of all pathogens; the availability of donated human cadaver nerve material to make the Avance grafts could be an issue if demand exceeds supply. Furthermore, substantial nerve injuries such as those of the brachial plexus will require longer and/or complex graft shapes beyond the structural capacity of Avance grafts. Consequently, in the long-term, next generation NRCs will likely be the ideal treatment option for complex PNR.

Enhancing the performance of peripheral NRCs is an ever expanding intense field of research with an extensive range of biomaterials and cutting-edge technologies available to the tissue-engineering scientist. We initially proposed the question, is there a role for CNTs in repairing peripheral nerves? At this stage, the application of CNTs for PNR is in its infancy; the full potential of this material is yet to be realised and issues of biocompatibility must first be resolved definitively. However, with such an unusual and desirable combination of properties amenable for PNR, it is highly likely CNTs will be utilised in future NRCs to enhance their strength, conductivity and axon regeneration promoting properties (**Figure 5**).

Whilst current materials used for NRCs such as collagen and PGA are suitable for shorter nerve lesions, there is still a need to develop materials to enhance the mechanical structure of NRCs whilst facilitating precise refinement of the degradation profiles of NRCs for larger and more complex nerve lesions. Fiber reinforcement is a very common technique used to enhance the mechanical properties of materials. CNTs clearly have excellent mechanical properties and if incorporated into NRC walls, could be an ideal reinforcing material to enhance structural properties whilst slowing, but still retaining the biodegradability of the surrounding matrix to limit constriction of the repaired nerve.

Although the studies reviewed in section 4.2 provide promising insights into the application of CNTs for PNR, further development of material processing techniques and CNT surface modifications are needed to improve the integration of CNTs within biomaterial matrices to impart improved mechanical properties to NRCs. A greater understanding of how CNTs influence degradation profiles of biodegradable materials and improved methods for fine-tuning biodegradability are also essential. CNTs could be incorporated into NRC walls in a concentration gradient increasing from proximal to distal. This would allow the biodegradation of the NRC to be concurrent with the expected rate of regeneration from the proximal to the distal nerve stump and limit constriction of the proximal end of the nerve that might otherwise occur in NRCs of uniform biodegradability.

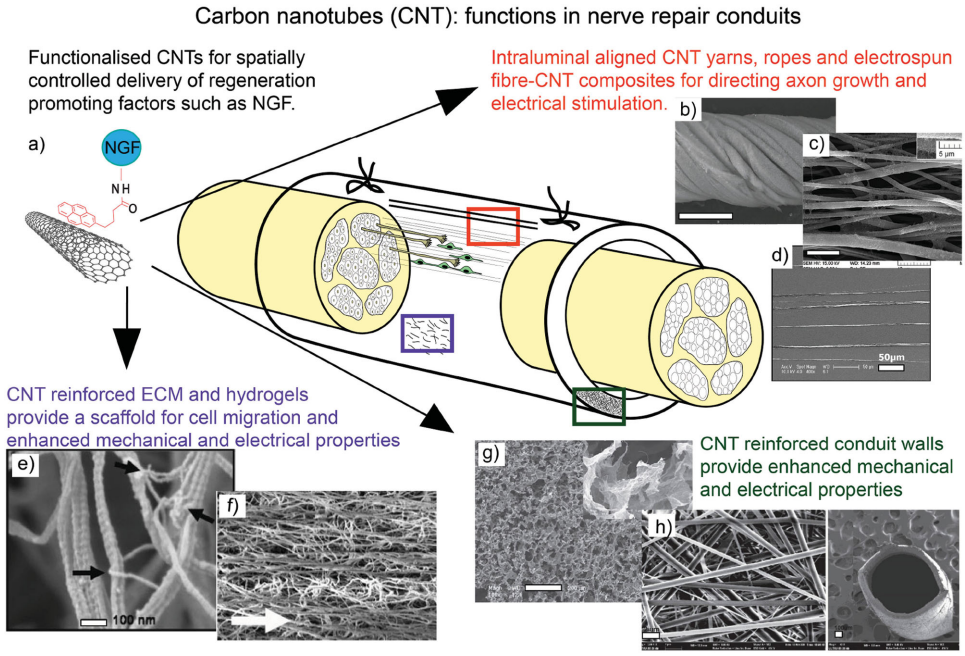

Figure 5. Schematic illustration of CNT utilisation within PNR conduits. a) schematic showing a CNT non-covalently functionalised with NGF for controlled drug delivery; b-h scanning electron micrographs of b) CNT rope, scale bar 200 μm c) CNT coated electrospun poly (L-lactic acid-co-caprolactone) nanofibers, scale bar 5 μm d) CNT yarns, scale bar 50 μm e) CNT-collagen gel, black arrows indicate CNTs, scale bar 100 nm f) strain induced alignment of a CNT-collagen gel, white arrow indcates direction of strain g) highly porous CNT – poly (L-lactide) nanocomposite scaffold, scale bar 200 μm h) CNT reinforced collagen/poly (ε-caprolactone) electrospun nerve repair conduit, left scale bar 2 μm, right scale bar 100 μm. Reproduced with permission.[54] Copyright 2012, American Chemical Society. Reproduced with permission.[80] Copyright 2011, Elsevier. Reproduced with permission.[56,150,201] Copyright 2005, 2008, 2012, John Wiley and Sons. Reproduced with permission.[90,104] Copyright 2014 2015, IOP publishing.

