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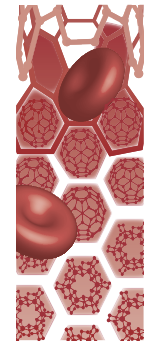

Nanoparticles for neurotrophic factor delivery in nerve guidance conduits for peripheral nerve repair

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Peripheral nerve injuries are a major source of disabilities, and treatment of long nerve gap autografts is the gold standard. However, due to poor availability and donor-site morbidity, research is directed towards the development of regenerative strategies based on the use of artificial nerve guidance conduits (NGCs). Several properties and characteristics of the NGCs can be fine-tuned, such as the architecture of the conduit, the surface topography and the addition of bioactive molecules and cells to speed up nerve regeneration. In this review, US FDA-approved NGCs are described. The recent works, in which polymeric, magnetic, silica-based and lipidic NPs are employed to introduce growth factors (GFs) to NGCs, are overviewed and discussed in depth herein.

Plain language summary: Nerves present in the extremities of the body are often injured, and this can lead to disabilities. To treat this problem, nerve sections from other body parts can be used, but the main disadvantage of this technique is poor availability and donor-site morbidity. To tackle these difficulties, research is focused on the development of artificial nerves, which are known as nerve guidance conduits (NGCs). This review article focuses on advances in this field, which is mainly related to the optimization of the material for conduit synthesis, on architecture and topography, and on how the functionalization of the NGCs with bioactive molecules can support nerve regeneration at the injured site. Currently commercialized NGCs are presented, and an in-depth discussion on strategies comprising neurotrophic factors administered alone, or included in the NGCs using nanoparticles, is also provided.

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The goal of tissue engineering (TE) and regenerative medicine is the fabrication of biomaterials with the ability to stimulate naturally the desired tissue regeneration. Tissues are complex systems, and due to their intrinsic complexity, their self-regenerating capacity becomes impaired during the post-traumatic recovery period. The nervous system presents a complex physiology, where neurons are the main functional unit, leading to a limited capacity of self-regeneration. Injuries of the peripheral nervous system (PNS), known as peripheral neuropathies, are a major source of disabilities and occur with high frequency. After an injury, sensations can suffer distortion, and an impairment of muscle mobility can occur, leading to painful neuropathies [1].

Despite advances in microsurgical technologies, the treatment of peripheral nerve injuries (PNIs) remains a challenge and complete recovery of nerve function after surgery has never been achieved. Moreover, the muscle suffers histological changes due to nerve degeneration and reinnervation. Muscle atrophy is followed by a loss-of-function recovery after reinnervation, and for functional recovery, the time-lapse between the traumatic event and the regeneration of the nerve and its functional activity plays a key role in eventual functional recovery [2]. Because of the time lag between the injury and the surgical procedure, nerve ends might retract, and in many cases, there

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might be a loss of nerve segment owing to injury. Nerve suturing cannot be performed in such cases because of the tension that might be created in the nerve segment, which would lead to a poor regeneration outcome. When nerves are injured and retracted, restoration of their function does not happen spontaneously, and continuity of the nerve needs to be reestablished first. It is possible to overcome this problem if the two nerve ends are approximated using grafts [1].

Autografts are considered the golden standard for nerve repair and are being used clinically; however, they can present several problems, such as site morbidity, mismatching on nerve size and neuroma formation in the implant site. Other autologous tissues have been also investigated, such as blood vessels, muscles or tendons. These tissues are subjected to a decellularization process to overcome the problems presented by the autologous nerves, but compatibility problems usually arise, and complications such as injuries in a second site are a major drawback [3].

Progress in TE has resulted in the development of artificial nerve constructs that can guide and facilitate the axonal nerve growth, overcoming the issues that autologous tissues presented. A nerve guidance conduit (NGC) is a tubular structure intended to bridge the gap of the sectioned nerve, protecting it from the surrounding tissue (scar formation in this case) and guiding the axonal regeneration from the proximal to the distal nerve stump [3].

PNI & recovery

When a PNI occurs, there may be an associated hemorrhage; the nerve ends often retract, and due to the inflammatory response, fibrin starts to deposit, resulting in fibrosis and scarring. Peripheral nerves comprise of multiple longitudinal arrangements of fascicles, called nerve fibers [4]. Axons are elongated in the fibers to conduct the individual neurons and are classified as myelinated and unmyelinated; motor nerves possess myelinated axons, whereas sensory nerves are typically composed of unmyelinated axons. Schwann cells (SCs) are associated with myelinated axons because they produce the laminin-rich myelin sheets, composed of proteins and fatty acids. The rapid impulse propagation through saltatory conduction is enabled by the fatty membrane that the myelin sheets form, isolating the axon [5].

Phenotypic changes in the neurons, due to interruption of the axonal continuity, affect the myelin sheaths and trigger inflammatory pathways, which damages more motor and sensory neurons. Muscular denervation is secondary to the injury, and the length of the time that elapses until reinnervation of the muscle determines whether the myofibrils and motor endplates are permanently lost, which would result in atrophic changes in the muscle [6].

Several factors influence the self-renewing capacity of nerves after an injury occurs: the age of the injured individual, the type of injury observed and the integrity of the neural cell body of the injured nerve influence the patient's recuperation. In compression injuries, the most noticeable characteristic is the focal demyelination at the compression site without axonal or connective tissue damage. In crushing lesions, the basement membranes of SCs that cover the fascicles and the nerve fibers are not disrupted, which guarantees axonal regeneration guided by the SCs [7]. If nerve transaction occurs, the lesion is more severe, and regeneration will depend on the gap generated in the nerve. The larger the gap, the greater the challenge. When physical separation occurs, the proximal and distal sections of the nerve lose communication; thus, the distal portion begins the degenerative phenomenon, called Wallerian degeneration, which is mediated by macrophages, recruited to remove the myelin sheets and the generated cellular debris. To repair the transacted axons, specific proteins are produced to guide the regeneration and bridge the nerve ends. The cell body regenerates the axon from the proximal to the distal end, and the structure that formed is called growth cone, where SCs organize themselves to form so-called bands of Büngner, which provide a guidance cue for the regenerating axons (Figure 1) [8]. Macrophages together with SCs also promote the optimal environment for axonal regeneration, as they release necessary extracellular matrix (ECM) proteins, cytokines, chemokines and neurotrophins [8].

Nerve guidance conduits

To develop conduits for peripheral nerve repair (PNR), research has focused on the development of hollow conduits. This first generation of artificial NGCs has been successful for short nerve gaps but has been less successful in recovering functionality of the regenerated nerves [9]. Thus, more complex NGC development is taking place. Ideally, NGCs need to have several characteristics; they must be biocompatible, biodegradable, flexible, mechanically stable and have ECM-like properties to avoid the immune response. They also need to promote tissue remodeling, be useful for implantation over a joint and have structural integrity and biological performance [10]. To date, various strategies have been developed, and research has been mainly focused on the development of polymeric structures (e.g., nanofibers and hydrogels) and biofunctionalization [11].

