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Coupling biophysical stimuli with functional scaffolds to overcome the current limitations of peripheral nerve regeneration: a review

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Peripheral nerve injuries are common occurrences that can lead to the loss of sensibility and function, strongly impairing the patient's quality of life. The current techniques acting on nervous tissue regeneration rely on grafts, which are autologous or synthetic nerve guidance conduits produced by tissue engineering methods. However, even using these procedures, functional recovery is limited to a success rate of around 50%, which indicates the need for improvement in the peripheral nerve regeneration approach. Scaffolds with biomimetic characteristics and functional properties are increasingly being developed based on nanotechnology principles. Moreover, different external biophysical stimuli can be applied to achieve even better results. This review discusses the limiting factors that preclude complete nerve recovery and addresses four biophysical strategies to improve regeneration: electric, magnetic, light, and ion-release-based stimulations. The literature has shown that combining these techniques with nanomaterial-based nerve guidance conduits yields an improved nerve repair process. Furthermore, understanding the biological mechanisms underlying regenerative principles of nerve repair can drive new strategies of nerve tissue engineering under biophysical stimuli, overcoming current limitations of peripheral nerve regeneration.

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1. Introduction

The peripheral nervous system (PNS) is an extensive and relatively unprotected tissue compared with other vital tissues and organs protected by the bones. Consequently, peripheral nerves are susceptible to physical injuries, such as stretching, laceration, or compression, which may occur in traffic, construction, or electrical accidents, war wounds, and chronic diseases.[1] The annual incidence of peripheral nerve damage in developed countries ranges between 10 and 20 per 1000 people, resulting in high costs for patients and the healthcare system, with annual expenditures in the USA exceeding 150 billion dollars.[2,3] In Brazil, an epidemiological study found a high prevalence of nerve injuries in the upper limb, where approximately 50% of observed cases required surgical intervention for nerve repair.[4]

Although the PNS has an intrinsic ability for regeneration, the success of repair is limited and depends on factors such as the severity of the injury, time elapsed until treatment, patient age, and type of nerve affected.[5] Incomplete repair leads to severe consequences for the patient, including neuropathic pain, loss of motor, sensory, or autonomic functions, and overall reduced quality of life associated with self-perceived depression.[6] Thus, strategies have been developed to treat more severe injuries where spontaneous regeneration is insufficient. The basic approach in nerve tissue regeneration is neurorrhaphy, which consists of suturing the ends of damaged nerves. However, the gap size limits such a technique, given that injuries greater than 0.5 cm result in excessive tension in the nerve segments, leading to regeneration failure.[1]

Grafting is well used in peripheral nerve regeneration (PNR), with autologous graft being the current gold standard. It can be applied to treat moderate (0.8 up to 3 cm) and severe injuries (>3 cm), with a maximum gap of 5 cm. Other grafts, such as decellularized allografts or xenografts, can be recommended for more significant gaps, even though they may trigger an immune response, requiring immunosuppressants.[7] Other disadvantages of autologous grafts, beyond size limitation, include limited availability and morbidity of the donor

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tissue, the need for two surgical procedures, and incompatibility between donor and recipient tissue.[8] Despite being regarded as the best technique for PNR, this treatment results in a functional recovery success rate of only 50%.[9]

Tissue engineering presents an alternative for PNR by using biomaterials as scaffolds for tissue repair.[10] These materials can be shaped into tubes, known as nerve guidance conduits (NGC), that are used to reconnect segments when the structure is completely transected. The first developed NGCs were hollow and made of inert materials, mainly silicone, serving as structural support and protecting the regeneration area.[11] This method, known as tubulation, eliminates the need for autologous tissue donation and supports regeneration by reducing scar tissue infiltration, increasing the accumulation of growth factors, and guiding axon growth.

However, the success of NGCs is limited to gaps up to 4 cm, and functional recovery shows results comparable to or even worse than those obtained with autologous grafts.[12] Advancements in understanding nerve anatomy and physiology, the development of new biomaterials, and the application of nanotechnology and multi-functional approaches have allowed for increased complexity and success rate of conduit tubes.[13] One strategy that has been drawing the scientific community's attention is biophysical stimulation. It is successfully applied for nerve regeneration due to the excitable nature of the cells and signaling pathways that can improve nerve repair.[14] External stimuli, such as electrical, magnetic, light, and ionic release, can improve regeneration by promoting neurite extension and alignment, regulating cell activity, and altering the release of growth factors.[15–18] These strategies can also be coupled with NGCs to promote a multidimensional approach for better regeneration. Notwithstanding, combining nanotechnology with these simulations can further improve the scaffold performance because of the enhanced functional properties that synergistically affect the stimulation effect.[19]

This review focuses on how biophysical stimulation and functional scaffolds can overcome the current limitation of PNR, highlighting the mechanisms responsible for improved regeneration. The biophysical stimuli covered here are (i) electrical, (ii) magnetic, (iii) light, and (iv) ionic release. Considering the stimulus *per se* or combined with NGCs, the regeneration mechanism is briefly discussed, paying special attention to multi-functional strategies employing nanotechnology. We thoroughly analyzed relevant studies, showing the pros and cons. Ultimately, we summarized the perspectives for PNR in the light of biophysical approaches aligned with nanotechnology.

