Nanotechnology in peripheral nerve repair and reconstruction

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ARTICLE INFO

ABSTRACT

The recent progress in biomaterials science and development of tubular conduits (TCs) still fails in solving the current challenges in the treatment of peripheral nerve injuries (PNIs), in particular when disease-related and long-gap defects need to be addressed. Nanotechnology-based therapies that seemed unreachable in the past are now being considered for the repair and reconstruction of PNIs, having the power to deliver bioactive molecules in a controlled manner, to tune cellular behavior, and ultimately guide tissue regeneration in an effective manner. It also offers opportunities in the imaging field, with a degree of precision never achieved before, which is useful for diagnosis, surgery and in the patient's follow-up. Nanotechnology approaches applied in PNI regeneration and theranostics, emphasizing the ones that are moving from the lab bench to the clinics, are herein overviewed.

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Contents

1. Introduction

Neurological defects are among the most common and demanding clinical situations despite decades of research in the neurological field [1]. The reason for this relies in the complexity of the nervous system functions, structure and anatomy, which makes it more challenging to treat as compared to other tissues in the human body [2].

The nervous system comprises two components: i) the peripheral nervous system (PNS), and ii) the central nervous system (CNS). The PNS consists of the regions of the nervous system outside the CNS (brain and the spinal cord). The PNS includes cranial nerves, spinal nerves and their roots and branches, peripheral nerves (PNs), and neuromuscular junctions. Opposing to the CNS, the PNS is not protected by a hard bone layer or by the blood-brain barrier, making it much more disposed to traumatisms or any kind of injuries [3]. Therefore, PN injuries (PNIs) are considered a huge clinical burden, being the incidence 1 in 1,000 individuals per year [4]. In fact, PNI is associated to $150 billion health-care expenses per year in the USA alone [5]. These costs are though underestimated, since "bed-days" and lack of productivity also account for monetary losses, worldwide. This scenario tends to worsen with the increasing world population and respective average lifespan. Considering those, an additional number of injuries tend to appear and consequently a high number of treatments and surgeries will be required to allow the restoration of the damaged nerves.

In this scope, nanotechnology and nano-based materials have attracted a significant amount of interest from the scientific and medical community. These types of materials are considered a reliable alternative to tackle the main hurdles in PN regeneration (PNR). Although the CNS is vastly protected and therefore less prone to injuries, it has a limited ability to regenerate because of the succeeding scar tissue development which can be created by a vast range of cell types, such as fibroblasts, neuroglia, monocytes, and endothelial cells [6]. In contrast, PNIs are considerably more common, but the PNs have a greater regeneration potential when compared to the nerves of CNS. This is because PNS glial cells, i.e. Schwann cells (SCs), tend to adapt to a regenerative phenotype and have the capacity of triggering neuronal regenerative processes, although usually slow and in a partial manner [7]. Given to their exposure, PN damages can be caused by many types of events, such as traumatic injuries, complications on surgeries, congenital defects and war wounds. Concerning the traumatic injuries, they can also vary significantly and include penetrating injuries, crush, traction, ischemia, and less common mechanisms such as thermal, electric shock and radiation [8]. Another common injury mechanism is compression, which may involve mechanical deformation, as well as ischemia. A vast range of diseases can also be the root cause of PNIs, as is the case of diabetic peripheral neuropathies [9,10].

In respect to the type of injury, small and long gaps must also be differentiated. This concerns the severity of the injury, as well as the outcome and the degree of recovery for the patient.

In humans, for small gaps, which usually measure less than 30 mm, hollow lumen conduits can be used with rather good outcomes. However, reconstruction of gaps longer than 30 mm still remains a challenge nowadays. Nerve autografts and allografts still remain the clinically available options and the ones that provide the best chances of recovery [11]. However, when considering long gaps, most of the barriers to recovery remain unsolved, namely the slow regeneration rates and specificity of target innervation, which means that the distal stump is too far from the proximal. The degeneration of the target end-organ is another obstacle to a proper and functional regeneration [12]. Therefore, the challenge presented by long gaps is the reason why innovative nanotechnology-based strategies are sought to be a good technological possibility. Fig. 1 summarizes the main possible applications of nanotechnology approaches in an attempt to tackle some of the hurdles of PNIs.

Despite those seemingly good chances of recovery when using autografts, incomplete recuperation from PNIs usually lead to multiple negative consequences, which comprise numbness of affected members, chronic pain, diminishing of sensory and/or motor function and a disturbing permanent disability of the patients. It is a fact that these outcomes are unsatisfactory for the demands of today's patient lives, since only 25% of patients regain proper motor function and less than 3% recuperate sensation in a full extent [13]. The outcome is a major loss of patient's quality of life, both physically and mentally, and considering the above-mentioned facts, it is urgent to visualize PNIs as a worldwide pertinent clinical problem [14]. Given the stated importance of PNI, the use of nanotechnology to help address and solve some of the challenges posed to clinical management of PNI is a field worthy to be explored and properly funded, and certainly will growth in the coming years.

2. PNIs: Fundamentals, current treatment options and challenges

In order to make a preliminary evaluation, followed by a precise diagnosis and proceeding with the proper treatment of nerve injuries, it is imperative to have plain knowledge of nerve anatomy (Fig. 2), as well as the neurobiological mechanisms that occur immediately after injuries. That is of utmost importance, since strategies to improve the outcomes following nerve injuries are often based on such biological mechanisms [15].

The PNS is composed of motor and sensory neurons. Their cell bodies can be found within the spinal cord and their elongated cytoplasmic extensions, called axons, link and transmit the signals to the distant corresponding organ [5]. Fig. 2A schematically represents the anatomy of the peripheral nerve. Anatomically, each individual axon is protected by the

Fig. 1. Nanotechnology applied to peripheral nerve regeneration and reconstruction. Carbon nanomaterials, nanoparticles, nanofibers, nano-topographic cues and theranostics and nano-imaging are some of the areas in nanomedicine that should are being explored in order to improve tissue biofunctionality.

endoneurium, a layer of collagen and elastic elements. A group of endoneurium protects axon groups into nerve fascicles, which are sheathed by the perineurium, mainly composed of connective tissue. Finally, several fascicles are gathered together by the epineurium. External to this layer is the mesoneurium, containing the blood supply to the nerve. A fine network of capillaries exists at the endoneurial level [1,5,16]. Any break or defect in this stratified structure fallouts in a programmed and permanent cell death, unless rapidly and thoroughly reestablished [17,18].

The several degrees of injury to PNs are detailed in Table 1, which were firstly described by Seddon [19], and later by Sunderland [20]. The Seddon classification is divided into three categories according to the gravity of the injury: (i) neurapraxia, (ii) axonotmesis, and (iii) neurotmesis. By its turn, Sunderland classification comprises five different categories: first, second, third, fourth and fifth degree.

There are also three different types of PNs that should be considered for reconstruction after an injury: (i) the motor neuron, (ii) the sensory neuron, and (iii) the mixed PNs [21]. Although all the three types of nerves are essential to carry a normal lifestyle, the motor and the
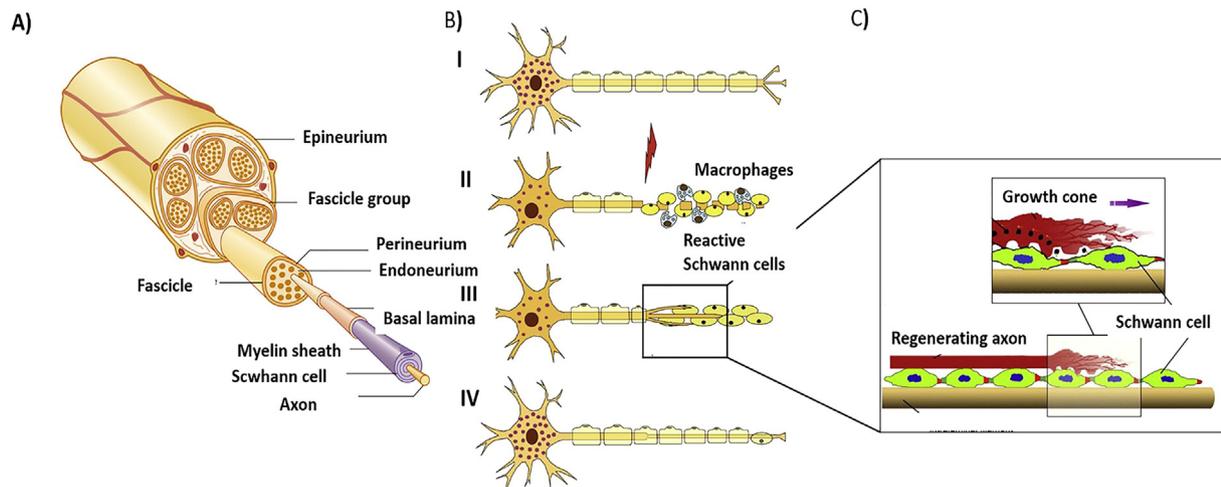
Fig. 2. Schematic representation of the anatomy of peripheral nerve and degeneration and regeneration after an injury. (A) Scheme of peripheral nerve anatomy); (B) Degeneration and regeneration after peripheral nerve injury. (I) Normal neuron; (II) Wallerian degeneration. Schwann cells proliferate, and macrophages invade the distal nerve segment and phagocytosis of degrading materials; (III) Schwann cells in the distal end align in bands of Bungner; and (IV) Axonal re-joining with distal end and organs; and (C) Axonal sprouts advance entrenched with aligned Schwann cells. Reprinted and adapted from (A) [351]; (B) [18]; (C) [14].

Table 1
Seddon and Sunderland classifications of nerve injuries according to the pathophysiology of the nerve.

Seddon classification	Sunderland classification	Process	Degree of recovery
Neurapraxia	I	Local myelin damage usually secondary to compression	Full recovery
Axonotmesis	II	Axon severed but endoneurium intact (optimal circumstances for regeneration)	Full recovery
	III	Axon discontinuity, endoneurial tube discontinuity, perineurium and fascicular arrangement preserved	Wallerian degeneration, recovery incomplete
	IV	Loss of continuity of axons, endoneurial tubes, perineurium and fasciculi; epineurium intact	Wallerian degeneration, recovery incomplete
Neurotmesis	V	Complete physiologic disruption of entire nerve trunk	Wallerian degeneration, recovery incomplete

mixed type of nerve are the ones that have major clinical importance in the reestablishment of the movement of the limbs. However, up to date, mostly sensory nerves are involved in the repair strategies due to easier harvesting, and relatively lower donor-site morbidity.

Immediately after injury, the regeneration process of PNs runs in sequenced phases and different phenomena can occur at different levels on the injury site (Fig. 2B), encompassing both proximal and distal sites [22]. In the proximal position, separated axons and cell bodies degenerate via a programmed cell death pathway called chromatolysis [23]. In the distal injury end, a process called Wallerian degeneration occurs at 24 to 48 h after injury and all nerve components, such as distal axons and adjacent myelin starts to degenerate [24]. The goal of that phenomenon is related to the clearance of undesired debris. SCs phagocytize axonal and myelin debris, until only empty endoneurial tubes remain. SCs, which are the ensheathing glial cells of the PNS, are crucial for normal nerve function, nerve repair and constitute 90% of nucleated cells within PNs [25,26]. After debris removal, they fill the empty endoneurial tubes and organize in characteristic bands or tubes of Bungner, and by this mean supporting the re-growth of axons. Not only SCs have a crucial role, but also macrophages are recruited to the area releasing growth factors and cytokines. They will stimulate SCs and fibroblast proliferation and are responsible for the axonal regeneration process [27]. Ahead in the process, in the proximal injury end, a growth cone emerges following the path formed by the band of Bungner, which is of fundamental importance for the advance of the regenerating axon (Fig. 2C) [28].

Considering the current state-of-the-art on nerve pathophysiology, only minor advances in medical and operating techniques have been made in the past years. Epineural repair remains the preferred surgical reconstruction when it is accomplished by direct tension-free suture of both well-vascularized nerve ends [29]. Whenever that approach is not possible, due to excessive tension caused by elongating the nerves, and a gap remains, these lesions do not spontaneously regenerate. In such cases, autologous nerve grafts (ANG) become the gold standard treatment for nerve restoration [30]. However, ANG cannot guarantee an absolute functional outcome for the patient. Problematic consequences, such as unavailability of appropriate-sized nerves and donor site morbidities, sensibility abnormalities and painful conditions are also frequently associated to this type of approach [30].

An alternative option to use in nerve transplantation is cadaveric allografts [31]. However, like ANG, there are pros and cons regarding the use of allografts. The selection of the type of graft is based on the patient's needs and typically it involves a systemic immunosuppression therapy in the patient for long periods of time [32].

For these reasons, increasing efforts have been made over the last decades in the search for effective alternatives to ANG. Tissue engineered tubulization has been a widely explored alternative to bridge the nerve gap and throughout the years many types of nerve guidance conduits (NGC) were proposed, being some of them already approved by the Food and Drug Administration (FDA) [33,34]. Since mature neurons are not susceptible to mitosis phenomena, it is crucial to support the re-growth of the existing cell bodies, providing a protective environment and guiding paths. In this way, it is possible to direct

axons from the proximal do the distal site, permitting the proper linking of the damaged synapses connections. In brief, the protection of the injury site and performance as a guidance substrate are the two main reasons why tubulization is used in PNIs.

In the early use of conduits made of synthetic materials in the clinics, they were mainly composed by silicon tubes and could only repair injuries up to 10 mm. Some disadvantages on the use of that conduits included total lack of biodegradability, which led to inflammation and chronic foreign body reaction, as well as lack of swelling capacity, which would compress the nerve, thus hindering the regeneration process [35]. In order to overcome such difficulties, biodegradable nerve guidance conduits have been proposed, some of which are FDA-approved and being currently used in the clinical setting [34]. Nevertheless, despite the major advances achieved with the use of silicon conduits, most NGCs are hollow tubes and can only repair short nerve gaps.

In 2014, however, there was a breakthrough regarding this subject. The first conduit integrating a luminal filler acting as three dimensional (3D) guiding and supporting scaffold was approved. NeuraGen® 3D nerve guide matrix is composed by collagen I, whereas the luminal filler comprised of collagen I and glycosaminoglycan chondroitin-6-sulfate, with aligned porosity acts as a topographical cue for regenerating axons. In vivo data demonstrated significantly improved regeneration as compared to an empty conduit [36].

In the past decades, numerous NGC made of both synthetic and natural biomaterials have been described. Regarding the synthetic materials, these are still considered very promising since the majority of the FDA-approved nerve guidance conduits are composed of materials such as polyglycolic acid (PGA), polylactic-co-glycolic (PLGA), polycaprolactone (PCL) [34]. Polyhydroxybutyrate (PHB) has been reported to have neuroprotective features and support axons regrowth [37]. Among the natural-based biomaterials, polysaccharides such as chitosan (Cht) [38,39] and alginate [40] have been also considered as promising materials due to their worthy results in the scope of PNR. Still among the natural-origin biomaterials, extracellular matrix (ECM) endogenous proteins such as collagen [41], fibrin [42], laminin [43,44], and hyaluronic acid (HA) [45] have been highly investigated, since they all naturally exist in the human body.

Not only the type of biomaterial used in the NGC is important, but its architecture is also an important feature. There is still no consensus in the scientific community on whether the conduit should remain empty [46] or filled with any kind of materials [47], such as hydrogels [48], nanofibers [49] or films [50]. An empty lumen has the advantage of allowing free space for nerve regeneration, so that the axons may selectively re-innervate the appropriate target. Conversely, a lumen filled with any type of topographical and biological cues can provide a supporting structure that may favor cells ingrowths and guidance [1,51].

Apart from the use of NGC and fillers, systemic or localized delivery of neurogenic, anti-inflammatory and pro-angiogenic factors should be ideally performed. Among those, cytokines, drugs or stem cell therapies can be considered in order to enhance the neurofunctional outcomes [52]. It is important to point out that these strategies work by different mechanisms and distinct biochemical pathways. Therefore, a proper

combination of a promising NGC, guiding fillers, with cellular therapy and localized delivery of factors will most definitely match the results usually obtained with ANG. Although PNS has a certain ability to regenerate, other physiopathological phenomena impair nerve regeneration and these are presented as the great challenges that need to be overcome to succeed in PNR. Also, most of the strategies being developed are focused on the specific injury site [53]. Other scientific solutions should be sought in order to address problems that emerge after the injury itself and focus on maintaining distal axonal and muscular target integrity. Also, it must be guaranteed the survival of cell bodies in the proximal site, occurrence that remains largely unexplored and would essentially contribute to maintain the natural physiology after injury.

Therefore, other therapies or approaches should be taken in consideration to address simultaneously what happens in the nerve gap itself, but also, upstream and downstream of that location. This is where novel nanotechnologies, including for instance, nanoparticles (NPs), nanoimaging and carbon nanomaterials start to take a key role in cutting-edge approaches that might be able to solve problems that could not be addressed with traditional methods. Taking a closer look to some nanotechnologies, one can immediately understand their potential in PNR. In one hand, the refinement of NGC's technologies lies in the incorporation of nanotechnological techniques. On the other hand, nanotechnological approaches can be used on their own [54].

Because of their properties and competences, NPs have an immense broad of applications in PNR. They can not only carry every type of growth factor or biomolecules of interest to the desired place but can also deliver it with the appropriate kinetics for an optimal result. This has been a problem that scientists have been trying to address for years, since mimicking the natural events by the correct delivery of biological factors is crucial for an efficient regeneration [55]. Further than that, they comprise the basic nanoimaging tools, such as MRI (magnetic resonance imaging) allowing visualization techniques never achieved before in the clinics [56]. Carbon nanomaterials, such as carbon nanotubes, are another exciting example of multiple-advantageous materials for PNR, encouraging the scientists to achieve goals that take PNR to another level. Since PNS relies on electric conduciveness, not only can they be used for coating electrodes for interfacing neurological prosthesis, but they can simply integrate tubular conduits making them also electrically conductive [57]. In addition to providing a permissive substrate for neuronal growth, patterned carbon nanotubes substrates are also able to control the direction of neurite outgrowth and participate in the organization of neural networks, as the misdirection of growing axons is still an intriguing problem for surgeons and scientists.

First of all, engineering a NGC should aim at facilitating cellular spreading and growth of damaged nerve tissues in 3D [58]. Other than that, it is crucially important that the material envisioned to be used to construct the NGC is cytocompatible and has pronounced biomechanical properties. If an engineered NGC does not present a proper cytocompatibility, may not contribute to the growth of damaged nerves, but would instead be the reason of acute inflammation and even infection [59]. It must exhibit good biocompatibility with low inflammatory and immunogenic reactions [14]. It must also be biodegradable, and ideally degrade in the same rate as nerve regenerates. Otherwise, a quick degradation might trigger an inflammatory response [58,60]. Regarding the mechanical properties, the NGC should provide sufficient mechanical strength to prevent the NGC rupture during the patient's movements and physically support neural tissue regeneration. Concurrently, the NGC should have appropriate elasticity to be able to lessen tensions in the damaged area [59]. Furthermore, the materials used to construct NGC should prevent the migration of fibroblasts that will lead to the formation of glial scar tissue around the implant, which could reduce the healing chances [61].

The permeability of a conduit is also an important parameter to consider in the TCs design as both nutrients and oxygen must diffuse into the site of regeneration. Otherwise, cells inside the conduit, especially if it is a long conduit, will be under a deleterious ischemic environment

which can result in cellular hypoxia and lack of proper nutrients. Ideally, electrical conductivity would be preferred for a NGC used in neural tissue engineering (TE) in order to mimic the electrical properties of nerves and at the same time excite the neuron communication [62]. Succinctly, an ideal NGC should be biocompatible, biodegradable, flexible, compliant, porous, neuroconductive and with suitable surface and mechanical properties. However, even if all these criteria were successfully gathered in a developed NGC, there are some complications that are difficult to avoid and that reduce the chances of regenerative success. This section unfolds some of the most important physiopathological phenomena (e.g. inflammatory response, fibrosis or scar tissue formation, and lack of proper vascularization) that prejudice PNR and should be addressed to achieve a successful nerve recovery as overviewed in Table 2. A schematic of such events can also be seen in Fig. 3.

Neuroinflammation is an important phenomenon in nerve degeneration and consequent regeneration and is intrinsically associated with PNIs. It is a lively and active process that changes throughout the sequence of recovery. Both the timing and grade of inflammation will either result in the improvement or obstruction of the regeneration process. This process also significantly varies between individuals, which makes it harder to control [63].