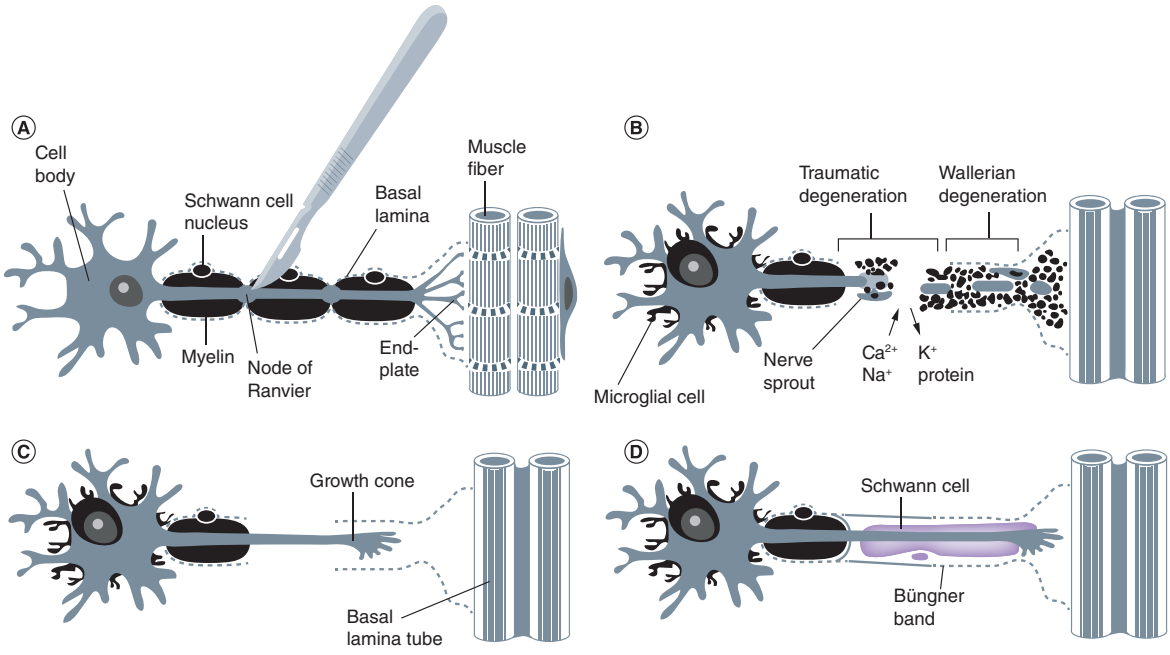

Figure 1. Peripheral nerve injury and repair. (A) Transection of the injured axon. **(B)** Degeneration in the injury site and Wellerian degeneration. **(C)** Regeneration growth cone to the basal lamina tube. **(D)** Alignment of the Schwann cells to form Büngner bands.
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Table 1. FDA-approved nerve guidance conduits.

Name	Company	Material	Filling	Nerve gap	FDA approval
NEUROLAC and NEUROLAC TW	Polyganics BV, Rozenburglaan, Netherlands www.polyganics.com/	Poly(DL-lactide-ε-caprolactone)	Hollow	≤20 mm	2005
NEUROTUBE	Synovis Micro, AL, USA www.synovismicro.com/	Poly(DL-lactide-ε-caprolactone)	Hollow	8–30 mm	1999
Nerbridge	Toyobo CO., LTD, Osaka, Japan www.toyobo-global.com/	PGA/collagen	Collagen	≤20 mm	2016
NeuraGen	Integra LifeSciences Corporation, NJ, USA www.integralife.com/	Collagen type I	Hollow	Overcomes flexing the proximity	2001
NeuroMatrix and NeuroFlex	Stryker Global Headquarters, MI, USA www.stryker.com/	Collagen type I	Hollow	≤30 mm	2001
Reaxon Direct	Medovent GmbH, Mainz, Germany www.medovent.de/	Chitosan	Hollow	≤10 mm	2015
Reaxon Nerve Guide				≤26 mm	
AxoGuard Nerve Connector	Axogen, FL, USA www.axogeninc.com/	Porcine submucosa ECM	Porcine submucosa ECM	≤5 mm, overcomes flexing the proximity	2003
Avance Nerve Graft		Human nerve allograft	ECM	Variable	2018

ECM: Extracellular matrix.

A successful peripheral nerve graft should be able to guide axon growth toward the severed distal nerve and provide sufficient cues to help the sprouting axons re-enter the correct fascicle to reinnervate the targeted muscle. The nerve conduits must be noncytotoxic, nonimmunogenic and easy to manufacture. They should also guide axon regeneration and isolate it from the scar tissue [12].

Numerous NGCs, made of either synthetic or natural materials, have been developed during recent decades. US FDA-approved NGCs are summarized in Table 1 [13].

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Natural materials, such as collagen, gelatin, hyaluronic acid (HA), laminin, fibrin or silk fibroin, have gained the attention of the field because of the properties they present; they are biodegradable, biocompatible and nontoxic [14]. Natural-based polysaccharides, such as the chitosan (Cht) or alginate, have also been studied, with good outcomes [15]. The greater disadvantage of employing natural materials for NGC fabrication is the weak mechanical properties that they have. However, with adequate modification of the materials, it is possible to enhance the physicochemical properties of NGCs. Crosslinking is the most widely used modification, which simply links different polymer chains between them, often providing the graft with better mechanical properties, such as improving the tensile strength or the crush resistance, among others [16,17]. Also, another popular technique is the use of composite materials. It is possible to blend natural with synthetic materials to achieve a stronger NGC [18].

Regarding natural-material conduits, the Integra LifeSciences Corporation has in the market NeuraGen, a conduit made of collagen type I, indicated to regenerate nerve gaps whose closure can be achieved by flexing the proximity. Conduits made of collagen type I are also commercialized by the Stryker company, the NeuroMatrix and NeuroFlex. The latter is interesting because it can bend up to 60°, but both can be used to overcome discontinuities of a length <30 mm. The commercialized product AxoGuard Nerve Connector, manufactured by the company Axogen, has the benefit that 1.5-cm length, which allows for an easier surgery for the implantation of the conduit because of the larger area that the implant has for suturing. Axogen conduit is made of porcine submucosa ECM and can be transplanted to the patient when the nerve gap is shorter than 5 mm. However, it is contraindicated for patients sensible to porcine-derived materials. The Avance Nerve Graft is another conduit manufactured by the same company, but it is a human nerve allograft; the main drawback of this implant is that it cannot be guaranteed to be completely free of pathogens. The German company Medovent GmbH sells two conduits, Reaxon Direct, which is designed for small defects up to 10 mm, where the surgical procedure usually consists of suturing of the two nerve ends, and Reaxon Nerve Guide, made of Cht, which can repair PNIs up to a defect length of 26 mm.

To obtain the same shape of the nerve tissue, synthetic polymers can be obtained in form of porous tubes, meshes or foams [19]. Among the synthetic materials several polyesters such as polylactide acid (PLA), polycaprolactone (PCL), polyglycolic acid (PGA), polylactic-co-glycolic acid (PLGA) or poly(caprolactone-co-lactide) (PCLA) and their copolymers are the most used materials [20]. Because these materials are thermoplastic, they allow fabrication of different-shaped tubes and form through different synthetic techniques such as molding, printing, precipitation, coating or extruding [19].

Several FDA-approved NGCs are made of polyesters. It is the case of the several products developed by the company Polyganics. They have commercialized NEUROLAC and NEUROLAC-TW (thin wall) guide conduits, made of a combination of PLA and PCL, the poly(DL-lactide-ε-caprolactone) (PLA-PCL copolymer), which can overcome an axon gap of up to 20 mm. The company Synovis Micro markets a conduit made of PLA-PCL NEUROTUBE, which can be used to regenerate nerve gaps in between 8 and 30 mm. Nerbridge is another conduit from the Toyobo company. They are synthesized from a mixture of PGA and filled with a newly developed medical collagen from Nippon Meat Packers, Inc. The main drawback of these materials is their lack of biocompatibility, which can be overcome by blending the synthetic polymers with natural materials [19,20].

Neurotrophic growth factors

PNR is a complex process, mediated by SC secretion and upregulation of a number of growth factors (GFs). The GFs includes the NGF, GDNF, LIF, BDNF, GAP-43 and NT4. NT4 plays a significant role in axon regeneration [1]. Since the efficiency of using artificial NGCs to overcome long nerve gaps has been demonstrated, several approaches are being developed to incorporate GFs such as the neurotrophic factors NT-3, NGF, BDNF, CNTF or VEGF to functionalize the conduit to promote nerve regeneration [21]. Table 2 summarizes research results about the role that NT-3, NGF, BDNF, VEGF, GDNF and bFGF play in PNR and their effects when administered exogenously.