2. Peripheral nerve regeneration: state-of-the-art and key challenges

The PNS involves ganglia, which contain nerve cell bodies and satellite cells, and nerves, composed of axons, glial cells (Schwann cells), connective tissue, and blood vessels. Anatomically, nerves have a complex hierarchical structure, and any defect or injury needs to be quickly and meticulously repaired to avoid functional loss.[2] After traumatic events, morphological and metabolic changes occur in the cells composing nervous tissue. Injuries too close to the soma may lead to cell death, likely through apoptosis. Otherwise, chromatolysis begins, leading to histological changes that result in increased metabolism aimed at producing proteins and other components of the axonal cytoskeleton at the expense of neurotransmitter production.[20]

Peripheral nerve injuries (PNI) cause the degeneration of the distal segment axon and myelin sheath. This event, known as Wallerian Degeneration, generally occurs 24–48 hours after injury.[21] Schwann cells (SCs) that remained on the endoneurial tube after Wallerian Degeneration and recruited macrophages are responsible for removing the resulting fragments. Both macrophages and SCs release growth factors and cytokines that regulate and sustain regeneration by stimulating the proliferation of other peripheral nerve cells.[20]

After PNI, considerable modifications occur in the microenvironment to achieve regeneration. When the SCs lose contact with the axon, they alter their phenotype to one favorable for regeneration, called repair SCs.[22] In this condition, they express several molecules, including growth factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and transforming growth factor β (TGF-β), enzymes, and extracellular matrix (ECM) molecules. These biomolecules maintain the SCs, which have a survival period of only a few months in the distal endoneurium and assist in nerve tissue regeneration.[23] Once reconnection with the target tissue is achieved, SCs can be reverted to a myelinating phenotype. This process is controlled by the contact between axons and different signaling molecules, leading to the formation of the myelin sheath around axonal fibers, completing the regeneration process.

After nerve damage, SCs migrate within the endoneurial tubes and guide the regenerating axons. However, when a gap is formed between nerve stumps, SCs need support for their directional growth. The initial reconnection of nerve ends is achieved by developing the bridge structure, consisting of cells, primarily macrophages, neutrophils, fibroblasts, endothelial cells, and extracellular matrix (ECM) components that reconnect the nerve segments.[15]

Then, after the hypoxia caused by the injury, macrophages increase vascular endothelial growth factor (VEGF) production, and there is a significant infiltration of vascular endothelial cells that form blood vessels in the region. Only after this process can repair SCs from both stumps migrate through the vascularized bridge structure and promote axonal directional growth.[24] At the proximal segment, degeneration occurs up to the first node of Ranvier. The axons form growing cones guided by SCs on the bridge until the distal stump, where SCs form the bands of Bungner, which are the tube-like structures formed by repair SCs in the endonerial tubes, aiming to reconstruct the damaged nerve.[20]

In proximal injuries, chronic denervation can occur, *i.e.*, the absence of contact between the nerve structures and axons causes SCs to atrophy, basal laminae to deteriorate, and Bungner bands to disappear.[25,26] This creates an unfavorable