Developments in luminal fillers for NRCs have provided techniques and materials to generate a range of longitudinally orientated filaments, fibers and features for enhancing the anisotropic organization of regenerating axons, a major missing feature from current NRCs. Electrospun fibers have been the most popular luminal fillers to date demonstrating good efficacy in in vivo studies. Combining these fibers with CNTs could enhance the axon growth promoting properties of these fibers further by providing nanoscale surface roughness that can augment growth cone interactions with the fibers. CNT coatings would also provide a significantly greater surface area for funtionalisation with growth-promoting molecules to directly facilitate interactions with migrating growth cones to promote PNR.

Although early studies suggested CNTs may not provide a good substrate for rapid and extensive neurite regeneration, the key to exploiting CNT properties for PNR in the future will clearly be to functionalise them with ECM molecules such as laminin.[58,80,82] This concept was successfully demonstrated by Jin et al. in 2011, who reported superior neurite outgrowth from postnatal peripheral neurons on PLCL electrospun fibers coated with laminin adsorbed CNTs in comparison to PLCL-laminin adsorbed fibers without CNTs.[80] The mechanism by which CNTs stimulate neurite outgrowths is poorly understood and requires further investigation. However, the reported increase in FAK expression with increase in neurite outgrowth suggests that the CNTs enhanced the presentation of laminin to integrin receptors owing to the greater surface area imparted by the nanotextured CNT layer. This study provides good evidence of the feasibility and efficacy of utilising CNTs to enhance axon regeneration in a configuration directly relevant to in vivo NRCs i.e., coated aligned electrospun fibers as luminal fillers. CNT-functionalisation with laminin and other nerve regeneration relevant ECM proteins may be the way forward in expediting the development of CNT-based PNR in the medium to long-term.

The cytotoxicity of CNTs is still of major concern in the development of CNT-based NRC. The general biocompatibility of CNTs with neural tissue, particularly in vivo, is currently poorly understood. Establishing whether or not CNTs are biocompatible with neural tissue is key to the research efforts aimed at exploiting CNTs for PNR. Such studies will likely provide key insights leading to the identification of the CNT configurations that are most suitable for preservation of biocompatibility for PNR. The ideal CNT configuration(s) for PNR therefore, requires more research to inform the development of CNT-based NRCs.

The systemic toxicity of CNTs is also presently a controversial issue and although insights are slowly forthcoming, the lack of unequivocal proof of the safety of CNT exposure to neural tissue via the circulatory system is a significant hurdle to clinical application in NRCs. In response to safety concerns, the FDA Center for Biologics Evaluation and Research (CBER) is undertaking its own investigations into the mechanisms of CNT toxicity to blood vessels and blood cells. The intense interest in the development of CNTs for a vast array of biomedical applications should ensure financial investment in overcoming these toxicological issues and advancement of CNT-based devices towards clinical trials.

NRCs must be made, at least in part, from biodegradable materials to ensure the regenerating nerve is not constricted. Thus, NRCs containing CNTs embedded in biodegradable composites could present a risk of systemic exposure to CNTs during the degradation process. The use of knitted or long rope-like CNT scaffolds with retention of CNTs at the site of implantation should circumvent the potential risk of CNT entry into the circulatory system. Such scaffolds may therefore initially be preferable for development towards clinical trials for complex PNR. However, if adverse reactions occur and the CNT device required removal, this could cause severe damage to the nerve, as the entire length of nerve within the conduit would likely require resection. These potentially significant safety risks make it unlikely that the initial clinical trials involving CNTs will be for development of NRCs for PNR.

In the short-term, clinical trials involving CNTs will most likely receive approval when the benefit of using CNTs outweighs the risks involved and/or where no other therapies are available. For example, end of line treatments for cancer such as CNT-based drug delivery vehicles or CNT facilitated tumour targeting. Alternatively, approved trials may involve the use of implants that contain CNTs, but where the CNTs are encapsulated, unable to distribute systemically, and are easily removed, for example, in biosensors or CNT-based electrode arrays. Successes in these trials will undoubtedly open doors to the clinical application of CNTs in NRCs.

The on-going intense research into the application of CNTs for complex PNR will likely produce significant insights and a comprehensive characterisation of the efficacy of CNTs in nerve repair. This will hopefully inform the design of bespoke CNT-based NRCs that can proceed to clinical trials and FDA approval in the long-term.

Acknowledgements

This work was supported by funds from the following organizations: University of Brighton, the National Physical Laboratory, Engineering and Physical Sciences Research Council (GR/T18387/01) and University College London/University College London Hospitals Biomedical Research Center which receives funding from the National Institute for Health Research.

Received: October 27, 2015
Revised: January 10, 2016
Published online: March 29, 2016

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