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Review Article

Matrices, scaffolds, and carriers for protein and molecule delivery in peripheral nerve regeneration

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ABSTRACT

Local application of exogenous agents with neurotrophic properties enhances the regenerative capacity of injured neurons, especially following reconstructions of long nerve gaps and delayed nerve repairs. Recent advances in biomaterials and biomedical engineering have provided options for the sustained and controlled release of macromolecules to the peripheral nerve. Here, we review five methods for delivering macromolecules to the peripheral nerve including mini-osmotic pumps, hydrogel-based delivery systems, nerve guidance conduits, electrospun fibers, and nerve wraps. In addition to controlling the release of bioactive macromolecules, the ease of clinical use and versatility in implantation at a variety of "real-world" anatomical locations are key factors in designing an ideal delivery system. The incorporation of both mechanical and biological cues into such devices also helps optimize these systems.

1. Introduction

1.1. Evolution of controlled macromolecule release

Since the 1960's, controlled macromolecule release has been a highly-researched topic in the medical field as healthcare workers and researchers became more aware of dose-delivery problems and sought solutions (Hoffman, 2008; Langer & Peppas, 1981). Such problems include the high frequency of dose delivery whereby, because the bioactivity of a delivered therapeutic agent declines over time, the agent must be administered repetitively to achieve a desired effect. Patient-to-patient variability in metabolism of the molecule is also a major concern that affects the frequency of its administration. Furthermore, when the dose is administered beyond the maximum/minimum safe and effective levels, its efficacy may decrease and the risk of toxicity may ensue (Langer & Peppas, 1981). The idea of controlling macromolecule release is to extend the bioactive period through one administration and to manipulate the rate of release where the zero-order release rate is optimal (Huang & Brazel, 2001). Some advantages of this include the localization of drug delivery to the desired biological site thereby reducing associated side-effects from systemic release, retained bioactivity of drugs that the body may otherwise consume rapidly if freely circulated, and improved patient compliance (Langer, 1990).

Many delivery systems have been developed to achieve the goals of controlled release for various applications, insulin delivery being one of the first. Diabetes is becoming endemic worldwide (Venkat Narayan, 2005). As a result, the need for reliable and patient-friendly insulin delivery devices increases exponentially with many efforts directed at developing an artificial pancreas using technologies. These include sensor-augmented pumps and closed-loop control algorithms that detect glucose levels and adjust insulin administration continuously (Cobelli et al., 2011; Shah et al., 2016). Wearable and implantable artificial pancreases are commercially available but, improvements in the control algorithms should be made to increase the accuracy, reduce lag-time, and cope with system disturbances (Cobelli et al., 2011). These

Abbreviations: SCs, Schwann cells; GDNF, Glial cell line-derived neurotrophic factor; BDNF, Brain-derived neurotrophic factor; NGF, Nerve growth factor; CNTF, Ciliary neurotrophic factor; SFI, Sciatic functional index; PLGA, Poly(lactic-*co*-glycolic acid); LA, Lactic acid; GA, Glycolic acid; PCL, Poly(ε-caprolactone); [P(HEMA-*co*-MMA)], poly(2-hydroxyethyl methacrylate-*co*-methyl methacrylate); PCLEEP, ε-caprolactone and ethyl ethylene phosphate; PDGF, platelet-derived growth factor; CP, Common peroneal; ANA, Acellular nerve allograft; EDL, Extensor digitorum longus muscle; SLS, 3D structured light scanning

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devices exemplify efficient controlled drug release and set the precedence for design of other delivery systems.

Administration of immunosuppressant agents following organ transplantation is another active clinical field that relies on the controlled delivery of molecules. Organ transplantation is a life-saving procedure that owes its success to the integration of immunosuppression. With the development of immunosuppressive drugs such as Cyclosporin, Tacrolimus (FK506), and Rapamycin, organ transplants now have greater long-term survival because the agents suppress the immunological rejection mechanisms of the host immune system in response to foreign tissues (Castillo, 2014). Traditionally, these drugs are prescribed at high doses for life-long systemic administration through either intravenous or oral routes (Fisher et al., 2015). However, systemic administration causes many debilitating side-effects that include opportunistic infections, nephrotoxicity, and neurotoxicity (Naesens et al., 2009). These very serious and global side-effects are an urgent challenge, leading researchers to investigate and develop methods by which immunosuppressant delivery is controlled and effective only at the site of the organ allograft to reduce the side-effects. Aerosol delivery of immunosuppressants following lung-transplantation shows promise in being a localized delivery method for the prevention of the rejection of chronic and acute lung-transplants (Corcoran, 2009). Other strategies to direct immunosuppressant release include implantable polymeric or targeted particle delivery systems. In a mouse model, De Oliverira et al. demonstrated that a Tacrolimus-loaded poly (ε-caprolactone) (PCL) subcutaneous implant was able to reduce local angiogenic and inflammatory reaction to an implanted non-biocompatible foreign object whilst preventing toxicity and protecting kidneys, liver, and immune functions (De Oliveira et al., 2017). Fisher et al. reviewed the use of micro- and nanoparticles to provide local or systemically-administered targeted delivery of immunosuppressants (Fisher et al., 2015). They summarized studies that showed how encapsulation within the polymeric particles could provide sustained release, be surface-modified to affect the cells of interest, and be effective at modulating immune response following organ transplantation (Fisher et al., 2015).

The advantages of controlled macromolecule release are substantial: minimally-invasive methods result in higher patient compliance than repeated drug administration (Anselmo & Mitragotri, 2014), release systems can be modified and tuned to provide sustained release, decreasing the frequency of intervention (Robert (Langer & Folkman, 1976)), and devices can be directly applied to the pathological site or introduced systemically with specific cell targets, thereby increasing the bioavailability, effectiveness, and site-specificity of the therapeutic molecule (Allen & Cullis, 2004). These effects have been harnessed and widely researched for application in areas such as cancer treatment (Allen & Cullis, 2004), modulation of the immune system for organ transplantation or autoimmune diseases (Fisher et al., 2015), and treatments of ophthalmic diseases (Jaffe et al., 2000). Furthermore, the knowledge and developments made over the years in controlled macromolecule release are highly-relevant and applicable to solving challenges in peripheral nerve regeneration, as considered and discussed in this review.

1.2. Peripheral nerve regeneration

1.2.1. Tissue response to peripheral nerve regeneration

After peripheral nerve injuries that disrupt the continuity and contact of the axons with the cell body of neurons, the distal nerve stump that is isolated from the cell body undergoes Wallerian degeneration due to disconnection from the metabolic resources of the cell bodies (Waller, 1850). Within minutes to a few hours, calcium ions entering through sealed membranes of both proximal and distal nerve stumps mediate proteolysis that results in the degeneration of axon segments several hundred micrometers proximal and distal to the injury site (Gordon, 2015; Kerschensteiner et al., 2005). In the nerve fibers of the axotomized neurons, those neurons whose axons have lost their target connectivity (Fig. 1), the nerve degeneration proceeds to the first Node of Ranvier. In the denervated distal nerve stumps, the nerve fibers break down with progressive myelin and axon degeneration within the first few days following transection. The Schwann cells (SCs) shed their myelin lipids and divide as they begin their phagocytosis of the myelin and axonal debris (Perry et al., 1995). The phagocytosis is largely taken over by the macrophages that, within three days, flood from blood vessels into the distal nerve stump through the permeabilized blood-nerve barrier (Avellino et al., 1995; Martini et al., 2008; Perry et al., 1995). Because the myelin protein debris contains inhibitory molecules for axonal growth (David et al., 1995), axonal and myelin debris clearance by SCs and macrophages is crucial for successful peripheral nerve regeneration. In addition to clearing myelin debris, SCs and macrophages produce cytokines which play a role in axonal growth (Gordon, 2015). The SCs align themselves along the endoneurium as

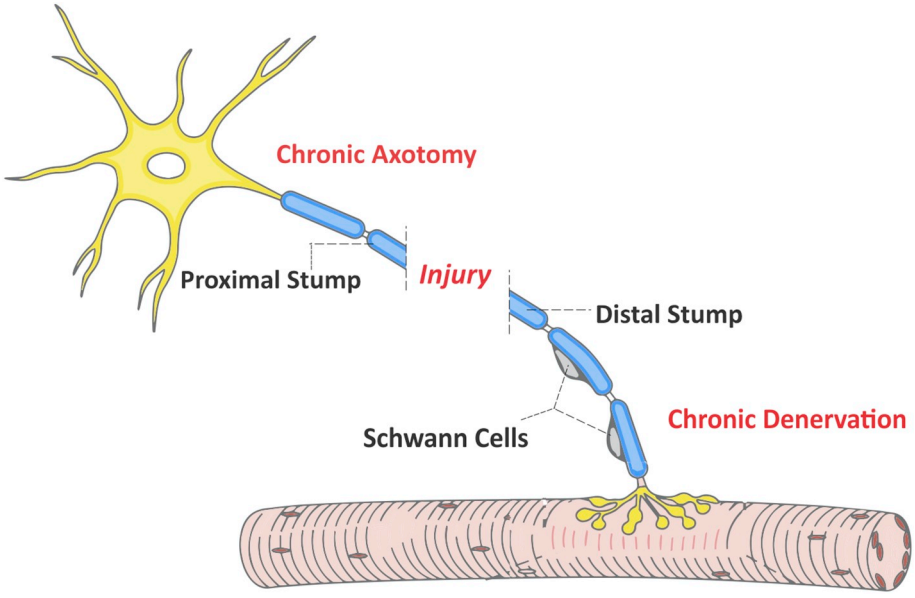

Fig. 1. Chronic axotomy and chronic denervation. Chronic axotomy occurs in the proximal stump when neurons are no longer in contact with their target end-organs. Chronic denervation occurs in the distal nerve stump when the Schwann cells are no longer connected with the neuronal cell bodies and viable axons.

the Bands of Büngner where they guide regenerating axons from the proximal nerve stump (Chan et al., 2014; Fu & Gordon, 1997; Gordon, 2015).