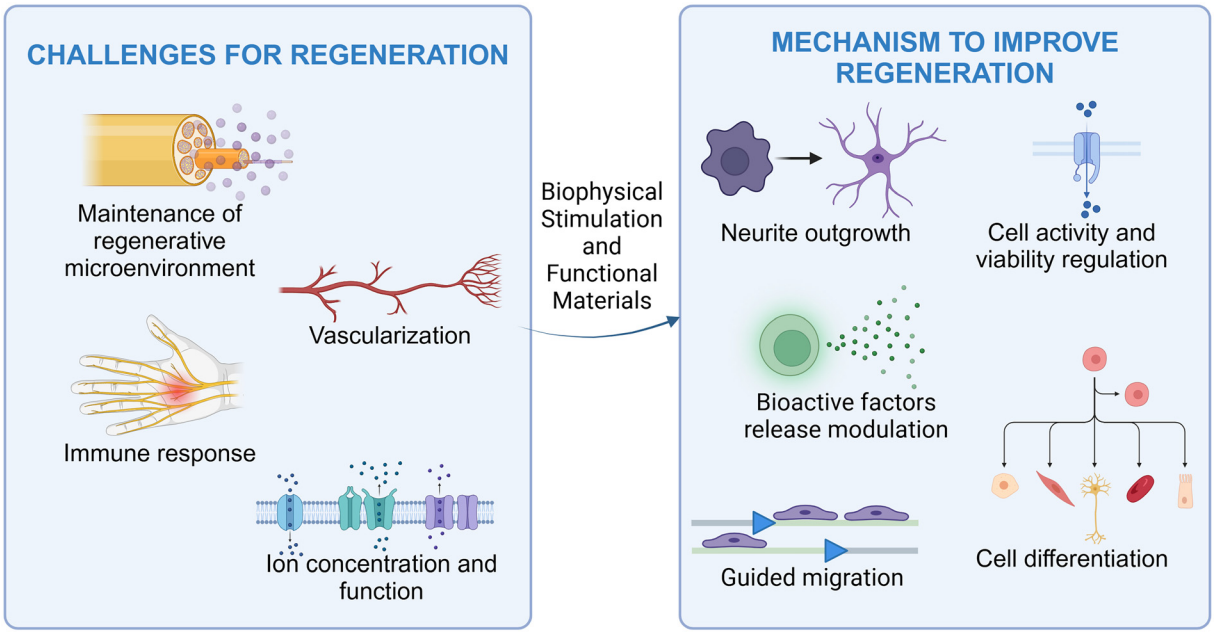

Fig. 1 Summarized challenges for nerve regeneration and mechanisms for improvement through biophysical stimulation and functional materials.

environment for axonal growth, potentially leading to nerve atrophy and preventing complete regeneration.[27] Typically, this process begins eight weeks after injury. By 6 months, there is almost no regenerative stimulus for axons, which is one of the reasons why functional recovery may not be achieved.

Macrophages are another crucial cell in PNS repair, recruited by SC, releasing cytokines at the injury site.[27] Macrophages are important for triggering phenotypic changes in SC through the release of growth factors and sustaining this change *via* a feedback loop of cytokines produced by both cells.[28] Additionally, macrophages promote the directional growth of SC, as they are components of the bridge and release VEGF.[15]

Two types of macrophages play different roles in nerve regeneration. M1 macrophages are essential for Wallerian degeneration due to their pro-inflammatory phenotype, while M2 macrophages promote regeneration and have an anti-inflammatory phenotype. The M1 phenotype can impair regeneration through excessive inflammatory responses, thus hindering the restoration of appropriate conditions for nerve repair.[15] Along with inflammation, producing reactive oxygen species (ROS) by mitochondria can, in excess, lead to dysfunction and neuronal cell death.[28] Therefore, controlling the immune response at appropriate levels is essential for successful regeneration.

For regeneration to be considered complete, functional tissue recovery must be achieved in addition to structural repair. During injury, the electrical signaling ability of the nerves is impaired and only returns after complete tissue regeneration. As a result, there is an imbalance in the bioelectric conduction of PNS. This change leads to the creation of an electric field through a cascade of events initiated by a cellular calcium influx (known as galvanotaxis), which can guide macrophages and SCs to the injury due to the field direction and intensity, which is proportional to the severity of the injury.[29] The re-establishment of bioelectric signaling requires proper ion channel function and ionic concentrations. During injury, there is an increase in potassium ion concentration, which prevents the maintenance of the standard resting potential, reducing action potential amplitude and duration, and may also cause severe complications such as neural hypersensitivity and neuropathic pain.[30,31] On the other hand, there is also an increase in axonal calcium ion concentration, which triggers a pro-regenerative chain of events by activating neuron metabolic pathways that increase the production of growth factors, such as BDNF. However, very high concentrations can also impair the cellular microenvironment.[32,33]

Thus, the main challenges in PNR are: (i) maintaining the regenerative phenotype of SCs until complete repair; (ii) vascularization of the regenerating nerve; (iii) controlling excessive immune responses; (iv) re-establishing optimal ion concentrations and the electrical functioning of the tissue, as represented in Fig. 1. Therefore, NGCs have been designed to overcome these challenges. In the following sections, the scientific advances in biophysical stimuli and their combination with functional materials, especially nanomaterials, will be addressed, emphasizing electrical, magnetic, light, and ionic release stimuli.