NT-3

NT-3 is the third member of the neurotrophin family and has been shown to support the survival and differentiation of existing neurons. Moreover, it can promote neuron growth and differentiation. NT-3 expression varies throughout the life span; during maturation the level decreases, and it appears in higher concentrations in regions of the CNS where proliferation, migration and differentiation are ongoing [22]. At the level of the PNS, NT-3 induces survival and differentiation in sensory and parasympathetic neurons, and it has been reported to support the survival of motoneurons *in vitro* and rescues them from naturally occurring cell death [23].

Table 2. Neurotrophic factors effects in peripheral nerve repair.

Growth factor	Endogenous effects	Exogenous effects	Ref.
NT-3	- Supports survival of existing sensory and parasympathetic neurons and promotes growth and differentiation - Supports survival of motorneurons *in vitro*	- Beneficial effects in motor and particularly proprioceptive neurons by promoting their survival and differentiation - It can rescue SCs and motor and proprioceptive neurons from cell death	[23,24]
NGF	- Synthesized and released by tissues that are highly innervated by sensory and sympathetic neurites - Essential for the survival, differentiation and maintenance of primary sensory and sympathetic neurons	- In injured motoneurons, the concentration of NGF has been shown to plas an important role on neurite regeneration - Stimulates neural survival and differentiation	[25–27]
BDNF	- SCs massively express BDNF after nerve injury in the distal denervated nerve part to offer a supply of the factor to the regenerating BDNF-sensitive neurites.	- Has an effect on some subtypes of peripheral sensory neurons as demonstrated *in vitro*.	[28,29]
VEGF	- Regulates the angiogenesis by inducing the proliferation, migration and permeability of endothelial cells - Plays a role in the development of neurons, which express different amount of VEGF and its receptors during nerve cell development	- Studies *in vivo* have supported the hypothesis that exogenous VEGF has a positive effect on axonal regeneration and on proliferation and migration of SCs	[30–32]
GDNF	- Endogenous GDNF promotes the sprouting of transacted axons, from the proximal end to the denervated stump, but in chronically axotomized motoneurons, they progressively fail to regenerate due to the reduction of neurotrophic factor synthesis - Key factor for motorneuron survival and has an influence on sensory and autonomic neurons	- Promotes axonal growth	[34,35]
bFGF	- Maintenance of tissue repair - Expressed in low levels in the PNs, where SCs are the major source, but also invading macrophages after the lesion strongly express it; highly expressed bFGF by macrophages can induce SC proliferation and inhibition of myelination during axonal growth	- Has protective and restorative effects - Initial studies showed that in a sciatic nerve lesion, the growth factor can promote neurite growth and extension and induce vascularization at the regenerating site by crossing the gap formed between the distal and proximal nerve ends	[36]

PN: Peripheral nerve; SC: Schwann cell.

Exogenous NT-3 has also shown beneficial effects in motor and particularly proprioceptive neurons by promoting their survival and differentiation. Other works making with chicks and adult mice models have demonstrated that it can rescue SCs and motor and proprioceptive neurons from cell death [24].

NGF

NGF is essential for the survival, differentiation and maintenance of primary sensory and sympathetic neurons [25]. Although this factor was discovered because of its action during development, it is now known to function throughout life. Usually, it is synthesized and released by tissues that are highly innervated by sensory and sympathetic neurites [26].

For some time, much works has shown the potential of the exogenous administration of NGF after nerve injury, although this effect does not reflect the NGF physiology. After axotomy, sensory neurons have been shown to become insensitive to endogenous NGF physiological concentrations, and this sensitivity is only restored once reinnervation of the peripheral targets is achieved. However, it has been shown in injured motoneurons that the concentration of NGF plays an important role on neurite regeneration. NGF binds to the TrkA receptors present at nerve terminal and is internalized in a receptor complex manner; it is then transported along the axons to the cell body, where initiates the signaling cascades intracellularly stimulating neural survival and differentiation [27].

BDNF

BDNF has an essential role in the development and survival of neurons, synaptic plasticity and cognitive function. Its dysregulation is associated with several neurodegenerative disorders, such as the Alzheimer's disease. In contrast to NT-3, BDNF increases its expression with maturation, and the concentration is lower in developing regions [22]. It has an effect on some subtypes of peripheral sensory neurons and also on many neurons type derived from the CNS, as demonstrated *in vitro* [28]. SCs express massively BDNF after nerve injury in the distal denervated nerve part, in order to offer a supply of the factor to the regenerating BDNF-sensitive neurites [29].

VEGF

The VEGF is an angiogenic factor that is known to be involved in the development and maintenance of the vascular system. It regulates angiogenesis by inducing the proliferation, migration and permeability of endothelial

cells [30]. VEGF acts through its interaction with neuropilin 1 and two types of phosphotyrosine kinase receptors, flt-1 and flk-1, which, apart from endothelial cells, have been also found in neurons and SCs [31]. It was demonstrated that VEGF plays an essential role not only in vasculogenesis but also in the development of neurons, which express different amounts of VEGF and its receptors during nerve cell development [32]. Several studies *in vivo* have supported the belief that exogenous VEGF has a positive effect on axonal regeneration and on proliferation and migration of SCs [31].

GDNF

GDNF has been shown to be a potent survival factor for dopaminergic midbrain and spinal cord neurons. It also acts outside the nervous system as a morphogen for the development of the kidney and by regulating the differentiation of spermatogonia [33]. When a traumatic injury occurs, the transcription of this factor is upregulated in the spinal cord, the striatum and the hippocampus, so it is of special interest for the treatment of neurodegenerative disorders [33]. Endogenous GDNF promotes the sprouting of transacted axons, from the proximal end to the denervated stump, but in chronically axotomized motoneurons, they progressively fail to regenerate due to the reduction of neurotrophic factor synthesis [34]. It is possible to overcome this by administering the GDNF to promote axonal growth [34]. GDNF is a key factor not only for motoneuron survival but also has an influence on sensory and autonomic neurons [35].

FGF-2

FGF-2 is part of the FGF family, involved on the mitogenesis of mesoderm and neuroectoderm-derived cells [36]. This family also regulates proliferation and differentiation in embryonic development and maintenance and tissue repair in adulthood [36]. Exogenously applied FGF-2 has protective and restorative effects in the PNS [36]. Initial studies showed that in a sciatic nerve lesion the growth factor can promote the neurite growth and extension and induce vascularization at the regenerating site by crossing the gap formed between the distal and proximal nerve ends. FGF-2 is expressed in low levels in the PNs and SCs are the main source of FGF-2 mRNAs. In a study of a crushed rat sciatic nerve model, it was demonstrated that FGF-2 not only is expressed by SCs, but invading macrophages also strongly express it. Also, another study revealed the influence of FGF-2 on the mitogenesis of SCs, suggesting that the highly expressed FGF-2 by the macrophages can induce SCs proliferation and inhibition of myelination during axonal growth [36].

Nanoparticles incorporated to NGCs

Finding regenerative agents and nanoformulations that can promote neuronal repair and differentiation is highly important, but the main problem of using GFs is that they have a short biological half-life and thus degrade quickly without improving nerve regeneration. GFs can be conjugated or complexed with other materials to form nanoparticles (NPs), and a slower, more lasting release can be achieved. NPs made of several materials have been functionalized to be used for neuronal regenerative purposes. These NPs are summarized and their references included in Table 3. In this section, the recent advances in NPs to include GFs within the lumen of the NGCs are described, where polymeric, magnetic, silica-based and lipidic carriers are englobed.

Polymeric NPs

Control over the delivery of the neurotrophic factors is essential to regulate their bioactivity; the ideal delivery is composed of a sustained release over a physiologically relevant timeframe while the dose is minimized. Lackington *et al.* have worked on a PLGA NP delivery system for NGF and GDNF to avoid supraphysiological doses of the GFs (Figure 2), which can lead to axon and SCs entrapment, impeding the regeneration of the neurons [37]. Much research has shown the high potential of exogenous NGF administration after nerve injury [38].