Axotomy of the neurons, a state in which the neurons lose their target contacts, leads to morphological changes in the neuronal cell body known as chromatolysis. These include swelling of the neuronal cell body, shift of the nucleus to an eccentric position, and disorganization of the basophilic granules (Nissl bodies) (Lieberman, 1971). These morphological changes reflect an altered gene expression state which, in motoneurons, is a switch from a signaling mode to a growth mode with up-regulation of many growth associated genes that include neurotrophic factors, cytoskeletal proteins, and neuropeptides, and downregulation of the cytoskeletal protein, neurofilament (Gordon, 1983; Gordon, 2015). The downregulation of neurofilament in the axotomized neurons is responsible for the reduced fiber diameter and reduced conduction velocity of the nerves proximal to the injury site (Hoffman et al., 1984). Once the disrupted membrane is sealed, axons grow out from the proximal nerve stump, many of which aborted (Cajal, 1928; Morris et al., 1972a; Morris et al., 1972b). The axonal outgrowth across a surgical site of nerve transection and repair, and into the distal nerve stump, occurs over a protracted period of up to 30 days in a rat (Brushart et al., 2002). Once the axons enter into the distal nerve stump, axonal regeneration proceeds at a rate of about 1–3 mm/day (Jacobsen & Guth, 1965) with up to 20 axon branches regenerating within the distal stump (Aitken et al., 1947). The switch to the transmitting mode in the axotomized neurons includes the upregulation of neurofilaments and in turn, the recovery of the fiber diameter, that occur only when regenerating nerve fibers make functional contacts with their target organs (Gordon & Stein, 1982; Tetzlaff et al., 1988). Many of the axon branches may polyneuronally reinnervate muscle endplates (Angelov et al., 1996; Brown et al., 1981; Cleveland & Hoffman, 1991; Hennig & Dietrichs, 1994; Rich & Lichtman, 1989) but over time, the excess branches slowly withdraw (Gordon, 1983; Mackinnon et al., 1991).

1.2.2. Delayed nerve-target contact after nerve injury

Functional recovery is poor frequently, despite the growth response of injured neurons and the supportive growth environment provided by the SCs in the distal nerve stumps (Kline & Hudson, 1995). Even when injured nerves are surgically repaired with little or no delay, the protracted period in which regenerating axons cross the injury site and proceed to regenerate, results in the neurons lacking contact with target organs, a state of chronic axotomy (Fig. 1). The SCs, target muscles, and sensory organs become chronically denervated (Fig. 1) prior to reformation of functional contacts (Fu & Gordon, 1997). Gordon and colleagues (Fu & Gordon, 1995a; Fu & Gordon, 1995b; Gordon et al., 2011) used a cross-suture technique of Holmes and Young (Holmes & Young, 1942) and a nerve autograft, to evaluate the effects of progressively long periods of axotomy, SC denervation in the distal nerve stump, and of denervated muscle, independently of each other. In each case, there was exponential decline in regenerative capacity of the motoneurons within 4 to 12 months with only 33% of axotomized motoneurons regenerating their axons through freshly denervated SCs and into freshly denervated muscles (Fu & Gordon, 1995a; Gordon et al., 2011). The decline in the number of the motoneurons that regenerated through chronically denervated 12 mm autografts to ~40% occurred more rapidly as did the decline to ~20% within four months after chronic SC and muscle denervation (Gordon et al., 2011; Sulaiman & Gordon, 2002). The likely basis for the decline in nerve regeneration after chronic axotomy is the transient expression of growth supportive genes (Gordon & Borschel, 2016). These include the cytoskeletal proteins, tubulin and actin, the neurotrophic factors such as brain-derived neurotrophic factor (BDNF), and glial derived neurotrophic factor (GDNF) (Gordon & Tetzlaff, 2015; Gordon et al., 2015; Höke et al., 2002; Jubran & Widenfalk, 2003) The transient expression of the many neurotrophic factors and the receptor p75 by denervated SCs (Brushart et al., 2013; Höke et al., 2006; You et al., 1997), progressive SCs atrophy, and reduction in their numbers (Siironen et al., 1995) are all factors that account for the reduced regeneration through the chronically denervated nerve stumps. The progressive reduced numbers of satellite cells that replenish nuclei in the multinucleated and chronically denervated muscle fibers (Anzil & Wernig, 1989; Viguie et al., 1997) explain the incomplete recovery of the chronically denervated muscles after reinnervation (Fu & Gordon, 1995b; Gordon et al., 2011).

1.3. Local administration of exogenous agents

Supplementing the injured peripheral nerve with proteins and molecules that have neurotrophic and neuroregenerative properties as a replacement for the reduced expression of intrinsic growth factors and neurotrophic factors with time and regeneration distances, holds promise in enhancing outcomes following severe cases of peripheral nerve injury. Exogenous neurotrophic factors including BDNF, GDNF, and nerve growth factor (NGF) that were delivered locally in appropriate doses promote axon regeneration and enhance functional recovery in rat models of delayed nerve repair (Boyd & Gordon, 2003a; Boyd & Gordon, 2003b; Jubran & Widenfalk, 2003). Exogenous delivery of molecules with neuroregenerative properties such FK506 (Gold et al., 1994; Gold et al., 1995; Toll et al., 2011) and ibuprofen (Madura et al., 2011) also have promising pro-regenerative effects. However, the associated systemic toxicity and non-specific delivery of the molecules to tissues other than the injured nerve (Apfel et al., 2000; Tung, 2010; Wallemacq & Reding, 1993) have prevented their clinical application to improve peripheral nerve regeneration in patients. Several biomedical engineering approaches are being developed currently to deliver therapeutic molecules to the site of the nerve injury, as reviewed below. Their promise for clinical application is also presented.

1.4. Outcome measures of peripheral nerve regeneration

The outcome measures used to determine the effectiveness of the biomedical devices in promoting regeneration of injured peripheral nerves are critical. There are several outcome measurement techniques but there are problems with some of these and, moreover, we lack a standardized set of measures to assess peripheral nerve regeneration following injury and repair. The most frequently used outcome measure remains the counting of regenerated nerve fibers with most of the studies using rat models of peripheral nerve injury, in particular sciatic nerve crush or transection (Wood et al., 2011). It is important to consider whether an experimental intervention may promote axonal sprouting disproportionally to increasing the number of the neurons that regenerate their axons, or even worse, *only* promoting the outgrowth of more sprouts and *not* increasing the numbers of neurons that participate in the regenerative process after nerve injury.

During the natural course of axon regeneration from the proximal into the distal nerve stump, many axonal branches are emitted from the parent axons in the proximal nerve, the number averaging five in rats with a maximum of 20 reported in the distal stump of rabbit nerves and five in rat distal stumps after crush injuries (Aitken et al., 1947; Mackinnon et al., 1991). Moreover, each sensory neuron generates approximately double the number of regenerating nerve fibers as compared to each motoneuron (Redett et al., 2005). These nerve fiber counts do not necessarily reflect how many neurons regenerated these fibers unless the number of the neurons that contribute to the regenerating nerves are enumerated distal to the injury site, within distal stumps, nerve grafts, or conduits (Pfister et al., 2011). Erroneous conclusions have been drawn especially under conditions where the administration of exogenous agents influences axonal outgrowth, including neurotrophic factors (reviewed by (Gordon, 2009)). Evidence of increased numbers of retrogradely labeled motoneurons that regenerated axons into the distal stump taken together with electron microscopic evidence of increased numbers of axons growing out from

Table 1
Summary of quoted methods to deliver macromolecules locally to peripheral nerve.

Delivery vehicle type	Delivery material component(s)	Biodegradability	Delivered macromolecule(s)	Duration of drug release[a]	Mechanism of drug release	Outcomes	Reference
Mini-osmotic pump	Alzet osmotic pump, polyethylene 60 tubing cannula	Not biodegradable; explant required	BDNF/CNTF	28 days	Osmotic pressure differential and continuous release	Improved SFI at midpoint and preservation of axon diameters suggesting correlation to improved functional outcome.	(Lewin et al., 1997)
	Alzet osmotic pump, silastic tubing cannula	Not biodegradable; explant required	BDNF/GDNF	28 days	Osmotic pressure differential and continuous release	Increased number of regenerated motoneurons after chronic axotomy with combined BDNF/GDNF delivery.	(Boyd & Gordon, 2003)
Hydrogel-based delivery System	Fibrin gel with heparin-binding domain	Biodegradable	Basic fibroblast growth factor	30 days	Affinity based release	*In vitro* enhancement of neurite extension in a dose-dependent manner. Maintenance of the growth factor's biological activity.	(Sakiyama-Elbert & Hubbell, 2000)
	Fibrin gel	Biodegradable	Neurotrophin-4	No data	Passive diffusion	Enhanced sciatic function index, and fiber maturity in a rat sciatic nerve transection and immediate repair model.	(Yin et al., 2001)
	Fibrin gel with PLGA microspheres	Biodegradable	GDNF	7–28 days	Passive diffusion and bulk erosion	Improved axon regeneration, fiber maturity, and functional recovery in a rat delayed nerve repair model. Improved axon regeneration, and fiber maturity in a rat large nerve gap model.	(Wood et al., 2013a,b; (Tajdaran et al., 2016)
	Fibrin gel with PLGA microspheres and particulate drug	Biodegradable	FK506	7–28 days	Passive diffusion and bulk erosion	*In vitro* enhancement of neurite extension. Maintenance of the growth factor's biological activity.	(Tajdaran et al., 2015)
Nerve Guidance Conduit	Ethylene vinyl acetate conduits with microchannels	Biodegradable	GDNF	30 days	Water penetration of microchannels and passive protein diffusion	Increased number of myelinated axons and regenerated motoneurons in rat facial nerve immediate injury and repair model.	(Barras et al., 2002)
	(P(HEMA-co-MMA)) conduits with NGF-microsphere lumen coating, NGF-solution lumen coating, or NGF liquid-liquid centrifugal cast lumen coating	Biodegradable	NGF	28 days	Passive diffusion and bulk erosion	Liquid-liquid centrifugal casting technique is effective for sustained/local delivery of NGF from conduit lumen.	(Piotrowicz & Shoichet, 2006)
	Silicone conduit with heparin affinity-based release system	Non-biodegradable	GDNF or NGF	4–12 weeks (*in vivo* conduit incubation period)	Cell-mediated degradation of heparin-bound growth factors for release	GDNF-filled conduits produced larger nerve fibers, greater recovery in twitch/tetanic forces, and improvement in behavioral tests after the 12-week endpoint.	(Wood et al., 2009; Wood et al., 2010)
	PCL conduits with silk fibroin embolization agent	Biodegradable	NGF	12 weeks	Concentration gradient diffusion release	Similar SFI, compound muscle action potentials, and myelinated nerve fiber/diameters to autograft controls. Better outcome results than uniform gradient release conduits.	(Tang et al., 2013)
	Chitosan	Biodegradable	FK506	8 weeks (*in vivo* conduit incubation period)	Passive diffusion and bulk erosion	Improved axon fiber maturity, motor neuron functional recovery, and elevated BDNF and TrkB levels for regeneration.	(Li et al., 2010) and (Zhao et al., 2014)
	PLGA	Biodegradable	FK506	20 days	Release through diffusion holes	Retained drug bioactivity and extension of neurite length.	(Labroo et al., 2016)
	Y-tube silicone conduit with lumen concentration gradient	Non-biodegradable conduit with biodegradable lumen	NGF and GDNF	3 weeks	Concentration gradient diffusion release	Improved sensory and motor axon regrowth guidance through respective paths. Improved gait duty cycle.	(Johnson et al., 2015)
	PCLEEP	Biodegradable	NGF	3 months			(Chew et al., 2005)