3. Electrical stimulation

PNR can be improved with electrical stimulation (ES), especially in those cases where the distance between the proxi-

mal and distal segments is long, considering the ability of nerve tissue cells to respond to such stimulation.[34] The mechanism responsible for the PNR improvement using ES is attributed to (i) changes in the permeability of ion channels, increasing intracellular calcium concentration, and (ii) signaling transduction through membrane receptors, regulating the expression of BDNF, cyclic adenosine monophosphate (cAMP) in the neurons, expression of M2 phenotype in macrophages, and NGF expression by SC.[35] Altogether, these changes lead to asymmetric activation of signaling molecules and changes in the cytoskeleton, which is required to guide axon regeneration, which depends on cell direction in response to the applied electrical field. Those events change survival, migration, proliferation, and differentiation of cell processes.[36,37] For example, *in vitro* tests have shown an increase in dorsal root ganglion (DRG) neurite outgrowth by 30% under electrical prestimulation, and SCs secrete a higher concentration of NGF (up to 11 times) when stimulated by 50 mV mm^{-1}.[38] Also, human neural crest stem cells (NCSC) demonstrate more significant differentiation into SCs when exposed to ES (200 mV mm^{-1}, 20 Hz, and 100 μs).[39]

Low mitochondrial population and trafficking affect ATP supply and may lead to peripheral nerve degeneration and cell death. Albin *et al.* evaluated the effect of pulsed ES on axonal growth and mitochondrial trafficking using DRG cells and a chemotherapy-induced peripheral neuropathy model.[40] ES treatment at low frequencies (10 and 100 Hz) led to an improvement in mitochondrial trafficking and axonal growth in healthy cells. For the treated model, two drugs were used: paclitaxel, a microtubule-targeting drug that affects axonal trafficking, and oxaliplatin, a DNA-targeting drug with a higher toxicity effect on mitochondria. ES application in models treated with drugs resulted in neuroprotective action, with decreased degeneration and increased mitochondrial trafficking using the same frequencies. These results indicate a possible similarity between the neuroprotective mechanisms activated by ES in both cases. It is suggested that enhanced mitochondrial trafficking may be associated with increased expression of BDNF and greater availability of ATP at the lesion site.

Although clinical protocols of ES for PNR vary among studies, a standard approach is an intraoperative procedure using frequencies of 20 Hz for one hour, yielding promising outcomes. Patients with cubital tunnel syndrome, a compressive neuropathy, treated by a clinical protocol (<30 V, pulse duration of 0.1 ms) had better axonal regeneration and improved grip and pinch strength.[41] A critical factor for ES in clinical practice is the requirement of general anesthesia, given that local anesthesia blocks channels that preclude the effect of ES on the cells.[42,43]

Even though there is extensive literature on the beneficial effects of ES on PNR, studies of setup parameters and intermittent application are required owing to the lack of a guide for clinical or pre-clinical protocols. Adams *et al.* evaluated the effect of pulse intensity and duration through a computational model.[44] The results were validated with embryonic chicken dorsal root ganglion cells using pulses of 70 to 45 000 V m^{-1}, from 10 μs to 100 ms. An increase in the concentration of Ca^{2+} was shown in the cell body of neurons with each pulse, which was proportional to the intensity and duration of the pulses. Despite this result, clinical studies suggested that currents lasting longer than 500 μs are uncomfortable for patients.[45]

Another critical parameter is the time duration of exposure to ES. Despite the beneficial results of brief stimulation, continuous stimulation for more than three hours exhibited results similar to the non-stimulated groups.[46] Ju *et al.* investigated the efficiency of intermittent ES (25 Hz/0.1 ms pulses for 30 min) in implantable devices and by the transcutaneous method in rats. The group with the implantable device, capable of wireless stimulation, had a faster functional recovery with a lower sciatic functional index (SFI), having larger axon and muscle fiber diameters.[47]

The use of ES is often associated with the postoperative period. However, studies indicate that ES used in preoperative procedures, such as elective surgeries to treat chronic nerve injuries, can significantly improve regeneration.[48] Lesion conditioning typically occurs 7 days before surgery, favoring upregulation of pro-regeneration molecular pathways, such as growth-associated protein (GAP-43), BDNF, phosphorylated CREB, and changes in SCs. This approach improves regeneration by decreasing the time to axonal growth and increasing the number of regenerating fibers. Moreover, an inflammatory response is avoided, an advantage when compared to crush conditioning.[35] Thus, developing materials that allow brief intermittent *in situ* ES with less invasiveness can be an interesting approach.