The immune system plays a critical role during nerve regeneration and an inflammatory response is always involved after an injury. As SCs release cytokines and chemokines, different immune cell types are recruited to the critical place in the first hours after the traumatic event, including granulocytes (neutrophils and mast cells) and agranulocytes (monocytes/macrophages and lymphocytes) [50,64]. Controlling the inflammation that occurs in the neurodegenerative scene is a highly complex problem that requires patient-specific interventions. Recently, investigations in the area of nanotechnology have been trying to address that problem [65]. Considering the neuroinflammation process as something to take advantage of, and by marking macrophages with specific NPs, one could retrieve information about the biodistribution of drugs, treatment efficacy and assessment on possible

Table 2
Phenomena prejudicing peripheral nerve regeneration.

| Phenomena impairing PNR	Related features	Cells/molecules involved
Inflammatory response	- Inflammatory response is always involved after an injury - Timing and inflammation severity l influence regeneration process - Process varies between individuals	- Macrophages that can be either M1 (anti-inflammatory) or M2 pro-inflammatory - IL10 (anti-inflammatory cytokine)
Fibrosis or Scar tissue formation	- Intraneural fibrosis will prevent the passage of axons from the proximal segment to the distal segment- Extraneural fibrosis limits the physiological movement of nerves during the patients' movement - Excessive fibrosis results in painful adhesions	- Fibroblasts (fibrotic tissue deposition) - IL-10 (support scarless healing)
Lack of vascularization	- The relationship between vascularization and neural TE has been recognize as early as 1990 - Lack of vascularization leads to ischemia and consequent cell death by lack of oxygen and nutrients	- VEGF - Macrophages
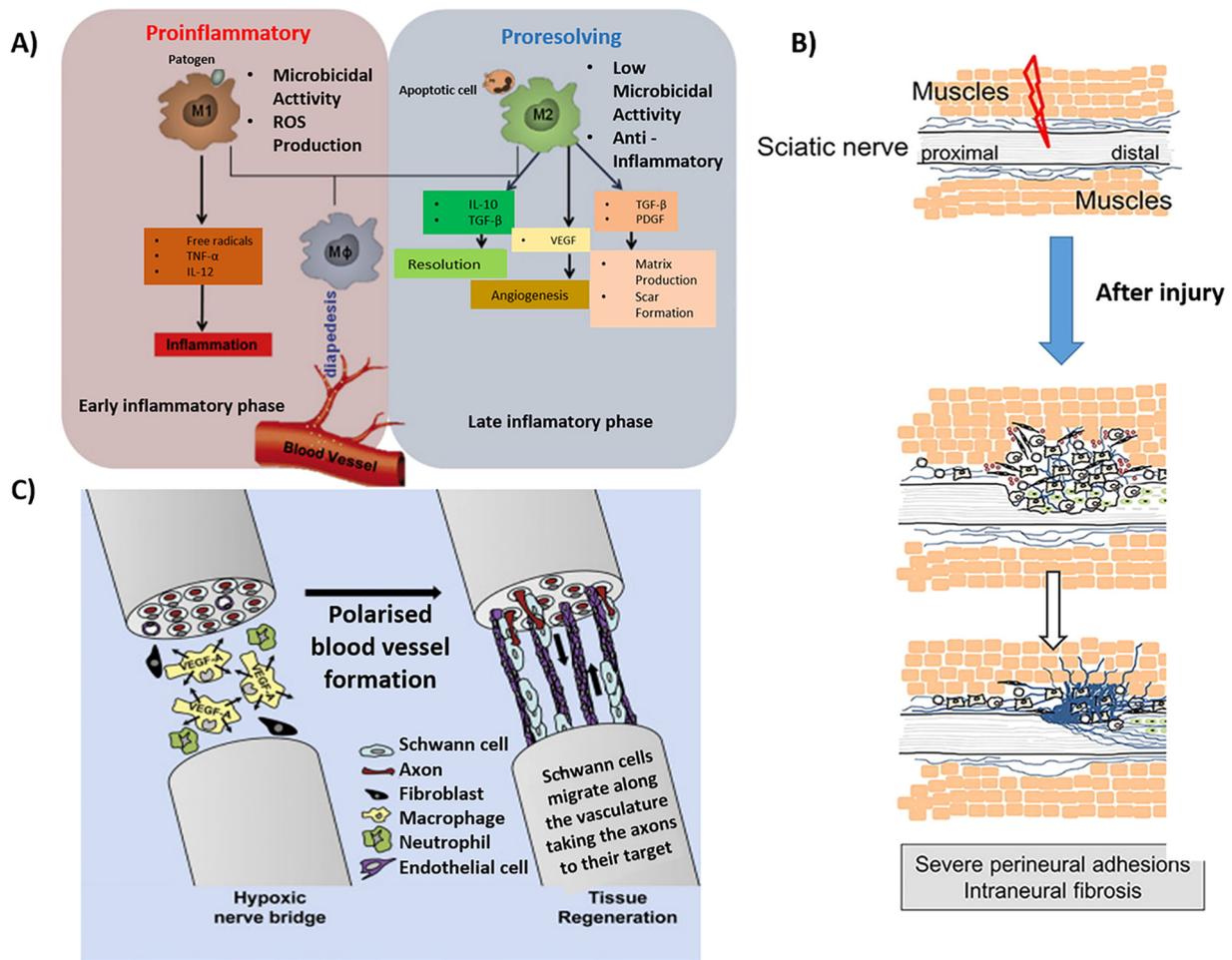
Fig. 3. General phenomena impairing peripheral nerve regeneration, such as inflammation, excessive scar tissue or lack of vascularization. (A) Macrophage plasticity in tissue repair, where M1 pro-inflammatory macrophages can change phenotype into M2 pro-regenerative macrophages; (B) After injury, fibroblasts that are attracted to the injury site deposit excessive collagen that leads to perineural adhesions and intraneural fibrosis; and (C) Macrophage-induced blood vessels guide Schwann cell to cross the nerve gap. Reprinted and adapted from (A) [67]; (B) [320]; (C) [76].

toxicity in a real-time manner, with consequent improved therapeutic intervention.

Along with SCs, macrophages play the most crucial role after an injury, intrinsically connected to Wallerian degeneration. These cells can assume two phenotypes (Fig. 3A): (i) pro-inflammatory, being designated as M1, and (ii) anti-inflammatory/pro-healing, being in this case designated as M2, phenotypes, which change during the course of the injury [66,67]. However, endogenous mechanisms exist that intend to control PNI inflammation, such as the release of cytokine interleukin 10 (IL-10) [68]. Also, SCs remyelination of regenerated axons stimulates macrophage removal. Still, these mechanisms are not efficient enough and prolonged or chronic inflammation becomes deleterious and a challenge during PNR [69].

Fibrosis or scar tissue formation around the implant is another expected and natural occurring event after an injury [70]. However, depending on its extension, it might be critical for nerve regeneration since excessive deposition of ECM and consequent remodeling lead to the formation of permanent scars. Fibrosis can hinder regeneration in two ways: (i) intraneural fibrosis will prevent the passage of axons from the proximal segment to the distal segment, and (ii) extraneural fibrosis limits the physiological movement of nerves during the patients' movement, leading to pain and functional limitation (Fig. 3B) [71]. Moreover, it was reported that IL-10 supports scar less healing in postnatal tissue, acting as an adjuvant in combating the excessive fibrosis formation that prevents the correct process of regeneration [72].

Lack of proper vascularization, i.e. the deficiency of angiogenesis process in the injured site, has lately been considered as one of the main factors limiting nerve regeneration. The relationship between vascularization and neural TE has been recognized as early as 1990 [73]. After nerve reconstruction, the nerve is subjected to ischemic conditions, which potentially alters the regenerative environment and outcome across the bridge or graft [74]. Tissues subjected to prolonged ischemia are associated to abundant pathological processes, including cell damage and dead, due to the lack of oxygenation and nutrients' supply. This has been considered an explanation for the poor regenerative outcomes of engineered grafts, since a delayed re-vascularization has been observed, in comparison to normal autografts that re-vascularize much faster [75]. However, the beginning of the regeneration process (e.g. Wallerian degeneration and SCs arrangement in band of Bungner) is initiated by hypoxia [76]. Such phenomena are selectively sensed by macrophages that start to secrete vascular endothelial growth factor (VEGF), inducing a polarized vasculature that relieves from hypoxia. SCs then use the blood vessels as physical guiding cues to migrate through the gap and bring regenerating axons along the way (Fig. 3C).

3. Nanotechnology approaches for functionalization of tubular conduits

With the rising application of nanotechnology in every sector of our daily lives, the demand for the development of novel nano-scaled

(<100 nm) approaches is growing at an incredibly rate [77]. The use of nanotechnology and nano-based materials is widely spread in a variety of sectors, such as healthcare [78], electronics [79], cosmetics [80], agriculture [81] and the continuous improvement and innovation in the field is perceived as an extraordinary opportunity for significantly improve treatment and diagnosis of PNIs [82]. Thus, the application of precisely engineered nanomaterials that can be used in all stages of illness: prevention, diagnosis and therapy, including neurological pathologies is now attracting a great deal of attention [83]. The ultimate advantage of nanomaterials applied in medicine is based on their ability to interact with biological systems, which takes place at a molecular level with a high degree of specificity. This translates on the ability of such materials to migrate through cell membranes, amplified solubility, steadiness and bioavailability of biomolecules, thereby enhancing their delivery competence [83].

Therefore, besides the incorporation of nanomaterials in scaffolds with the aim of boosting their properties, the application of nanomaterials alone can be done for bioimaging [84] and targeted delivery of desired specific molecules into cells [70]. Another major advantage of nanomaterials is their nano-roughness resemblance to ECM [85]. In this sense, nanomaterials reproduce what happens at a nano-scale level in the human body, being therefore regarded as the ultimate biomimetic materials.

Although, nanotechnology has played a huge role in PNR in recent years, both problems of short and long gaps are being simultaneously addressed by means of developing advanced nanosystems (e.g. aligned nanofibers [86,87] or carbon nanotubes [88–90]) that can act as guiding cues and direct and stimulate the correct re-growth of axons. Advances in nanomedicine field applied to PNR are expected to have a major impact in the patient's recovery and quality of life, since novel therapeutic strategies are evolving and reflecting the interdisciplinary and integrated treatment strategies that bring together nanotechnology and TE [91].

Among the variety of available nanomaterials existing and the hundreds of thousands of publications in the area, the most promising works reporting on nanomaterials and offering remarkable advances in the field of PNR were selected and are reviewed herein.

3.1. Carbon nanomaterials

Carbon nanomaterials were projected as encouraging candidates for many industrial and technical purposes many decades ago. Worldwide commercial interest in carbon nanomaterials is reflected in its extreme production every year. Diverse commercial products ranging from batteries, motorized components, sporting goods, any kind of structural material and even agricultural products have nanomaterials in their composition now-a-days [92,93]. Only after that initial burst in other areas, their potential for biomedical applications was realized [94]. That was due to their great properties that were envisioned as very useful when combined with other biomaterials in TE approaches [95].

Carbon nanostructures were proposed as promising candidates to develop neural scaffolds. When considering neuronal regeneration, electrical stimulation, as well as appropriate electrical conductivity of the applied biomaterials, have been considered one main advantage since the natural PNS is capable of easily transmitting electrical impulses [96,97]. Furthermore, carbon materials are mainly composed of the very basic element existing in all things, e.g. carbon. Currently, the best-known carbon nanostructures belong to four different categories: carbon nanotubes (CNTs), graphene, carbon nanofibers (CNFs), and nanodiamonds (ND). Fig. 4 depicts some of the most interesting results concerning the section of carbon nanomaterials, which will be discussed in depth.

3.1.1. Carbon nanotubes

Carbon nanotubes (CNTs) represent one of the most studied allotropes of carbon. CNTs are sheets of graphite rolled into cylindrical tubes, built from a hexagonal arrangement of sp^2-hybridized carbon atoms in nano-scale dimensions and were first introduced by Iijima [98]. They can be mainly divided in two categories depending on the number of shells: single-walled carbon nanotubes (SWCNTs), with diameter ranging between 0.8 and 2.0 nm and multi-walled carbon nanotubes (MWCNTs), with diameter ranging from 2.0 and 100 nm. Scheme of such CNTs can be seen in Fig. 4AI.

There are numerous methods for CNTs production, that mainly include electric discharge [98], laser ablation [99] and chemical vapor deposition [100]. Some of the exciting features of CNTs include their extreme mechanical, thermal, magnetic, optical, electrical, surface, and chemical properties. The combination of these characteristics enlarges the range of possible biomedical applications where they can be applied [101]. As previously mentioned, besides being in the nano-scale range, the most attractive feature of such materials relies on their ability to display metallic and superconducting electron transport properties. However, original CNTs do not own the necessary solubility for being used in biomedical applications. Therefore, it is critical to functionalize CNTs not only to make them more soluble, but to allow their integration into many organic, inorganic, and biological systems and applications. Two main strategies have been identified to allow the application of CNTs under physiological conditions, which rely on covalent and noncovalent functionalization [102]. With such modifications, CNTs can be used in several biomedical applications, such as drug delivery, diagnostics, biosensors, biomedical imaging, as well as TE and regenerative medicine.

The diameter of an individual CNT (1–100 nm, depending on the number of walls) is comparable to that of a single protein complex, such as a microtubule (25 nm diameter), which forms the basic structural element of the neuronal cytoskeleton [103]. However, these carbon nanomaterials have many extraordinary features to be applied in neuronal repair, namely: (1) are chemically stable and inert, being also biocompatible due to their carbon composition; (2) have nanoscale topography mimicking neural tissues (e.g. neurites); (3) enable intracellular permeation for delivery of biomolecules; (4) interact with neuronal cells, i.e. by means of promoting cell attachment, differentiation and growth by overall supporting neurite elongation and branching being internalized by neuronal cells, as PC12, Schwann or SH-SY5Y and to guide them; (5) enable to expose neuronal cells to different kinds of stimulus, according to the properties of the applied carbons materials; (6) are cell-friendly allowing neuronal cells adhesion and proliferation; and (7) have bulk electrical properties in accordance with native neuronal tissues [101,104].

The exact role of CNTs in PNR have not been defined yet but is mainly related to enhancing axon growth rates via electrical stimulation and contact guidance cues, as well as providing structural reinforcement of NGCs. Also, CNTs could be used for localized delivery of growth-promoting molecules [105].

The first report on the application of CNTs in the field of neuronal research was published in 2000 by Mattson et al. [106]. In that work, embryonic rat-brain neurons were grown on MWCNTs and it was observed that on unmodified nanotubes, neurons extend only one or two neurites. By its turn, nanotubes coated with the bioactive molecule 4-hydroxynonenal, allowed neurons to develop multiple neurites and display extensive branching. These findings establish for the first time the feasibility of using CNTs as substrates for nerve cell growth.

Ever since, several works have already demonstrated the significant and profound effects of CNTs in PNR, opening the path for further research [105,107–111]. In Table 3, the main uses of CNTs in PNR are summarized [88–90,112–120].

In 2014, Roberts et al., [112] have produced horizontally aligned carbon nanotubes (HACNT) on both film substrates and flat glass coverslips. The HACNT substrates were found to support the growth of primary embryonic rat motor neuron for up to eight days in serum-free culture. Importantly, major neurites were more likely to be oriented
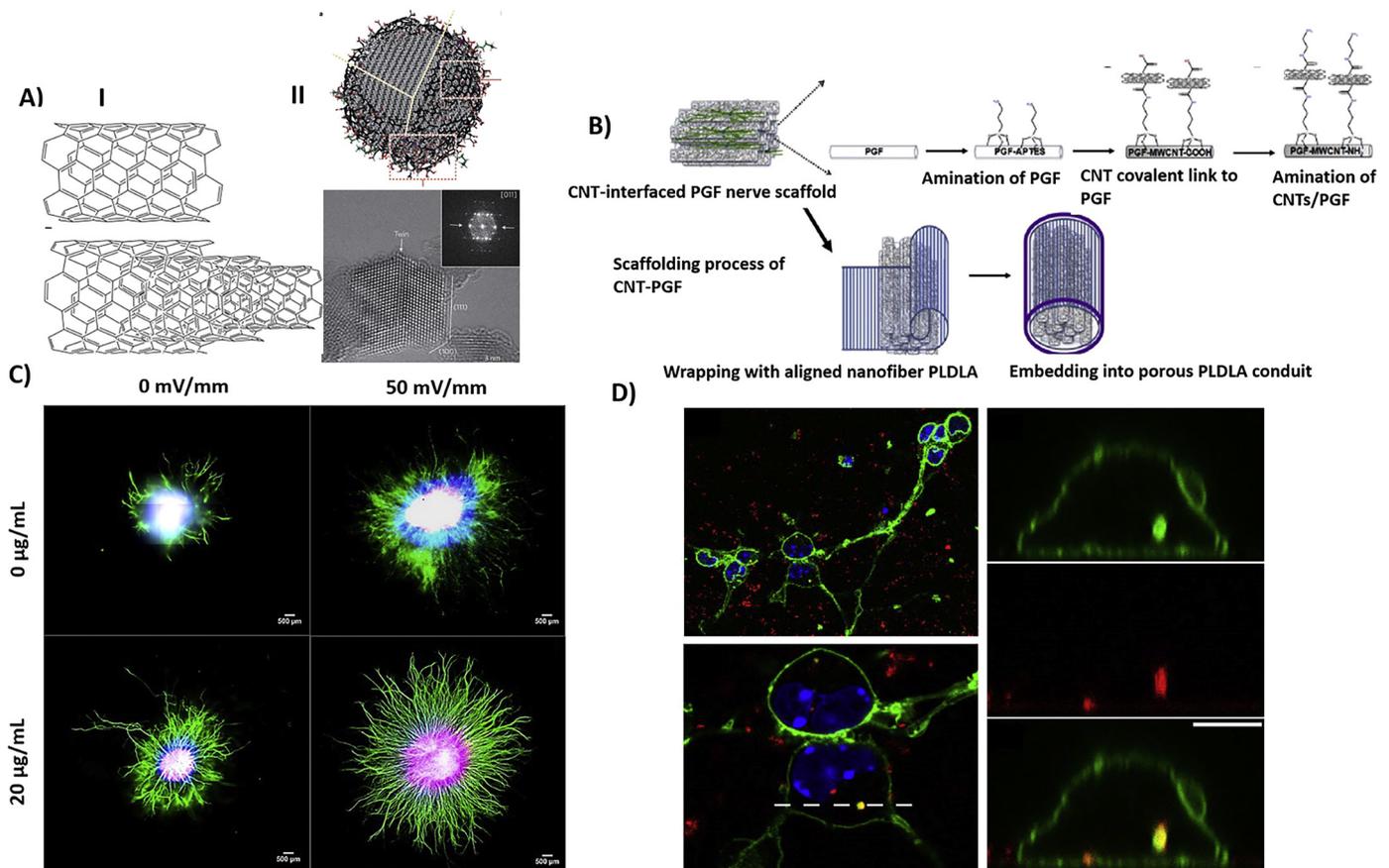
Fig. 4. – Carbon nanomaterials structure and their application of in peripheral nerve regeneration. (A) (I) Single and multi-walled carbon nanotubes schematic structure. (II) Nanodiamond schematic structure. (B) Schematic representation of the aligned PGF bundle interfaced with carbon nanotubes for neurite outgrowth. (C) DRGs neurite outgrowth can be promoted by 50 mV/mm electrical stimulation or inclusion of 20 μg/mL SWCNTs. The occurrence of both has a synergistic effect. (D) Fluorescent nanodiamonds internalized into dissociated neurons. Confocal image of dissociated mouse cortical neurons cultured in the presence of 20 μg/mL fluorescent Nanodiamonds, stained with Alexa Fluor 488-conjugated concanavalin-A and DAPI. Reprinted and adapted from (A) (I) [352] and (II) [151]; (B) [89]; (C) [115]; (D) [158].

parallel to the aligned CNTs. In the same year, Yu et al., [88] combined the superior properties of MWNTs with collagen/PCL nanofibers produced by electrospinning. The aim was to evaluate the mechanical effects and efficacy of MWNTs on the electrospun nerve conduit, as well as their biocompatibility and toxicology when applied in vivo. Their studies revealed that carboxyl MWNTs could greatly alter the nanofibers' physicochemical properties, such as hydrophilicity, mechanical properties and degradability. The carbon materials were also able to support SCs adhesion and elongation in vitro. In in vivo studies, MWNT-enhanced collagen/PCL conduits were shown to effectively promote nerve regeneration of sciatic nerve defect in rats and prevent muscle atrophy without promoting body rejection or serious chronic inflammation, evidencing the potential of this biocomposite NGC.

In 2015, Gupta et al. [90], achieved the challenging task of aligning MWCNTs in a Cht scaffold, producing an anisotropic conductive MWCNTs-Cht composite scaffold fabricated using electric field alignment technique. Not only the mechanical properties greatly improved with the incorporation of 0.5 wt% of aligned MWCNTs, but electrical conductivity increased 100,000 times along its direction. When tested in vitro, 50–60% of HT-22 hippocampal neurons were aligned in the MWCNTs alignment direction of the scaffold with increasing viability over time. With the same materials, Nawrotec et al., [113] developed a new straightforward method for obtaining Cht–CNT implants enriched with calcium ions in the form of tubular hydrogels. Both SWCNTs as well as MWCNTs can be incorporated by an electrodeposited phenomenon. Additionally, hydroxyapatite was added once it has been proved to increase neuronal growth due to the increase of calcium

concentration. L929 fibroblasts and mHippoE-18 hippocampal cells were tested in pro-inflammatory tests to reveal the cytocompatibility of such NGC intended for PNR.

Both Cht and chitin have been proposed as components of composites containing CNTs, despite their poor solubility. Wu et al., [114] developed chitin/CNTs composite hydrogels by dispersing CTNs in chitin previously dissolved in sodium hydroxide/urea aqueous system. The resulting hydrogels exhibited nanofibrillar network with outstanding mechanical properties. Three different concentrations of CNTs were used, being the 5 wt% the most successful concentration showing promising results in terms of biocompatibility when using PC12 and RCS96 cells.

In the work of Ahn et al., [89] aminated CNTs were chemically linked onto the surface of aligned phosphate glass microfibers (PGFs), as shown in Fig. 4B. Then, CNT-interfaced PGFs (CNT–PGFs) were successfully placed into 3D poly(L/D-lactic acid) (PLDLA) porous tubes, by wrapping the CNTs-PGFs onto a PLDLA nanofiber mesh and embedding in a porous PLDLA tube afterwards. This composite scaffold, although effective in restoring motor functions as observed by electrophysiological studies, it did not provide better conditions than those of ANG.

Koppes et al., [115] also envisioned to use SWCNTs to manipulate the bulk electrical properties of a collagen type I-10% Matrigel™ composite hydrogel. The researchers tested the effect of the presence and absence of SWCNTs, as well as the beneficial effects of electrical stimulation or the lack of it. In fact, electrical stimulation and SWCNTs-loaded biomaterials resulted in a 7.0-fold increase in outgrowth relative to the unstimulated, nanofiller-free controls, as seen in Fig. 4C.

Table 3
The use of carbon nanotubes applied to peripheral nerve regeneration.

Type of carbon nanotube	Cell type and/or animal model	Main results	References
MWCNTs	Primary embryonic (E15) rat Motor Neuron	Major neurites are more likely to be oriented parallel to the aligned CNTs	Roberts et al. [112]
Nanofibers reinforced with MWCNTs	Schwann cells; sciatic nerve defect in rat	*In vitro* support of Schwann cell adhesion and elongation and *in vivo* promotion of sciatic nerve regeneration in rat	Yu et al. [88]
Chitosan-aligned MWCNTs composite	HT-22 hippocampal neurons	Improvement of mechanical properties, electrical conductivity and *in vitro* alignment of hippocampal neurons	Gupta et al. [90]
Chitosan-MWCNTs	L929 fibroblasts and mHippoE-18 hippocampal cells	Both SWCNTs and MWCNTs can be incorporated with an electrodeposited phenomenon and cytocompatibility was proved between these materials and cells	Nawrotek et al. [113]
Chitin/CNTs nanofibrilar structure	PC12 cells and RCS96 cells	Three different concentrations of CNTs were used, being the one with 5 wt% the most successful one. PC12 and RCS96 cells were used to test the biocompatibility with promising results	Wu et al. [114]
CNT-interfaced Phosphate Glass Fibers	PC12 cells, DRGs; sciatic nerve defect in rat	Carbon nanotubes were successfully interfaced on phosphate glass fibers for nerve guidance and then implemented into a 3-D scaffold which possessed physicochemical integrity with good cell viability and neuronal interactions	Ahn et al. [89]
SWCNTs in collagen type I-10% Matrigel™ composite hydrogel	DRGs	Presence of SWCNTs was beneficial due to increased electrical conductivity	Koppes et al. [115]
Nanocomposite where MWCNTs, were dispersed in a poly-L-lactic acid matrix	SH-SY5Y cells	The combination of the nanocomposite scaffold and such peptides proved to synergistically boost neuronal differentiation of SH-SY5Y	Scapin et al. [116]
Poly-PEDOT and MWCNTs coated on the electrode surface	Coated and un-coated electrodes were implanted on rats DRGs	*In vivo* model involving stimulation of DRGs Coated electrodes revealed healthier neurons after stimulation	Kolarcik et al. [117]
Gold-CNTs electrode	Electrode place in rat sciatic nerve	Amongst the several formulations, the electrode made of gold-CNTs has significant lower impedance and dramatically increases the signal recording resolutions	Xue et al. [118]
Residual rubber shielded MWCNTs	NIH3T3 fibroblasts and human neuroblastoma cell line, SH-SY5Y	Neuroblastoma cells revealed amplified filopodia growth in comparison to unetched surfaces and silicone rubber	Tegtmeier et al. [119]
MWCNTs-PDMS	Schwann cells and DRGS	Two-fold increase in the viability and proliferation of the neural cells and Schwann cells using the PDMS/MWNT sheet	Kang et al. [120]

In a more complex approach, Scapin et al., [116] developed a nanocomposite scaffold, where MWCNTs were dispersed in a poly-L-lactic acid matrix, contributing to provide electrical cues and mimic neural topography. Furthermore, to mimic guidance cues and the natural neuronal environment, biomimetic peptides reproducing active motifs from L1 and Leucine-rich repeat and immunoglobulin domain-containing protein 1 (LINGO1) proteins were incorporated. These peptides control neurite outgrowth, adhesion, myelination and axon guidance. The combination of the nanocomposite scaffold and such peptides proved to synergistically boost neuronal differentiation of SH-SY5Y.