PLGA is a biodegradable and biocompatible synthetic polymer that has been widely used to encapsulate GFs [39]. PLGA NPs containing NGF or GDNF were prepared through solvent evaporation and incorporated to a NGC made of collagen and HA by soaking the conduit into a cocktail of both NPs in culture media. *In vivo* evaluation confirmed the efficiency of the optimized NGC containing NGF- and GDNF-functionalized PLGA NPs to enhance the regeneration over a 15-mm sciatic nerve defect in male Lewis rats, compared with clinical gold standard and autografts [37]. The authors also reported a synergistic effect of NGF and GDNF and observed a delayed delivery of the GFs, with a physiological flux that resembles to the response these GFs have after an injury [37]. The aim of encapsulating GFs is also to obtain a longer bioactivity of the molecules. Zuidema *et al.* focused their research on

Table 3. Nanoparticles for growth factor encapsulation for peripheral nerve repair.

	Material	Growth factor	Outcomes	References	Ref.
Polymeric NPs	PLGA	NGF and GDNF	*In vivo*, regeneration of 15-mm sciatic nerve defect in rats was enhanced when the PLGA NPs containing NGF and GDNF are incorporated to the NGC.	Lackington *et al.*	[37]
	PLGA	NGF, BDNF, GDNF and bFGF	Improved axonal and motor and sensory neuron regeneration *in vivo* when GFs are incorporated to NPs	Santos *et al.*	[43]
	PLGA	NGF	Enhanced neurite growth and axonal orientation on PC12 neural cells in *in vitro* experiments	Lee *et al.*	[41]
	PLGA	bFGF	Greater recovery of the electromuscular activity in a 10-mm sciatic nerve defect in Sprague–Dawley rats	Si *et al.*	[42]
	PLLA / IONPs	NGF	Directed regeneration of neurites in E9 chick dorsal root ganglia	Zuidema *et al.*	[40]
	Cht	NGF	Bounded NGF to Cht NPs enhanced MSC differentiation into neurons	Mili *et al.*	[46]
	Cht	NGF	Increased differentiation of human adipose-derived stem cells to Schwann cells and higher myelination	Razavi *et al.*	[47]
Magnetic NPs	Fe_3O_4	NGF	- Improved neurite outgrowth of PC12 sympathetic ganglion neuronal cells. - Higher levels of expressions of differentiation gene markers - Possible to guide the NPs with a magnetic modular device *in vivo*	Marcus *et al.*	[55,56]
	Fe_3O_4	NGF and VEGF	*In vivo* acceleration of neuron regeneration and faster motor function recovery	Giannaccini *et al.*	[58]
	Fe_3O_4/Au core/shell	NGF	Enhanced PC12 cell differentiation and neurite length when dynamic magnetic fields are applied	Yuan *et al.*	[67]
	Fe_3O_4/quercetin	NGF	- Higher branching of PC12 cells and improved outgrowth of neurites - Enhanced expression of ß3-tubulin	Katebi *et al.*	[68]
	Fe_3O_4	GDNF	No conclusive results *in vivo* when the GDNF is conjugated to the IONPs	Fregnan *et al.*	[70]
Silica NPs	MSNs	NGF	*In vitro* studies on PC12 cells have shown an improved outgrowth of neurites	Cho *et al.*	[73]
	MSNs	NGF	Higher expression of GAP43 protein on PC12 cells sutured on top of collagen hydrogels with NPs	Lee *et al.*	[74]
	MSNs	NGF	Enhanced PC12 cell differentiation to neurons and a higher outgrowth of neurites	Sun *et al.*	[75]
Lipid NPs	Heparinized CSL NPs	NGF	Enhanced differentiation *in vitro* of induced pluripotent stem cells to neural cells	Kuo *et al.*	[80]

CSL: Cationic solid lipid; IONP: Magnetic iron oxide NP; NP: Nanoparticle; PLGA: Poly(lactic-co-glycolic) acid; PLLA: Poly(L-lactic acid); MSN: Mesoporous silica NP.

composite NPs for NGF release. The prepared NPs combining NGF-containing poly(L-lactic acid) (PLLA) NPs with magnetic iron oxide NPs (IONPs) through the modification of water–oil–water double emulsion technique to have a controlled and localized release of the molecule [40]. These composite NPs were incorporated into PLLA-aligned microfibers to form gradients of NGF to study the neurite outgrowth of E9 chick dorsal root ganglia and were demonstrated to be a promising strategy to direct the growth of regenerating neurites [40]. This work is of special interest because it combines the use of Magnetic NPs (MNPs) with a coating of poly-L-Lysine (PLL), which reduces the toxicity of the MNPs and also carries NGF on the surface.

Core-shell NPs made of a PLGA shell and NGF core through electrospraying have been used by Lee *et al.* [41]. By using the electrospraying technique, a large quantity of GFs can be encapsulated on the core to cover it with polymers (PLGA in this specific work). They developed a PEG 3D-printed NGC where the NPs are embedded as part of the ink, and studied PC12 neural cell growth in the conduits, comparing the results with cells grown on the same NGCs without the NPs but with sprayed NGF [41]. The results demonstrated that PC12 neurite extension is enhanced and axons are oriented along the longitudinal axis of the conduits when the NGF is encapsulated with the PLGA NPs. The results were compared with neurite extension when NGF is sprayed directly on the conduit [41].

bFGF has a short half-life and is deactivated quickly in body fluids. To overcome this issue, Si *et al.* synthesized PLGA NPs containing bFGF. They incorporated the microspheres into 15-mm silicon tubes to bridge a 10-mm sciatic nerve defect in Sprague–Dawley rats [42]. Their objective was to achieve a controlled release of bFGF to

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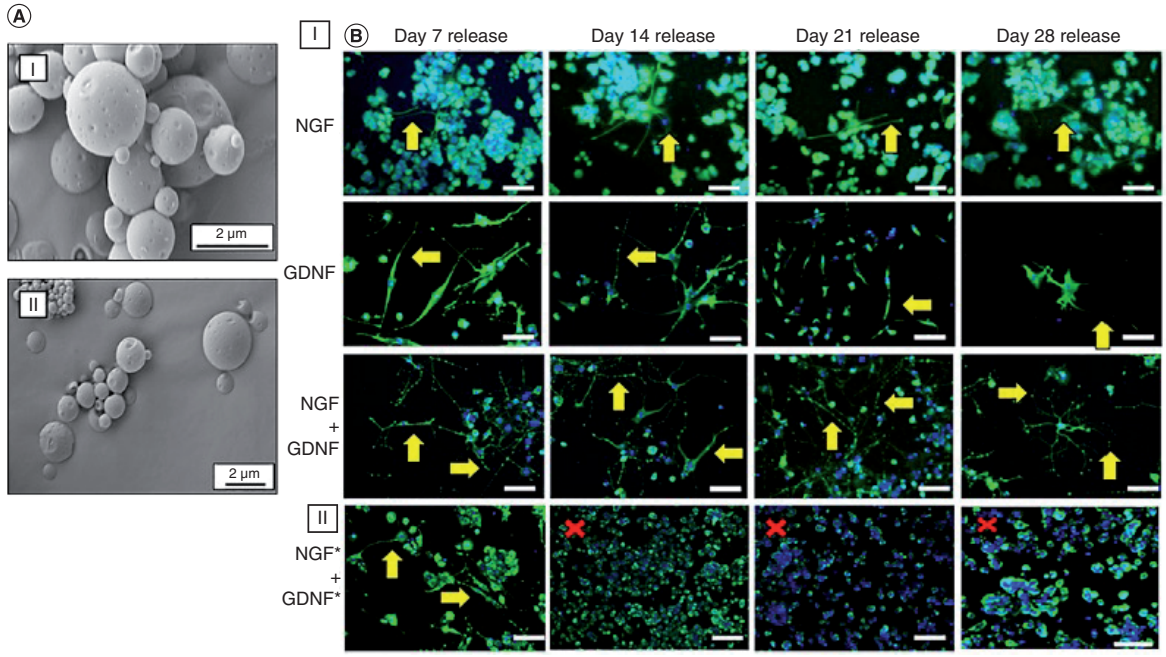