(*continued on next page*)

Table 1 (*continued*)

Delivery vehicle type	Delivery material component(s)	Biodegradability	Delivered macromolecule(s)	Duration of drug release[a]	Mechanism of drug release	Outcomes	Reference
Electrospun fibers					Passive diffusion and bulk erosion	Retained bioactivity, stimulation of PC12 cells to neurons.	
	Aligned PCLEEP fibers	Biodegradable	GDNF	8 weeks	Passive diffusion and fiber degradation	Increased number of myelinated axons, cross sectional nerve area, and electrophysiological recovery compared to empty nerve conduit controls over a 15 mm rat sciatic nerve gap model.	(Chew et al., 2007)
	PCL	Biodegradable	NGF	28 days	Passive diffusion and bulk erosion	Retained bioactivity and induced neurite outgrowth.	(Valmikinathan et al., 2009)
Nerve wrap	PCL, PLGA 6535, PLGA 8515	Biodegradable	Neurotrophin-3, BDNF, PDGF	10 weeks	Passive diffusion and bulk erosion, varying degradation rates of different polymer layers	Improved SFI and mechanical allodynia results. Topographical guidance of cell adhesion and guided nerve regrowth.	(Hong et al., 2018)

[a] *In vitro* release periods unless otherwise specified.

the proximal nerve stump, demonstrated that exogenous GDNF and low doses of BDNF promote extensive outgrowth of axons into the distal nerve stump (Boyd & Gordon, 2003a). Excessive axon outgrowth in a facial nerve transection model was reduced by neutralizing antibodies to several growth factors, in particular BDNF (Streppel et al., 2002), consistent with the data of Boyd and Gordon (Boyd & Gordon, 2003a). Thus, both axon and neuron counts are important in assessing the effects of exogenous agents on nerve regeneration.

Functional outcome measures including analysis of reinnervated muscle forces and movement are important for many reasons. The measures include determination of the direction taken by the regenerating axons and the extent of muscle reinnervation (Guntinas-Lichius et al., 2005; Streppel et al., 2002). The functional outcomes are important with movement of rat whiskers after facial nerve injuries being an elegant example of demonstrating misdirection of regenerating axons (Streppel et al., 2002). The numbers of regenerating axons per neuron may complicate interpretation of functional measures of muscle reinnervation because excess numbers of the axons per facial neuron result in polyneuronal innervation of motor endplates simultaneous with some functional recovery of whisking (Streppel et al., 2002). Recovery of whisking actually increases when microtubules are stabilized with taxol and the polyneuronal reinnervation reduced, despite the excess numbers of regenerating axons (Grosheva et al., 2008). In addition, the increased expression of neurotrophic factors in blinded rats is associated with reduced polyneuronal innervation (Grosheva et al., 2016). Analyses of locomotion as another functional outcome measure differ widely and have frequently over- or under estimated the success of nerve regeneration in reinnervating denervated targets (see (Wood et al., 2011)).

2. Overview of current technology for local macromolecule delivery to the peripheral nerve

Recently, researchers have sought to enhance the regenerative potential of peripheral nerves through local and controlled-release of proteins and drugs that have the capacity to promote nerve regeneration. There are several implantable devices with the capacity to release therapeutic agents directly to the nerve injury site. These are itemized in Table 1 and include those devices such as implantable osmotic pumps that release their contents over time either systemically or *via* silastic tubes (Boyd & Gordon, 2003; Lewin et al., 1997). Other devices are composed of polymer biomaterials that deliver macromolecules by different means, the most common being degradation of the carrier and diffusion of the therapeutic macromolecules.

2.1. Osmotic pump infusion systems

Mini-osmotic pumps were one of the first implantable localized delivery systems used to study the efficacy of therapeutic agents in promoting peripheral nerve regeneration. Typically, the therapeutic component is housed in a rigid cylindrical reservoir, separated from a surrounding layer of an osmotic driving agent by a movable partition, and protected from the extracellular fluid by a semi-permeable membrane, allowing only the permeation of water (Theeuwes & Yum, 1976). Once implanted, a net flux of extracellular water across the semi-permeable membrane into the osmotic driving agent chamber applies pressure on the partition, thereby infusing the reservoir fluid through a delivery orifice proportionally (Theeuwes & Yum, 1976). Thus, these systems are useful in providing sustained release of the agent over a desired therapeutic window, as determined by the properties of the components.

Lewin et al. demonstrated that continuous infusion for 28 days, *via* a mini-osmotic pump, of both BDNF and ciliary neurotrophic factor (CNTF) to the repair site of the transected rat sciatic nerve, significantly improved functional outcomes, using walking-track analysis as the measure: the mean [±standard error (SE)] of the Bain-Mackinnon-

Hunter sciatic functional index (SFI) 40 days after the repair was −67.1 ± 9 as compared to −96.5 ± 14 when BDNF was infused alone. Yet there was no significant difference between these measures at 80 days with the effect of the combined treatment and BDNF alone being the same. One explanation given was that the CNTF effect is progressively attenuated (Lewin et al., 1997). The combined BDNF and CNTF treatment also resulted in significantly better recovery of regenerated fiber diameters than when BDNF was administered alone, suggesting a correlation between nerve fiber size and improved functional outcome (Ikeda & Oka, 2012). However, there was no significant difference in the number of regenerated nerve fibers (Lewin et al., 1997). Their conclusion was that continuous and localized delivery of BDNF and CNTF was more effective in promoting functional recovery after immediate nerve repair than the BDNF treatment alone (Lewin et al., 1997).

The use of an Alzet mini-osmotic pump to deliver BDNF and/or GDNF to the site of the repair of a transected rat hindlimb nerve allowed the demonstration of a progressive increase, as a function of time, in numbers of two month chronically axotomized motoneurons that regenerated their axons. The numbers increased to a maximum at four weeks following continuous delivery of GDNF and a synergy between low dose BDNF and any dose of GDNF was observed (Boyd & Gordon, 2003). Importantly there was a bimodal dose-response effect of BDNF but not GDNF with high doses being inhibitory due to BDNF binding to trkB and p75 receptors, respectively (Boyd & Gordon, 2002; Boyd & Gordon, 2003). Significant enhancement of nerve regeneration with osmotic-pump delivery of GDNF or BDNF was not observed following immediate nerve repair due to the saturated levels and optimized support of endogenous neurotrophic factors immediately after nerve transection (Boyd & Gordon, 2003).

Though the mini-osmotic pump systems have been shown to be effective in providing controllable and continuous delivery of neurotrophic factors at the site of nerve injury and surgical repair in rats, they are limited mainly by problems such as fibrosis occurring at the connection site of the pump (Lewin et al., 1997) because they are not biodegradable. There are also risks of wound infection when the pumps are surgically removed post-treatment. The pumps are bulky making it difficult to implant the device into regions with lower soft tissue volume (*e.g.* hand or face) and to maintain its position. Thereby any potential patient compliance is likely to be compromised. Hence, the clinical applicability of the mini-osmotic pump is unlikely. Researchers have sought other methods by which macromolecules may be delivered in a controlled and local manner.

2.2. Neurotrophic factor expression via gene delivery

Gene therapy is a potential method to deliver proteins with neuroprotective and neuroregenerative properties to injured peripheral nerves. Gene therapy can be defined as the introduction of a foreign, therapeutic gene into living cells in order to treat a disease (De Winter et al., 2013). This gene delivery to the peripheral nerve has been primarily achieved by genetic modification of motoneurons and SCs by adeno-associated viral vectors and lentiviral vectors respectively, with the aim to promote neuronal survival and nerve regeneration (Ruben (Eggers et al., 2016)). Injection of lentiviral vectors into SCs has the potential to promote the expression of cell adhesion molecules and cytokines, including neurotrophic factors capable of promoting extension or guidance of axons (De Winter et al., 2013; Lavdas et al., 2011).

Currently, the lentiviral vector is the most successful vector type for SC transfection (De Winter et al., 2013; Mason et al., 2011). It provides gene expression for as long as several months after injection of the vector into the distal nerve stump with only a mild and transient inflammatory response (Nayak and Herzog, 2010). However, despite the prolonged GDNF delivery from GDNF gene transfected SCs, regenerating axons become entrapped in swirling patterns resulting in unwanted formation of neuromas (Blits et al., 2004; Eggers et al., 2008; Tannemaat et al., 2008).

Gene therapy is limited to delivery of proteins (Mason et al., 2011) and thus, is not capable of delivery of most therapeutic molecules. This necessitates development of drug delivery strategies which can deliver any molecule within a required period. To this end, degradable biomaterials capable of drug delivery are likely to be better alternatives for controlled release of growth factors and therapeutic molecules at the site of nerve injury and surgical repair.

2.3. Hydrogels in drug delivery applications

Advances in hydrogel technologies have spurred development in many biomedical applications over the past decades. Since the establishment of the first synthetic hydrogels in 1960 (Wichterle & Lím, 1960), the growth of hydrogel technologies has advanced biomedical fields such as those of pharmaceutics (Kashyap et al., 2005) and biomedical implants (Corkhill et al., 1989). Hydrogels provide ideal scaffolds for tissue engineering and drug delivery systems as they are typically biocompatible, biodegradable, and have tunable mechanical properties to match those of native tissues. Moreover, hydrogels can act as suitable drug delivery systems since they lack strong hydrophobic interactions that can disturb protein molecular structure (Lin & Metters, 2006). Naturally derived hydrogels such as fibrin, collagen, chitosan, hyaluronic acid, and cellulose derivatives have been used extensively (Biondi et al., 2008) along with chemically synthesized hydrogels including polyethylene glycol and polyvinyl alcohol (Peppas et al., 2000). These polymeric hydrogels have received considerable attention as suitable drug delivery systems for the central and peripheral nervous systems (Biondi et al., 2008).