Kolarcik et al., [117] aimed to construct a neural electrode capable of *in situ* stimulation of the rat dorsal root ganglion (DRG). For that, authors used the conductive polymer poly(3,4-ethylenedioxythiophene) (PEDOT) and MWCNTs, which were coated on the electrode surface and doped with dexamethasone. In fact, results showed that coated microelectrodes have lower *in vitro* and *in vivo* impedance values. Significantly less neuronal damage was observed with coated electrodes as compared to non-coated controls. The inflammatory response with the PEDOT/CNT-coated electrodes was also reduced, demonstrating the advantage of including MWCNTs in such devices.

In the field of electrodes for recording PNs signals, Xue et al., [118] developed a novel polyimide-based C-shaped neural interface electrode. Amongst the several formulations, the gold-CNTs made electrode had significant lower impedance values and dramatically increased the signal recording resolution, when compared to CNTs-free electrodes. Tegtmeier et al., [119] developed an active neural implant by immersing MWCNTs in silicone rubber and re-etching the surface. Since rubber presents toxic substances, residual rubber was reduced to an estimated layer of only 13 nm covering the CNTs and thus securing them to the electrode material. Impedance measurements confirmed the etching success. NIH3T3 fibroblasts and human neuroblastoma SH-SY5Y cell

lines were used to prove the adequate biocompatibility of the neural implant. Inclusively, neuroblastoma cells also revealed amplified filopodia growth in comparison to non-etched surfaces and silicone rubber.

Kang et al., [120] studied the applicability of MWCNTs mixed with polydimethylsiloxane (PDMS) sheet to promote primary neuronal cells proliferation, by incorporating MWCNTs in PDMS using a simple printing transfer method. The results showed increased mechanical properties and roughness, as well as superior electroconductivity when compared to PDMS sheets alone. The developed material increased adhesion and proliferation of primary DRGs, as well as SCs, in a higher extent when compared to poly-l-lysine (PLL) coated dishes, which are commonly used to increase cellular attachment.

The downside of the use of carbon nanomaterials for PNR relies in their toxic nature [121]. Their physicochemical properties could make them toxic for living organisms or the environment [122]. Mechanisms of toxicity include membrane damage, DNA damage, oxidative stress, changes in mitochondrial activity and altered intracellular metabolic routes [122].

CNTs have a highly hydrophobic surface and a non-biodegradable nature that contributes to their reduced biocompatibility, limiting their application in the biomedical field, with growing concerns about their chronic toxicity [122].

Presently, there are controversial results regarding carbon nanomaterials toxicity, as some publication reported that carbon materials are toxic for neuronal-related cells. For instance, MWCNTs seem to inhibit neuronal differentiation of PC12 cells [123] and SH-SY5Y [124]. Moreover, Wu et al., [125] reported that exposure of DRG cultures to MWCNTs compromises regenerative axonogenesis [125]. Additionally, respiratory toxicity is the main concern when carbon nanomaterials are used [126,127]. Nevertheless, many other works report non-toxic effects both in *in vivo* and *in vitro* [88,114]. Meanwhile, it was reported

that MWNTs are likely to be a more neural-friendly interface than SWNTs, since they allow for a wider external surface and effective functionalization [128]. What is important to state is that before transposing carbon-based nanotechnology to the clinics, scientists must be critical in assessing its possible toxicity.

3.1.2. Graphene

Graphene belongs to one of the allotropic forms of carbon. This two-dimensional planar monolayer nanomaterial composed of SP^2-bonded carbon atoms is by norm arranged in a honeycomb form with carbon to carbon interatomic length, also possessing a number of amazing mechanical, optical and conductive properties [129]. The intensive research on the biological applications of graphene and its derivatives is based on its several fascinating properties, such as high specific surface area (2,630 m^2/g), exceptional electric conductivity, thermal conductivity (~5,000 W/m/K), mechanical strength (Young's modulus, ~1,100 GPa), intrinsic biocompatibility, low cost and scalable production, and facile biological/chemical functionalization of graphene oxide [130]. In its elementary form, graphene can also be used to aid in PNR. However, due to the fact that the surface of graphene lacks functional groups, it is difficult to dissolve in solvent and can easily agglomerate. Therefore, in many works, graphene oxidized (GO) is used, since its surface is rich in functional groups containing oxygen [131]. Table 4 overviews the works showing the application of graphene in PNR [132–142].

Li et al., [132] developed a GO/polyacrylamide (GO/PAM) composite hydrogel fabricated by in-situ free radical polymerization. The GO/PAM composite hydrogel with 0.4% GO could effectively enhance the

attachment and proliferation of SCs and release higher amounts of growth factors and larger matrix adsorption than other samples with different amounts of GO.

Baniasadi et al., [133] were responsible for the development of a porous conductive Cht/gelatin scaffold containing polyaniline/graphene (PA-Graphene) nanocomposite. The effects of PA-Graphene content showed that the electrical conductivity and mechanical properties increased proportionally to the increase in PA-Graphene loading, while the porosity, swelling ratio and *in vitro* biodegradability decreased. Overall, the construct containing 2.5 wt% of PA-Graphene revealed to be the most suitable in terms of SCs adhesion, and therefore the most promising for PNR.

Das et al., [134] reported for the first time a method that allows the differentiation of mesenchymal stem cells (MSCs) into a SC-like phenotype through electrical stimuli, without the need of additional chemical growth factors. Such differentiation was carried out on a flexible, inkjet-printed graphene interdigitated electrode. This work opened doors to the scalable nano-manufacturing of graphene and graphene-based circuits with complex geometries on virtually any substrate, including flexible and degradable polymers.

Nanofibers are considered a very promising type of scaffold for the integration of graphene. Zhao et al., [135] synthesized polypyrrole functionalized graphene (PPy-Graphene) by using for the first time a simplistic but effective polymerization-enhanced ball milling method, which is an environmental-friendly, easy to be controlled and efficient method for preparing functionalized graphene. Such strategy can be envisioned for application as a retinal electronic implant to repair the damage of optic nerve, which is mainly derived from the atrophy,

Table 4
The use of graphene applied to peripheral nerve regeneration.

Type of graphene nanomaterial	Cell type or animal model	Main results	References
Graphene/hydrogel	Schwann cells	GO/PAM composite hydrogel with 0.4% GO enhanced the attachment and proliferation of Schwann cells	Li et al. [132]
Conductive Graphene nanoparticles	Schwann cells	Construct containing 2.5 wt% of PAG revealed to be the most suitable in terms of Schwann cell adhesion	Baniasadi et al. [133]
Graphene-based eletrode	MSCs	Differentiation of MSCs into Schwann cell-like phenotypes through an electrical stimulus, without the need of additional chemical growth factors	Das et al. [134]
Polypyrrole functionalized graphene (PPy-Graphene)	Retinal ganglion cells	PPy-Graphene based aligned nanofibers were fabricated for guided growth and electrical stimulation of RGCs. Significantly enhanced viability, neurite outgrowth and anti-aging ability of RGCs were observed after electrical stimulation	Zhao et al. [135]
GO-coated PLLA electrospun nanofibers	Schwann cells, PC12 cells	The GO coating improved properties of the simply aligned PLLA nanofibrous mesh, including surface roughness and hydrophilicity. Also, it significantly promoted SCs growth and regulated cell orientation, and induced PC12 cells differentiation and neurite growth	Zhang et al. [136]
Electrospun graphene-silk fibroin composite	L929 cells, Schwann cells	10% of graphene in the nanofibrous mesh was selected as the optimized one, as this specific concentration allowed cell viability and cell attachment	Zhao et al. [137]
Electrospun silk fibroin scaffolds coated with reduced GO	PC12 cells	The coating with GO improved the adhesion of cells. The use of SF/GO scaffolds combined with electrical stimulation promoted the differentiation into neural phenotypes more than treatment with NGF	Aznar-Cervantes et al. [138]
Hybrid Gr nanosheets-sodium alginate (SA)/polyvinyl alcohol (PVA) (Gr-AP) fibrous scaffold	PC12 cells	Successfully developed hybrid graphene incorporated alginate/PVA fibrous scaffolds in which the best amount of graphene was 1 wt%	Golafshan et al. [139]
Graphene oxidized combining with decellularized rat sciatic nerve defect (allograft)	Rat sciatic nerve defect	GO nanomaterial could combine with allogeneic sciatic nerve decellularized scaffold to facilitate nerve regeneration	Wang et al. [140]
Graphene oxide foam layers with thicknesses of ~15–50 μm	hNSCs	Rolled GOFs were developed as electrically conductive 3D-scaffolds with desirable large-scales and utilized in directional growth of neural fibers under electrical stimulations	Akhavan et al. [141]
poly(ε-caprolactone) tubular prosthesis associated with nanoparticles of carbon and graphene	Rat sciatic nerve defect	Nanocomposite PCL tube enables nerve repair and results in better regeneration	Assaf et al. [142]

apoptosis or death of retinal ganglion cells (RGCs). Following the polymerization reaction, PPy-Graphene based aligned nanofibers were fabricated and designed to act as guiding cues and permit electrical stimulation of RGCs. This successful approach significantly enhanced viability, neurite outgrowth and anti-aging ability of RGCs after electrical stimulation.

Zhang et al., [136] also fabricated GO nanosheets with topological structure of aligned nanofibrous for PNR applications. However, these differ from the above since the GO nanosheets were coated onto aligned and aminolyzed poly-ʟ-lactide (PLLA) nanofibrous scaffolds produced by electrospinning. The GO coating improved several properties of the simply aligned PLLA nanofibrous mesh, including surface roughness and hydrophilicity. Further than that, it significantly promoted SCs growth, regulated cell orientation and induced PC12 cells differentiation and neurite growth.

Zhao et al., [137] were able to obtain graphene/silk fibroin (SF) based nano-membranes acquired by electrospinning technique. Graphene percentage varied from 0% to 20%, however a concentration of 10% of graphene in the nanofibrous mesh was selected, since it allowed cell viability and attachment, supporting the survival and growth of SCs with no significant cytotoxic effects.

In a similar approach, Aznar-Cervantes et al., [138] reported promising results that indicate the potential use of electrospun SF scaffolds coated with reduced GO for neural tissue engineering. The stimulus provided by the reduced GO alone induced a significant differentiation level of PC12 cells to neuronal-like phenotype, which can even increase by application of electrical stimulation. By this manner, the neurite outgrowth was more pronounced when electric currents are applied to the cell cultures when compared to the traditional treatment with nerve growth factor (NGF).

Golafshan et al., [139] developed hybrid graphene incorporated alginate/PVA fibrous scaffolds with varying amounts of graphene (up to 5 wt%), using an electrospinning approach for engineering nerve tissue. The authors found that the incorporation of 1 wt% of graphene in the nanosheets enhanced the toughness and strength of the scaffolds by 4- and 3-fold, while tensile modulus did not significantly vary. Moreover, the addition of graphene upon 1 wt% resulted in a significant reduction in impedance value, meaning higher electrical conductivity, which is advantageous for PNR.

Wang et al., [140] innovatively prepared GO through improving Hammer's Method and combining it with decellularized scaffold of a sciatic nerve of rats. The GO/decellularized scaffold was used to bridge a 10 mm gap of injured sciatic nerve. Such operation was conducted by using the oscillation mixing method. It has been proved that GO nanomaterial could be associated with allogeneic sciatic nerve decellularized scaffold to induce nerve regeneration. The regeneration parameters evaluated, i.e. sciatic nerve action potentials, thickness of myelin sheath, diameter of axon and dominated muscle rehabilitation level, revealed that the regeneration of the nanomaterial group were significantly higher than the blank group.

Graphene foams have successfully been developed and suggested as 3D scaffolds providing an adequate support with the desired topographies for differentiation of cells [143]. Akhavan and colleagues, [141] firstly fabricated graphene oxide foam (GOF) layers with thicknesses of ~15–50 μm and density of ~10 graphene oxide. Then, the GOF layers with desirable scales were rolled to obtain 3D scaffolds with capability of inducing directional proliferation and differentiation of neurons along the main axis of the rolls. After seeding human neural stem cells (hNSCs) and proceeding with electrical stimulation, increased hNSCs proliferation rates and accelerated differentiation into neurons was observed, as compared to the differentiation into glial cells.

Assaf et al., [142] combined both graphene and carbon NPs to enhance the properties of a tubular conduit made of PCL. Despite the final conduit transparency and adequate mechanical properties, which allowed the correct alignment of the stumps, the gap was not a critical gap presenting only 3-4 mm between the nerve ends, therefore not permitting a correct evaluation of the results.

3.1.3. Carbon nanofibers

Carbon nanofibers (CNFs) are other type of carbon nanomaterials, such as vapor grown carbon nanofibers [144] and carbonization of polymer-based nanofibers produced in the electrospinning process [145] that are manufactured using different methods. These nanofibers are characterized as non-microporous graphitic materials with a high surface area (100–200 m^2/g), high purity and tunable surface chemistry [146]. They have been investigated for numerous applications due to their unique physical properties, such as high strength, low density, metallic conductivity, tunable morphology, chemical and environmental stabilities, as well as compatibility with organo-chemical modification [147].

Being the less explored carbon nanomaterials, especially for PNR applications, CNFs were developed by Shilpee and colleagues, [148] using electrospinning technique. Their effect on SCs fate was studied when seeded on amorphous carbon substrates, depending on the evolution of intracellular oxidative stress. That results are encouraging, as evidenced that CNFs own a conducting nature and their fibrous structure can provide directional growth to axons, facilitating SCs proliferation and growth. The level of reactive oxygen species was not high enough to induce apoptosis in all the culturing periods tested on carbon fibrous and film substrates.

Wei Zhu et al., [149] developed novel carbon nanofibrous scaffolds by annealing electrospun mats at elevated temperature, whose graphitic structure generated by annealing rendered superior electrical conductivity. When subjected to electrical stimulation, neural stem cell proliferation was promoted, while an up-regulated neuronal gene expression level and increased expression of microtubule-associated protein 2 were detected, thus demonstrating an improved neuronal differentiation and maturation.

3.1.4. Nanodiamonds

Nanodiamonds (NDs) were termed for the first time by researchers in World War II in 1963, as detonation investigations with carbon-based explosives were being implemented and 4–5 nm diamond particles accompanied by graphite and other non-diamond carbon particles were found [150].

NDs are an evolving type of carbon nanomaterials which holds a distinctive set of properties, either at a chemical, physical, and biological level. Its generic structure can be appreciated in Fig. 4AII. Above all, they are considered to be completely non-toxic, making them suitable for biomedical applications [151]. Such intrinsic physicochemical properties are indispensable and allow designing innovative therapies in several areas of the medical field, such as delivery of therapeutic molecules, and many applications in TE and imaging [152].

In what concerns drug delivery, NDs are promising materials because of their high surface area to volume ratio and surface chemistry, which permits the stocking of molecules in their amine groups, as well as in other polar moieties. The phenomenon occurs by physical adsorption, allowing drugs and other molecules of interest to be noncovalently linked to NDs [151]. This simplistic loading process is effective and does not involve the usually hard and time-consuming processes of chemical modification [153].

Another major advantage of NDs is that they are promptly internalized by cells but do not undergo an immediate exocytosis. Therefore, bioactive molecules stay within the cell for an improved therapeutic effect [154].

Similarly to other carbon nanomaterials, NDs have also been used as nano-fillers to strengthen the mechanical properties of composite implantable biomaterials.

The number of advantages of NDs increased, as the optical properties of fluorescent NDs (FNDs) allowed their use as imaging probes. Importantly, the FNDs were able to retain high photostability and longer

Table 5
The use of nanodiamonds applied to peripheral nerve regeneration.

Type of nanodiamonds	Cell type or animal model	Main results	References
Amine functionalized ND	NG108-15 neuroblastoma cells, SCs, DRGs	Amine-terminated surface NDs proved to be a suitable substrate since it promoted neuronal cell adhesion, proliferation and neurite outgrowth	Hopper et al. [157]
Fluorescent ND	Hippocampal neurons and DRGs	Fluorescent ND did not induce cytotoxicity in primary neurons from CNS or PNS. However, interfere with neuronal morphogenesis	Huang et al. [158]
ND ink	Not applicable	ND solutions can be used instead of proteins and factors that are commonly used for cell guiding patterning	Tong et al. [159]
3D-nanostructured boron doped ND	Spinal cord and hippocampal cell cultures	3D-nanostructured BDD offers good performances for neural recording and stimulation	Piret et al. [160]
Nitrogen included ultra nanocrystalline diamond	Rat cortical neurons	The fabricated neural interfaces exhibit high efficacy, long-term stability and a healthy neuron/electrode interface	Tong et al. [161]
Micro-textured nanocrystalline diamond	Inner-ear neuron of human and mouse origins and ReNcell (a human neural progenitor cell line)	The findings demonstrate that regenerating auditory neurons show a strong affinity to the NCD pillars, and the technique could be used for neural guidance and the creation of new neural networks	Cai et al. [162]

fluorescent periods as compared to other fluorophores used in cellular imaging [155]. These properties permit the location of such NPs within living organisms.

Finally, the high biocompatibility of NDs when compared to the above-mentioned carbon nanomaterials (e.g., SWCNTs, MWCNTs, graphene), translates into a great advantage for NDs and increases the probability of such carbon materials to be translated to the clinical practice [156]. Although NDs' mechanical and electrical properties seem suitable for PNR applications, there are not many works publications describing the use of this promising technology. These properties combined with the aforementioned ones, make NDs an encouraging environment for neuronal networks. Table 5 summarizes the most relevant reports describing the use of NDs in PNR [157–162].

Interestingly, Thalhammer et al., [163] first tested a NDs monolayer coating surface to promote the formation of functional neuronal networks, achieving extraordinary results that proved that detonation-derived NDs exhibit encouraging similarity to protein-coated materials in what concerns neuronal cell attachment, neurite outgrowth and functional network formation. Furthermore, cell-autonomous neuronal excitability and functionality of the resulting electrical networks demonstrated the potentiality of this type of coating.

More recently, Hopper et al., [157] were able to produce amine functionalized NDs, which were studied as substrates for neuronal cell culture. The obtained results indicated that these functionalized NDs are beneficial for *in vitro* neural cell culture and can be regarded as possible coatings for *in vivo* neural implants. The NDs were able to support the growth of primary SCs and DRGs neurons with the correct phenotype over three weeks of *in vitro* culturing.

In the same year, Huang et al., [158] reported for the first time the influence and application of FNDs on the nervous system. Neurons were treated with FNDs particles, which did not induce cytotoxicity in primary neurons from either CNS or PNS. Furthermore, the neuronal uptake of FNDs was confirmed, as seen in Fig. 4D.

Nevertheless, it was found that FNDs caused a decrease in neurite length in both CNS and PNS neurons. These outcomes proved that FNDs exhibit low neuronal toxicity but in fact, have the power to interfere with neuronal morphogenesis. This must be considered when the application of FNDs involves the growing of neurites, such as in PNR.

In a different approach, Tong et al., [159] presented a method for fabricating a diamond platform with the goal to direct neural cell adhesion. Micro-contact printing was used for selective NDs seeding and chemical vapor deposition was conducted subsequently to form NDs patterns. As a result of this work, it was observed that NDs solutions can be used in replacement of proteins and factors that are commonly used for cell patterning with good alignment results.

In the field of neural implants and neuroprostheses, Piret et al., [160] aimed at developing a platform that exhibits high capacitance and appropriately contacts with neurons to provoke real neural responses at low voltages. For such, they developed a 3D-nanostructured boron doped NDs platform. This interface allowed neural cell attachment, survival and neurite extension, after experiments with spinal cord and hippocampal cell cultures. Particularly, the platform allowed the detection of low amplitude in the range of 10–20 μV local-field potentials. These properties are found to be extremely valuable for the fabrication of diamond neural interfaces with the predictable long-term steadiness of diamonds at the same time supporting the life of healthy neurons.

In the same field, Tong et al., [161] reported on a new high capacitance material fabricated using nitrogen – ultra nanocrystalline diamond (N-UNCD), which was treated with oxygen plasma, resulting in increased charge injection capacity. Rat cortical neurons were used *in vitro* to measure its biocompatibility. Surface roughness was found to be critical for healthy neuron growth, with best results observed on surfaces with a roughness of approximately 20 nm. Overall, the authors provided a method of producing a ND electrode that is optimized for both high charge injection capacity and neuronal biocompatibility.

In the field of cochlear implants for patients with profound hearing loss due to neuronal deficits, Cai et al., [162] developed micro-textured nanocrystalline diamond (NCD) surfaces on cochlear implants electrode arrays, where the surface consisted in micrometer-sized pillars (size $5 \times 5 \mu m^2$). When using human and murine inner-ear ganglion neurites and a neural progenitor cell line, the results showed that these cells can attach to patterned NCD surfaces without any ECM coating. These findings demonstrated that regenerating auditory neurons have a solid affinity to the NCD pillars and the technique could be used for neural guidance and the creation of new neural networks.

3.1.5. Carbon nanomaterials toxicity

Although the popularity of carbon nanomaterials has been increasing due to their high biocompatibility and consequent potential in the area of biomedicine (e.g. drug delivery, bio imaging and tissue engineering), its likely cytotoxicity has raised some concerns [164]. Because of their unique and innovative physico-chemical characteristics explored previously, an intensive use of such NP is expected, resulting in the accumulation of carbon molecules in the environment.

Currently, one of the main alarms of the utilization of carbon nanomaterials falls in the scope of toxic effects on reproductive system. For instance, GO has shown potential side effects on both females and their offspring. The results obtained give evidence to extremely high toxic effect of GO on females at late pregnancy stages [165].

Regarding SWCNT, this nanomaterial has shown side effects at both low high concentrations: when administered at high concentrations,

the number of miscarriages was significantly augmented. Groups exposed to small dosage had apparent defects of embryonic development [166].

When dealing with MWCNT, many studies focusing on mammal's embryonic development [167], delay of pregnancy time, offspring's quality [168] and even male reproductive system [169], no significant anomalies had been detected.

Pulmonary toxicity has also been demonstrated for this type of materials. Several processes of toxicity include inflammation [170], injury [171], fibrosis [172] and pulmonary tumor [173].

Although the previous studies provide valuable insights into the toxicity of carbon materials at different levels, there is still a lack of reproducible and accurate results that can be used to make a final statement on their use and safety. Although red flags arise when toxicity is found in rodent models, studies need to be correctly designed in order to assess the risk of exposure for nature and human health.