3.1. Conductive materials

Conductive scaffolds can maintain electrical conduction between the proximal and distal regions of the lesion, thus acting as cellular support, favoring the transduction of electrical signaling and modulating cell adhesion. It is well known that nervous tissue cells have innate electrical properties resulting from the difference in ion concentration between the inner and outer sides of the cell membrane, with a resting potential of −70 mV. Changes in the cell membrane polarization lead to electrical activity responsible for cellular function, such as changes in the concentration of intracellular potassium and sodium or the release of neurotransmitters, in addition to communication with other cells and skeletal muscles (Fig. 2(i)).[49–51] Besides the ES, conductive substrates can also increase the expression of neurotrophic factors by SCs, accelerate axon elongation, and improve the differentiation of stem cells into nerve cells,[51,52] which altogether increases the success of nerve repair.[53]

Hydrogels are widely used materials in tissue engineering due to their unique properties, such as biocompatibility, high water content, porosity, softness, plasticity, large surface area, and the ability to simulate a natural ECM. Some conductive materials, such as carbon-based composites and various polymers, can be incorporated into the hydrogel matrix, aiming for tunable properties that can present many native tissue func-

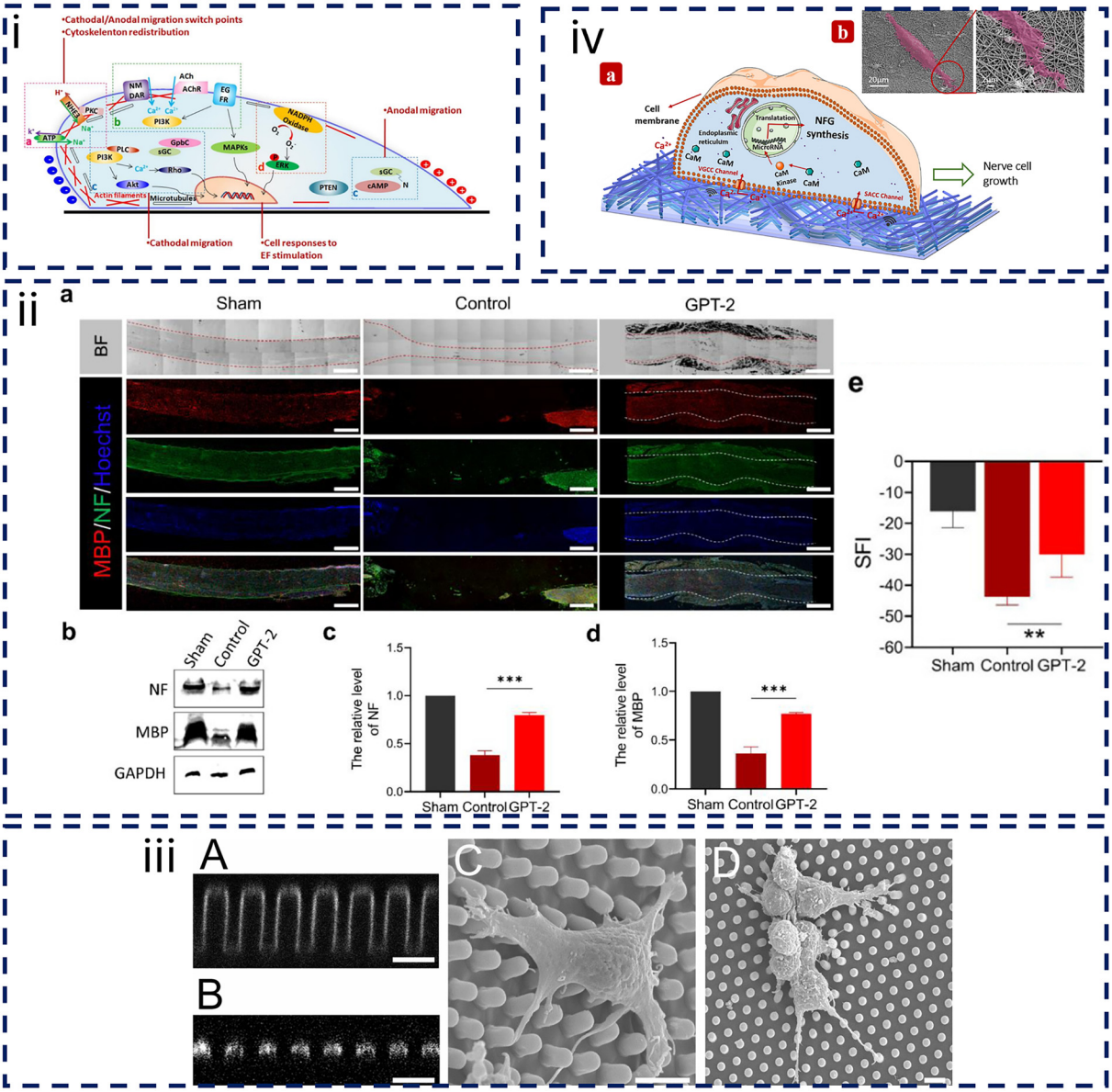

Fig. 2 (i) Signaling web in electrical field stimulated cells.[51] Copyright 2020, Elsevier. (ii) Regeneration of the sciatic nerve 2 weeks after implantation. (a) Immunohistofluorescence images of sciatic nerve obtained from animals in the sham group at 2 weeks. (b) Representative images for the western blot of the NF and MPB protein bands. (c and d) Quantitative analysis of western blot. (e) SFI of different groups (**$p < 0.01$).[54] Copyright 2023, Elsevier. (iii) Characterization of Ncad—Fc-coated micropillars and C2 spreading on μFSAs (A and B) x–z confocal sections of immunofluorescent labeling of Ncad—Fc immunoadsorbed onto μFSAs. (C and D) SEM observations of C2 cells loaded for 3 h onto N-cadherin-coated μFSAs.[55] Copyright 2006, John Wiley and Sons. (iv) (a) Illustration of a possible pathway for the growth of cells as induced by the piezoelectric and external charges of PEDOT/CS nanofibers. (b) SEM images of BNCs cultured on PEDOT/CS nanofibers for 1 h ES.[56] Copyright 2020, Elsevier.

tions.[57] Conductive polymers, such as polyaniline (PANi), poly (3,4-ethylenedioxythiophene) (PEDOT), and polypyrrole (PPy), are interesting options for exploring the electrical properties of the peripheral nervous tissue. Various PANi-type coatings can modulate the adhesion and proliferation behavior of PC12 cells, especially when coated with PANi nanoparticles.[58] Xu *et al.* developed a hydrogel that, after being coated with PANi particles, acted as a platform for differentiating stem cells into nerve and glial cells when exposed to ES.[59] However, the absence of PANi degradation caused a cytotoxic response and the formation of reactive oxygen species (ROS).[60] Although PANi shows potential characteristics for nerve regeneration, further studies on alternative morphologies are desirable to mitigate potential drawbacks.