Fibrin gel produced from cross-linked fibrinogen and thrombin with factor XIIIa, forms naturally in the wound healing process and has been used as a tissue sealant (Amrani et al., 2001; Dunn & Goa, 1999) and a growth factor delivery vehicle for peripheral nerve repair (Sakiyama-Elbert & Hubbell, 2000; Wood et al., 2013b; Yin et al., 2001). Similar to some of the other naturally derived polymers, fibrin supports cell adhesion and growth and its physical properties can be tuned by the fibrinogen/thrombin formulation in the design of a material well tolerated within physiological environments (Willerth et al., 2006).

However, for drug delivery purposes, natural hydrogels such as fibrin alone, cannot sustain the delivery of therapeutic proteins over a seven day period (Wood et al., 2013b) because the majority of the hydrogel structure constitutes water and lacks the ability to impose a significant barrier against diffusion of embedded therapeutic agents. This condition is exacerbated in the case of hydrophilic proteins such as GDNF as the embedded agents because they can diffuse out of the hydrogel network rapidly. Therefore, another drug delivery vehicle must be incorporated into the hydrogel-based drug delivery systems to allow more sustained drug release.

2.4. Use of polymeric microspheres as a drug vehicle

One solution to achieve sustained rather than short release profiles for therapeutic proteins is a composite system of polymeric microspheres incorporated into the fibrin gel in order to control the time course of delivery of the proteins. In such a system, the fibrin gel localizes the delivery of the factors to the site of interest and the microspheres sustain the release (Caicco et al., 2013; Tuladhar et al., 2015; Tajdaran et al., 2015; Wang et al., 2013; Wood et al., 2013a).

Polymeric microspheres were first developed in the 1970s when they were initially devised as carriers for vaccines and anticancer drugs (Ravi Kumar, 2000). Since then a large number of polymers have been investigated as candidates for synthesizing these colloidal particles for different drug delivery applications. These polymers are categorized based on their chemical structures that characterize their degradation type and period. The degradation of polymeric microspheres is dependent on the hydrophobicity of the polymer. Hydrophilic polymer

chains absorb water at a faster rate than chain hydrolysis and, consequently, the microspheres will degrade by bulk erosion (Körber, 2010).

Poly(esters), for example, are bulk-eroding hydrophilic polymers in which the rate of water uptake exceeds the rate of hydrolytic chain cleavage. Many poly(esters) are biodegradable and can be used to encapsulate a variety of ingredients for biological applications, thus making them one of the largest classes of polymers used in the controlled delivery systems. Examples include radioactive imaging and therapy (Mumper & Jay, 1992), protein and small molecule delivery (Kim & Park, 1999), gene and viral delivery (Li & Huang, 2004), and cell delivery (Chun et al., 2004).

Among poly(esters), block copolymers of poly(lactic-*co*-glycolic acid) (PLGA) can be considered as one of the most widely used polymers for drug delivery applications because PLGA is one of the few biodegradable polymers with FDA approval for biological use (Chun et al., 2004; Mundargi et al., 2008). PLGA offers a wide range of tunable characteristics such as intrinsic viscosity and rate of degradation (Mundargi et al., 2008) that may be used to encapsulate drugs with a wide range of properties and that achieve a variety of release profiles for drugs (Mundargi et al., 2008). Studies have shown that the period of encapsulated drug release can be adjusted from one or two weeks to several months by varying the relative ratios, the chain length of the copolymer blocks, lactic acid (LA) and glycolic acid (GA), the overall molecular weight of the copolymer, and the terminal functional groups on PLGA (Pollauf et al., 2005). In addition, the resulting PLGA microspheres can range in diameter from about 200 nm to approximately 600 μm (Cohen-Sela et al., 2009; Morita et al., 2000). The small dimensions of the microspheres allows their use in various delivery system including the injectable forms (Baumann et al., 2009; Caicco et al., 2013; Stanwick et al., 2012; Wang et al., 2013).

Wood et al. engineered a PLGA microsphere system that was incorporated within the fibrin gel which allowed localized and sustained release of GDNF at a site of a peripheral nerve transection and repair in rats (Wood et al., 2013a; Wood et al., 2013b). Changing the GDNF loading and the PLGA chemical properties, including the polymer's inherent viscosity and molecular weight, resulted in an adjustable duration of release of the GDNF *in vitro*. The use of PLGA with higher inherent viscosities or molecular weights lengthened the *in vitro* cumulative release duration from 10 to >30 days (Fig. 2A) (Wood et al., 2013a) in confirmation of the findings of others who used different encapsulated proteins in the microspheres (Yeo and Park, 2004). In the Wood et al. study, the engineered GDNF delivery systems with 2- and 4-week release formulations were then evaluated in rats where the common peroneal (CP) nerve was transected and repaired after a delay of two months. Whilst only the 2-week formulation significantly increased the number of regenerated nerve fibers (myelinated axons; Fig. 2B), both 2- and 4-week GDNF release formulations promoted the reinnervation of the target musculature with more CP nerve fibers reinnervating the extensor digitorum longus (EDL) muscle within 12 weeks after a 2 month delayed CP nerve repair (Wood et al., 2013b). As shown in Fig. 2C–D, stimulation of the CP nerve fibers eliciting significantly higher isometric twitch and tetanic forces in the EDL muscles. These findings extend our findings of GDNF local release significantly increasing the number of retrogradely labeled motoneurons that regenerated their axons into the distal nerve stump and across an acellular nerve allograft (ANA). (Tajdaran et al., 2016; Wood et al., 2013b).

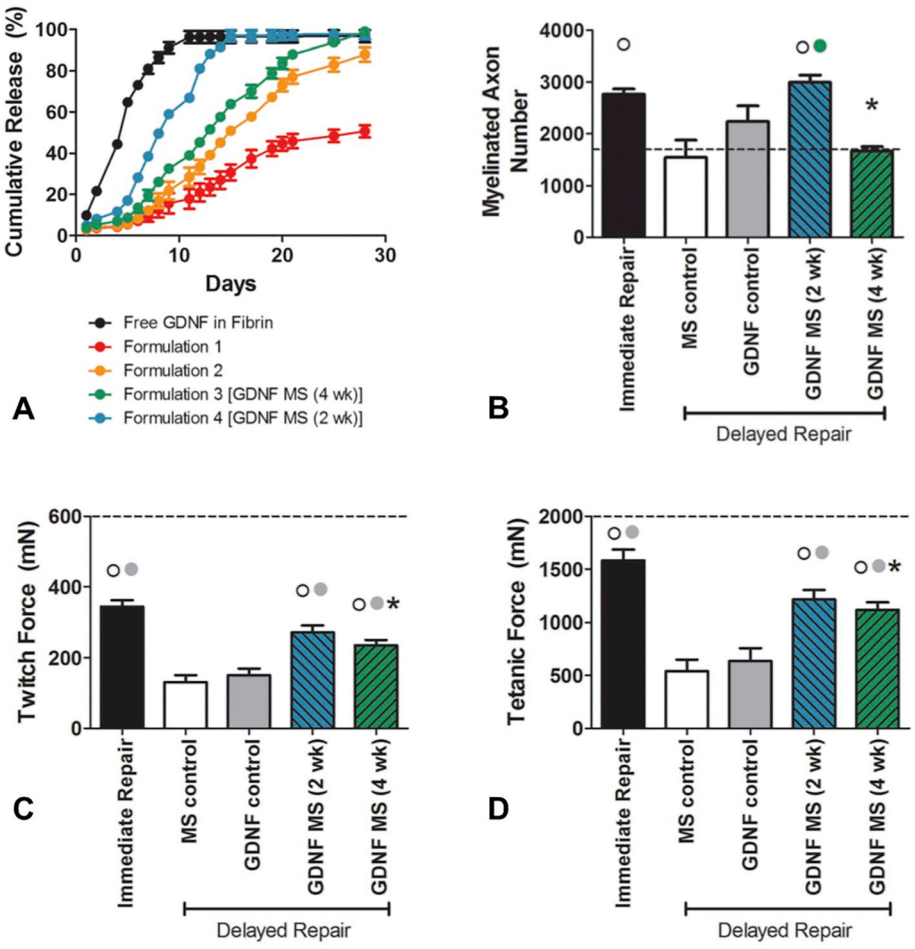

Fig. 2. A fibrin gel based GDNF delivery system containing GDNF microspheres improves axonal regeneration and enhances functional recovery following peripheral nerve repair that was delayed for 2 months prior to coaptation of the common peroneal (CP) nerve. (A) The release of GDNF from fibrin gels loaded with microspheres containing GDNF was decreased by constructing microspheres (MSs) with higher PLGA inherent viscosities or molecular weights (formulations 1 > 2 > 3 > 4). Microspheres used as experimental groups to be tested in rats were chosen (formulations 4 and 3) based upon their release of GDNF for 2–4 weeks in vitro, respectively. (B) Quantitative histomorphometric analysis of nerves 20 mm distal to the repair site, 12 weeks following the delayed repair of the CP nerve and the implantation of the drug delivery system. The groups in which the transected CP was repaired immediately and the groups in which CP nerve repair was delayed and GDNF MSs (2-week formulation) were placed, had increased myelinated axon counts as compared to the MSs with and without GDNF (4-week release formulation). Maximum evoked isometric twitch (C) and tetanic (D) contractile forces of the extensor digitorum longus (EDL) muscle was recorded 12 weeks after delayed nerve repair and drug delivery system implantation. The rats in the experimental groups with GDNF MSs had significantly increased twitch and tetanic forces as compared to those in both control groups. The reinnervated muscles recovered as much twitch and tetanic forces when the delayed CP nerve repair site was exposed to the 2-week release GDNF formulation as the muscles after the immediate repair (with no GDNF administration). The data (n = 6) represent mean ± SEM and * indicates statistical significance compared to Immediate Repair, ○ compared to microsphere control, ● compared to GDNF control, and ● compared to GDNF microspheres (4 weeks; $p < 0.05$). Normal, uninjured values are represented by the dashed line. This figure was adapted from (Wood et al., 2013b). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The efficacy of the fibrin-gel based GDNF delivery system was particularly striking with *all* the motor and sensory CP neurons regenerating their axons 10 mm distal to an inserted 10 mm long ANA within 8 weeks, regardless of whether the microspheres released the GDNF for 2 or for 4 weeks at the ANA coaptation sites ((Tajdaran et al., 2016); Fig. 3A–B). There was an obvious increase in the density of the axons visualized with green fluorescent protein (GFP) within the ANAs treated with the fibrin gel loaded with GDNF microspheres (Fig. 3C–E). The frequency of the larger diameter fibers in the GDNF microspheres treated ANAs matched the frequency in the rats in which an isograft was placed as the positive control group and was statistically greater as compared to the negative control groups of no drug delivery system (DDS) and microspheres (MS)s that did not contain GDNF (Fig. 3F). Importantly, there was minimal sprouting of axons from the proximal nerve stump in the GDNF treated nerves (Fig. 3G) in contrast to the more robust sprouting that was reported when GDNF was delivered with a mini-osmotic pump (Boyd & Gordon, 2003). Whilst muscle reinnervation was not determined, the results for the increased muscle reinnervation corresponding with more motor nerves regenerating their axons after delayed CP nerve repair (Fig. 2C–D) indicate that it is likely that muscle reinnervation after regeneration of nerves through the ANA was hastened by the GDNF administration.