3.2. Nanoparticles

In the last years, researchers have been proposing the use of NPs for neural regeneration approaches [174]. There are several benefits offered by NPs, such as their small size, physical properties that diverge from the bulk, surface functionalization and chemical stability. They also differ from other nano-sized particles in their electrical charge, magnetic and optical properties [175]. These nano-sized biomaterials are mainly envisioned as carriers for targeted and controlled delivery of drugs and other biological molecules such as growth factors, both *in vitro* and *in vivo*. Antibodies, labeling probes, hydrophobic or hydrophilic molecules, DNA and oligonucleotides are other types of molecules that can be linked to NPs, allowing a tailored application for the desired purpose, such as drug delivery or cell tracking and monitoring [176,177]. This versatility is mostly a result of the wide range of approaches that can be used for NPs functionalization. Furthermore, NPs are widely used to enhance the properties of scaffolds, as mechanical properties and degradation rate in several TE applications.

When it concerns to neuronal regeneration, NPs can be used with different purposes, as enhancing neuronal differentiation [178], stimulating neuronal regeneration and survival [179], manipulation of neuronal electrical activity [180,181] or allowing imaging and theranostics [182]. NPs will be divided according to their composition in two main categories, inorganic and organic, and the most significant reports overviewed herein.

3.2.1. Inorganic NPs

Inorganic nanomaterials are being widely exploited for nerve regeneration applications. Within this field, gold (Au), zinc (Zn), silver (Ag) and silica-based NPs (SiO$_2$-NPs) are the ones that have been attracting a great deal of attention. Table 6 summarizes the most significant works describing the use of inorganic NPs applied in PNR [179,183–186,188–204,208].

3.2.1.1. Magnetic NPs.
Magnetic elements in the nanometer scale (e.g. iron, nickel, cobalt and their oxides), are being used in different biomedical applications [205]. One characteristic that is modified when iron becomes a nano-scale material is its magnetism. Reducing the size of iron as a bulk material to the nano-scale level results in superparamagnetic behavior. Superparamagnetic iron NPs (SPIONs) are non-magnetic particles until they are exposed to a strong magnetic field. If the magnetic field is removed, they revert to a non-magnetic state again [187]. Several approaches have been using these magnetic NPs (MNPs) in the last few years [206,207]. The main advantages rely on the fact that MNPs-loaded cells can be directed to specific sites in response to an external magnetic field gradient and the possibility of imaging through MRI. Fig. 5 captures some of the most interesting results related to the use of MNPs applied to PNR.

In 2014, Riggio et al., [183] developed and validated a non-invasive approach for physical guidance of nerve regeneration based on the synergic use of MNPs allied to the appliance of magnetic fields. For such, *in situ* polymer coating was done with polyethylenimine of iron oxide MNPs, which were further functionalized with NGF. These MNPs demonstrated to be able to trigger PC12 differentiation into a neuronal phenotype. Above all, these authors evidenced that mechanical tension created by the MNPs and the magnetic field can induce "stretch growth" of neurites process initiation, as depicted in Fig. 5A.

Marcus et al., [184] studied the interactions of four different types of iron oxide MNPs with PC12 cells and have shown that cell uptake is highly sensitive to the MNPs type and incubation conditions. The researchers demonstrated that uncoated maghemite MNPs led to maximal cellular penetration, thus proposing these particles as efficient candidates for magnetic-based neuronal manipulations. Once again, MNPs were demonstrated to be effective in controlling neurite growth orientation of primary neurons along the process of neural network formation using a magnetic field.

Huang et al., [185] developed a way to improve the therapeutic potential of SCs, which consisted in magnetically drive SCs to migrate across the astrocyte-SC boundary to interact with astrocytes (Fig. 5B). To achieve that, SCs were firstly magnetized with PLL-coated SPIONs. As a result, magnetized SCs exhibited enhanced migration along the direction of force in the presence of a magnetic field. It was also demonstrated that SPION-mediated forces could act as powerful stimulants to enhance the migration of SCs across the astrocyte-SC boundary, via integrin-mediated mechano-transduction.

Using the same kind of strategy of combining magnetically responsive MNPs and a magnetic field, Liu et al., [186] prepared MNPs–Cht/glycerophosphate membranes and scaffolds and explored whether such magnetic nanocomposites would regulate SCs biological activities via application of magnetic fields. It was observed that magnetic nanocomposites containing 10% MNPs were able to support cell adhesion and spreading and further promote proliferation of SCs under magnetic field exposure. Interestingly, a magnetic field applied through the 10% MNPs scaffold significantly increased the gene expression and protein secretion of brain-derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3) and VEGF. Later on, the same authors proved that the same scaffolds had modest magnetization and proper degradation rate, making them suitable for cellular distribution and new axon regeneration [208]. After loading the scaffold with SCs and exposure to a magnetic field, it was observed a synergistic improvement of nerve regeneration and functional recovery *in vivo*, by increasing the distribution efficiency and viability of SCs.

Tseng et al., [188] combined the cell transplantation strategy with the use of spheroids and the incorporation of SPIONs. The authors investigated the effect of the substrate-derived MSC spheroids versus single cells on the regeneration of transected rat sciatic nerve (Fig. 5D). This effect was evaluated after injection of Fe$_3$O$_4$NPs-labeled MSC spheroids into microporous nerve conduits made of polylactide acid (PLA). With this strategy, MRI could be employed to trace the distribution and migration of these spheroids *in vivo*. Furthermore, authors also delivered BDNF gene to MSC spheroids and evaluated the efficacy of BDNF-transfected spheroids on nerve regeneration across the gap defect, which showed significant functional recovery.

Liu et al., [189] explored for the first time the use of hydroxyapatite-coated MNPs to enhance neuronal regeneration in injured nerves. For that purpose, the experiments were carried out on cultured rat DRG neurons and the results showed that n-HA-coated magnetic NPs (Fe$_3$O$_4^+$n-HA) can effectively increase cell viability and promote axonal elongation. Interestingly, the authors have also demonstrated by Western blot that Netrin-1 axonal guidance cues significantly increase after n-HA-coated magnetic NPs treatment.

Gianaccini et al., [190] reported on the ability to immobilize NGF and VEGF on MNPs for controlled release, both *in vitro* and *in vivo*. Bioactivity tests were performed to induce a neuronal-like phenotype in PC12

Table 6
The use of inorganic nanoparticles applied to peripheral nerve regeneration.

|	Type of nanoparticle	Cell type or animal model	Main results	References
Magnetic NPs	PEI-coated Fe$_3$O$_4$ NP	PC12 differentiation	MNPs direct the neurite outgrowth preferentially along the direction imposed by an external magnetic field	Riggio et al. [183]
	Maghemite (γ-Fe$_2$O$_3$) fluorinated magnetic NP and Magnetite (Fe$_3$O$_4$) core particles with different coatings	PC12 cells and SHSY-5Y cells	MNPs control neurite growth orientation of primary neurons along the process of neural network formation	Marcus et al. [184]
	SPIONs	SCs and astrocytes	SPION-mediated forces could act as powerful stimulants to enhance the migration of SCs across the astrocyte-SC boundary	Huang et al. [185]
	MNP (Fe$_3$O$_4$)–chitosan mixture was fabricated into membranes or scaffolds	SCs	Magnetic nanocomposites containing 10% MNPs were able to support SCs adhesion and spreading under magnetic field exposure. Same concentration of MNPs, increased the gene expression and protein secretion of BDNF, GDNF, NT-3, and VEGF	Liu et al. [186]
	MNP (Fe$_3$O$_4$)–chitosan scaffold (same author as previous)	SCs; rat sciatic nerve defect	The magnetic chitosan scaffold synergized with the magnetic field enhanced the viability of SCs. The same scaffold loaded with SCs in addition to the magnetic field promoted nerve regeneration and functional recovery	Liu et al. [208]
	SPIONs	MSCs single cells or spheroids; rat sciatic nerve defect	Compared to MSC single cells, the pristine or BDNF-transfected MSC spheroids significantly promoted the functional recovery of animals	Tseng et al. [188]
	Nanohydroxyapatite (n-HA) coated Fe$_3$O$_4$ magnetic NP	DRGs	(n-HA) coated Fe$_3$O$_4$ can successfully increase cell viability and promote axonal elongation. Also, Netrin-1 axonal guidance cues rise significantly after treatment with n-HA-coated Fe3O4	Liu et al. [189]
	Polyethylenimine -coated (Fe$_3$O$_4$)NPs	PC12; zebrafish; rat sciatic nerve defect	MNPs functionalized with NGF and VEGF accelerated the regeneration process and recovery of motor function	Giannaccini et al. [190]
	SPIONs	DRGs	NGF releasing PLLA/iron oxide composite-NPs can direct neurite outgrowth and composite-NPs can be positioned by external magnetic field gradients	Zuidema et al. [191]
	SPIONs	L929, DRGs	With the aid of SPIONs and a magnetic field, the micro-structure of an hydrogel could be aligned, which in turn aligned the neurites of the DRSs	Rose et al. [192]
Metallic NPs	Gold nanorods	NG108-15 neuronal cells	NG108-15 cells were cultured with both bare and coated Au NRs and then irradiated with 1.2–7.5 W/cm(2) at 780 nm, which showed a neurite length increase of up to 25 µm versus control	Paviolo et al. [193]
	Gold nanorods	NG108-15 neuronal cells	When NG108-15 neuronal cells were exposed to the NIR light of 780 nm laser diode, it was found to induce intracellular Ca^{2+} transients	Paviolo et al. [194]
	Polyethylenimine -coated gold-NPs	PC12 cells	Pulsed current stimulation induced neurite outgrowth of PC12 cells to the AuNPs coated surfaces, thus proving the potential of AuNPs as an electrically conductive matrix for nerve regeneration	Adel et al. [195]
	Gold-NP adsorbed in electrospun silk fibroin nanofibers	SCs; rat sciatic nerve injury	AuNP-silk fibroin nanofiber noticeably decreases the resistance of an electrically insulating material like silk	Das et al. [196]
	AuNPs modified with 6-mercaptopurine (6MP) and a neuron-penetrating peptide	SH-SY5Y cells	Seeded SH-SY5Y cells in a surface coated with 6MP-AuNPs-RDP, are capable of internalizing NP, which led to a significant increase of neurite growth	Xiao et al. [197]
	Gold nanoparticles/Polyvinylidenefluoride (PVDF) composite electrospun mat	PC12 cells	Au NPs/PVDF composite nanofibers have the ability to stimulate the growth and adhesion of neuronal cells	Motamedi et al. [198]
	Chitosan-zinc oxide nanocomposite conduit	Rat sciatic nerve defect	Chitosan-zinc oxide nanocomposite conduit resulted in acceleration of functional recovery and quantitative morphometric indices of sciatic nerve	Iman et al [199]
	PCL electrospun matrix with variable quantities of zero valent zinc NPs	Human primary fibroblasts and U87 brain glioblastoma cell line	Low concentrations of Zn-NPs promoted neuronal regeneration and showed relatively non-toxic characteristics to fibroblasts	Aydemir et al. [200]
	Silver NPs	SH-SY5Y cells	The analysis of single neurite level reveals increased neurite formation and growth, better than on the AuNP and ZnONP substrates or smooth substrates	Alon et al. [179]
Silica NPs	SiO$_2$-NPs	Guinea pigs	Significantly greater survival of SGNs in cochleae that received BDNF. Supraparticles were well tolerated	Wise et al. [201]
	SiO$_2$-NPs encapsulated within a lipid bilayer and modified with the atoxic subunit B of the cholera toxin (CTB)	Motoneuron-like NSC-34 cells and L6 muscle cells	SiO$_2$-NPs functionalized with CTB showed greater motor neuron uptake when compared to unmodified lipid bilayer (60% vs. 15%, respectively)	Porras et al. [202]
	Aligned poly lactic- PLGA and PLGA/gelatin nanofibrous scaffolds embedded with mesoporous SiO$_2$-NPs	PC12	Cellular proliferation on PLGA and PLGA/gelatin with SiO$_2$-NPs were higher than that on the aligned pure PLGA scaffolds	Mehrasa et al. [203]
	SiO$_2$-NPs encapsulated with Tetrodoxin	Rat sciatic nerve defect	The proved sustained release properties of hollow SiO$_2$-NPs contributed to the extension of nerve block and enhanced safety by slowing release	Liu et al. [204]
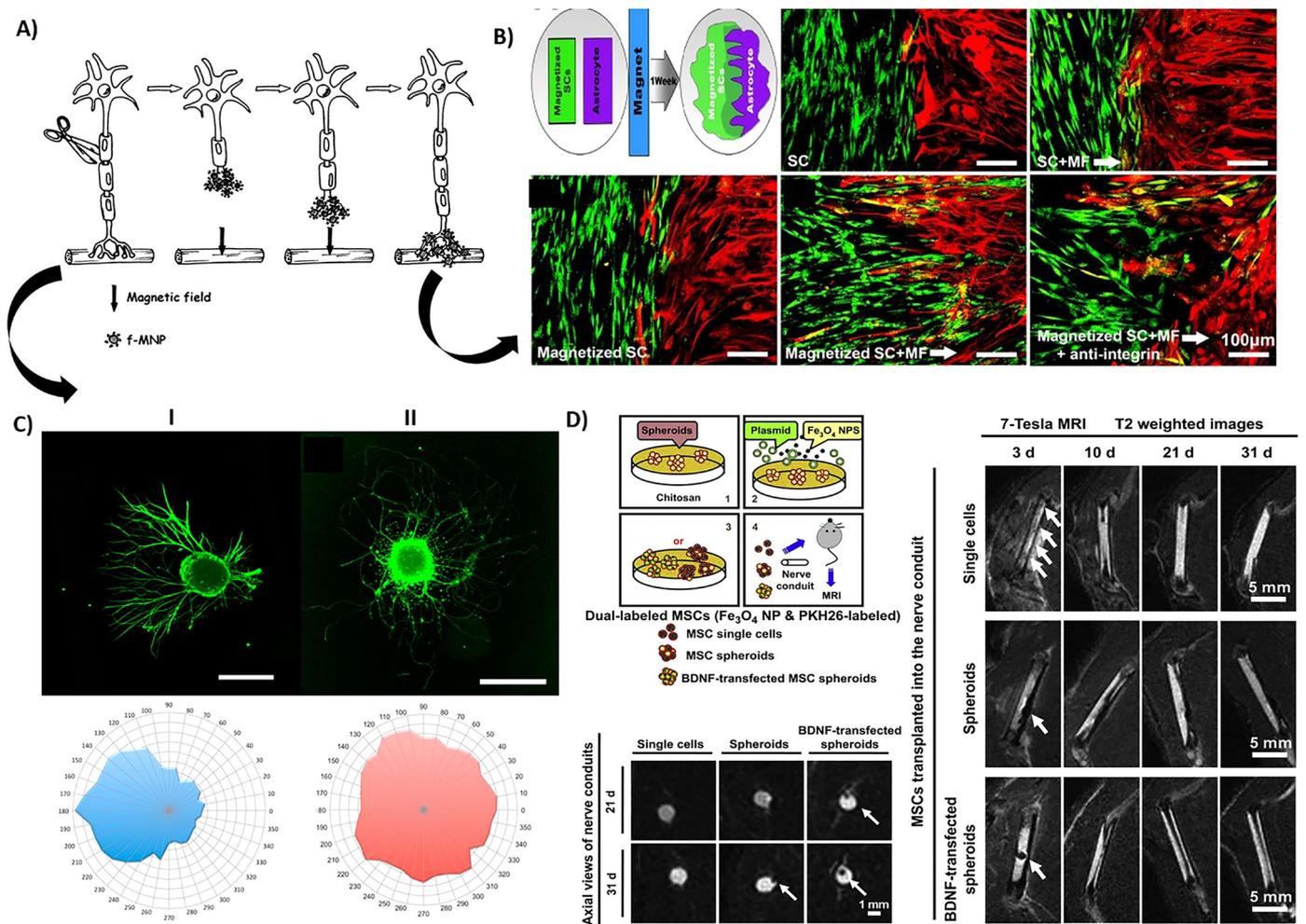
Fig. 5. Magnetic nanoparticles applied to peripheral nerve regeneration. (A) MNPs bind to the injured nerve and a magnetic field is applied, creating mechanical tension. This stimulates nerve regeneration in the direction forced by the magnetic field. This physical guidance is envisioned to direct more efficiently the regeneration of the injured nerve from the proximal toward the distal stump; (B) Schwann cells-astrocytes confrontation assay. Magnetized Schwann cells (in green) and astrocytes (in red) were seeded in parallel stripes and were allowed to migrate and intermingle for 1 week under applied magnetic field; (C) NGF gradients generated by MNPs are able to direct the growth of extending neurites from chick DRGs. (I) NGF-cNPs directed the extension of neurites from DRG. (II) Control cNPs had no effect on the directional outgrowth of extending neurites, and respective polar histograms (below); and (D) Sagittal and axial MRI of experimental animals receiving MSCs in sciatic nerve gap. Animals were implanted with MSC-loaded nerve conduits and MRI images were taken at different time points post-implantation. Arrows indicate the visible location of Fe_3O_4 NPs-labeled cells. Scale bar: 1 mm. Reprinted and adapted from (A) [183]; (B) [185]; (C) [191]; (D) [188].

cells with NGF and neo-angiogenesis in a zebrafish model with VEGF. Afterwards, a neurotmesis of the rat median nerve was made in which the two nerve stumps were sutured to the ends of a synthetic conduit filled with the particles. In conclusion, there was an increase in the stability of growth factor concentration and delivery, mediated by MNPs. Its use is responsible for the enhanced neuroprotective effects of VEGF and NGF in the MNPs group as compared with the control group.

Zuidema et al., [191] established a composite-NPs to create NGF gradients at specific locations within a tissue culture dish to direct neurite extension. The composite nanoparticle was fabricated by combining NGF-releasing PLLA NPs with iron oxide MNPs, which were manipulated by the administration of an external magnetic field gradient, through the modification of water/oil/water double emulsion technique. NGF-composite-NPs was afterwards combined with aligned PLLA microfibers to create a biomaterial approach that utilizes both topography and chemotropic gradients to direct extending neurites. In brief, the authors have demonstrated that magnetic, NGF releasing PLLA/iron oxide composite-NPs can direct neurite outgrowth and that composite-NPs can be positioned by external magnetic field gradients, and release NGF for up to 6 days. This can be seen in Fig. 5C.

Also in combination with other materials, namely injectable hydrogels, Rose et al., [192] developed the first biomaterial that can

achieve *in situ* highly controlled and ordered structures after injection to guide cell and nerve growth. Those are magnetoceptive, anisometric microgels that were applied as building blocks to create a unidirectional structure, with the aid of magnetic fields. For such, microgels were doped with small quantities of SPIONs, allowing alignment by external magnetic fields in the milliTesla order, in which DRGs neurites were stimulated, to grow and align.

3.2.1.2. Other metallic NPs. Metallic NPs are predominantly striking because of their unique optical properties. Indeed, when metal NPs are irradiated by an external light field, they can generate a resonant coherent oscillation called "localized surface plasmon resonance", which depends on the NPs shape and aspect ratio [209].

With respect to metallic NPs, gold NPs (AuNPs) and gold nanorods (AuNRs) are the most widely explored. The integration of AuNPs in neurological research has the potential to lead to the discovery of new approaches to treat conditions that medicine and science previously failed to. This perspective arises from their exceptional properties, including optical response, chemical and physical stability, relatively low toxicity, and wide range of possible surface functionalization [210].

Paviolo and colleagues, [193] revealed a novel and extraordinary application for AuNRs associated with a low power laser exposure of
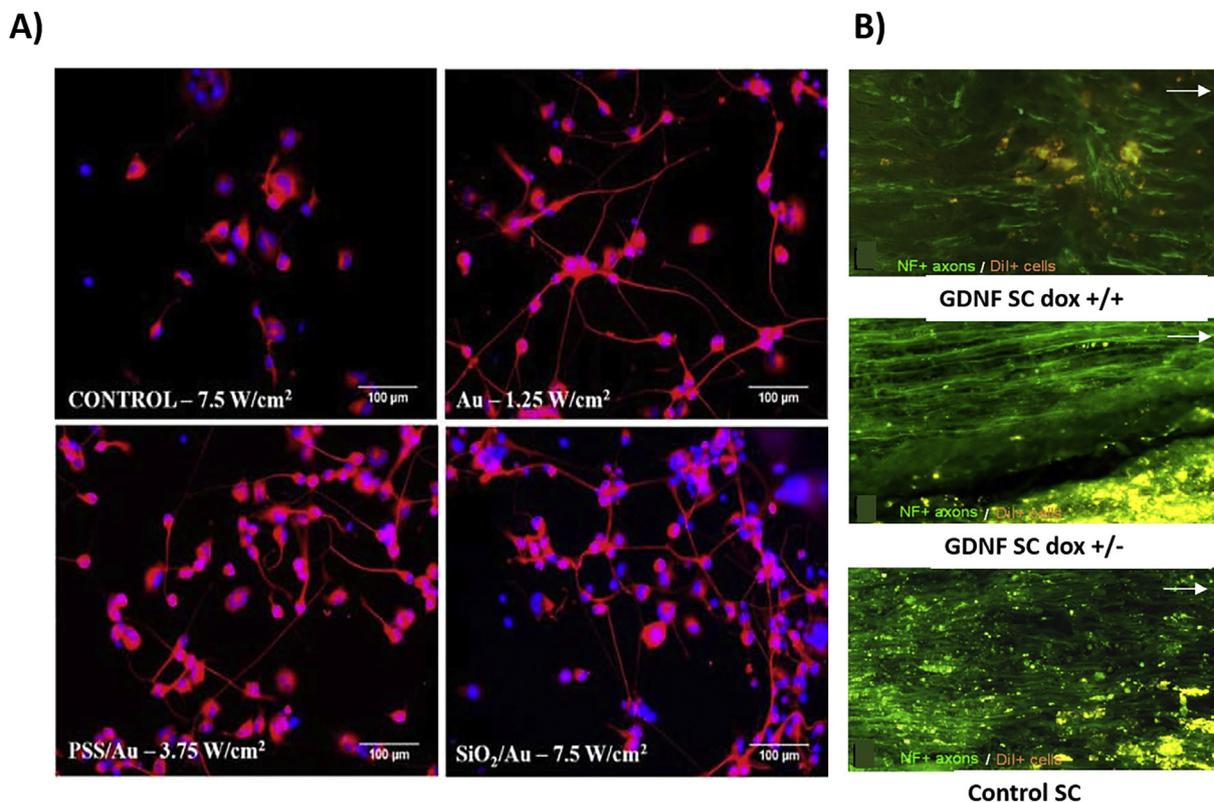
Fig. 6. The effect of other nanoparticles in peripheral nerve regeneration. (A) The use of gold nanoparticles in PNR: examples of epifluorescence images of NG108-15 neuronal cells cultured with Au, PSS/Au, and SiO$_2$/Au NRs and labeled with β-III tubulin (red) and DAPI (blue). The effect of PSS/Au and SiO$_2$/Au NRs on the percentage of neurons with neurites is evident; and (B) Doxycycline-inducible GDNF expression and respective regeneration scenarios in the distal sciatic nerves of experimental 3 weeks' time point. Continuous GDNF expression (dox +/ +) causes some axons to avoid the regenerative path; time-restricted GDNF expression (dox +/−) leads to unobstructed and profuse regeneration; White arrows indicate the direction of axonal regeneration (proximal to distal). Total magnification: 100x. Reprinted and adapted from (A) [193]; (B) [225].