Figure 2. Polylactic-co-glycolic acid nanoparticles for neurotrophic factor release. (A) Scanning electron micrographs of PLGA microparticles encapsulating **(I)** NGF and **(II)** GDNF. **(B)** Images showing the neurite outgrowth of PC12 cells cultured on NGCs filled with a hyaluronic-acid-based hydrogel containing **(I)** PLGA microparticles with neurotropic factors and **(II)** nonencapsulated neurotrophic factors. The yellow arrows positive neurite outgrowth, and red crosses represent the absence of it, demonstrating the advantages of encapsulating the neurotrophic factors to avoid their bioactivity loose.
PLGA: Poly(lactic-co-glycolic) acid; NGC: Nerve guidance conduit.
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the matrix, achieving a release that lasts 2 weeks. They performed electrophysiological studies, comparing results of empty silicon tubes, tubes containing bFGF and tubes containing the bFGF-PLGA microspheres. The authors saw an overall promotion on sciatic nerve regeneration and greater neuromuscular activity recovery when the tubes included bFGF-PLGA NPs [42]. Santos *et al.* also developed PLGA NPs containing several GFs (NGF, BDNF, GDNF and bFGF) to study their efficacy *in vivo* [43]. Silicone tubes filled with collagen to introduce the NPs containing the GFs or free GFs were evaluated to repair 6-mm sciatic nerve defect on female Sprague–Dawley rats. When the GFs were encapsulated in the PLGA NPs, the axonal regeneration of motor and sensory neurons was promoted, compared with conduits functionalized with free GFs [43]. Again, the authors concluded that the enhanced bioactivity of the GFs observed could be due to their encapsulation and their controlled delivery, thus keeping their bioactivity for longer time.

Among natural polymers, Cht has gained significant attention for encapsulating GFs [44]. Cht is a widely used biopolymer for pharmaceutical applications due to its biodegradable and biocompatible properties [45]. Mili *et al.* developed NGF-functionalized Cht NPs through the ionotropic gelation method and evaluated the release profile of the GF *in vitro*; an initial slow release occurred for the first 48 h, showing peak release after 8 days with a steady release state reached at 12 days [46]. Studies done on canine bone-marrow-derived mesenchymal stem cells (MSCs) demonstrated that the differentiation of the MSCs into neurons has a higher yield with NGF-functionalized NPs compared with a medium supplemented with unbound NGF [46]. A study by Razavi *et al.* was also based on the use of Cht NPs to encapsulate NGF and Au NPs [47]. The Au NPs have been demonstrated to have a positive effect on stem cell proliferation and differentiation [48], so in this work they were used with the objective of enhancing the differentiation process of human adipose-derived stem cells (hASCs) to SCs. They used the same process for NP synthesis – that is, the ionotropic gelation method to incorporate the factor and the Au NPs to the Cht NPs. *In vitro* studies demonstrated that hASC differentiation is increased and the myelinating capacity of the SCs is improved compared with cells treated with unfunctionalized Cht NPs (Figure 3) [47]. Jahromi *et al.* incorporated the previously studied NPs to PLGA conduits containing rat ADCSs and demonstrated their potential use for

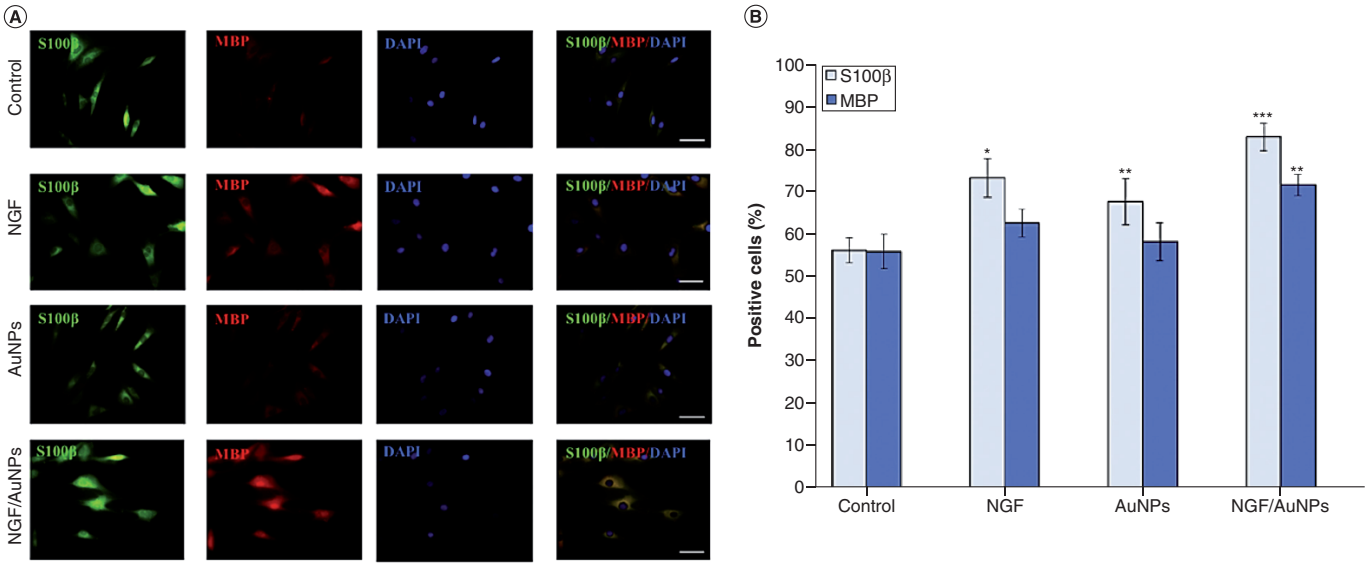

Figure 3. Chitosan nanoparticles for neurotrophic factor release. (A) Immunocytochemical staining for Schwann cell (SC) marker S100b (Green) and myelinating ability (MBP) (red) for differentiated cells in tissue culture plates (control), with the addition of NGF loaded chitosan nanoparticles (Cht NPs) (NGF), with the addition of Au NP-loaded Cht NPs (AuNPs) and with Cht NPs loaded with NGF and (NGF/AuNPs). All nuclei were stained with DAPI. Scale bar is 100 mm. **(B)** Comparative analysis of the mean percentages of S100b and MBP markers. These results demonstrate that the synergistic effect of NGF and Au NP release enhances SCs differentiation.
*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$.
Au NP: Gold nanoparticle.
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nerve repair after injury [49]. In a more recent work, Seyedebrahimi *et al.* incorporated BDNF to the same NPs, rather than incorporating NGF [50]. These NPs were then encapsulated with rat adipose tissue-derived stem cells (ADSCs) in a fibrin matrix for BDNF delivery, and the efficiency of the system *in vivo* was studied [51]. They were demonstrated to have a positive effect on axonal growth; if the number of axons is compared, the amount is higher within the graft that contains the NPs compared with the nonfunctionalized grafts, suggesting enhanced differentiation of the ADSCs [51].

Magnetic NPs

Magnetic NPs (MNPs) are unique in that they can be manipulated through the use of magnetic fields and are usually formed with two components: a magnetic material (e.g., iron, nickel and cobalt) and a chemical component with a functionality, which can have biocatalytic or with biorecognition properties. They have been extensively used for biomedical applications including magnetic targeting of genes or drugs, magnetic resonance imaging, diagnosis, DNA purification and cell separation to name a few [52]. The most frequent NPs for biomedical applications are composed of iron oxide (Fe_3O_4), although Stergar *et al.* have reviewed the biomedical use of NiCu MNPs, which are not common for applications in this field [53].