Tajdaran et al. (Tajdaran et al., 2015) also engineered a drug delivery system for sustained and controlled release of the bioactive and lipid soluble drug, FK506, that has major side effects when administered systemically (Tung, 2010; Wallemacq & Reding, 1993; Yamazoe et al., 2014). FK506 was incorporated in fibrin gel in solubilized, particulate, and PLGA microspheres encapsulated forms (Fig. 4A–C). As for GDNF (Fig. 2A), the *in vitro* release profile of FK506 ranged from 7 to 28 days from the drug. However, in the case of FK506 that was incorporated in fibril gel in three forms (Fig. 4D), the rate of release was most rapid for the solubilized form, and then the particulate form. The most prolonged period of release was seen with the FK506 microsphere incorporated form (Tajdaran et al., 2015).

This study is of considerable clinical interest because the particulate form of the FK506 containing drug delivery system has the better clinical potential than the FK506 microspheres and as such, has a higher commercial value because of the simpler synthesis process. The particulate delivery system sustains bioactive FK506 release up to two weeks without the need of encapsulation within PLGA. By eliminating the need for encapsulation, the drug loading and encapsulation efficiency of FK506 within the delivery system increases significantly. The higher drug encapsulation efficiency is due to the direct incorporation of fresh FK506 within the fibrin gel, whereas, during the encapsulation process of FK506 in PLGA microsphere, up to 30% of the initial drug mass can be lost (Tajdaran et al., 2015).

Sustained and prolonged release of water soluble molecules in the particulate form is not possible due to their fast dissolution rate. Therefore, incorporation of these molecules within polymers such as PLGA is required to obtain prolonged release profile. To overcome the limitations of protein encapsulation within PLGA microspheres, including protein denaturation and low protein encapsulation efficiency (Wischke & Schwendeman, 2008), an encapsulation-free controlled release of proteins has been engineered (Pakulska et al., 2016). This

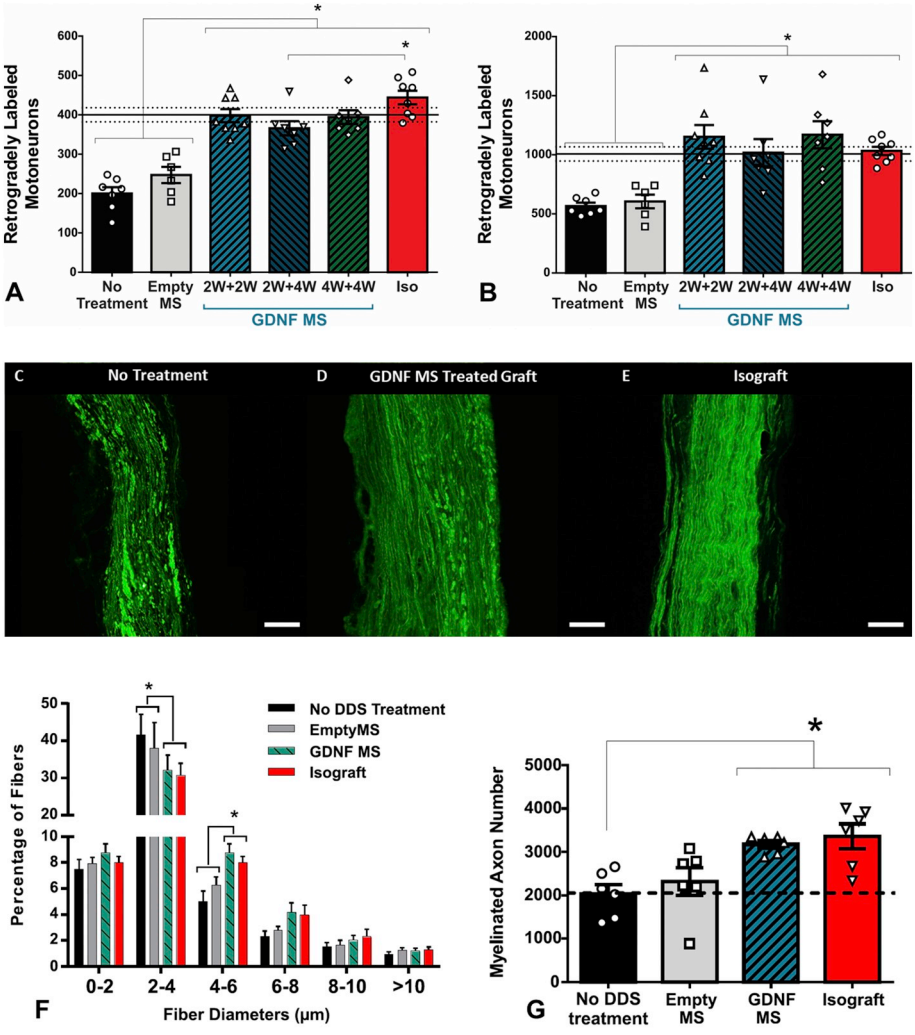

Fig. 3. Fibrin-gel based GDNF local drug delivery system (DDS) enhances common peroneal (CP) nerve regeneration across acellular nerve allografts (ANAs). Experimental groups consisted of grafts receiving fibrin gels loaded with 2-week release formulation GDNF MS, or 4-week release formulation GDNF MS at both suture sites. Another experimental group received 2-week release formulation GDNF MS at the proximal suture site and 4-week release formulation GDNF MS at the distal site. To assess motor and sensory neuron regeneration, retrograde labeling of neurons was performed 10 mm distally from the distal repair site 8 weeks following experimental treatment. The experimental groups receiving fibrin gels loaded with microspheres containing GDNF showed that all the motor (A) and sensory (B) neurons regenerated their axons through ANAs, as did the neurons regenerating their axons through an isograft of the same length. The period of GDNF release from the drug delivery system did not influence the extent of nerve regeneration. (E) Longitudinal nerve graft sections (30 μm each) indicated that GDNF treatment using the microspheres enhanced allografts' axons alignments and increased the axon density, to the same extent as the isografts. (F)The GDNF microspheres were also as effective as isografts in shifting the nerve fiber frequency distributions to higher values with greater fiber size recovery. (G) Histomorphometric analysis of the cross-sections of regenerated CP nerve fiber size was consistent with the elevation of numbers of the neurons that regenerate their axons through the ANA (see text for further details). Data represent the mean ± standard error of the mean. Scale bar: 300 μm Normal uninjured values ± standard error are represented by the dashed line. *$p < 0.05$. This figure was adapted from (Tajdaran et al., 2016).

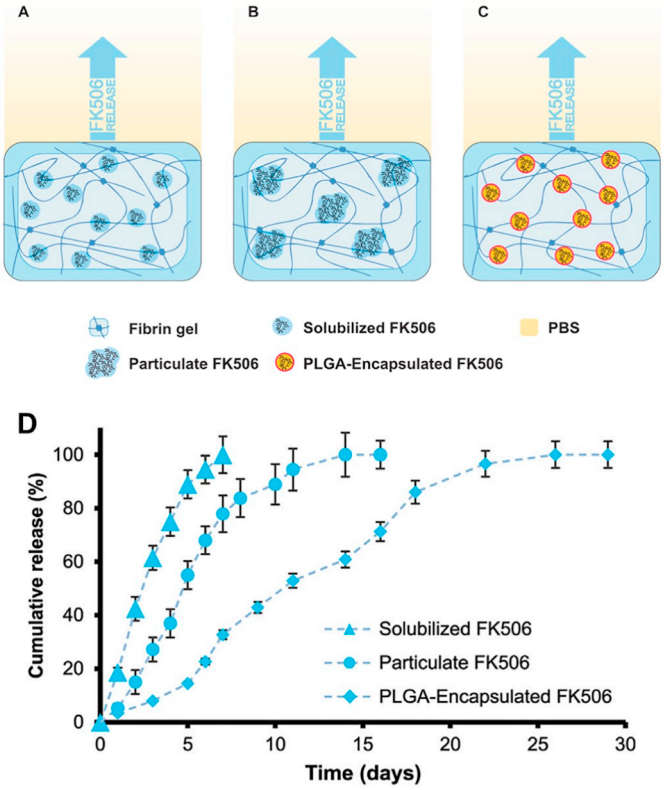

Fig. 4. Three methods of incorporating FK506 in the fibrin gel: (A) solubilized FK506, (B) particulate FK506, and (C) PLGA-encapsulated FK506. The arrows denote the release of FK506 from the fibrin gel. (D) *In vitro* cumulative mass release of FK506 in solubilized (▲), particulate (●), and PLGA encapsulated (♦) forms from fibrin gel shows incorporation of solubilized FK506 into the gel provides release for up to five days. The FK506 encapsulation in PLGA microspheres extends the sustained release to 28 days. FK506 content in the release samples was determined using mass spectrometry. (Mean ± standard deviation, $n = 3$ per release study). This figure was adapted from (Tajdaran et al., 2015).

new system takes advantage of the electrostatic interaction of proteins with PLGA spheres to provide burst-free and prolonged release of bioactive proteins (Pakulska et al., 2016). Obviating the need of drug encapsulation within PLGA spheres is a paradigm shift in the controlled delivery field, which can provide the basis for a fundamental change in the way we use PLGA for protein delivery.