NG108-15 neuronal cells after uptake of such NPs. Surprisingly, when these cells were irradiated with a 780 nm laser (IR or near-IR), the number of neurons and respective neurites augmented. A scheme of such work can be observed in Fig. 6A. Later on, the same authors [194] explored one of the mechanisms of such phenomenon. They described the fabrication of AuNRs that were up-taken by NG108-15 neuronal cells. Cells were then exposed to the near-infrared (NIR) light of 780 nm laser diode, which was found to induce intracellular Ca^{2+} transients. In order to explain such phenomena, the authors hypothesized that the temporary heating resultant from the light excitation of the localized Surface Plasmon Resonance (SPR) of the NRs could serve either to generate changes in the cellular membranes capacitance by activating temperature sensitive ion channels in the cell membrane or to diminish the intracellular calcium storage in the organelles.

In a study reported by Adel et al., [195] AuNPs were able to induce neurite outgrowth of PC12 cells, after another kind of stimulus to which these particles are sensitive to: pulsed electrical stimulation. The authors firstly evaluate the deposition of 39 nm AuNPs onto polyethyleneimine pre-coated surfaces and then studied the effect of the pulsed electrical stimulation. That study showed that pulsed current stimulation induced neurite outgrowth of PC12 cells adhered to the AuNPs coated surfaces, thus demonstrating the potential use of AuNPs as electrically conductive matrix for nerve regeneration.

Being aware that the presence of AuNPs enhances the conductive potential of the materials, Das et al., [196] reported on a novel SF-Au nanocomposite conduit which was tested in a neurotmesis grade sciatic nerve injury rat model over a period of eighteen months, after pre-seeded with rat SCs. To produce this scaffold, AuNPs were adsorbed onto silk fibers and further transformed into a nanocomposite sheet

by electrospinning. The results show that AuNPs-SF nanofiber noticeably decreases the resistance of an electrically insulating material like silk.

Xiao et al., [197] reported on the production of AuNPs modified with 6-mercaptopurine (6MP) and a neuron-penetrating peptide (RDP) as a neurotrophic agent. The 6MP is an anti-inflammatory drug which has been used to functionalize the surface of AuNPs to form 6MP-modified AuNPs (6MP-AuNPs) through an Au-sulfur bond. Additionally, with the purpose of increasing the neural cell uptake efficiency of 6MP-AuNPs, the RDP was linked to the particle surface to form a 6MP-AuNPs-RDP conjugate. When SH-SY5Y cells were transplanted to a surface coated with 6MP-AuNPs-RDP it was concluded that the conjugate attached to the cell surface and was then internalized into cells, which in turn led to a significant increase of neurite growth.

Motamedi et al., [198] fabricated and characterized AuNPs/polyvinylidenefluoride (PVDF) composite electrospun mat with enhanced piezoelectricity in which the Au colloidal NPs were prepared via laser ablation of metallic targets in liquid media. After an extensive physicochemical characterization, the in vitro cytocompatibility, as well as the attachment and morphology of PC12 cells cultured on the electrospun composite was evaluated. It was demonstrated that laser ablated AuNPs can be used together with PVDF nanofibers, with proper organizational properties and increased piezoelectricity.

Zn is another metal that can be conjugated with NPs to form electrically conductive matrices. Having in mind that numerous studies have reported that the mechanical and electrical properties of different polymers have improved when Zn oxide NPs are incorporated into polymeric matrices, Iman et al., [199] reported the fabrication of a Cht-Zn oxide nanocomposite conduit and its effect on functional recovery of

transected PNs. Results of this study indicated that the use of a Cht-Zn oxide nanocomposite seems to have several distinct advantages for the treatment of PNs, because it is inert and does not induce extensive scarring or degeneration after implantation. Also, Aydemir Sezer and colleagues [200] attempted to use for the first time a PCL electrospun matrix with variable quantities of zero valent ZnNPs, aiming at using it as conductive and biodegradable luminal fillers. Human primary fibroblasts and U87 brain glioblastoma cell line were used for evaluation of cytotoxicity, metabolic activity and proliferation. The results showed that low concentrations (5% and 10%) of ZnNPs were non-toxic to fibroblasts and promoted neuronal regeneration. The fact that electrical conductance became approximately nine times greater in PCL/Zn5 samples than in PCL, and material characterization and mechanical analysis indicated that the material is compatible with soft tissues and is suturable, makes it a great candidate for PNR applications.

Alon et al., [179] reported the use of AgNPs as regenerative agents to promote neuronal growth. Neuroblastoma cells were seeded on surfaces coated with AgNPs. The effect on the development of the neurites during the initiation and the elongation growth phases was evaluated, with the observation that AgNPs function as favorable anchoring sites. Indeed, the growth on the AgNPs-coated substrates leads to a significantly enhanced neurite outgrowth with three times more neurites than cells grown on uncoated substrates, and two times more than cells grown on substrates sputtered with a plain homogenous layer of Ag, which thus evidences the benefit of using AgNPs.

3.2.1.3. Silica NPs. Moreover, still in the scope of inorganic NPs, SiO_2-NPs play an important role. SiO_2 is known to be biocompatible, with an outstanding chemical permanence and defined properties [211]. There are, however, an extensive assortment of SiO_2 nano-formulations that can be applied in biomedical applications, in many different purposes, such as: (i) common biomedical imaging and integration as imaging contrast agents, (ii) application in ablative technologies, and (iii) application in controlled drug delivery. The reason rely on the fact that SiO_2-NPs can be produced using multiple synthetic techniques that allow to accurately regulate their physical and chemical characteristics [212].

Wise et al., [201] was able to improve auditory nerve survival with nano-engineered SiO_2-NPs aiming at delivery of neurotrophic factors. The authors investigated the safety and efficacy of BDNF-SiO_2-NPs as drug delivery system for the cochlea. For that, they bilaterally implanted the system into the basal turn of cochleae in deeply deafened guinea pigs. The results showed a significantly greater survival of spiral ganglion neurons in cochleae that received BDNF-SiO_2-NPs as compared to the contralateral control cochleae. Also, they were well tolerated within the cochlea, with a tissue response that was localized only at the site of implantation in the cochlear base.

Gonzales Porras et al., [202] described a novel delivery system to motor neurons using mesoporous SiO_2-NPs encapsulated within a lipid bilayer and modified with the non-toxic subunit B of the cholera toxin (CTB). This subunit was described to binds to gangliosides present on neuronal membranes. The aim was to increase the interaction between muscle fibers and motor neurons in case of neuromuscular disorders. It was found that the whole system containing SiO_2-NPs functionalized with CTB showed greater motor neuron uptake when compared to unmodified lipid bilayers, when tested using motoneuron-like NSC-34 cells.

By its turn, Mehrasa et al., [203] resorted to the electrospinning technique to produce a nanocomposite of aligned PLGA and PLGA/gelatin nanofibrous scaffold embedded with mesoporous SiO_2-NPs. Cultivation of PC12 cells on such scaffolds demonstrated that the introduction of mesoporous SiO_2-NPs into the matrices leads to improved cell attachment and proliferation, as well as longer cellular processes. DAPI staining results indicated that cell proliferation on the PLGA 10 wt% mesoporous SiO_2-NPs and the PLGA/gelatin/10 wt% mesoporous SiO_2-NPs scaffolds were outstandingly higher than that on the aligned pure PLGA scaffolds, reaching values of 2.5 and 3 folds, respectively.

Liu et al., [204] looked at the problem of anesthetizing PNs for surgeries and other procedures. That is because the drug delivery systems that have been used to prolong the duration of local anesthetic effect are generally thought of as being essentially systems that release local anesthetics in the surrounding area of the nerve. The authors have demonstrated that 28 nm hollow SiO_2-NPs-containing Tetrodoxin, which is a powerful anesthetic, can penetrate into the nerve. This phenomenon contributes to the increase in the number of successful nerve blocks as well as the prolongation of nerve block. The proved sustained release properties of hollow SiO_2-NPs also contributed to the extension of nerve block and enhanced safety by slowing release. The discovery of a system that can penetrate in PNs could be useful in delivering not only local anesthetics, but also a range of other therapeutics.

3.2.2. Organic NPs

3.2.2.1. Polymeric NPs. Polymers have been considered appealing materials due to their bulk physical properties, tunable structural design and architecture as well as customized biodegradability [213]. There are countless flexible synthesis methods of polymers and the polymer chains allow functionalization with a wide range of molecules. In this regard, the final polymer products can be made of different compositions and properties, envisioning a wide range of applications and strategies. When in the nano-scale, polymer NPs have the amazing capacity of a high drug loading capability [214]. Table 7 summarizes the relevant reports dealing with the use of organic NPs applied in PNR [215–228].

Cht is one of the most explored polymers in the field of TE and regenerative medicine. To further improve the property of promoting PN regeneration of Cht materials, several authors have prepared and explored such polymer in the form of NPs [229–231]. Mili et al., [215] prepared the NGF encapsulated Cht NPs (NGF-ChtNPs) by ionotropic gelation method with tripolyphosphate as an ionic cross-linking agent and evaluated the neuronal differentiation potential of canine bone marrow derived mesenchymal stem cells. With a NPs size of 80–90 nm and a NGF loading efficiency of 61%, the NGF-ChtNPs were found to be cytocompatible to MSC. Furthermore, NGF-ChtNPs can release bioactive NGF with the ability to transdifferentiate mesenchymal stem cells into neurons.

Li et al., [216] produced Cht porous hybrid scaffolds with varying amounts of Calcium titanate ($CaTiO_3$), which is well known for its high dielectric constant, conductivity properties and luminescence. Primary SCs directly cultured onto Cht/$CaTiO_3$ hybrid scaffolds with a suitable concentration of $CaTiO_3$ NPs, which was distinguished to be with 5 mg of NPs, could evidently stimulate the attachment, proliferation and biological function maintenance of SCs.

In a very advanced and innovative strategy, Lopes et al., [217] explored the use of a microfluidic based DRG neuron culture to test the uptake and transport kinetics of gene carrying trimethylated Cht (TMCht)-based NPs actively targeted to neurons. For such, the NPs surface was functionalized with the nontoxic and neurotropic C-terminal 54 kDa fragment of the TeNT heavy chain. The purpose was to develop a way of directly deliver any kind of molecules to a relatively inaccessible injection site of PNs and DRG, all of this while mimicking a peripheral *in vivo* route of administration. This microfluidic-based neuron culture showed to be of added value in the evaluation of cell–nanoparticle interactions, namely Cht NPs and PNs.

One year later, the same authors [218] explored another interesting way to mediate the targeted delivery of therapeutic genes to DRG neurons, through the development of biocompatible and biodegradable NPs, using the same optimized TMCht-based NPs. They proposed the use of such NPs to mediate targeted gene delivery to peripheral neurons upon a peripheral and minimally invasive intramuscular administration. To do so, they grafted the NPs with the non-toxic carboxylic fragment of the tetanus neurotoxin to allow neuron targeting and were explored to deliver a plasmid DNA encoding

Table 7
The use of organic nanoparticles applied to peripheral nerve regeneration.

	Type of nanoparticle	Cell type or animal model	Main results	References
	NGF encapsulated chitosan nanoparticles	Canine bone marrow derived MSCs	NGF-CNPs are capable of releasing bioactive NGF with the ability to transdifferentiate mesenchymal stem cells into neurons	Mili et al. [215]
	Chitosan porous hybrid scaffolds with varying amounts of Calcium titanate (CaTiO₃) NP	SCs	5mg of NP, could evidently stimulate the attachment, proliferation and biological function maintenance of Schwann cells	Li et al. [216]
	Trimethylated chitosan (TMC)-based NP	Microfluidic based DRG neuron culture	This microfluidic-based neuron culture showed to be of added value in the evaluation of cell–nanoparticle interactions, nanoparticle axonal transport and safety	Lopes et al. [217]
	Thiolated trimethyl chitosan (TMCSH) grafted with the non-toxic carboxylic fragment of the tetanus neurotoxin (HC)	Mice crush injury nerve model	TMCSH-HC/BDNF NP use stimulated the release and expression of BDNF, which led to a superior functional recovery after injury. Furthermore, there was an improvement in crucial pro-regenerative actions	Lopes et al. [218]
	Hyaluronic acid (HA) doped-poly (3,4-ethylenedioxythiophene) (PEDOT-HA) NP into a chitosan/gelatin (Cs/Gel) matrix	PC12 cells	8% PEDOT-HA/Cht/Gel scaffold had a higher cell adhesive efficiency and cell viability than the other conductive scaffolds and could support PC12 cells adhesion, survival, and proliferation. Cells in conductive scaffold expressed high synapse growth gene of GAP43 and SYP	Wang et al. [219]
Polymeric NPs	PEDOT-HA/PLLA film	PC12 cells	Electrical stimulation of 0.5 mA for 2 h significantly promoted neurite outgrowth with an average value length of 122 ± 5 μm and enhanced the mRNA expression of GAP43 and SYP in PC12	Wang et al. [220]
	Hydroxyapatite nanoparticle-containing collagen type I hydrogel	SCs; sciatic nerve defect in rats	significantly enhanced functional behavior of the rats compared with the collagen hydrogel without NP	Salehi et al. [221]
	(NGF)-loaded heparinized cationic solid lipid nanoparticles (NGF-loaded HCSLNs)	(NGF-loaded HCSLNs) effect was studied in induced pluripotent stem cells (iPSCs)	NGF-loaded HCSLNs can be used for differentiation of iPSCs into neurons and NGF-loaded HCSLNs with EQ 1 had higher viability of iPSCs than NGF-loaded HCSLNs with SA	Kuo et al. [222]
	(Rop)- polyethylene glycol-co-polylactic acid (PELA) NP	Rat postoperative pain model	Analgesic effect for nerve blocking for over 3 days after single administration, compared do the 3 h of normal administration	Wang et al. [223]
	3-D biomimetic scaffold, with tunable porous structure and embedded core-shell NP	PC12 cells and primary rat cortical neurons	It was found that 3-D scaffolds with NGF NP greatly increased the neurite outgrowth of PC12 cells and primary cortical neurons	Lee et al. [224]
Dendrimers	PAMAM and phosphorus dendrimers	Transgenic cells were next injected into transected and repaired sciatic nerves of isogenic Lewis rats to assess GDNF expression	Cell-based GDNF therapy was shown to increase the extent of axonal regeneration, while controlled deactivation of GDNF effectively prevented trapping of regenerating axons in GDNF-enriched areas, which was associated with improved functional recovery	Shakhbazau et al. [225]
	(PAMAM)–NH2 dendrimers of fourth generation – pLVTHM/PAMAM nanocomplex	SCs seeded onto GAG matrix	SCs migration into the GAG-matrix tubes separately from proximal and distal ends of transected sciatic nerve	Shakhbazau et al. [226]
	PAMAM dendrimers of generation 5 modified with RGD, YIGSR, or IKVAV peptides	Study on the neurite outgrowth of PC12 cells after inducing NGF differentiation	The adhesive peptides were successfully conjugated to PAMAM dendrimer to promote cell-material interaction and subsequently cell attachment	Maturavongsadit [227]
	Dendrimers covalently linked to near-infra red cyanine-5 fluorescent dye (D-Cy5)	Injected the molecules both intravitreally and systemically (in the rats) or just systemically (in the monkey) after induction of NAION	Systemic administration of dendrimers allows selectively targeting of the ischemic optic nerve lesion	Guo et al. [228]

for BDNF in a PN injury model. This complex loaded NPs stimulated the release and expression of BDNF, which in turn led to a superior functional recovery after injury. Furthermore, there was an improvement in crucial pro-regenerative actions, like the increased expression of neurofilament and growth-associated protein GAP-43 in the injured nerves as well as significantly higher density of myelinated in the distal stump of injured nerves.

Wang et al., [219] produced a novel porous conductive scaffold that was prepared by incorporating conductive HA doped with PEDOT (PEDOT-HA) NPs into a Cht/gelatin matrix. As expected, the incorporation of PEDOT-HA into the scaffold increased the electrical and mechanical properties while decreasing the porosity and water absorption.

The results revealed that 8% PEDOT-HA/Cht/Gelatin scaffold had a higher PC12 cell adhesive efficiency and cell viability than the other conductive scaffolds. Furthermore, cells in the scaffold with 8 wt% PEDOT-HA expressed higher synapse growth gene of GAP43 and SYP compared with Cht/Gelatin control group suggesting that 8%PEDOT-HA/Cht/Gel scaffold is an attractive cell culture conductive substrate which could support neuronal cells survival.

In a different approach, but using the identical conductive material, PEDOT, the same authors [220] produced the same kind of NPs, (HA)-doped PEDOT NPs but instead of Cht, prepared PEDOT-HA/PLLA composite films, which were subjected to different current intensity to elucidate on the effect of electrical stimulation on neurite outgrowth of PC12 cells. Electrical stimulation of 0.5 mA for 2 h significantly promoted neurite outgrowth with an average value length of 122 ± 5 μm. It also enhanced the mRNA expression of growth-associated protein (GAP43) and synaptophysin in PC12 cells as compared with other types of stimulation. These results suggest that PEDOT-HA/PLLA film combined with electrical stimulation may be an attractive candidate for enhancing nerve regeneration.

Moving to another kind of biomaterials as matrix for embedding NPs, Salehi et al., [221] aimed to enhance the efficacy of PNR by combining Ha-NPs with the diameter of 212 nm in a collagen type I hydrogel, extracted from rats' tails. *In vitro*, primary rat SCs cultivation on the prepared hydrogel demonstrated a significantly higher cell proliferation than the tissue culture plate. *In vivo*, the prepared nanocomposite of collagen hydrogel was administrated on the sciatic nerve crush injury in

rats showing significantly enhanced functional behavior when compared to the collagen hydrogel without NPs.

In a different approach, Kuo et al., [222] were able to produce (NGF)-loaded heparinized cationic solid lipid NPs (NGF-loaded HCSLNPs) using heparin-stearic acid conjugate, cacao butter, cholesterol, stearylamine (SA) or esterquat 1 (EQ 1) as reagents. The immunochemical staining of neuronal nuclei revealed that NGF-loaded HCSLNs can be used for differentiation of induced pluripotent stem cells (iPSCs) into neurons and NGF-loaded HCSLNs with EQ 1 had higher viability of iPSCs than NGF-loaded HCSLNs with SA, proving that EQ 1 may be promising formulation to regulate the membrane charge of iPSCs during neuronal differentiation.

Once again addressing the problem of anesthetizing PNs, Wang et al., [223] designed a study to develop and test long-acting NPs and observe the analgesic effects for nerve block in a rat postoperative pain model. For such, they developed long-acting anesthetic ropivacaine-NPs using polyethylene glycol-co-polylactic acid (PELA). Having in mind that the analgesic effect of ropivacaine lasts 3–6 h after intrathecal injection for single use, the ropivacaine-PELA NPs produced an analgesic effect for nerve blocking for over 3 days after single administration.

Lee et al., [224] used a groundbreaking technique to fabricate 3-D biomimetic scaffolds. Stereolithography is a laser-based 3-D printing system capable of fabricating aligned micro- and macro-size 3-D constructs via a layer by layer assembly method: the scaffolds were in the shape of square grid as the base pattern, creating a 3-D porous scaffold with internal pores and channels. To complement this technology, BSA–PLGA NPs and NGF-PLGA NPs were embedded into the previous matrix. It was found that 3-D scaffolds with NGF NPs greatly increased the neurite outgrowth of PC-12 cells and primary cortical neurons. The authors believe that is not only because of the bioactive factor, but also because of the increase of the nano-roughness added by the incorporation of NPs.

These nano-sized molecules have been extensively considered as promising candidates for application as drug-delivery carriers in all biomedical fields. They are highly-branched and symmetrical, branching out from a central core and subdivided into hierarchical branch units, external capping units, with unique structural properties [232]. Their important characteristics ascend from their unique and extremely well controlled architecture, size and surface properties as compared to traditional linear polymers.

Such characteristics offer dendrimers the exciting possibility of accommodating other molecules in their interior, which characterizes its crucial advantage of acting as non-covalent drug-encapsulating agents [233].

These spherical, biocompatible and biodegradable polymeric-based NPs were reported in the late 70s, by Vögtle group, however, they can still be considered as new drug solubilizers [234].

To our knowledge, little has been done with the dendrimer nanotechnology in PNR and repair. On the other hand, there is a vast literature on the use of dendrimers applied in the central nervous system [235–239].

Shakhbazau et al., [225] reported on proof-of-concept work focused on the application of provisional GDNF expression system in injured PNs. For such, they firstly engineered primary cultured SCs using dendrimers or lentiviral transduction with the vector providing doxycycline-regulated GDNF expression. Transgenic cells were next injected into transected and repaired sciatic nerves of isogenic Lewis rats to assess GDNF expression. With this study, they were able to demonstrate for the first time the genetic modification of SCs with use of dendrimer/plasmid complexes. They have also shown for the first time that dendrimer-driven expression of neurotrophic factors is compatible with doxycycline-regulated system and ensures tight control of GDNF release, as seen in Fig. 6B.

The same authors, [226] hypothesized that a collagen-glycosaminoglycan matrix could provide a synthetic way to mimic the SC Basal Lamina (SCBL), since SCBL is a particularly potent substrate for neurite promotion. Also, the authors wanted to confirm the compatibility of such matrix with a virus-free genetic engineering approach, as the use of dendrimers. For such, collagen and chondroitin-6-sulfate proteoglycan suspension was dispensed into the collagen conduits, which were tested in a sciatic nerve defect in rats. Their transfection experiments confirmed polyamidoamine (PAMAM) dendrimers ability to transfect SCs and the penetration of dendrimer complex into the 3D matrix structure.

The 5th generation PAMAM dendrimers were used by Maturavongsadit et al., [227] with the goal of enhancing cellular responses, namely the promotion of increased cellular adhesion. The real aim of the work consisted in both assess the possibility of functionalizing the dendrimers with several cell-binding motifs, such as RGD, YIGSR, or IKVAV peptides and determine how it affects cellular responses. The RGD, YIGSR, or IKVAV functionalized PAMAM coated substrate could promote evident neurite outgrowth of PC12 cells on all of the peptides-modified PAMAM coated substrates by day 1. Quantitative image analysis of the neurite lengths on all of the peptide-modified PAMAM surfaces were in range of 18–20, 50–60, and 90–115 μm on day 1, 2 and 4, respectively, which were significantly higher than the neurite length on the control surfaces.

In an attempt to address non-arteritic anterior ischemicoptic neuropathy and knowing that dendrimers should be directed and collected in inflammatory cells upon systemic administration, Guo et al. [228] tested the difference between locally and systemic injection of traceable dendrimers covalently linked to near-infra red cyanine-5 fluorescent dye. The results revealed that systemic dendrimer administration provided the best penetration in the optic neuropathy lesion site when injected shortly after induction, leading to the conclusion that systemic administration of dendrimers allows selectively targeting of the ischemic optic nerve lesion.

3.2.3. Biologically derived NPs

Intercellular transfer of macromolecules through vesicles, known as exosomes, has become the subject of increasing interest as a novel means for intercellular crosstalk. Exosomes can be considered one kind of NPs. However, unlike the ones mentioned in the sections before, these are biologically derived.