IONPs loaded with NGF have shown that this NP can be magnetically targeted and can release the product in a controlled manner [54]. Marcus *et al.* have covalently conjugated NGF to MNPs and studied the effect of the novel complex on the differentiation of PC12 sympathetic ganglion neuronal cells (Figure 4A) [55]. When they compared neurite outgrowth of cells incubated with NGF-NPs and free NGF, a significant improvement was observed when cells are treated with the conjugated factor. Also, the complexity of the neuronal branching trees was promoted, and differentiation gene markers showed higher levels of expression. These results are based on studies they did on NGF degradation rates, which was shown to be slower when conjugated with the IONPs [55]. The same NPs were later evaluated as a magnetic target; using a magnetic modular device, they studied the ability to guide the NPs *in vivo* (Figure 4C). NPs were administered in the sciatic nerve by injection and intravenously. Placing the magnet next to one of the eyes, they saw a significant accumulation of IONPs in the retina of mice [56]. In a more recent work, Li *et al.* showed the potential use of mouse NGF-conjugated IONPs for PNR using an applied external magnetic field [57]. They synthesized magnetic mouse NGF-poly(lactic acid glycolic acid) copolymer NPs, and the

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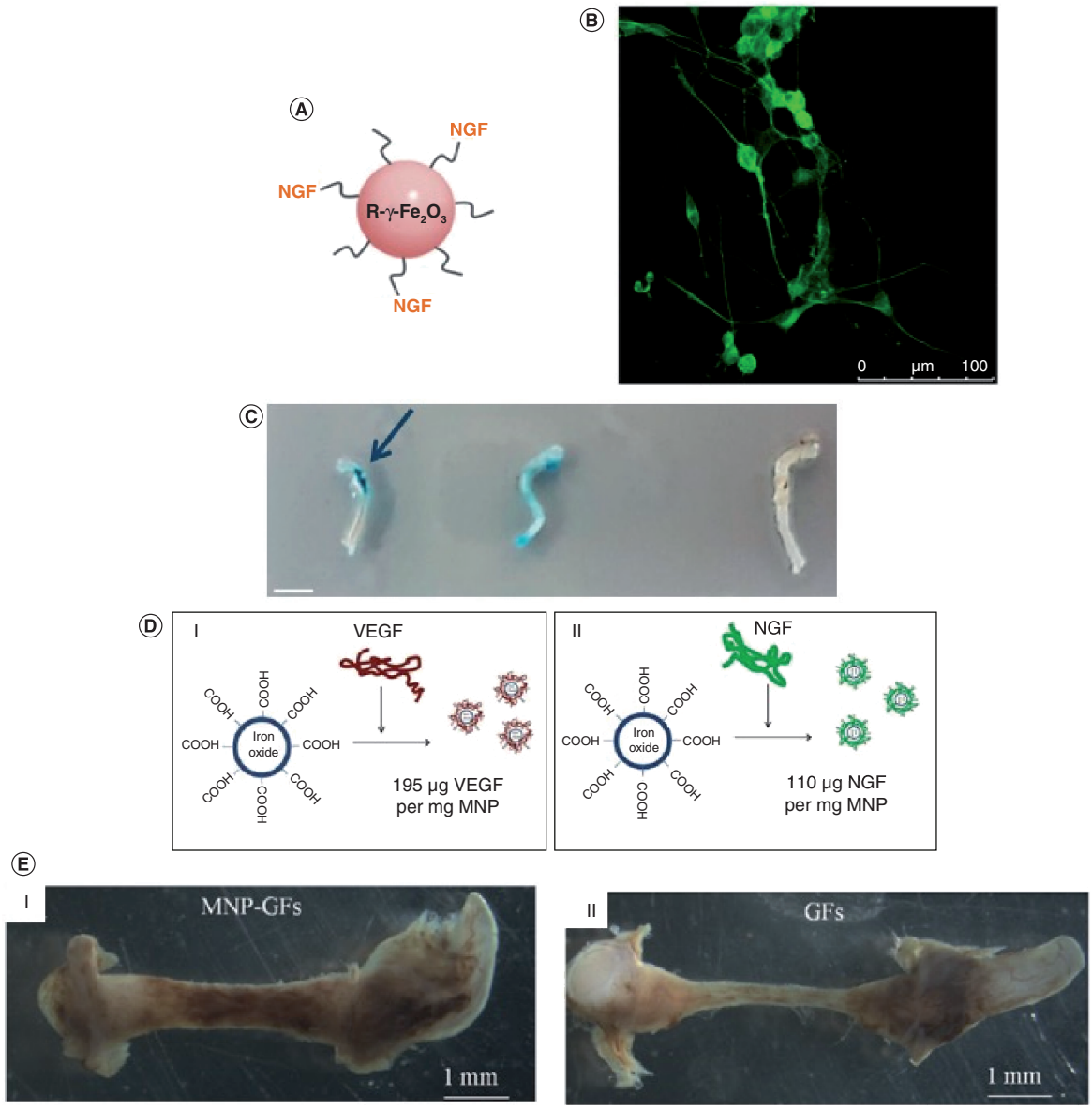

Figure 4. Magnetic nanoparticles for neurotrophic factor release. (A) Schematic illustration of NGF-conjugated NPs. Uniform fluorescent (rhodamine isothiocyanate) IONPs were synthesized by nucleation; a thin film of γ-Fe_2O_3 is grown on their surface and then coated with HSA via precipitation, and NHS-ended PEG is covalently conjugated to interact with the primary amino groups of the NGF [55]. **(B)** Fluorescence imaging of PC12 cells after their differentiation for 4 days mediated by the NPs. **(C)** *In vivo* targeting of NGF-IONPs with magnets in sciatic nerve model: the IONPs are injected. Left: nerve injected with the IONPs and use of a magnet to follow the injection externally. Middle: injection of IONPs without an external magnet. It can be seen that the IONPs are distributed along the nerve. Right: Control – no injection of IONPs. Scale bar = 4 mm. **(D)** Schematic illustration of PEI-coated IONPs functionalized with VEGF and NGF synthesis by precipitation. **(E)** Explants of the upper limb of rats is treated with conduits containing **(I)** IONP-growth factors (GFs) and **(II)** GFs 12 days after surgery. The results show that explants treated with IONP-GFs have structured matter throughout the entire length of the implant and, most importantly, contain an outgrowth of regenerated axons that connect the proximal to the distal end of the transacted nerve. MNP: Magnetic nanoparticle; IONP: Iron oxide nanoparticle; GF: Growth factor.
(A–C) Reproduced from [56] © Marcus M *et al.* (2018).
(D & E) Reproduced with permission from [58] © John Wiley and Sons, Inc. (2017).

GF distribution *in vivo* was studied, demonstrating the possibility of aggregating the drug by magnetic targeting to promote functional recovery [57]. Giannaccini *et al.* also used NGF and VEGF to functionalize their IONPs (Figure 4D), injecting the NPs to synthetic NGCs and an acellular allograft [58]. The authors produced a neurotmesis in Sprague–Dawley rats and removed 5 mm of nerve. Conduits were transplanted, and it was demonstrated that the use of the GF-conjugated NPs accelerates the regeneration of neurons and promotes motor function recovery, compared with the incorporation of free GFs to the NGCs (Figure 4E) [58].

It has been demonstrated that MNPs are an effective nanocarrier for NGF. The main drawbacks of using 'naked' MNPs are that they are unstable in a neuronal environment, aggregate and are toxic for the cells, resulting in a short half-life and being cleared by the macrophages via phagocytosis [59,60]. MNPs can be protected to avoid this situation; for this purpose, they can be coated with various materials such as polymers or metals [61]. Gold is considered one of the best candidates because it is known to be a metal with a high mechanical strength, stability and biocompatibility [62]. Also, the superparamagnetic nature of the MNPs is retained after Au coating [63]. It has been demonstrated that Au-coated substrates can enhance neurite outgrowth and differentiation on PC12 cells [64], and the usefulness of Au NPs for neural TE applications has already been reviewed [65]. Yuan *et al.* demonstrated the biocompatibility of Au-coated superparamagnetic iron oxide (SPIO) NPs [66]. They also studied whether NGF-functionalized SPIO-Au core-shell NPs stimulate neuron growth and differentiation on PC12 cells when an external magnetic field is applied. The results confirmed their hypothesis: application of dynamic magnetic fields enhanced cellular differentiation and neurite length elongation [67].