2.5. Molecule-releasing nerve conduits

Even after a clean nerve transection in patients, the nerve stumps retract such that the gap must be bridged by a nerve construct (Millesi, 1986). Nerve autografts, in particular sural nerve removed from the leg, are the first choice but the removal of the nerve not only leaves a scar and reduces sensation in the foot but may be a source of morbidity. As a result, many types of nerve conduits have been explored as a bridging construct to guide axonal regrowth after surgical repair of injured nerves (reviewed by (Lundborg, 2004; Pfister et al., 2011)). Briefly, these conduits are formed using various processing methods to shape polymers into cylindrical tubes of differing lengths and diameters. Researchers have explored the use of many synthetic and natural polymer materials for the construction of these devices to take advantage of their inherent material properties. These include biodegradability, biocompatibility, mechanical durability and flexibility, and their ability to be processed (Table 1). Studies have shown that nerve conduits alone have the mechanical properties for effective guiding of nerve regeneration over short nerve-gap distances (< 3 cm) but they lack the natural microstructure and cellular content that allows cell-mediated and sufficient diffusion of chemical cues for growth of regenerating nerves over longer distances (Kemp et al., 2008). Therefore, incorporation of drugs and exogenous neurotrophic factors into conduits through covalent-binding (Chen et al., 2005), affinity-based binding to small peptides (Lee et al., 2003; Wood et al., 2009), and loading into the walls (Barras et al., 2002; Chew et al., 2007; Piotrowicz & Shoichet, 2006) or lumen (Lee et al., 2003; Wood et al., 2009; Yang et al., 2005), has been studied to provide chemical cues in addition to the mechanical support offered by the conduits.

2.5.1. Growth factor release from nerve conduits

The interaction of endogenous neurotrophic factors that are naturally sourced from surrounding SCs and basal lamina, is prevented during regrowth through artificial conduits (Kemp et al., 2008). Therefore, researchers have strived to provide these chemical cues by loading growth factors into the conduits. Barras et al. used histomorphometry and retrograde labeling of the neurons that regenerated their axons to compare the effectiveness of locally-released GDNF and neurotrophin-3 (NT-3) from the walls of 1 cm long ethylene vinyl acetate conduits that bridged an 8 mm gap between the nerve stumps of a 5 mm transected rat facial nerve; the microchannels in the conduit allowed controlled neurotrophin release over the course of 6 weeks (Barras et al., 2002). The surgically repaired nerves regenerated ~70% of the number of myelinated axons in the non-lesioned nerve group with controlled GDNF release in contrast to 10% of the axon numbers in the same control group when NT-3 was released. There was a corresponding greater number of motoneurons that regenerated their axons and the maturity of the regenerated nerve fibers was also higher for the GDNF treatment group as compared to the NT-3 treatment group. The authors thus concluded that GDNF was superior to NT-3 in promoting axon growth from motoneurons, attributed to the lower sensitivity of the motoneurons to NT-3 as well as an inhibitory effect of NT-3 on myelination. This indicated the potential benefits of extended-release of GDNF in promoting nerve regeneration although the study did not address the question of appropriate reinnervation of facial nerve targets.

Piotrowicz and Shoichet also described methods in which NGF was incorporated into walls of poly(2-hydroxyethyl methacrylate-*co*-methyl methacrylate) [P(HEMA-*co*-MMA)] conduits (Piotrowicz & Shoichet, 2006). NGF was either encapsulated in MSs, applied as a conduit wall coating by soaking the conduits in a NGF-containing solution, or coated on the conduit wall using a secondary liquid-liquid centrifugal casting technique (Piotrowicz & Shoichet, 2006). Sustained release of NGF over 28 days was only observed for the MS-encapsulated and liquid-liquid centrifugal casting NGF coatings but, the former method was limited in that there was a maximum extent that the MSs could be loaded (Piotrowicz & Shoichet, 2006). The liquid-liquid centrifugal casting technique had the greatest potential as a method of incorporating NGF or other growth factors as a conduit wall coating for sustained release (Piotrowicz & Shoichet, 2006). However, unfilled conduits lack the advantage of having the microstructure to guide axonal regrowth and support cell adhesion throughout the construct.

Other studies in rats have shown the benefits of loading the growth factors within a conduit lumen. Wood et al. used a silicone conduit containing a luminal filler with an affinity-based release system of heparin-binding proteins to sequester GDNF or NGF, encased in fibrin gel to bridge a 13 mm sciatic nerve gap, as shown in Fig. 5A (Wood et al., 2009; Wood et al., 2010). The affinity-based release mechanism depends on the infiltration of cells through the conduit and cell-mediated degradation of the binding proteins to alter the release rate of GDNF or NGF (Wood et al., 2009). Evaluation of the sciatic nerve fiber density and the percent neural tissue within the conduit seven weeks after surgery, together with the numbers of retrogradely labeled motoneurons that regenerated through the conduit (Fig. 5B), and the normalized specific force of EDL muscles evoked by maximal sciatic nerve

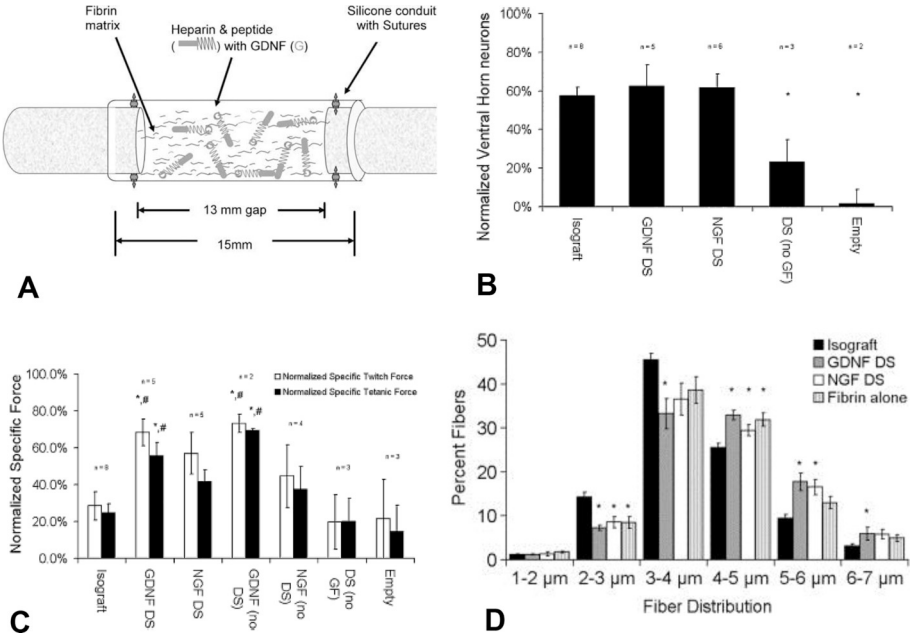

Fig. 5. Summary of quantitative results showing that an affinity-based GDNF release nerve conduit can enhance sciatic nerve regeneration following nerve injury. (A) Diagram of a constructed nerve conduit with affinity-based delivery system (DS), where a 13 mm nerve gap was surgically repaired with a 15 mm silicone conduit that was sutured on both ends to the proximal and distal stumps. The conduit lumen is composed of a fibrin matrix with or without the heparin-bound growth factor delivery system. A bi-domain peptide is crosslinked at one domain to the fibrin matrix, while the other is bound to heparin which binds the growth factor and is released through cell-mediated degradation. (B) Retrograde-labeled ventral horn motoneuron counts normalized to contralateral motoneuron counts for each rat, 12-weeks post-injury. The percentage of ventral horn motoneuron that regenerated axons was similar in the GDNF DS, NGF DS, and isograft groups. These groups contained significantly more labeled motoneurons than the empty and DS (no growth factor) groups. (C) Extensor digitorum (EDL)specific force s 12-weeks post-operatively, normalized to uninjured and normal EDL muscle-specific forces. EDL muscles were stimulated proximal to conduit/graft to produce maximal twitch and tetanic muscle forces which were then divided by muscle cross-sectional area to derive specific forces. Only GDNF groups showed significant specific twitch and tetanic specific forces compared to empty, delivery system without growth-factor, and isograft groups. (D) Histomorphometry results of the distribution of myelinated fiber size distributions at the conduit midline. The percentage of large-diameter regenerating nerves (4–5 and 5–6 µm) were greater in the GDNF and NGF DS groups as compared to the isograft control group but the GDNF DS had a significantly higher percentage of the largest fibers (6–7 µm) as compared to the isograft. Data (n = 12) is represented as the mean ± SEM, where * and # represents statistical difference ($p < 0.05$) compared to isograft or empty delivery system (no GF) groups, respectively. This figure was adapted from (Wood et al., 2009; Wood et al., 2010).

stimulation 12 weeks after surgery (Fig. 5C), demonstrated that the neurotrophic factor containing nerve conduits were equal or better than isografts inserted between the proximal and distal nerve stumps (Wood et al., 2009; Wood et al., 2010). In addition, a shift of the size distribution of regenerated nerve fibers within the conduit in response to the inclusion of either GDNF or NGF (Fig. 5D), concurred with the positive effect of the factors on the other parameters of success of nerve regeneration (Wood et al., 2009). The behavioral tests that included the sciatic functional index and the success of the rats gripping their toes on a wire mesh, did not demonstrate superior recovery, possibly due to misdirection of regenerating nerve fibers and/or the development of toe and ankle contractures. The conclusion that there was improvement in the number of successful grips 12 weeks after surgery in all but the empty conduits is weak based on the data presented despite the suggestion that the lumen-filled conduits provided a permissive substrate for adhesion that was missing in the empty conduit (Wood et al., 2010). Additionally, incorporation of fibrin in the conduit lumens acts as a cell-adhesion platform to encourage SC migration. Yet, despite the evidence for the conduits in which growth factors were trapped, the silicone is non-biodegradable in addition to the possibility of nerve compression and scar tissue formation (Wood et al., 2009; Wood et al., 2010).

Uniform release of growth factors may not be the most effective method of promoting nerve regeneration as the growth cones emitted from the proximal nerve stump are sensitive to chemotactic cues during the regeneration process (Tang et al., 2013). Introducing concentration gradients within the controlled release nerve conduits is a strategy by which directional regeneration can be optimized. Tang et al. demonstrated the effectiveness of a neurotrophic factor gradient by creating a concentration gradient of NGF immobilized by silk fibroin and surface coated within a poly(ε-caprolactone) (PCLA) conduit of 15 mm length that was inserted into a rat sciatic nerve gap of 14 mm (Tang et al., 2013). The concentration gradient was induced by placing the conduits upright in a reaction vessel and then pumping the NGF solution parallel to walls of the vessel, thereby creating a differential exposure effect (Tang et al., 2013). Outcome measures of myelinated nerve fiber counts and diameters, evoked compound muscle action potentials, and of functional recovery (using SFI) after sustained release for 12 weeks, were the same as those for insertion of an autograft, outperforming uniform release conduits (Tang et al., 2013). These findings certainly have potential clinical relevance.