By definition, exosomes are small membranous vesicles or nanovesicles with 40–100 nm in size, with a density of approximately 1.13–1.19 g/cm^2 [240].

Regarding their development, exosomes are produced via the internal budding of endosomes to form multi-vesicular bodies that fuse with the plasma membranes to release exosomes into the surrounding environment. Based on the cell types they are originated from, they contain a wide range of factors, such as proteins, lipids, RNA, mRNA and miRNA [241]. These elements that are carried by exosomes can be termed as "cargo" and will be delivered either to the surroundings cells or taken to have action in more distant cells. With this, it is understandable that depending on the cargo content, different phenomena can happen to the recipient cells, including its DNA reprograming [242]. Not only the cargo will have an impact on the receptor cell, but also the surface membrane of the exosomes contains proteins that act as makers [243]. Exosomes are therefore recognized as new form of intercellular communication between cellular components, but without the expected cell-to-cell direct contact. After understanding the way exosomes work, the potential of such NPs can be assumed for drug delivery and other therapies [244].

It is known today that exosomes are deeply involved in neuronal activity, from its protection, degeneration, development and even regeneration. Many studies have shown that CNS components are capable of releasing exosomes [245–247]. For instance, studies indicate the release of exosomes by neurons [248], microglia [249], astrocytes [250], oligodendrocytes [251], and neural stem cells [252]. However, its role in the PNS has only been showed much more recently. Fig. 7 embodies some exciting results obtained from research works focused on exosomes.
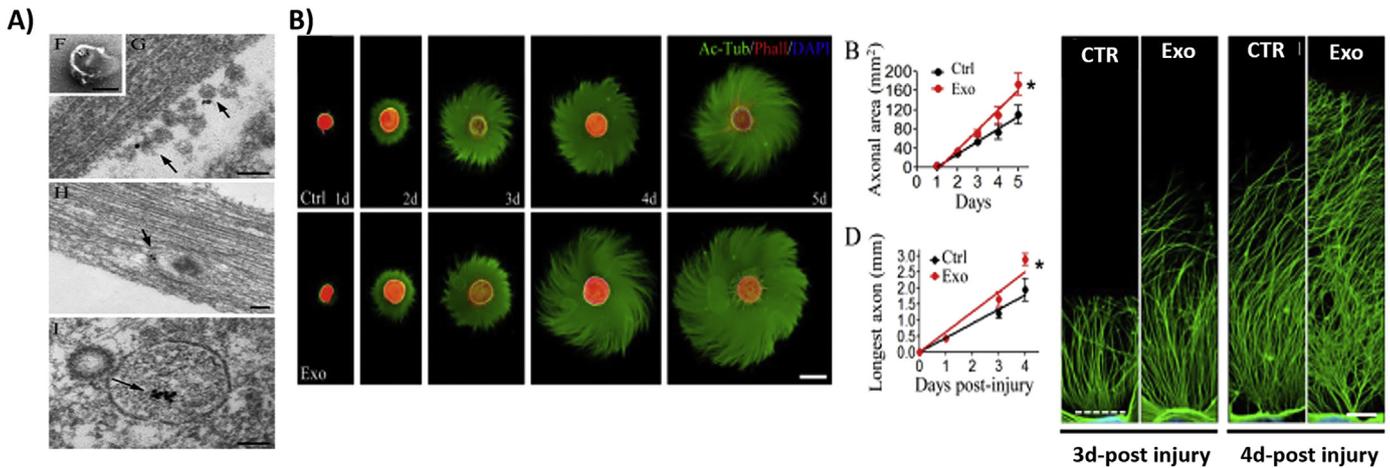
Fig. 7. The use of exosomes in peripheral nerve regeneration. (A) Representative transmission electron micrographs of Schwann cell-derived exosomes in DRG axons, after staining exosomes with p75[NTR]-gold labeling on their surface; (B) Axonal regeneration of DRGs after vehicle (Ctrl) or exosome (Exo) treatment for 5 days. DRGs are stained for acetylated tubulin (Ac-Tub, green), Phalloidin-Rhodamine (Phall, red) and nuclei (DAPI, blue). Scale bar: 50 μm. Reprinted and adapted from [256].

In 2008, Court et al., [253] proved that in damaged PNs, SCs deliver vesicles containing ribosomes into the axon and its contents are then released. Therefore, exosomes can deliver mRNA and ribosomes to injured nerves and promote local protein synthesis which is needed for the process of Wallerian degeneration and consequent regeneration. In this context, the same author [254] has shown that labelled ribosomes in the nerve are derived from the SCs. However, not all exosomes secreted by SCs have positive effects. In fact, SCs have been shown to secrete exosomes containing pathogenic prions in vitro, therefore existing the possibility that these pathogens might be released from the PNS to infect the CNS [255]. Recent literature on the relation between exosomes and PNR is reviewed and summarized in Table 8 [256–260].

In 2013, Lopez-Verrili et al., [256] focused on the regulation of axons by SCs mediated secreted vesicles and studied this means of communication between these two role players of PNS, to support neuronal and axonal regeneration after an injury. The authors explored the hypothesis that exosomes released from SCs would be uptaken by axons in a way to regulate intrinsic mechanisms of neuronal regeneration. They have in fact demonstrated, both in vivo and in vitro that SCs secrete exosomes and that they are selectively internalized by axons. To support their exciting findings, they further proved that SC-secreted exosomes, but not fibroblast-derived ones, markedly increase axonal regeneration. Images regarding this work can be seen in Fig. 7.

The same authors compared the effect of human menstrual MSCs (MenSCs) mediated by three different ways: cell–cell contact, by their total secretome or by secretome-derived extracellular vesicles on

neuritic outgrowth in primary neuronal cultures [257]. As exciting conclusion, they found that the contact of MenSCs with cortical neurons inhibited neurite outgrowth as their total secretome containing exosomes enhanced it. To complete their hypothesis of using exsomes derived from Stem cells, they found that extracellular vesicle fractions showed a distinctive effect. While the exosome-enriched fraction enhanced neurite outgrowth, the microvesicle-enriched fraction displayed an inhibitory effect, suggesting that exosomes derived from MenSCs could have possible applications in PNR.

To address the problem of neuropathic pain and knowing that it is highly likely that miRNA plays some key roles in the formation of pain, Hori et al., [258] performed a miRNA array analysis in the DRG of mice with sciatic nerve ligation and investigated the possible changes in DRG-associated miRNAs in exosomes derived from blood serum in mice. They demonstrated that miR-21, miR-431, and miR-511-3p in the DRG gradually increased in an IL-6-dependent manner during the development of neuropathic pain, but only miR-21 in exosomes extracted from blood was secreted in mice with sciatic nerve ligation. With these results, the authors believe that amplified blood exosomal miR-21 after sciatic nerve ligation may be seen as a diagnostic biomarker for neuropathic pain.

Still considering the subject of neuropathic pain, Simeoli et al., [259] show that DRG neuron cell bodies release extracellular vesicles, including exosomes containing miRs. They have demonstrated that pure sensory neuron-derived exosomes released by capsaicin are readily phagocytosed by macrophages in which an increase in miR-21-5p expression promotes a pro-inflammatory phenotype. This suggests that

Table 8
Exosomes in scope of peripheral nerve regeneration.

Type of nanoparticle	Cell type or animal model	Main results	References
SCs exosomes	SCs, fibroblasts, DRGS, Rat crush injury model	Dedifferentiated SCs secrete nano-vesicles known as exosomes which are specifically internalized by axons. They increase axonal regeneration both in vivo and in vitro	Lopez-Verrilli et al. [256]
MSCs's derived exosomes	Menstrual MSCs Bone Marrow MSCs, umbilical cord MSCs and chorion MSCs	MenSC exosomes showed superior effects on the growth of the longest neurite in cortical neurons	Lopez-Verrilli et al. [257]
Exosomes derived from blood serum in mice	Mice and partial sciatic nerve ligation model in rat	demonstrated that miR-21, miR431, and miR-511-3p in the DRG gradually increased in an IL-6-dependent manner during the development of neuropathic pain	Hori et al. [258]
DRGs exosomes	DRGs, nerve injury in mice	Up-regulation and release of miR-21 contributed to sensory neuron–macrophage communication after damage to the peripheral nerve	Simeoli et al. [259]
NG2+ cells exosomes	NG2+ cells, rat neurons	The release of Retinoic Acid in association with exosomes provided a permissive substrate to neurite outgrowth	Gonçalves et al. [260]

upregulation and release of miR-21 contribute to sensory neuron-macrophage communication after damage to the peripheral nerve.

Gonçalves et al., [260] identified a novel mechanism by which neuronal retinoic acid receptor β (RARβ) activation results in the endogenous synthesis of retinoic acid, which is released in association with exosomes and acts as a positive cue to promote axonal/neurite outgrowth. The excitement of this discovery relies in the fact that their data suggests the possibility of an advantageous therapeutic approach where a RARβ agonist could be used for a much shorter period than the one required for the full regeneration of the axon.

These biologically derived NPs are a promising field in the PNR area. One can envision the use of SCs autologous patient-specific exosomes to enhance nerve regeneration. However, the downside of this is the same as the use of autografts, as one healthy nerve needs to be sacrificed for such [261]. However, stem-cells play a major role here, since it is well know that Schwann-like cells can be obtained from both MSCS and Adipose Derived Stem Cells (ACSs) and so could their exosomes.

Nevertheless, the above-mentioned studies might result in significant improvements for the patients in the future when the use of exosomes is used in the clinical setting, however that vision is still a step ahead.

3.2.4. NPs Toxicity

Although related to various successful and innovative discoveries, the use of NPs is not completely safe and various reports have been considering this subject [262–265]. This is mainly due to the fact that such particles in the nano-size interact directly with cells and lead to the formation of reactive oxygen species, leading to oxidative stress [262]. In fact, and although the advantages of nanotechnology are undeniable, people are now generally unprotected against various nano-scale materials. This fact makes them extremely powerful, as they may assist in the clinical practice, but might also become a threat to human life [266]. Because of their size, it is expected that NPs with less than 10 nm behave similar to a gas and can easily enter human tissues [267].

Further cytotoxicity can be found in positively charged NPs, as they have the capacity to interact and induce disruptions in the living cells plasma membranes [268].

More specifically, aluminum-based NPs, which contribute to 20% of all nano-sized chemicals, were demonstrated to have cytotoxicity, as they disrupt cellular viability, alter mitochondrial function, increase oxidative stress, and modify tight junction protein expression of the blood brain barrier. Also, genotoxicity has been studied for these molecules, revealing dose-dependent genotoxic properties [269,270].

Silver NPs are considered one of the most cytotoxic NPs. When compared to others, these NPs have shown more toxicity in term of cell viability, generation of reactive oxygen species and lactate dehydrogenase (LDH) outflow. As they accumulate in different parts of the body, they have been identified in various organs, including lungs, spleen, kidney, liver, and brain [271].

Zinc oxide NPs have also been studied regarding their cytotoxicity on bacteria and mammalian cells. Their most common toxic effects are related to cell membrane damage and increased oxidative stress, which have been reported in various mammalian cell lines [272].

Often, the toxicity is provided not by the NPs themselves, but by the coatings many time used to facilitate the processes in biomedical applications, which is the case of polydimethylamine, found to have cytotoxic effects in cortical neurons isolated from chick embryos [273].

In the end, one must have precaution when using these nano-scale materials, since they can in fact interact with organisms in a cellular lever, especially in the nervous system. However, their benefits for the future of the science and medical community concerning PN treatment and repair are undeniable and many examples of that were described above.

3.3. Nanofibers

Nanofibers are fibers with diameters one or two orders of magnitude smaller than conventional fibers that closely mimic the ECM. These fibers have a uniquely large surface area-to-mass ratio and can be usually produced by electrospinning [274] and self-assembly [275] techniques.

Electrospinning is a simple and versatile electrodynamic practice in which a polymer solution can be spun by engaging a high potential electric field to obtain nano-scale long fibers, meaning it relies on the electrostatic repulsion between surface charges to continuously draw nanofibers from a viscoelastic fluid. It includes the quick evaporation of solvent and solidification of droplets to form fibers [276].

As the external electrical field is applied, the liquid in the metallic needle is induced to elongate at the tip of the needle and takes on the form of a cone which is extended in the form of a jet. A typical electrospinning apparatus contains four main components: i) a high voltage source (1–30 kV), ii) a metallic needle or capillary, iii) a syringe pump, and iv) a grounded conductive collector, that among many shapes, usually is a simple flat plate or rotating mandrel [277].

An incredible amount of materials can be used for electrospinning, including polymers, ceramics, small molecules, and their combinations [278]. Not only different biomaterials can be used, but the final smooth nanofiber meshes can be adapted to create nanofibers with a number of secondary structures, comprising porous, hollow, or core—sheath structures. Furthermore, nanofibers can be collected into ordered arrays or hierarchical structures by manipulation of their alignment, stacking, and/or folding. Therefore, it has been recognized at least for a decade now that nanofibrous scaffolds also offer great potential in the field of neural tissue engineering, as they are able to mimic native tissue tubular structures including axons, microtubules and ion channels [279,280].

Because of the potential related to the use of nanofibrous in PN tissue engineering, many interesting publications have emerged in the last few years. Extraordinary results obtained with reviewed random, aligned and conductive nanofibers can be seen in Fig. 8, as well as the electrospinning apparatus (Fig. 8A). Table 9 summarizes the reports on use of the different nanofibers applied in PN regeneration that will be further discussed [86,87,281–297].

3.3.1. Random nanofibers

As mentioned before, nanofibrous mats are very successful due to its resemblance to ECM. However, in a study conducted by Gnavi et al., [281] the influence of the fiber size on explanted cultures of SCs and DRGs was studied after producing micro- or nanofibers made of gelatin by electrospinning. In one hand, nanofibers promoted cell spreading and actin cytoskeleton organization, increasing cellular adhesion and the proliferation rate. On the other hand, both migration rate and motility, quantified by means of carrying out trans-well and time-lapse assays respectively, were greater in cells cultured on micro-fibers. That study clearly indicates that topography of electrospun gelatin fibers can be adjusted to modulate SC and axon organization (Fig. 8B).

In a noteworthy publication, Suzuki et al., [282] hypothesized whether an electrospun nanofiber sheet incorporating methylcobalamin (MeCbl), one of the active forms of vitamin B12 homologues, when delivered locally to the PN injury site, would contribute to promoting nerve regeneration. In vitro, cortical neurons were cultured in microfluidic chambers and it was seen that local administration of MeCbl to the axon compartment promoted axonal outgrowth equal to axon and administration of MeCbl. In vivo, when wrapping the nanofiber sheet around the PN injury, local administration of MeCbl promoted nerve regeneration and enhanced functional recovery.

In another study comprising random nanofibers, Mohamadi and colleagues [283] aimed at evaluating the sciatic nerve regeneration potential in the rat nerve transaction injury model followed by implantation of poly(e-caprolactone)/collagen/nanobioglass (PCL/collagen/NBG) nanofibrous conduits containing human endometrial stem cells
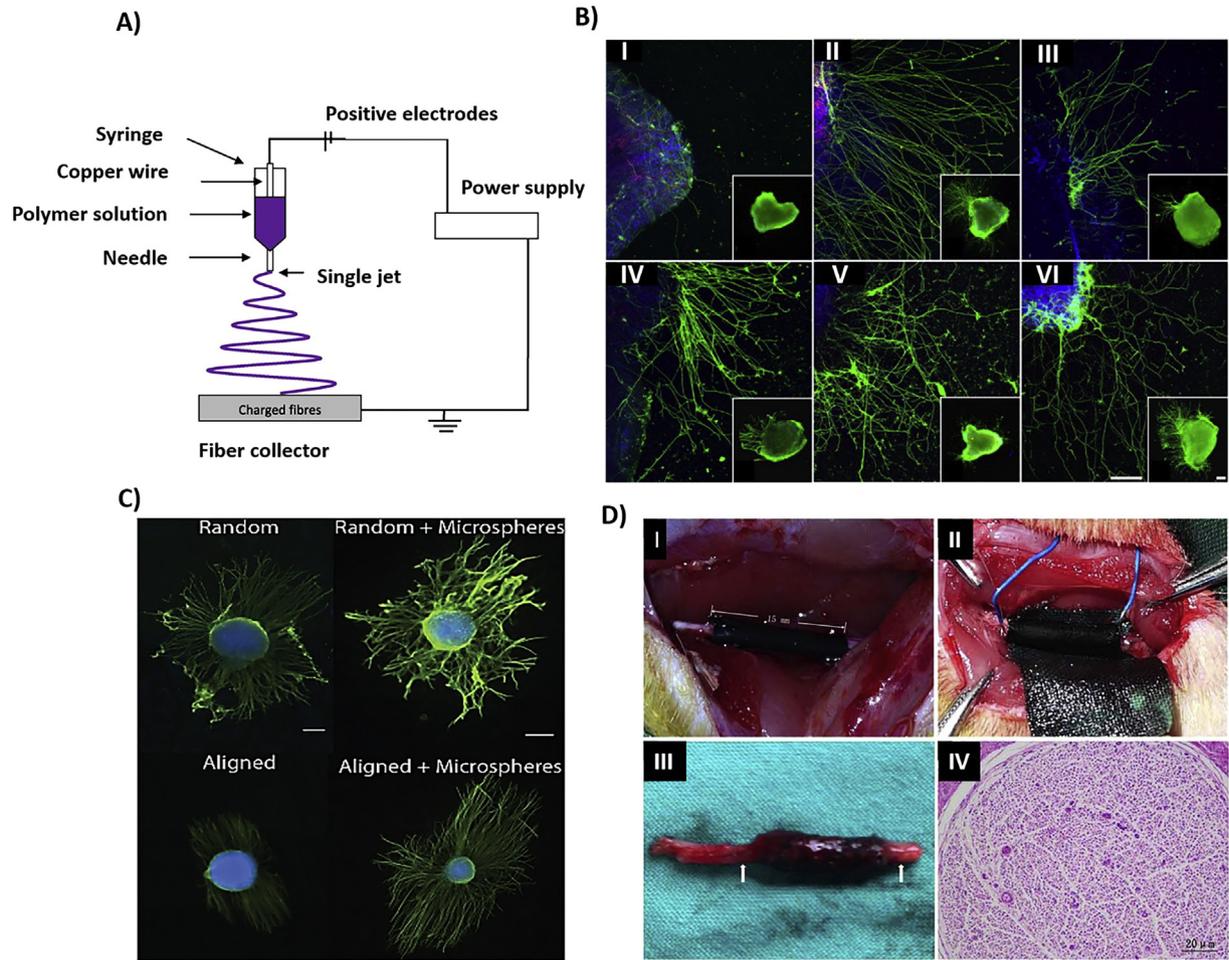
Fig. 8. – The application of nanofibers in peripheral nerve regeneration. (A) Schematic representation of the electrospinning process; (B) DRG axon outgrowth after fluorescent staining with β-tubulin (green) and DAPI (blue), when cultured on (I) Matrigel® coated coverslips without NGF (negative control conditions; (II) Matrigel® coated coverslips with 50 ng of NGF/ml in medium (positive control conditions); (III) DRGs seeded on 300 nm fibers; (IV) DRGs seeded on 600 nm fibers; (V) DRGs seeded on 1000 nm fibers; and (VI) DGRs seeded on 1300 nm fibers. Scale bar: 20 μm; (C) Representative images of DRGs cultured for 5 days on aligned and random scaffolds in the presence or absence of microspheres. Samples without microspheres were supplemented with 50 ng/ml of NGF. Aligned fibers direct neurite outgrowth. Scale bar: 250 mm; D) Use of PPY/PLCL combined with electrical stimulation in peripheral nerve regeneration. (I) The animal operation procedure immediately after 15 mm conduit implantation; (II) Circle electrode implantation; (III) Harvested regenerated nerve at 8 weeks post-implantation; and (IV) 8 weeks post-implantation histological section of the electrode contact site stained with H&E (white arrows indicate the electrode contact site). Reprinted and adapted from (A) [276]; (B) [281]; (C) [291]; (D) [297].

(hEnSCs). To do so, the authors first prepared the respective conduit by electrospinning. Afterwards, a fibrin gel containing hEnSCs with a concentration of 1x10^5 cells/mL was injected in the conduits and implanted. After extensive evaluation, the authors demonstrated that hEnSCs grafted inside a PCL/Collagen/NBG conduits have the potential to improve sciatic nerve regeneration, however without knowing the exact roles of hEnSCs in the process.

Huang et al., [284] combined the stage-wise approach of electrospinning to produce the outer conduit made of random nanofibers with the directionally freezing orientated collagen-chitosan (O-CCH) filler, since incorrect positioning or distribution of intraluminal fillers and consequent incorrect wiring of the regenerating axons might result in regeneration failure. After proper characterization, collagen/chitosan in the reason (1:1) was selected for filler fabrication and a wall thickness of 400 μm was selected for PCL sheath production. 3D-reconstruction further revealed that the O-CCH filler unveiled a longitudinally oriented microstructure with 85% of pores and the electrospun PCL porous sheath with pore sizes of 6.5 ± 3.3 μm that prevented fibroblast invasion. Overall, *in vitro* and *in vivo* studies up to 12 weeks indicated that the O-CCH/PCL scaffolds could promote axonal regeneration and migration.

Xia et al., [285] was able to incorporate VEGF and NGF on the surface and in the core of emulsion random electrospun PLLA nanofibrous scaffold, respectively, which was capable of dual delivery.

In this sense, VEGF and NGF had a sequential release pattern, in which most of the VEFG was released in the first few days as it was embedded in the surface of the meshes, but the NGF could be continuously released for more than 1 month, as it was captivated in the interior of the nanofibers. *In vitro*, the use of such scaffold could enhance the neural differentiation of iPSCs-derived neural crest stem cells (iPSCs-NCSCs). Additionally, this scaffold was applied to a critical sized defect in rat sciatic nerve model *in vivo*, which revealed a significant improvement of neovascularization as well as nerve healing after 3 months post-operation.

Sun et al., [286] reversed the previous strategy of using the nanofibers meshes as conduits and used it instead as a filler. Poly (L-lactic acid-co-ε-caprolactone)/SF (PLCL/SF) nanofiber sponges were used as fillers to prepare 3D nanofibers sponges containing conduit. The filler fabricated by electrospinning and subsequent freeze-drying demonstrated abundant macropores, high porosity and superior compressive modulus. *In vitro* cell viability studies indicated that the fabricated conduit could enhance the proliferation of SCs due to the macro-porous structure, since hematoxylin-eosin and immunofluorescence staining confirmed that these cells infiltrated into the nanofiber-sponges. Afterwards, the conduit was implanted in the rat sciatic nerve defect model to evaluate the effect *in vivo*, which in general performed better than the hollow conduit group.

Table 9

The use of nanofibers applied to peripheral nerve regeneration.