Pires *et al.*[61] developed a PEDOT and polystyrene sulfonate (PSS) film coated with laminin and evaluated their indirect cytotoxicity behavior in contact with L929 fibroblasts. With the use of ReNcell VM human progenitor cells, an increase in

neurite elongation and cell populations of neurons after ES was observed. Kang *et al.*[54] developed a conductive hydrogel based on gelatin, PPy, and tannic acid with self-regenerative properties. The PPy concentration modulated the conductivity of the hydrogel. *In vitro* tests showed that the stimuli of the conductive hydrogel yielded superior PC12 cell (nerve-like) viability, more significant neurite extension, and greater axon area in DRG compared to the control group. *In vivo* regeneration of mice sciatic nerve crush injury was also verified, indicating good regeneration, muscle recovery, as shown in Fig. 2 (ii), and improvement in SFI, although signs of inflammation were detected.

In addition to intrinsically conductive polymers, composites are frequently employed in fabricating conductive conduits; once dispersed phases can improve the biological properties of the matrix and the electrical characteristics of the final material. Polymers like collagen, chitosan, hyaluronic acid, poly(lactic acid) (PLA), and poly(glycolic acid) (PGA) are commonly used to produce such conductive materials. The dispersed phase must have electrical conductivity, enabling the neural pathway to be re-established, and include gold and silver nanoparticles, as well as carbon-based materials, such as carbon nanotubes and nanofibers, graphene, graphene oxide (GO), and reduced graphene oxide (rGO).[62]

Lee *et al.*[63] developed a sensor using a polyethylene glycol hydrogel and microstructured silver nanowires on a polyethylene terephthalate (PET) substrate. The composite containing silver nanowires was evaluated through *in vitro* models using nerve stem cells, and could guide the growth of neurites. Together with ES, it promoted the differentiation of the cells into neurons. Ding *et al.*[64] developed a scaffold based on collagen type I and gelatin with silver nanoparticles. The porous material was dipped in laminin, and *in vivo* in rabbits with 10 mm injury of the sciatic nerve, demonstrated that the insertion of silver nanoparticles increased the adsorption of laminin, the thickness of the regenerated myelin sheath, the nerve conduction velocity, and the potential amplitude. Despite the advantages of using silver nanomaterials, their concentration in biological media must be considered, as high doses can induce apoptosis and cell necrosis.[65]