Using the same approach of a gradient controlled release, a novel and clever 3D-printed custom bifurcated Y-shaped nerve conduit was created to investigate the ability of two different neurotrophic factors to preferentially direct the regeneration of motor and sensory nerve fibers into their respective pathways, as illustrated in Fig. 6A (Johnson et al., 2015). Localized NGF and GDNF were distributed in an increasing concentration gradient toward the distal end of the conduit to support sensory and motor nerve regeneration, respectively. Briefly, a sciatic mixed nerve bifurcation was transected and imaged using 3D structured light scanning (SLS), a 3D reconstruction of the transected site was then made to develop a precise model of the nerve pathway, and finally, the model was used to reverse engineer the Y-tube nerve conduit (Fig. 6B). The conduit was 3D printed from silicone deposited in a layer-by-layer manner to generate axially oriented microgrooves along the hollow conduit, as can be seen in Fig. 6C. GDNF and NGF were encapsulated in gelatin methacrylate hydrogel droplets and printed along the bottom of the lumen walls to form a spatially distributed concentration gradient (Johnson et al., 2015). The final Y-tube conduit was inserted between the 10 mm rat sciatic nerve segment, transected 0.5 mm proximally and distally to the bifurcation point, and shown to successfully guide regenerating nerves appropriately into one of the two pathways (Johnson et al., 2015). Significantly more sensory neurons regenerated their axons into the distal stump through the NGF than the GDNF pathway. The velocities of SC migration, measured by determining the horizontal distance travelled over 5-min time intervals of time-lapse videos obtained in a live cell perfusion chamber, were higher along the GDNF pathway. These results suggest the chemotactic cue gradients were effective at regenerating their respective sensory/motor nerves. Post-operative gait analysis revealed an overall improvement in functional recovery (Johnson et al., 2015).

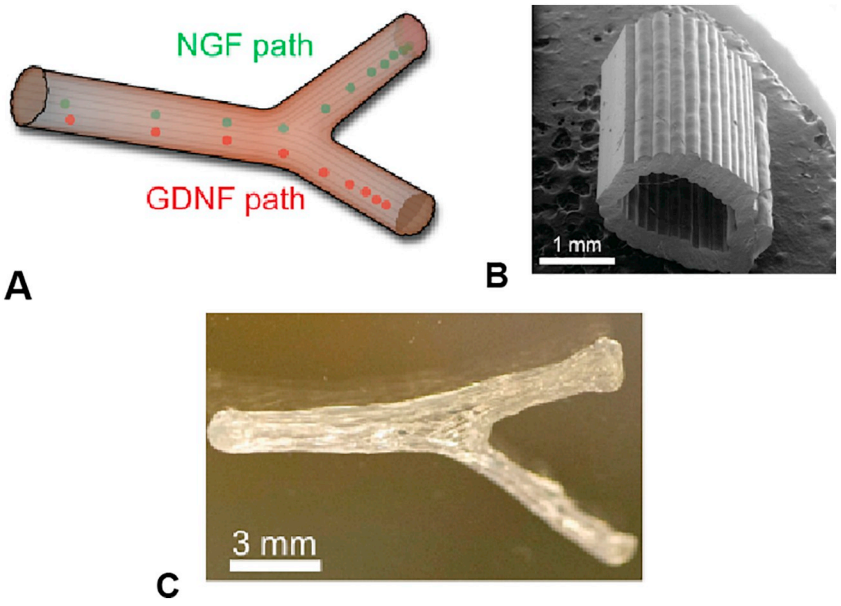

Fig. 6. Visual images and representations of the 3D printed hollow silicone Y-tube conduits developed by Johnson et al. for a 10 mm rat sciatic nerve bifurcation transection model (A) Diagram of path-specific growth factor (nerve growth factor (NGF) and glial derived neurotrophic factor (GDNF)) incorporation in increasing concentration, as denoted by the reducing distance between the dots for chemical support of the sensory and motor pathways in the sciatic nerve bifurcation model. (B) A scanning electron microscopic image of an axial section of the conduit in which the aligned microgrooves are clearly visible. (C) Microscopic image of the final Y-tube conduit that was generated from the reconstructed 3D model that matched to anatomical geometry of the original nerve. Adapted from (Johnson et al., 2015).

As shown by this study, path way specific controlled-release Y-tube conduits hold the potential for being effective, customizable, and feasible devices for assisting the regeneration of mixed nerves. The novel 3D structured light scanning approach used in this study was very beneficial in developing an anatomically accurate device. This has strong clinical implications in customization of the conduit for patient specific injuries and pathology. This may ultimately enhance treatment outcomes as the conduit may be easily printed to match the specific geometry required to bridge the nerve gap and tune the growth factor dosages, dependent on the degree of injury, to provide the optimal mechanical and chemical cues for patient specific nerve regeneration. However, the structured light scanning technique required that an intact or transected uninjured nerve segment be exposed and imaged prior to 3D modelling and reverse engineering of the conduit. Clinically, peripheral nerve injuries are often complex and a patient may present with many variants of the injury including an injury that leaves a large gap between proximal and distal nerve stumps, nerve compression, transection and neuroma formation, that cause the nerve geometry to differ from that of an uninjured nerve (Dellon, 1993). Therefore, because this 3D structured light scanning approach relies on the imaging of the nerve segment to be transected and repaired, it is unlikely to be applicable in cases where the geometry of the injured nerve is greatly altered or distorted, compromising the geometrical accuracy of the developed conduit. Furthermore, the process requires the injury site to be exposed prior to imaging and it was not made clear in the study of Johnson et al. (Johnson et al., 2015) if either the site was to be remained exposed or closed and re-opened at a later time for operation while the 3D modelling and conduit printing were completed. In both cases, this may not be clinically feasible since, in the first case, a long exposure and waiting period for the 3D printing would increase the risk of infection or dehydration to the surgical site and, in both cases, the efficiency of the surgery would be decreased.

Future studies could enhance the clinical feasibility of this approach by examining other imaging modalities in which an injured nerve could be imaged *in vivo* without requiring surgical exposure or imaging the contralateral uninjured nerve segment. Enhanced 3D modelling and processing algorithms may be employed to remove injured nerve defects and construct an optimal design for the customized nerve conduit that better matches the anatomical and geometrical features of an uninjured nerve. Regardless of the clinical aspects that this study could improve on, it is an excellent example of how peripheral nerve regeneration can be considered in a holistic manner. Specifically, both sensory and motor nerve regeneration were addressed, and biochemical and topographical cues were incorporated in the conduit to optimize nerve regeneration by better mimicking of native nerve tissue. Future synthetic conduit design should follow the direction of that of Johnson et al., as an optimal conduit should represent native nerve structure and enhance the regenerative capability beyond its natural capacity.

Allografts (nerve grafts taken from a human donor) whose cells have been removed and are hence, non-immunogenic, termed acellular nerve allografts (ANAs) are the most important biomaterial substitute for the conduits in which macromolecules, such as growth factors, could be, but have not as yet, been inserted. These ANAs retain the original structure of the nerve with empty endoneurial tubes through which regenerating nerves from a proximal nerve stump can regenerate into a distance distal nerve stump. The clinical efficacy of these ANAs is excellent for digital nerves (Rinker et al., 2017) and, although they are being used for repair of several different nerves, more evidence for their efficacy awaits. Combination of ANAs with localized release of GDNF in rats has shown superior nerve regeneration across a long nerve gap to the same extent as the idealized case of isograft treatment (Tajdaran et al., 2016). Based on the current preclinical evidence, development and commercialization of ANAs that are capable of localized macromolecule release, will provide a promising alternative in the current management of large nerve defects.

2.5.2. *Drug release from nerve conduits*

In addition to controlling the release of growth factors from the nerve conduits, release of pharmaceutical agents from these conduits has also been explored for peripheral nerve regeneration purposes. Li et al. explained that a biodegradable chitosan nerve conduit could be loaded with FK506 in the walls and sustain the release over 8 weeks (Li et al., 2010). Furthering this study, Zhao et al. found that the number and size of regenerated myelinated nerve fibers regenerating through the FK506 containing nerve conduit were significantly greater than those that regenerated through empty conduits after systemic FK506 administration (Zhao et al., 2014). There was a corresponding elevation in motor nerve conduction velocity in concert with elevated levels of BDNF and its trkB receptors, as determined through immunohistochemical staining of the levels 4–6 lumbar spinal cord (Zhao et al., 2014). Stimulation of the sciatic nerve elicited larger compound motor action potentials with the use of an FK506-loaded chitosan conduit, with the amplitude and velocity of the compound action potential after 8 weeks being approximately 73%, of the preoperative

uninjured muscle (Li et al., 2010).

An *in situ* study demonstrated FK506 release over 15 days from a conduit in which a FK506 reservoir was sandwiched between two concentric layers of PLGA tubes allowing controlled FK506 release by the number and sizes of diffusion holes made on the inner tube (Labroo et al., 2016). Their report of maintaining FK506 biological activity, using measurement of neurite outgrowth from dorsal root ganglion neurons, compares with the findings of Tajdaran et al. (Tajdaran et al., 2015), achieving sustained and tunable release of FK506 without any initial drug burst release from the fibrin gel-based delivery system containing FK506 MSs. The main shortcoming of the designed delivery system by Labroo and colleagues is the initial burst release of FK506 within the first 24 h. The authors claim that such initial FK506 burst release can be minimized easily due to the independency of the drug release from polymer degradation. However, no further information on how to mitigate the burst release issue was provided. In addition, Labroo and colleagues did not consider the fact that the diameter of the diffusion hole on the inner PLGA tube is dependent on PLGA polymer bulk erosion. Thus, FK506 release from the reservoir was unlikely in a controlled manner after 15 days due to the unpredictable effect of PLGA bulk erosion on geometrical changes to the diffusion hole.