	Type of nanofiber	Cell type or animal model	Main results	References
Random nanofibers	Gelatin micro- or nanofibers	SCs and DRGs	The topography of electrospun gelatin fibres can be adjusted to modulate SC and axon organization, since it affects parameters such as motility or cytoskeleton organization	Gnavi et al. [281]
	PCL and MeCbl Nanofibers	Cortical neurons; rat sciatic nerve crush injury model	Local administration of MeCbl at PN injury sites promoted nerve regeneration and enhanced functional recovery in a rat sciatic nerve crush injury model	Suziki et al. [282]
	poly (e-caprolactone)/collagen/nanobioglass (PCL/collagen/NBG) nanofibrous conduits transplanted with hEnSCs	Human endometrial stem cells (hEnSCs)	PCL/collagen/NBG nanofibrous conduit filled with hEnSCs is a suitable strategy to improve nerve regeneration after a nerve transaction in rat	Mohamadi 2017 [283]
	electrospun PCL porous conduit with O-CCH as a longitudinally oriented microstructure filler	DRG, Schwann cells; rat sciatic nerve defect	Functional results indicated that the CCH/PCL compound scaffold induced comparable functional recovery to that of the autograft group at the end of the study	Huang et al. [284]
	Emulsion electrospun poly (ʟ-lactic acid) (PLLA) nanofibrous	Induced pluripotent stem cells-derived neural crest stem cells (iPSCs-NCSCs)	VEGF and NGF in emulsion electrospun nanofibrous scaffold had a synergistic effect on regeneration of vascularized nerve tissue	Xia et al. [285]
	PLCL/SF nanofiber sponges	SCs; rat sciatic nerve model	The results demonstrated that nanofibers-sponge containing conduit performed better than the hollow conduit	Sun et al. [286]
	PCL and collagen/PCL blends incorporated in a gelatin matrix and inserted in collagen tubes	Rat sciatic nerves defect	When implanted in 15 mm sciatic nerve defect, animals containing collagen/PCL fibers had higher extent of recovery when compared to animals that had received empty implants, but not as good as with autologous nerve transplantation	Kriebel et al. [287]
Aligned nanofibers	Randomly oriented and aligned poly(methyl methacrylate) nanofibers	DRGs and SCs	When co-cultured, DRGs and SCs revealed that on aligned nanofibers, neurites and SCs had a higher chance of co-localization than on randomly oriented nanofibers	Xia et al. [288]
	Randomly oriented and aligned poly(methyl methacrylate) nanofibers	DRGs and SCs	When co-cultured, DRGs and SCs revealed that on aligned nanofibers, neurites and SCs had a higher chance of co-localization than on randomly oriented nanofibers	Xia et al. [86]
	Aligned PHBV nanofiber	rat sciatic nerve defect	Nanofibers in an aligned orientation favored stem cell growth and elongation and indicated that the FGF2-*miR-218* induction approach combined with the (PHBV)-3D nanofiber scaffolds facilitated the nerve regeneration	Hu et al. [87]
	Randomly oriented and aligned poly(L-lactic acid) nanofibrous scaffolds	iPSC-derived neural stem cells	Compared with randomly, the aligned PLLA nanofibers greatly directed neurite outgrowth from the iNSCs and significantly promoted neurite growth along the nanofibrous alignment	Lin et al. [289]
	Aligned electrospun polylactic acid (PLLA) microtube array membrane (MTAM)	Co-cultures of rat fetal neural stem cells (NSC) and astrocytes	MTAM is a better co-culture platform than the traditional Transwell system	Tseng et al. [290]
	Aligned electrospun Methacrylated hyaluronic acid nanofibers	DRGs	DRGs seeded o the scaffolds revealed that the combination of NGF released from the microspheres and the aligned nanofibers significantly enhanced neurite outgrowth	Whitehead et al. [291]
	PCL electrospun nanofibers were mixed with gelatin and further embedded in 3D printed hydrogel scaffold	Primary cortical neurons	It was possible to fabricate 3D neural tissue constructs by combining 3D bioprinting and electrospinning techniques and the neurons aligned in the aligned nanofibers	Lee et al. [292]
	3D hierarchically aligned fibrin nanofiber hydrogel (AFG)	SCs, DRGs; Rat sciatic nerve injury	The developed scaffold supports SCs cable formation and axonal regrowth within 2 weeks, with similar results to the autograft after short and long-term studies	Du et al. [293]
Nanofibers combined with conductive materials	Polyaniline and silk fibroin (PASF) nanofibers	SCs; sciatic nerve nerve defect	12 months after implantation it was verified enhanced neuro-regeneration	Das et al. [294]
	PPy(lactide acid) conductive fiber-film (CFF) coated with cell-derived extracellular matrix derived from L929 cells (CFF-ECM)	PC12 cells	The combination of ECM-CFF with electrical stimulation could improve the nerve regeneration by encouraging neural-cell adhesion, neurite growth and extension	Zhou et al. [295]
	PLA/PPy nanofibrous scaffold	human umbilical cord MCSs, SCs	PLA/PPy nanofibrous scaffold containing 15% PPy with sustained conductivity and aligned topography. Additionally, the direction of cell elongation on the scaffold was parallel to the direction of fibers	Zhou et al. [296]
	Conductive PPY/PLCL conduits were synthesized by polymerizing pyrrole coated on Poly (l-lactic acid-co-ε-caprolactone)	PC12 cells, DRG; Rat sciatic nerve defect	The PPY/PLCL conduits with electrical stimulation showed a similar performance compared with the autograft group, and significantly better than the non-stimulated PPY/PLCL conduit group showing great potential for PN regeneration	Song et al. [297]

Using the same strategy of applying nanofibers as a luminal filler, Kriebel et al., [287] developed a cell-free, 3D scaffold for axonal guidance for long-distance nerve repair in which the nanofibers of made of biodegradable PCL and collagen/PCL blends were incorporated in a gelatin matrix and inserted in collagen tubes, in a complex strategy developed earlier by the same author.

When implanted in 15 mm sciatic nerve defect, animals containing collagen/PCL fibers had higher extent of recovery (compound muscle action potentials, motor functions of the hind limbs) when compared to animals that had received empty implants, but not as good as with autologous nerve transplantation.

3.3.2. Aligned nanofibers

Many works have been focusing on horizontally aligned nanofibers, since in 2006 [298] and 2008 [299] it was proved the high potential of anisotropic topographies for PNR. This is because of the natural formation of the Bands of Bungner after an injury, which are oriented columns of laminin-1 and aligned SC. Regenerating fibers then enter the gap and follow these Bands of Bungner, reaching the distal end of the severed nerve, re-enervating the distal target, guided by the horizontal aligned cues.

Xia et al., [288] aimed at meticulously analyze the specifics of neurite growth of DRGs on randomly oriented and aligned poly(methyl

methacrylate) (PMMA) nanofibers and understand the relationship between neurites and nanofibers, as well as the alignment or random orientation of the nanofibers. The authors verified a relationship between neurites and nanofibers, as the neurites of DRGs were in close contact with the substrate nanofibers, and the neurites seemed to follow aligned nanofibers more than randomly oriented nanofibers. Most importantly, when co-cultured, DRGs and SCs revealed that on aligned nanofibers, neurites and SCs had a higher chance of co-localization than on randomly oriented nanofibers, which is of key importance in the process of nerve regeneration.

The same authors [86] described aligned electrospun PMMA nanofibers as a SC-loading scaffold *in vitro*, by monitoring the GFP-containing SCs cultured on nanofibers. They found that aligned nanofibers provided better support for the cells than did non-aligned nanofibers, as relationships between SCs and fibers could clearly be seen. Once again, the co-culture experiment showed on aligned nanofibers both SCs and DRGs adhered and elongated along the axes of the fibers, so they had a higher chance of co-localization is definitely beneficial to the ensure the process of myelination.

Hu et al., [87] were able to combine biomaterials and stem cells in an approach where neuronal differentiation of stem cells was based on the temporally sequential use of *miR-218* and Fibroblast Growth Factor 2 (FGF2) *in vitro,* (FGF2-*miR-218* induction approach). Furthermore, the authors applied this novel approach in repairing sciatic nerve damage *in vivo*.

The neuronally differentiated ASCs were integrated with the 3D aligned nanofibers and implanted in a 10 mm transected rat sciatic nerve defect *in vivo*. The results showed that, compared to randomly aligned nanofibers, the nanofibers in an aligned orientation favored stem cell growth and elongation. Furthermore, FGF2-*miR-218* induction approach combined with the poly(hydroxybutyrate-co-3-hydroxyvalerate)(PHBV) 3D nanofiber scaffolds facilitated nerve regeneration.

In a complex report also involving biomaterials and stem cells, Lin et al., [289] used mouse iPSCs generated from mouse embryonic fibroblasts with the non-integrating episomal vectors pCEP4-EO2S-ET2K and pCEP4-miR-302-367 cluster and differentiated them into NSCs as transplanting cells. Electrospinning was then used to fabricate randomly oriented and aligned PLLA nanofibers. Compared with randomly oriented, the aligned PLLA nanofibers greatly directed neurite outgrowth from the iNSCs and significantly promoted neurite growth along the nanofibrous alignment.

Tseng et al., [290] developed an innovative system where a substrate made of aligned electrospun PLLA microtube array membrane (MTAM) was successfully developed as a cell co-culture platform. Its architecture is based on one-to-one connected, ultrathin, nano-scale fibers that are set in an arrayed creation. To study the co-culture potential, rat fetal NSC and astrocytes were examined by relating the outcome of a typical transwell-based co-culture system and that of MTAM-based co-culture system. Greater cell viability of NSC was detected when cultured in electrospun PLLA MTAM and RT-PCR exposed a robust interaction between astrocytes and NSC confirming that MTAM is clearly a better co-culture platform than the traditional Trans-well system.

Making use of aligned nanofibers, Whitehead et al., [291] developed a conduit with all mechanical, chemical, and topographical cues destined for PNR. Methacrylated-HA was electrospun into aligned fibers, with poly-lactic-co-glycolic acid microspheres to deliver NGF. DRG seeded in the scaffolds revealed that the combination of NGF release from the microspheres and the aligned nanofibers significantly increased neurite growth (Fig. 8C).

To address some limitations of the regular 3D printing, Lee et al., [292] combined stereolithography and electrospinning techniques to fabricate a novel 3D biomimetic neural scaffold with a tunable porous structure and highly aligned nanofibers. PCL nanofibers were mixed with gelatin and further embedded in 3D printed hydrogel scaffold. Then printable hydrogel inks, composed of 40 wt% Polyethyleneglycol

(PEG), 60 wt% diacrylate (PEG-DA) and photo-initiator (0.5 wt% of PEG-DA concentration) covered the electrospun fiber. As expected, the results indicated that 3D printed scaffolds with electrospun fibers significantly improved NSC adhesion when compared to those without the fibers. Notably, the scaffold with PCL/gelatin fibers greatly increased the average neurite length and directed neurite extension of primary cortical neurons along the nanofibers.

Du et al., [293] was capable of producing a 3D hierarchically aligned fibrin nanofiber hydrogel through electrospinning and molecular self-assembly to resemble the architecture and biological function of the native fibrin cable mentioned before as bands of Bungner. In this produced biomaterial, nanofibers are laid in the surface of the hydrogel. Firstly, *in vitro* assays with SCs and DRGs showed fast and directional cell adhesion and migration, as well as alignment of neurites. Secondly, the aligned hydrogel was then used as a potential intraluminal substrate in a bioengineered chitosan tube to bridge a 10-mm long sciatic nerve gap in rats, revealing a beneficial microenvironment to support SCs cable formation and axonal regrowth within 2 weeks, with similar results to the autograft after short and long-term studies.

3.3.3. Nanofibers combined with conductive materials

Das et al., [294] synthetized and described for the first time PA-based nerve conduits, expressing the safety and efficacy of the conduits in PN injuries. For the production of such conduits, the nanocomposite was synthesized by electrospun a mixture of PA and SF, in which the silk nanofibers were uniformly coated with PA. Subsequently, by means of rolling of the electrospun sheet over a stainless-steel mandrel, tubular shaped nerve conduits are molded. It was verified that implanted PA-SF conduits seeded with SCs exhibited exceptional nerve conduction velocity, compound muscle action potential, motor unit potential, visible growth of healthy tissue along the nerve gap and thick myelination of axons 12 months after implantation, indicating enhanced neuro-regeneration, when compared to similar electrically conductive conduits.

Using PPy as the conductive material to blend in the nanofibers, Zhou and colleagues [295] could enhance neurite adhesion, alignment and elongation of PC12 cells on PPy-PLA conductive fiber-film (CFF) coated with cell-derived ECM derived from L929 cells (CFF-ECM). By combing the developed material with electrical stimulation, it was verified that PC12 cell adhesion rate, neurite-bearing cell rate, neurite alignment rate and neurite length on ECM-CFF were significantly larger than the corresponding values on bare CFF.

Using the same materials, PPy and PLA, Zhou et al., [296] embedded PPy into PLA nanofibers *via* electrospinning and fabricated a PLA/PPy nanofibrous scaffold. *In vitro* assays with human umbilical cord mesenchymal stem cells as well as SCs proved the scaffolds had good biocompatibility with such cells. Additionally, the direction of cell elongation on the meshes containing 15% PPy proved sustained conductivity and aligned topography.

Song et al., [297] investigated the abilities of direct current electrical stimulation through electrospinning conductive polymer composites composed of PPy and PLCL (PPY/PLCL) in PN regeneration. PC12 cells and DRGs cells cultured on PPY/PLCL scaffolds were stimulated with 100 mV/cm for 4 h per day. After that, the median neurite length and cell viability were measured in PC-12 cells and the levels of BDNF, GDNF and NT-3 were analyzed in DRG neurons, revealing that not only the quantity of PC12 cells increased, but also the median neurite length increase significantly after electrical stimulation. Regarding the neurotrophic factors, all of them were up-regulated, which was detected by western blot, RT-PCR and Elisa quantifications. When implanted *in vivo* and after electrical stimulation, the results revealed satisfying functional recovery and equivalent morphological recovery to nerve autografts (Fig. 8D).

After this review of the lately published works comprising both random and aligned nanofibers, as well as mixed with conductive polymers, one can easily see the huge potential of these nanotechnologies.

This is primarily related with three points: (i) its resemblance with ECM, to which PNS cellular components easily adhere, (ii) the advantage of this technology that allows to change its architecture to extremely aligned nanofibers, which is of crucial significance in PNR, as the process of nerve regeneration itself lies in the formation of aligned fibrin cables and ultimately possibly to augment nerve regeneration, and (iii) the electrospinning allows the easy incorporation of many kinds of substrates and molecules, such as vitamins, conductive polymers or other polymers that might act as co-adjuvants or stimulants in the process of regeneration.

3.4. Topographic cues at micro- and nano-scale

There is now sufficient body of evidence which states that artificial physical cues, such as topography, can have an important impact on the neuronal cell functions [300]. In fact, a relatively new theory states that the growth cone guidance follows the "substrate-cytoskeletal coupling" model [301]. Accordingly, "growth cones can move forward if they are capable of coupling intracellular motility signals to a fixed extracellular translocation substrate via cell surface adhesion receptors".

The power of topography and its influence on neuronal cells has been established as early as 1914, when *R. G. Harrison* first cultured embryonic frog in the threads of a spider web. Surprisingly, he observed that the cells specially stretched along the support given by the threads [302].

With the new production techniques of micro- and nano-topographies, new types of cell culture platforms can be established, and the influence of a panoply of topographical cues can be studied in what regards cellular functions, such as cytoskeleton morphology, proliferation or differentiation.

However, most of the structures that were studied and had the power to affect axonal growth and alignment were of micro-sizes [303–305].

In the field of topography, there are several methods available for the development of patterned biomaterial surfaces, ranging from simple manual scraping to highly controlled manufacture methods. Contact printing, [306] lithography, [307] microfluidic patterning [308] and electrospinning [284] are some of the techniques that allow a more precise and nano-scale physical cues to be achieved. Table 10 resumes the use of nano-scaled topographic cues applied in PN regeneration [309–313]. Fig. 9 depicts some of the most interesting achievements related to topographic and neuronal investigations.

In what concerns nanotopography, *Ferrari* et al. [309] studied the neuronal polarity of PC12 cells on cyclic-olefin-copolymer grooves of 350 nm depth and 500 nm groove width with varying ridge width. It was shown that cells would fluctuate from monopolar and bipolar to multipolar morphology, as the ridges width shifted, as seen in Fig. 9A.

The same authors [310] described an experience where PC12 cells were in contact with nano-gratings, which are periodic submwavelength structures, where there is an alternating of submicron lines of ridges and grooves, with 350 nm depth and with line widths and pitches of 500 nm were fabricated by nanoimprint lithography.

Cells were stimulated or co-stimulated with NGF and other neuronal factors like forskolin. It was found that the presence of Forskolin diminished the phenomena of neurite alignment to the nanogratings which was related to the inhibition of focal adhesion maturation necessary for the alignment to occur.

In a pioneer work, Foley et al. [311] addressed the question of which role topography might play in promoting neuritogenesis, since the phenomena of contact guidance has been well established before. For that, the authors cultured PC12 cells within an array of NGF concentrations on surfaces with ridge sizes ranging from 70 nm up to 1900 nm. Interestingly, it was found that with sub-optimal or lower concentrations of NGF, the neuritogensis was reinforced by topographic feature size. Also, contact guidance would happen to feature sizes as small as 70 nm. These results propose that topographic features can act compliantly with NGF signaling to regulate the formation and development of neurites.

Baranes et al., [312] used photolithography to produce matrices with line-pattern ridges in the nano-scale dimension. By using neurons isolated from the CNS of adult leeches, they found that majority of the neuronal processes that approach ridges of 75 nm and higher are affected by the ridge and change their original growth direction, as seen in Fig. 9B. Also, there are two parameters that, according to them, must be considered when predicting the probability of a neuronal process to be affected by the ridge: the ridges' height and the neuronal incoming angle, since for low ridges, the larger effective membrane surface due to acute incoming angles compensates the lack of adhesion surface.

CNT can also be considered as a kind of nano-scale surface texture. Megan et al. [112] was able to very that motor neurons on grow and aligned when seeded over thin films of horizontally aligned CNTs.

Mitra et al., [313] also worked with carbon materials, this time in a study that involved textured carbon substrates which were planned for investigation of neural cell performance and cytocompatibility. Nano-scale geometries in materials like resorcinol–formaldehyde gel derived carbon films and electrospun nanofibrous (200 nm diameter) mat derived carbon micro-patterns were tested with results showing that textural features from 200 nm (carbon fibers) were found to affect neurite outgrowth. Furthermore, regardless of the randomness of carbon nanofibers, they promoted preferential differentiation of N2a cells into neuronal lineage, showing the importance of the nano-scale topography.

With the above reviewed works, it is our feeling that it would be interesting to further explore the lower limit of neuronal sensitivity to nano-scale topographic cues and the resolution of that sensitivity.

Table 10
Nano-scale topographic cues applied to peripheral nerve regeneration.

| Type of nanomaterial	Cell type or animal model	Main results	References
Nanogratings with 350 nm depth and with line widths and pitches of 500 nm were fabricated	PC12 cells	Nanogratings with the smallest ridge size (500 nm) strongly favor bipolar cells and a transition to multipolarity is obtained increasing the ridge width to 1500 nm	Ferrari et al. [309]
Nanogratings with 350 nm depth and with line widths and pitches of 500 nm were fabricated	PC12 cells	Topographical guidance in PC12 cells is modulated by the activation of alternative neuronal differentiation pathways	Ferrari et al. [310]
X-ray lithography was used to pattern silicon wafers. The resulting period:ridge widths for the six patterned surfaces were as follows; 400:70, 800:250, 1200:400, 1600:650, 2000:850 and 4000:1900 (nm). Groove depths were 600 nm	PC12 cells	It was verified that the scale of substratum features can act supportively with sub-optimal growth factor concentrations acting as chemical cues to stimulate neurite extension	Folei et al. [311]
Lines are fabricated by standard photolithography methods	Neurons from adult medicinal leeches	Neuronal processes, which are of micron size, have strong interactions with ridges even as low as 10 nm in which the interactions depend on the ridges' height	Baranes et al. [312]
Varying micro and nano-scale geometries: resorcinol–formaldehyde (RF) gel derived carbon films and electrospun nanofibrous (~200 nm diameter) mat and SU-8 (a negative photoresist) derived carbon micro-patterns	Neuroblastoma cells and SCs	The in vitro studies on such carbon scaffolds using neural cells confirm that SCs are more adaptive towards topographic cues than undifferentiated N2a cells	Mitra et al. [313]
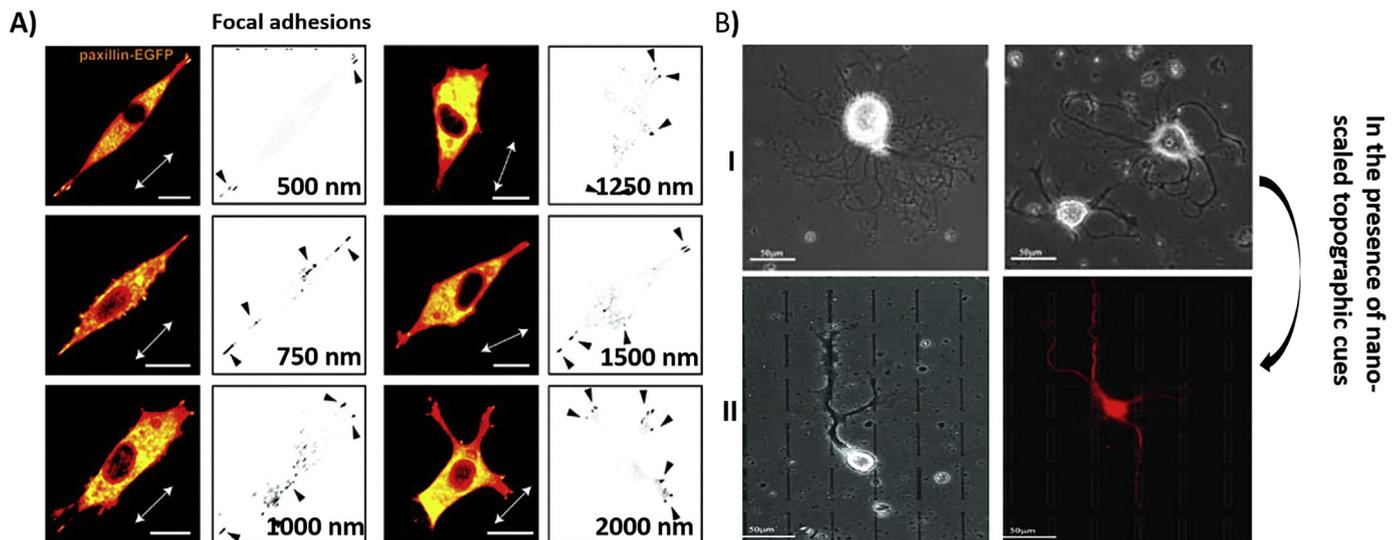
Fig. 9. The influence of nano-scaled topographic cues on cellular behavior. (A) Focal adhesion establishment by stimulating PC12 contacting nanogratings with increasing ridge width. EGFP fluorescent signal in PC12 cells differentiating on nanogratings with a ridge width corresponding to (I) 500 nm; (II) 750 nm; (III) 1000 nm; (IV) 1250 nm; (V) 1500 nm; and (VI) 1500 nm. White arrows indicate the direction of the nanografting. Scale bar: 10 μm; and (B) (I) Leech neurons growing atop control substrates pre-coated with concanavalin-A. No preferable growth direction can be detected; and (II) Leech neurons growing on nano-patterned substrates pre-coated with concanavalin-A. Pattern ridges of 25 nm in height. The neuronal branches attach to the ridges and the whole growth pattern is aligned according to the topographic cues. Reprinted and adapted from (A) [309]; (B) [312].