Song *et al.*[53] studied conduits composed of the shape-persistent polymer poly(lactide-*co*-trimethylene carbonate) (PLMC), gelatin, GO, or rGO in a hierarchical structure containing three smaller conduits. *In vitro* assays with Schwann and PC12 cells demonstrated excellent cell proliferation for the rGO composite. When associated with ES, signs of PC12 cell differentiation and greater neurite elongation were observed, and SCs expressed genes related to increased myelination. Sciatic nerve defects of 10 mm in rats demonstrated that the NGC yielded results of microvessel density, SFI, and muscle weight similar to the autologous nerve, with promising results for rGO. Despite their remarkable electrical and mechanical properties, studies have highlighted the dose-dependent toxicity of GO and rGO, with doses greater than 50 μg mL^{-1} showing decreased cell adhesion, induction of cell apoptosis, and presence of carbon inside the cell.[66]

3.2. Piezoelectric materials

One of the main challenges of ES relates to invasive procedures. To overcome this limitation, piezoelectric materials emerge as a promising wireless implantable alternative to electrical stimulation.[67] The piezoelectric effect was discovered in 1880 by Jacques and Pierre Curie and occurs mainly in non-centrosymmetric materials, resulting from the change in the crystalline structure when subjected to mechanical stress.[64] The pressure exerted causes a non-zero dipole moment, leading to the separation of charges inside the material, which migrate to the surface, forming an electric field (EF) and polarizing the material.[68,69] Even though stimulation is mechanical, the effect on the nervous tissue and the response mechanism is due to the ES.[70,71]

In living models, the piezoelectric effect can be activated in several ways, such as by body movements and blood flow[72,73] or by the traction force exerted by cells on the substrate, with average values from 15 to 30 nN (Fig. 3(iii)).[55,74] Although not yet fully understood, the regenerative mechanism of piezoelectric materials in cells can be attributed to the electric field induced by these materials.[75] This stimulus leads to changes in ion channels, membrane receptors, and ECM components, causing the asymmetric activation of signaling molecules and changes in the cytoskeleton in a manner dependent on the generated electrical field.[36] Thus, the possibility of polarization and control of the surface charges of piezoelectric materials appears to be a promising auxiliary alternative in treating nerve injuries.

Given these promising effects, the incorporation of piezoelectric materials in nervous tissue engineering has been gaining attention. Du *et al.*[56] evaluated the effect of a conductive and piezoelectric scaffold of PEDOT/chitosan manufactured by electrospinning and recrystallization in brain neuroglioma cells (BNC). The processing favored the piezoelectric effect of chitosan, generating a voltage of 0.93 V cm^{-3} when the scaffold was stimulated with ultrasounds (8.87 Pa at 1 Hz). During *in vitro* tests, the material showed greater BNC proliferation and neurite extension than pure chitosan. Applying an external EF of 400 mV cm^{-1} at 0.1 Hz improved cell differentiation and neurite extension of the cells in the scaffold. The resulting charges can open calcium channels, increasing the calcium concentration in the cell surroundings, thus causing a calcium wave to the cell interior, which increases the expression of BDNF and its receptor Tropomyosin receptor kinase B (TrkB). This leads to an increase in cAMP due to the inhibition of phosphodiesterases. These high levels of cAMP can increase the expression of genes associated with regeneration, such as Tα1 tubulin and growth-associated protein-43, in addition to favoring the reorganization of the cytoskeleton[80] and directional migration under an external electric field;[81] Fig. 2(iv) illustrates this metabolic chain.

Mao *et al.*[82] prepared an electrospun PCL/ZnO conduit and observed increased cell proliferation and NGF and VEGF release in SCs. *In vivo* tests with a 10 mm sciatic nerve defect in rats demonstrated nerve regeneration in 4 weeks with SFI