In work by Katebi *et al.,* IONPs were combined with the flavonoid quercetin and NGF [68]. Quercetin has been shown to act as a neuroprotective agent by interacting with proteins and protecting cells from oxidative stress [69]. The authors tried to enhance the effect of the factor in neuronal differentiation of PC12 cells and demonstrated enhanced neurite outgrowth and neuron branching [68].

GDNF is involved in motor neuron regeneration and remyelination. For these reasons, Fregnan *et al.* conjugated this neurotrophic factor to IONPs through the use of a dextran coating to stabilize the GF and extend its biological activity [70]. A hydrogel composed of HA and laminin was used to fill a Cht-made conduit, and functionalized with the GDNF-conjugated IONPs. The authors used Wister rats for the *in vivo* experiments and found no significant differences on regeneration when the GDNF was conjugated to the MNPs or free in the hydrogel [70].

Silica NPs

Silica nanostructures show a high biocompatibility, high loading capacity and stability. They are thus catalogued as safe materials for biomedical applications by the FDA [71]. Silica nanostructures are easy to prepare and can be acquire a porous inner structure through condensation methods. Particularly interesting are the mesoporous silica NPs (MSNs), which have been found to be useful for controlled and targeted delivery of drugs, proteins or genes. They can be coated with functional or stimuli-responsive caping agents and therefore play an important role in TE applications [72].

Spherical MSNs exhibit a large surface-to-volume ratio, and Cho *et al.* took advantage of this to incorporate NGF into the pores of the MSNs obtaining a high loading and a sustained and controlled release of the factor [73]. They synthesized MCM-41-type MSNs and the NGF was incorporated through stirring. The NPs were embedded within an electroactive polypyrrole (Ppy) film by electrochemical polymerization, and PC12 cells were grown on top of the composites. Results revealed improved outgrowth of neurites and added effectiveness in promoting neurite extension when electrical stimuli was applied to the system, as it was observed that the NGF release was also enhanced [73]. Lee *et al.* also incorporated NGF into MSNs; combined with a collagen hydrogel, the system was employed as a delivery system and a matrix to culture PC12 cells [74]. The release of the NGF from the MSNs was demonstrated to be sustainable for more than a week, increasing the release time when the NPs are embedded within the collagen gel. They demonstrated an upregulation of the expression of the GAP43 protein, which is related to the growth of cells, confirming the effectiveness of the delivery system for NGF [74]. Sun *et al.* also prepared MSNs with NGF and found improved differentiation of PC12 cells to neurons and a higher outgrowth of neurites compared with the use of 'naked' NGF [75].

Lipid NPs

When considering lipid-based NPs for drug delivery applications, the most popular lipidic structure are liposomes. Liposomes are formed by a lipidic bilayer membrane, self-assembled lipids that enclose an aqueous compartment. They can be prepared through several methods, such as solvent injection, sonication, membrane extrusion or

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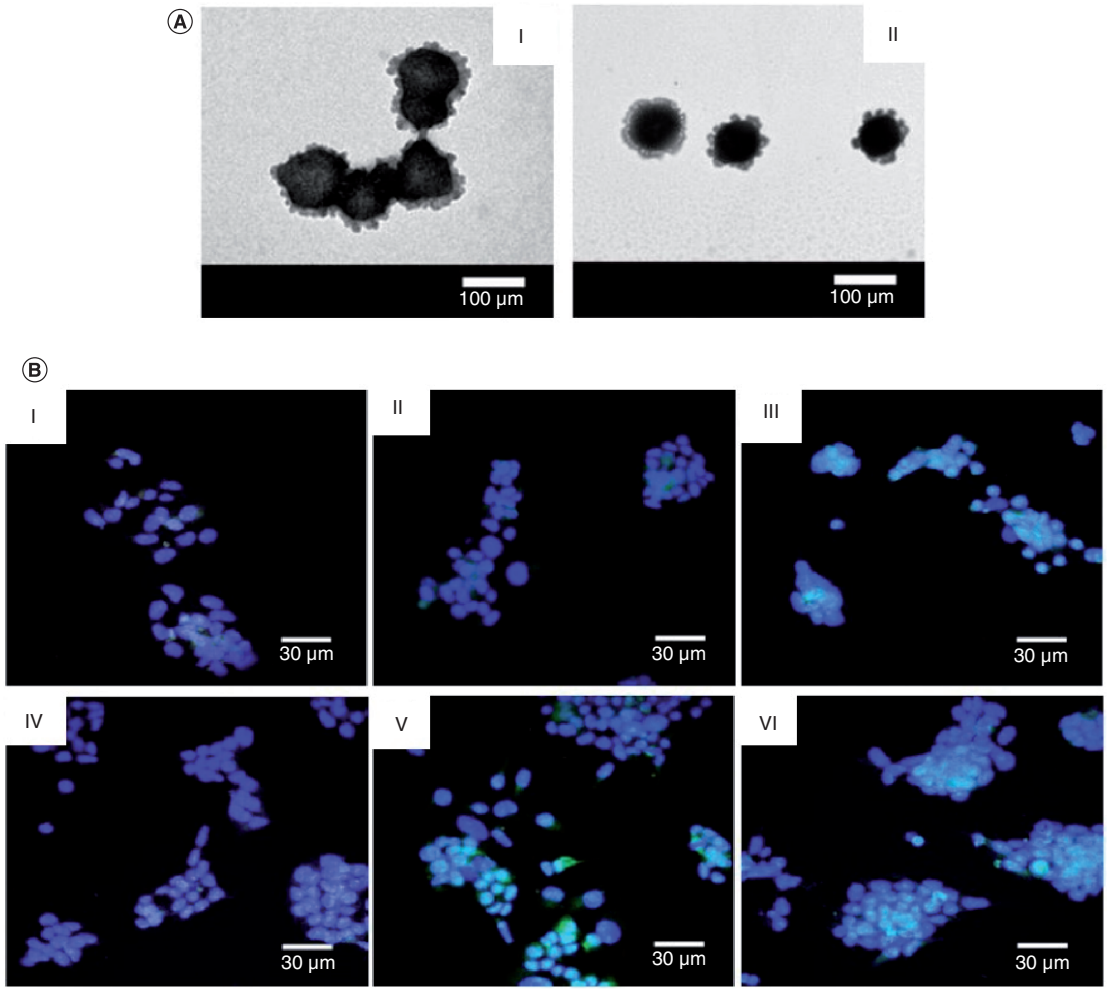

Figure 5. Lipid nanoparticles for neurotrophic factor release. (A) Transmission electron micrographs of NGF-loaded heparinized cationic solid lipid NPs with **(i)** stearylamine and **(ii)** esterquat 1. **(B)** Confocal microscopic images of stained SSEA-1 and NeuN expressed by induced pluripotent stem cells when they differentiate into neurons induced with the presence of NGF-loaded HCSLNs with **(i–iii)** esterquat 1 and **(iv–vi)** stearylamine. The slight purple results from the combination of blue and red, and white from the combination of blue, red and green, reveal the expression of SSEA-1. NeuN appears in green but can become pale blue color due to the combination of green and blue coming from the nuclei.
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microfluidic technology; the last is the most novel technique. Entrapment of hydrophilic moieties such as GFs or peptides in the inner aqueous core of the liposomes depends on the chemical characteristics of the liposome and its ability to entrap the biomolecule on its lipid bilayer [76]. Apart from liposomes, solid lipid NPs (SLNs) have also been used for GF delivery [77].