4. Nanotheranostics and imaging

The concept of theranostics relies in the combination of both therapeutics and diagnostics in one set. It is based in the image-guided therapy and also defining the treatment outcome at an early stage [314]. Theranostics should provide a visualization and tracking of imaging-labeled components not only for the diagnosis of the problem, but also possibly assessing the biodistribution of a therapeutic drug during treatment or specific molecular target [315].

Up to date, whenever an injury to PN is confirmed in the clinic context, the standard diagnosis is generally based on a fusion of simple clinical examination findings by the physicians and neuroelectrophysiology approaches [316]. Consequently, the diagnosis precision is often reduced since the two approaches mentioned before do not provide satisfactory information for the needed surgical repair, which in turn leads to improper treatments and poor outcomes for the patients, which results in life-long disabilities.

The fact that injuries vary according to patient populations, etiologies, age and complexity of the injuries are co-adjuvant of this problem. The alarming consequences of PNI are not because of the injury itself, but due of the poor diagnosis and delayed time of medical action [317,318].

Furthermore, whenever a treatment like surgery is applied, there is a complete lack of monitoring tools and reliable non-invasive strategies to assess the consequent degeneration or axonal regeneration into the distal segment [319].

Therefore, there is a pressing medical need for the development of reliable and reproducible, non-invasive imaging strategies [320]. Such imaging strategies must have adequate spatial and temporal resolution for the early and exact diagnosis assessment of PNI as well as continued monitoring after treatment [321]. Consistent, harmless, noninvasive and reproducible methodologies that aid in the determination of the injury degree and nature are required for the correct diagnosis, for instance to help in finding the exact location where changes in the normal nerve anatomy occurred. Once the diagnosis is correctly made, "regenerative tracking" and longitudinal monitoring of nerve regeneration, followed by treatment with neurotherapeutics holds great promise to achieve well-timed and precise treatments of PNI [322].

A few imaging techniques have been exploited for PNI, both in the scientific and pre-clinical settings, such as optical imaging, positron emission tomography computed tomography, magnetic resonance imaging, high resolution ultrasound and nuclear medicine [318,323–326]. Nevertheless, despite the promising advances in the literature, it remains clinically difficult to apply noninvasive imaging technologies, since most require general improvement to be considered as a standard method [327].

Now a days, there are two main methods used for imaging of PNS. The first is ultrasound, thanks to it wide availability and spatial resolution. Its use has clearly established indications and is particularly useful for PNS tumors. MRI is commonly considered as the second-line imaging method, aiming at providing decisive additional information thanks to its excellent contrast resolution. Both technologies are robust and widely spread in the clinic. Also, one has to consider the strong benefit of being able to examine the perineural environment, which cannot be evaluated by clinical examination alone or by electroneuromyography [328].

However, despite the panoplies of imaging methods mentioned before, it has been discussed previously that, in summary, MRI is the one that offers most promising results when in combination with nanotechnology, therefore is considered the best potential tool for PNI. MRI uses a robust magnetic field to generate a magnetization in the tissue, followed by a disruption of magnetization with a brief radio pulse [329].

In one hand, the resolution of the standard MRI is within the range of hundreds of microns which goes against the possibility of using it for visualizing axons which are from 5 to 10 μm. On the other hand, high definition MRI-based imaging techniques are highly specialized, very expensive and logistically restricted, so most often unreachable by patients who need it [330]. As a result, nano-systems tactics allied to MRI for molecular and cellular imaging after PNI must be highly considered. Furthermore, these systems that would allow visualizing the true cellular behaviors and molecular complex phenomena that take place after injuries of each patient would gratefully contribute to personalize medicine and provide a patient specific therapy. In our opinion, nanotechnology is the way to address this need.

NPs such as iron oxide NPs are an especially useful tool in nano-systems based imaging. They are highly sensitive contrast agents that can be up-taken by cells and their position changed by magnetic fields and located by MRI [331].

Perfluorocarbon (PFC) nanoemulsions are also considered as attractive nano-components as imaging platforms [332]. They are highly

Table 11
Theranostics and nanoimaging applied to peripheral nerve regeneration.

	Type of nanomaterial	Cell type or animal model	Main result	References
PNR imaging with MRI	USPIO labeling protein 0 (P0) and PMP 22 kDa	Not applicable	The developed MRI probe would allow for imaging the PN targets *in vivo*	Zheng et al. [321]
	USPIO conjugated with MRI	A model of neuropathic in rat sciatic nerve	Animals with neuropathic pain in the left hindpaw show increased trafficking of USPIO-laden macrophages to the site of sciatic nerve injury	Ghanouni et al. [334]
	USPIO, Molday ION Rhodamine B (MIRB)	Rat Neural Stem Cells	Although USPIO particles, such as MIRB, may have advantageous labeling and magnetic resonance-sensitive features for NSC tracking, a further examination of their effects might be necessary before they can be used in clinical scenarios of cell-based transplantation due to exaggerated MRI signal dropout in the animals	Umashankar et al. [335]
	SPION-labeled cells	GFP-expressing ADSCs; mice sciatic nerve defect	Histological analysis and immunohistochemistry confirmed the axon regeneration firstly revealed by MRI	Li et al. [336]
Imaging of neuroinflammation	Perfluorocarbon nanoemulsion, which is phagocytosed by inflammatory cells	Autoimmune encephalomyelitis rat	The *in vivo* MRI results were confirmed by extremely high-resolution ^{19}F/^{1}H magnetic resonance microscopy	Zhong et al. [337]
	Perfluorocarbon nanoemulsion, which is phagocytosed by inflammatory cells	Chronic constriction injury in rat	The results demonstrate that the infiltration of immune cells into the sciatic nerve can be visualized in live animals using these methods	Vasudeva et al. [338]
	Perfluorocarbon nanoemulsion	Rat sciatic nerve	^{19}F MRI allows *in vivo* visualization of inflammation in the peripheral nervous system. Inflammation is detected unambiguously with high spatial resolution. Quantification of the fluorine signal is possible with *ex vivo* ^{19}F spectroscopy	Weise et al. [339]

biologically inert and easily detected by MRI [333]. Table 11 summarizes the approaches envisioned for nanotheranostics and nanoimaging comprising nanotechnology in the field of PN regeneration [321,334–339]. Also, Fig. 10 reveals some of the most exciting results obtained in the field of theranostics and nano-imaging.

Zheng et al., [321] described a molecular and nano-scale imaging methodology, namely molecular and cellular MRI (MCMRI). Based on the fact that there are cellular and molecular changes on the nervous system, both at proximal and distal sites after an injury, the authors hypothesized that *in vivo* PN targets can be imaged using MCMRI with specific probes, based on injured nerve specific proteins as targets. That was achieved by using a molecular antibody fragment conjugated to iron NPs as an MRI probe. The options considered were protein 0 (P0) and PMP 22 kDa as PNs specific target proteins, since the exclusivity of the targets in PNs is the basis of this hypothesis.

To be able to get an image, such antibodies fragments were conjugated to ultra-small super paramagnetic iron oxide (USPIOs), which shortens the transverse relaxation of hydrogen proton and influence the imaging signal intensity and enhances the contrast. However, for this hypothesis to be successful, it is essential that the probes have good bio-distribution and are able to surpass the blood-brain barrier and have its deposition done in PNs.

In another study, Ghanouni et al., [334] used USPIOs conjugated with MRI to monitor macrophage trafficking, with the purpose of determining whether minocycline modulates macrophage trafficking to the site of PN injury *in vivo* and, in turn, results in altered pain thresholds. In fact, Animals with neuropathic pain in the left hindpaw show increased trafficking of USPIO-laden macrophages to the site of sciatic nerve injury, which might contribute to the anti-nociceptive effect.

Umashankar et al., [335] performed a comprehensive examination of rat NSC survival and regenerative function upon labeling with a specific kind of USPIO, named Molday ION Rhodamine B (MIRB), which allows for dual MRI and optical imaging. They observed that both MIRB doses (20 and 50 μg/mL) supported the robust detection of NSCs, over an extended period of time *in vitro* and *in vivo* after transplantation into the striata of host rats, using MRI and fluorescence imaging.

However, animals receiving the 50 μg/mL MIRB-labeled NSCs, had an immune response consisting of an increased number of CD68(+)-activated microglia, which appeared to have phagocytized MIRB particles and cells contributing to an exaggerated MRI signal dropout in the animals.

Li et al., [336] investigated the feasibility of the use of MRI to non-invasively track the role of ASCs in the repair of PNI *in vivo*. For such, the authors isolated, expanded and differentiated Green fluorescent protein (GFP)-expressing ASCs into SC-like phenotype (GFP-dASCs) at early passages and subsequently labeled with SPIONs. The results showed GFP-dASCs were efficiently labeled with SPIONs, without affecting their viability and proliferation. The labeled cells implanted into the mice sciatic nerve conduit exhibited a significant increase in axonal regeneration compared with the empty conduit and could be detected by MRI. Some images of such results can be seen in Fig. 10A.

In other approaches, the MRI technique has been mainly used to detect the migration of immune cells in living organisms, which contributes to the adequate understanding of the pathologies [337,340].

Also, the understanding of the neuroinflammation process is of extreme importance, as a comprehensive approach on this parameter will give clues about neuronal regeneration, since there is an intrinsic connection between the injury and the consequent process of inflammation. Also, the process of neuroinflammation changes during the course of the injury and respective restorative pathway. Therefore, it can be used to monitor the variable progress from individual to individual, using MRI as the most promising imaging technique. Fortunately, monocytes and macrophages are key players in the neuroinflammation process and are also extremely reachable to be used as cellular targets, since they usually phagocyte the imaging agents or NPs injected in the blood stream [341].

Zhong et al., [337] evaluated the ability to noninvasively image and quantitate disease pathology using emerging "hot-spot" ^{19}F MRI methods in an experimental autoimmune encephalomyelitis rat. Once again, PFC nanoemulsion was used and injected intravenously, which labeled predominately monocytes and macrophages in situ. Once again, the analysis of the spin-density weighted ^{19}F MRI data enabled
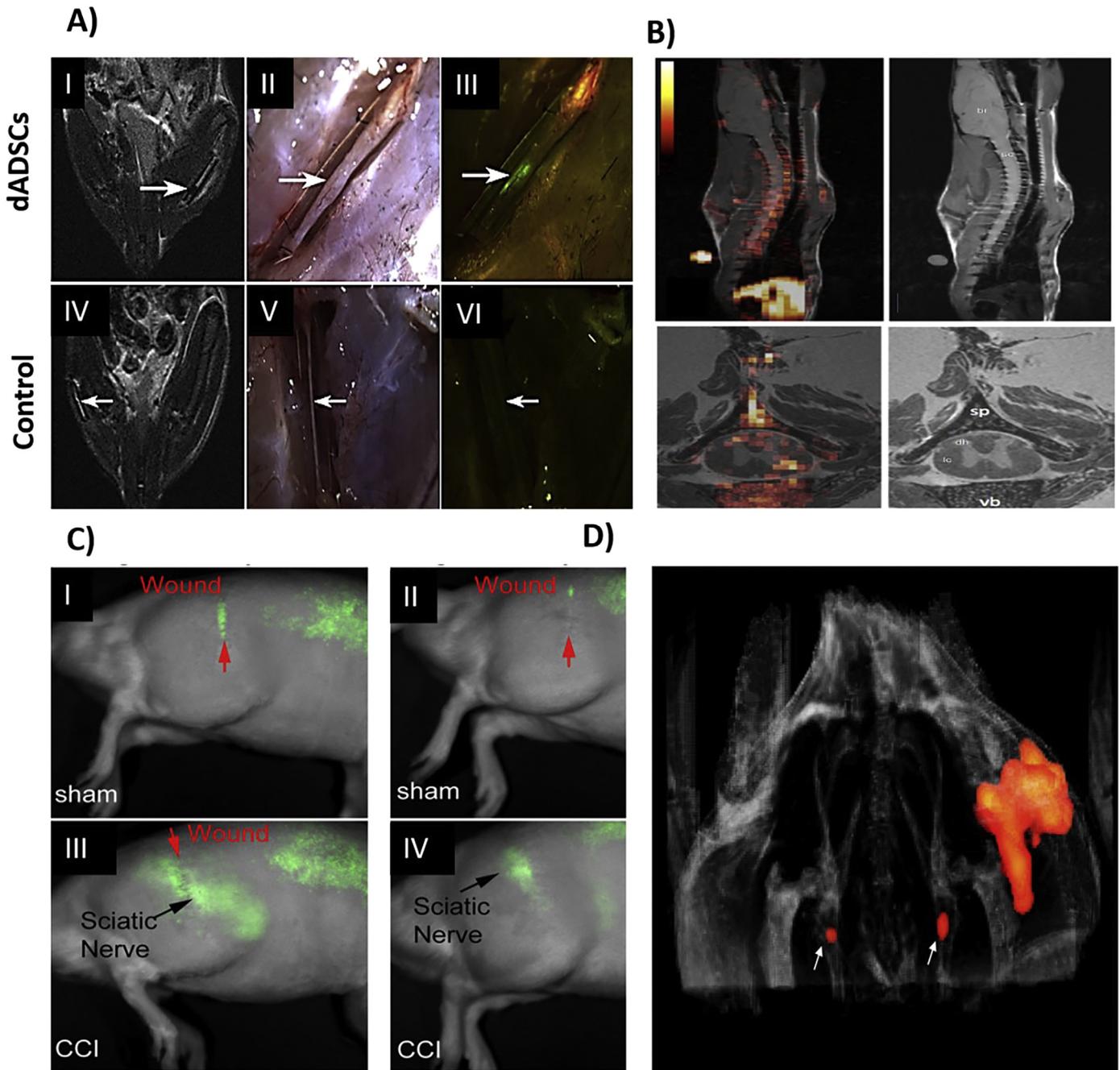
Fig. 10. The application of theranostics and nanoimaging in peripheral nerve regeneration. (A) Images of SPION-labeled GFP-dADSCs sciatic nerve. (I) MR images of operated sciatic nerve 28 d after transplantation; (II) Photograph of nerve that has been transplanted with GFP-dADSCs; (III) Fluorescent images of the nerve that has been transplanted with GFP-dADSCs; (IV) Positive control of I; (V) Positive control of II and (VI) Positive control of III. White arrows point to the regenerated sciatic nerve; (B) *In vivo* and *ex vivo* ^{19}F/^{1}H MRI of experimental autoimmune encephalomyelitis rat shows ^{19}F signal arising from the spinal cord and adjacent vertebral bone marrow, representing accumulation of inflammatory phagocytes; (C) Perfluorocarbon nanoemulsion, which is phagocytosed by inflammatory cells, was used in a rat pain model. The nanoemulsion is a near-infrared (NIR) lipophilic fluorescence tracer. The images demonstrate that in live rats, NIR fluorescence is concentrated around the affected sciatic nerve or in the area where incision was made, both reporting the site of macrophages location; and (D) *In vivo* ^{19}F MRI performed 5 days after induced nerve injury. In each rat, the fluorine marker uptaken by macrophages could be exclusively found in the left affected thigh, to where macrophages migrated. Two small bilateral "hot spots" are illustrated close to the spine which represent draining lymph nodes where marked macrophages also accumulate; and Reprinted and adapted from (A) [336]; (B) [337]; (C) [338] (D) [339].

quantification of the apparent macrophage burden in the central and peripheral nervous system and other tissues, as can be seen in Fig. 10B.

Vasudeva et al., [338] hypothesize that the infiltration of immune cells into the affected sciatic nerve could be monitored *in vivo* by molecular imaging. To test this hypothesis, an intravenous injection of a novel PFC nanoemulsion, which is phagocytosed by inflammatory cells, was used in a rat chronic constriction injury model in the sciatic nerve. To monitor the events, the nanoemulsion carries two distinct imaging agents, a near-infrared (NIR) lipophilic fluorescence reporter and a ^{19}F

MRI (magnetic resonance imaging) tracer. They have demonstrated that NIR fluorescence is concentrated in the area of the affected sciatic nerve as well as the ^{19}F MRI signal, as can be seen in Fig. 10C. That was proven to be true when histological examination revealed significant infiltration of CD68 positive macrophages.

Weise et al., [339] established ^{19}F MRI for cell tracking in the PNS of rats. In their experiment, in order to induce neuroinflammation, lysolecithin was injected directly into the left sciatic nerve which lead to demyelination followed by severe infiltration of monocytes/macrophages. In

fact, and as a proof of concept, systemic administration of PFC led to a fluorine signal along the proximal stretch of the affected sciatic nerves in *in vivo* [19]F MRI which was not seen on the right healthy side. An *In vivo* [19]F MRI performed 5 days after induced nerve injury can be seen in Fig. 10D.

Despite the reviewed promising advances, noninvasive imaging of PNI remains in its infancy in the clinic. In this specific field, it is essential that pharmaceutical industries take a leading role so that translation of innovative technologies is accelerated from the bench to the clinics.

4.1. Nanotheranostics toxicity

In the nanotheranostics field, PFCs have attracted a great deal of attention in the last decade. Two main factors contribute to the high positive impact of PFCs: (i) the lack of natural endogenous background signal *in vivo* and (ii) the high NMR sensitivity of the ^{19}F atom. These make PFC nanoemulsions ideal agents for cellular and magnetic resonance molecular imaging [342].

The wide range of drugs that PFCs can incorporate represent another advantage. Its core is surrounded by a lipid monolayer that can be functionalized to contain various agents for imaging or therapeutic action [343]. Despite the high toxicity presented by many drugs of interest, namely anti-tumor drugs, the use of such nanoemulsions can increase the degree of tumor targeting while diminishing its undesired secondary effects [344].

Analyzing PFCs toxicity, the tissue clearance time varies based on the chemical compound. These tissue half-lives range from 4 days to 65 days [345]. Clearance of PFCs takes place not through metabolism, but through slow dissolution back into the circulation by the lipid carriers in the structure being, in a last step, removed through expiration [345].

In a study developed by Zhong et al., [346] PFCs showed uptake at 24 h primarily in the liver, followed by spleen and lung, with minimal uptake in kidney and heart, with no binding to the brain. Indeed, no negative change was seen in animal body during the time of the study, even with multiple nanoemulsion administrations.

At this point of research, all the findings point to the potential of nanoemulsions for accurate organ/cell targeting while imaging at the same time, with no apparent toxicity, being considered one of the greatest advantages in the medical field.

Still in the field of theranostics, iron oxide contrast agents, well known as USPIOs, have been combined with magnetic resonance imaging for cell tracking. There are two main reasons why these nanoparticles need to be coated. The first is related to the fact that iron nanoparticles interact with each other in two forms: magnetically and via van der Waals' interactions, leading the NP to flocculate [347].

The second reason is related to their cytotoxicity, as uncoated NP have been shown to have up to a six-fold increase in cytotoxicity compared to dextran-coated iron NP. That cytotoxicity is mainly related to the creation of ROS, reduction of cellular proliferation and induction of cell death [348].

When it comes to USPIOs elimination by the body, they are phagocytosed by the reticuloendothelial system from the blood pool. These nanoparticles are typically taken up by macrophages and phagocytic cells in the liver, bone marrow, and spleen. Afterwards, the lysosomal compartments of macrophages gradually degrade the iron nanoparticles [349].

USPIO's have been reported to have an intrinsic toxicity that can be overcome by means of performing a surface coating with a biocompatible polymer, therefore increasing its potential to be used in the medical practices. Such demonstration has been made in a study by Neuwelt et al. [350], where it was concluded that USPIOs agents are a viable a more safe option for patients which cannot use the typical Gadolinium based contrast agent due to chronic kidney disease.

5. Final remarks and future perspectives

Peripheral nervous injuries are a public-health problem and recent achievements within the nanotechnology area, in particular to what concerns the development of advanced nanomaterials are expected to revolutionize novel therapies and provide improvements to current diagnostic and treatments.

Furthermore, with nanotechnologies, the medical community is able to perform a more precise determination of the extent of the neuronal damage, which enables a better diagnosis, thereby leading to a proper treatment and improvement of the consequent outcomes, instead of just using the old functional evaluation of the damaged nerve.

It is believed that current pre-clinical successes of nanotechnology can and should be adopted and adapted to the science of PNs regeneration and reconstruction. Not only that, but the described technologies should have a future in the clinics, since their benefits are immense. There are several significant PNR challenges which have not been fully addressed yet, despite decades of research. Poor vascularization, guided tissue regeneration, excessive fibrosis and neuroinflammation are some of those hurdles

Carbon nanomaterials, which are characterized by their excellent electrical properties, are of full interest when considering that electrical stimulation is beneficial for PNR. Allied to the fact that it is possible to conjugate other molecules and that carbon nanomaterials can be arranged in specific topographies, the outcomes are overall improved when they are used as neuronal cellular substrates, either alone or in conjugation with other materials.

Nanoparticles have the widest spectrum of benefits, since their purposes are endless: they can be used to just improve any materials' bulk properties, to deliver growth factors in a controlled and dependent manner or be used to label and track cells. This is useful not only to better understand the mechanisms of naturally occurring PNR, but also to provide a permissive and encouraging environment for the regenerative process.

Due to the aligned and nano-scaled nature of PNS components, nanofibers and nano-topographic cues are essential as physical cues and provide the guidance that PNs requires for a successful regeneration.

Finally, nanotheranostics and imaging allow to improve not only the diagnostic process, but also the course of treatment after PNIs. Recently developed contrast agents allow clear visualization of PNR phenomena, such as inflammation, pain and the regeneration itself, depending on the targeted cells and contrast agents used.

All of the above-mentioned nanotechnologies have a promising role in the clinics, since the outcomes are always improved in their presence. However, at this stage, it is fundamentally important to assess each nanotechnology safety, efficacy and quality, in order to be able to make that fundamental clinical translation.

The aforementioned sections overviewed the current nanomaterials, strategies and different approaches that scientists have been working on, that gives an insight into the dynamics of damage and regeneration of PNs and their cellular components.

In our perspective, nanomedicine is a field that can highly contribute to solve many problems related to the regeneration of partial nerve damage verified in most patients. In one hand, the design of TCs implantable devices comprising either carbon nanomaterials, NPs or nanofibers can provide a wide range of benefits, as a better substrate for neuronal cells, topographical cues that guide axons, adhesion points and optimized drug delivery. From a physio-pathological point-of-view, those features are necessary when envisioning a future medical device for PNR, for which such technologies should keep being sought. On the other hand, theranostics are the tools of future in any medical field, which permit real time imaging and a personalized treatment. When correctly developed and applied, as seen in a few publications herein reviewed, theranostic approaches can fill the gaps that currently

exist in the clinics and potentiate the technological translation from the bench to the clinics. We believe that by combing several nanotechnological strategies together, every step of the patient care since he enters the trauma room will be eased, improved and personalized. That is the consequence of better and faster diagnosis, precise treatment and follow-up.

Acknowledgements

Funding

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