Whilst controlled release nerve conduits show promise for nerve regeneration using mechanical and biological cues in a variety of nerve gap length models, some shortcomings still remain in using conduits to bridge large nerve defects. Advances in the construction of nerve conduits have moved toward using resorbable biomaterials and away from non-biodegradable synthetic polymers, such as silicone, which cause fibrotic reactions. However, the biomaterial degradation and macromolecule-release rates should be taken into consideration when developing nerve conduits and the applications, to prevent premature reabsorption or molecule release.

2.6. Nerve wraps

A major limitation of pre-formed nerve conduits is the potential for compression on the nerve as it regenerates, reducing regenerative potential and possibly resulting in scar and even neuroma formation. FDA approved and commercially available nerve wraps such as NeuraWrap™ or NeuroMend™ composed of Collagen type I, have been developed to protect the site of nerve injury and repair and to allow for nerve regeneration without scarring (Kehoe et al., 2012). The wraps, often consisting of an outer robust layer that resists mechanical deformation and an inner semi-permeable layer for nutrient transportation (Kehoe et al., 2012), are flat sheaths. These may be wrapped around the site of an end-to-end nerve repair (Jeans et al., 2007; Zhu et al., 2018) and other sites of nerve injury and repair. The wraps degrade with time and there are unpublished observations that scarring is prevented. Jeans et al. demonstrated that a biodegradable glass fiber nerve wrap stabilized with Tisseel glue around a median nerve end-to-end suture in sheep improved nerve regeneration and led to reinnervation and maturation of muscle endplates comparable to uninjured and epineurial repair groups (Jeans et al., 2007). This was determined using transcutaneous stimulated jitter which can measure the latent period between motor axon stimulation and muscle fiber contraction (Jeans et al., 2007). However, the regenerated nerves conducted more slowly than normal with significantly smaller axon diameters and myelin thickness as compared to a normal nerve after being allowed to fully recover over a period of seven months (Jeans et al., 2007). Nerve wrap treatment had the same results as when the nerve was repaired with epineurial suture (Jeans et al., 2007). The authors concluded that nerve wraps stabilized with Tisseel glue result in the same functional outcomes as direct epineurial suture repair but that they have greater clinical benefits in their ability to decrease surgery duration, the need for microsurgical instruments, and the risk of nerve fascicle fibrosis that epineurial suturing may cause (Jeans et al., 2007). Though the wrap has the potential for being a feasible and simple device to assist in nerve repair, the lack of adequate functional recovery in studies to date warrants the need for additional strategies. The inclusion of chemical and other physical cues may optimize nerve regeneration with use of nerve wraps, as discussed below.

The current technology may be improved by incorporating the biological cues shown to be beneficial in nerve conduit design (see Section 2.5). Electrospinning is a novel biomaterial development method in which polymer solutions are extruded at a constant flow rate and subject to a high voltage (Sill & von Recum, 2008). The polymer solution becomes charged and is expelled as polymer fibers when the electrostatic repulsion forces overcome the solution surface tension. Thereafter the fibers are collected at an oppositely-charged or grounded electrode. The resulting matrices are porous scaffolds with fiber diameters in the micro- or nano-meter range and varying physical properties can be obtained (Sill & von Recum, 2008). The tunable porosity of these scaffolds is beneficial for the transport of nutrients and waste and for cell infiltration, where optimized permeability allows the matrix to behave like native nerve tissue and has been shown to improve nerve regeneration (Belkas et al., 2004; Kehoe et al., 2012). Furthermore, the directionality of the polymer fibers may be designed to be randomly-oriented or anisotropic. Both orientations have advantages and disadvantages, with the random fibers displaying greater mechanical properties and anisotropic fibers displaying the ability to provide topographical cues for guiding axonal regeneration (Hong et al., 2018; Kim et al., 2016). Thus, researchers have sought to exploit the advantages of electrospun polymer fibers in combination with controlled release of therapeutic molecules for the development of multifunctional nerve wraps.

Therapeutic agents have long been loaded into biomaterial scaffolds, particularly into electrospun fibers for several applications including nerve regeneration (Sill & von Recum, 2008). Controlled release of macromolecules from nerve wraps is a relatively new field that builds on the early development and characterization of biomaterials (López Cebral et al., 2017). For example, Chew et al. showed that NGF release could be sustained for three months while retaining neurotrophic bioactivity when NGF was stabilized and incorporated with bovine serum albumin in poly(ε-caprolactone-*co*ethyl ethylene phosphate) (PCLEEP) electrospun fibers. The released NGF promoted neurite outgrowth from PC12 cells and differentiation into neurons, thereby confirming the bioactivity of NGF following encapsulation in electrospun fibers (Chew et al., 2005). These findings were corroborated in a later study where BSA-stabilized NGF in electrospun PCL fibers provided controlled release for 28 days (Valmikinathan et al., 2009). These preliminary material characterizations and their effectiveness to sustain and release bioactive NGF holds promise in developing nerve wraps with controlled release of therapeutic molecules.

The efficacy of three layers of a novel multilayered electrospun nerve wrap to release neurotrophic factors and to support functional recovery 10 weeks after a rat sciatic nerve crush injury was recently reported (Hong et al., 2018). The different polymer layers had varying degradation rates which controlled the rate of release: a base layer of aligned-PCL fibers, the secondary layer of PLGA 6535 encapsulating NT-3 and BDNF, and a tertiary layer of PLGA 8515 encapsulating platelet-derived growth factor (PDGF). The latter two PLGA layers released their trophic factors at the fastest and slowest rate, respectively, the rationale being that NT-3 and BDNF have beneficial effects early *in vivo* to assist with cellular proliferation prior to differentiation for axonal growth, while PDGF assists with regeneration in the later stages. The aim of the sequential release of the various growth factors was to simulate the chemical cue provision in the native nerve regeneration mechanism. The functional outcome measurements, including the SFI and the relative absence of mechanical allodynia, demonstrated the effectiveness of controlling the release of different trophic factors in addition to providing a rationale for developing such a device with electrospun fibers. Furthermore, the outer aligned-PCL fibers may have had influence on the topographical guidance of cell adhesion and

guided growth, as observed from histochemical analysis (Hong et al., 2018). The inclusion of topographical and controlled release of chemical cues in a single electrospun fiber nerve wrap mimics the native regenerative environment, providing the nerve with the optimal factors required for regeneration. Specifically, the sequential and sustained release of multiple growth factors is beneficial since the regeneration capability is enhanced through exposure to chemical cues at various points during the regeneration of the injured nerves (Boyd & Gordon, 2003b). The biodegradability and porosity of the nerve wrap allow minimal inflammation and increased penetration of supporting cells and nutrients, respectively. These reasons exemplify the great potential that controlled release nerve wraps with microstructure have in being auxiliary support devices to aid in peripheral nerve regeneration following nerve crush injuries or end-to-end suture repair of transected nerves.

Nerve wraps may be limited, however, in their ability to assist regeneration of long nerve gaps, as they are typically rolled and wrapped over nerve injury sites and either sutured or glued in place. This method may not act as a suitable construct for the bridging of long gaps and may be better for use as protection sheaths that release macromolecules and provide mechanical support for nerve regeneration. In the case of a long nerve gap, a controlled release nerve wrap may act as an accessory device in addition to implementation of a conduit or natural graft, like an ANA, to provide the supplementary biochemical cues and mechanical protection to enhance nerve regeneration through the guidance material.

A variant of the nerve wrap was developed in which electrospun poly(ε-caprolactone-*co*-ethyl ethylene phosphate) (PCLEEP) fibrous mats were loaded with GDNF, aligned longitudinally, and rolled and sealed to form 15 mm long hollow conduits (Chew et al., 2007). Most (~80%) of the GDNF was released within 2 months *in vitro*, with an initial burst release of 30% attributed to the localization of GDNF on the surface of the PCLEEP fibers. *In vivo,* this conduit promoted regeneration of transected rat sciatic nerve as assessed by higher numbers of regenerated nerve fibers and larger CMAPs as compared to conduits without GDNF, 3 months after surgery. The large GDNF burst release within the first day of implantation that severely reduced the available bioactive GDNF *in vivo* could be circumvented by molecular binding or encapsulation of GDNF in a core-shell electrospun fiber configuration that would reduce the burst release and provide a better sustained release profile. An even more important problem of the PCLEEP fiber conduit was that it was hollow where regeneration is known to fail over the 15 mm sciatic nerve gap that was chosen as a model of large nerve gaps. A recent review outlines the technological advances in interluminal filler design, such as unidirectional freezing and 3D printing, which provide promising methods of incorporating luminal microstructure within nerve guidance conduits. These would mimic native nerve internal topography and assist in nerve regeneration in addition to controlled release of neurotrophic factors (Wieringa et al., 2018).

3. Conclusions

Hydrogel-based drug delivery systems (Sakiyama-Elbert & Hubbell, 2000; Tajdaran et al., 2015; Wood et al., 2013b) and the nerve wrap (Hong et al., 2018) provide the best options for macromolecule delivery to the site of surgical repair of an injured nerve. These delivery systems have the versatility to be used in various clinical cases of nerve injury and repair. Due to their modular property, the hydrogels and nerve wraps are suitable for implantation at several different anatomical locations because both are not limited to a 'size fit' to the nerve in question. Furthermore, the biocompatibility and biodegradability of these two types of delivery systems eliminate the need for a secondary surgery to remove the implanted material, which is significantly important for clinical application.

The use of the acellular nerve allograft (ANA) capable of sustained exogenous factor release is an excellent approach to improve nerve regeneration in a long nerve gap injury and repair scenario (Tajdaran et al., 2016). The current conduit assisted nerve repair techniques, despite their considerable enhancement in creating patient specific and customizable 3D materials (Johnson et al., 2015), are not capable of supporting nerve regeneration to the same extent as the ANAs due to their lack of natural microarchitecture. Therefore, the combination of ANA bridging techniques and controlled release of therapeutic molecules may have the greatest clinical benefit because regenerating nerve fibers can be guided through the superior microstructure that the ANAs provide and enhanced by sustained and localized release of chemical cues.

Currently, the most concentrated research effort in the field of local macromolecule delivery to the peripheral nerve has been focused on development of the delivery systems and their materials. In order to ensure the success of the engineered systems following translation to the clinic, thorough investigation to establish the optimal local drug dose and drug release profile *in vivo* is necessary. Yet, the optimal local drug dose in humans remains to be elucidated. The dependency of local drug dose on the anatomical location, the severity of nerve injury, and the size of the injured nerve must be investigated for clinical use.

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