Angelova *et al.* developed anionic NPs. They employed PEGylated lipid NPs to encapsulate BDNF [78]. Most of the research regarding lipid NPs has been focused on treatments for neural diseases to deliver GFs, drugs or genetic material to the CNS [79]. With an application in mind for PNR, Kuo *et al.* synthetized NGF-loaded heparinized cationic solid lipid NPs (HCSLNs) made of a combination of heparin-stearic acid conjugate, cacao butter, cholesterol and stearylamine or esterquat 1 (Figure 5A) [80]. They evaluated several combinations to achieve differentiation of induced pluripotent stem cells (iPSCs) from mouse to neuron cells through the release of the NGF from the liposomes. They concluded that iPSCs cultured in the presence of liposomes with esterquat 1 for their differentiation showed higher viability in *in vitro* studies (Figure 5B) [80]. SLNs are under development, but there are no more significant works related their use for PNR applications.

Conclusion

NGCs are considered an alternative to autografts for the treatment of nerve PNR therapies. Given the incidence of PNIs and the growing aging population and because the supply of nerve autografts is limited, NGCs play an important role in PNR. As described in this review, NGCs can be made of different materials and with different designs. Commercially available NGCs have been used clinically to treat PNIs, but they are not designed to act as delivery vehicles for local administration of neurotrophic factors.

The goal of PNR is to achieve a functional conduit that can ensure fast nerve regeneration and complete neuromuscular activity of the denervated muscle after injury. Neurotrophic factor administration into the injured site or through their incorporation into NGCs has been considered an optimal approach to treat these injuries, but 'naked' GF administration can lead to a fast loss of activity and to degradation after administration or incorporation to NGCs. It was demonstrated that the use of NPs to encapsulate GFs gives them greater stability and preserves its activity. Several NP formulations have been included, where polymeric, magnetic, silica-based and lipid NPs and combinations of them have shown to be effective for GF encapsulation and release.

Polymeric NPs can be made of synthetic or natural polymers and are stable for encapsulating neurotrophic factors. They are versatile and, if natural, can exhibit superior biocompatibility, low toxicity and high cargo-loading ability. A primary advantages is their charge because it can be advantageous to have electrostatic interactions with the GFs and allow greater GF loading. Also, their biodegradability can allow a controlled release of the GF to achieve the necessary release and ensure complete nerve regeneration.

Applications for MNPs have been found in many biomedical areas. They have been demonstrated *in vivo* to increase the half-life of neurotrophic factors. An additional advantage they offer, compared with other NPs, is the magnetic behavior they exhibit, which allows guidance of NPs to the desired site for delivery of the cargo by external magnetic fields. However, the main drawback is their toxicity, which could be overcome by combining MNPs with other materials and developing coatings, such as the Au coatings described in this review. To retain the superparamagnetic properties of the MNPs, coating these NPs with polypeptides could be exploited to change their surface properties, make the MNPs less toxic and allow high GF loading not only in the core but also on the surface of the NPs. Compared with polymeric NPs, MNPs have the possibility of being functionalized only on the surface, with functional groups covalently attached; this could help with the immobilization of GFs on the surface and also give MNPs greater specificity for some cell types by immobilizing antibodies.

MSNs have been deeply studied for drug-delivery purposes because a large amount of material can be loaded in their inner structure; as discussed in this review, they also find an application in PNR. Their most remarkable advantage is that they exhibit a high inner volume, which can be tuned using different synthetic techniques and surfactants to achieve various mesoporous structures. These mesoporous structure give MSNs a high surface that can be used to adsorb or chemically immobilize GFs through their functionalization with chemical groups that can react and attach the factors covalently. Their tortuous inner structure allows slow release of any cargo loaded inside, which is of our interest to achieve a slow release of the GFs until nerve regeneration is ensured. Also, the outer pores can be closed by using polymers; if stimuli-responsive polymers are used, it is possible to have even more specific control of the release of the GFs encapsulated in the inner structure. A work done on lipid NPs for NGF-loading was also discussed in this review; heparinized cationic solid lipid NPs have been used and shown to be effective for the release of the neurotrophic factor and for promoting iPSC differentiation into neurons. The use of lipid NPs for PNR has not been greatly explored, possibly because their synthesis is time-consuming and not as reproducible as polymeric, magnetic or mesoporous NPs.

Overall, this review provides useful knowledge for the design of NPs that may have an important role on neurotrophic factor release inside the nerve guidance conduits used for PNR, providing more effective nerve regeneration.

Future perspective

Peripheral nerve treatment is still under development, and the field is evolving rapidly to achieve nerve recovery comparable to the use of autografts. The objective is to achieve an accurately designed 3D artificial nerve guidance channel. The biomaterials field is contributing to the improvement of NGCs as more knowledge is being gained, which helps to improve the rational design and synthesis of biocompatible NGCs and conduits. The main area for development should be the optimal conduits, which must mimic the nerve regarding the chemical and physical properties, cannot be degraded, should act as a support for nerve regeneration and fulfill requested mechanical properties such as blending and suturing. In this way, the best material for a biocompatible NGC synthesis should

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be defined to then act on the inner 'decoration' and functionalization of it. In this area, nanotopographical patterns are being studied to provide cues within the hydrogel that help direct axonal growth. If conduits are augmented with an inner, longitudinally aligned pattern, nerve regeneration would be possible, directed from the proximal to the distal side of the injured nerve.

The development of NGCs with compartments can be explored by introducing NPs with different neurotrophic factors that are stimuli responsive and necessary at different stages of nerve regeneration. Also, GFs can be strategically placed at different places of the NGC to have a synergistic effect on regeneration.

Apart from developing delivery systems for neurotrophic factors, research is also focused on other components that can be modulated to enhance nerve regeneration within the synthetic conduit. Cell biology is undergoing rapid advances, such as cell culture techniques and genetic engineering. This can provide new tools to improve nerve regeneration. Interestingly, research regarding peripheral nerve injury treatment is also focusing on electrostimulation of the deinnervated muscle to avoid atrophy; for that aim, electroconductive polymers or optogenetic neurons that can be electrically stimulated are being studied. This would help avoid muscle denervation while nerve regeneration occurs. Several polymers that are being studied for this purpose, and to make blends on the conduit, including poly(3,4-ethylenedioxythiophene) (PEDOT), which is known to be biocompatible. These new approaches need to be combined to achieve proper nerve regeneration and ensure muscle functionality after the regeneration process.

Executive summary

Peripheral nerve injury & recovery

- When nerve transaction occurs after an injury, nerve ends lose communication, and depending on the nerve gap generated, the two ends can be reunited by suturing; in long nerve gaps, they are bridged using grafts.
- Axonal regeneration is mediated by Schwann cells, which organize to form Büngner bands and provide guidance for regeneration.

Nerve guidance conduits

- Several nerve guidance conduits (NGCs) are approved by the US FDA. They are fabricated with natural or synthetic polymers, which offer advantages and disadvantages to the conduits.

Neurotrophic growth factors

- Several growth factors (GFs) are secreted and upregulated by Schwann cells to mediate in the regeneration process of nerves. Research is therefore focusing on their use for nerve recovery after an injury.

Nanoparticles incorporated to NGCs

- Nanoparticles are easy to incorporate into other materials such as NGCs, and several works have combined them with GFs, demonstrating the efficiency of conjugating GFs compared with their 'naked' use.
- The use of polymeric and magnetic NPs has been studied extensively; to a lesser extent, silica and lipid NPs have been studied as well.

Future perspective

- The incorporation of NPs with GFs into conduits is being studied as an approach for nerve repair. Thus, research is also focusing on the development of NGCs that can support axon regeneration and avoid muscle atrophy, which occurs due to denervation when an injury occurs.

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●● **Authors evaluate several combinations of lipid NPs for the release of NGF and the *in vivo* efficacy in differentiating induced pluripotent stem cells (iPCSs).**