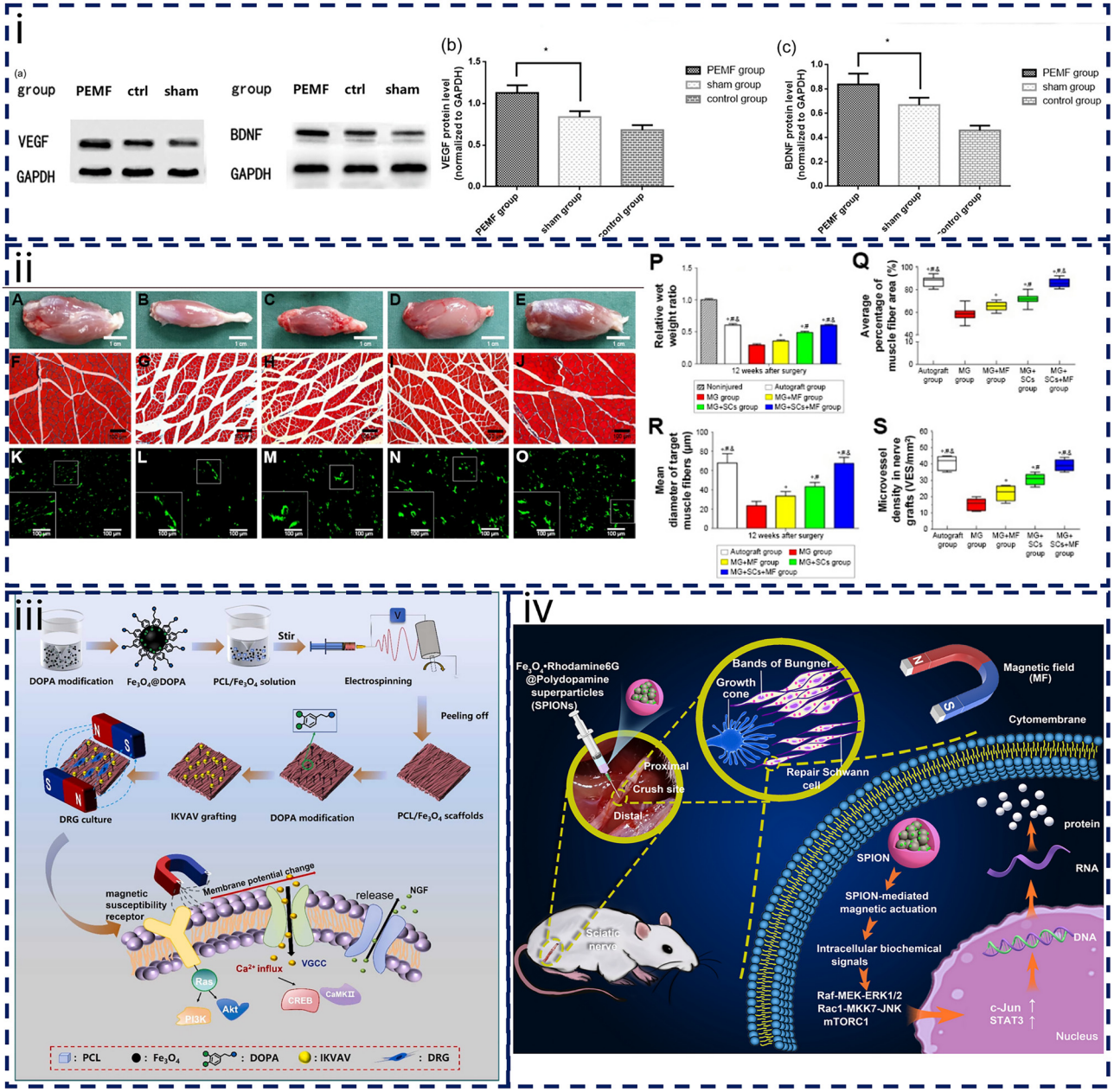

Fig. 3 (i) (a) Western blot of BDNF and VEGF 8 weeks after delayed nerve repair; (b) VEGF protein level; (c) BDNF protein level.[76] Copyright 2023, John Wiley and Sons. (ii) Weighting and histology of target gastrocnemius muscle and quantification of microvessel density in each group at 12 weeks post-operation. (A–E) The morphology of the gastrocnemius muscle. (F–J) The characteristic light photographs of the cross-sectional gastrocnemius muscle following Masson trichrome staining. (K–O) The characteristic photographs of MVD staining. (P) The relative wet weight ratio in each group is shown. (Q) The mean percentage area of muscle fibers in each group. (R) The average diameter of muscle fibers in each group. (S) The quantitative analysis of MVD in each group.[77] *International Journal of Nanomedicine*, 2017, 12, 7815–7832. Originally published by and used with permission from Dove Medical Press Ltd. (iii) Schematic preparation of IKVAV functionalized magnetic PCL/Fe_3O_4 orientation scaffolds.[78] Copyright 2024, Elsevier. (iv) Schematic illustration of SPION-mediated magnetic actuation promoting nerve regeneration by inducing and maintaining repair-supportive phenotypes in SCs.[79] Copyright 2022, Springer Nature.

parameters equivalent to suture, with improved myelin sheath regeneration under piezoelectric action. Increased NGF and VEGF expressions were identified after 28 days of intervention, demonstrating that the piezoelectric effect can promote the recruitment of SCs and the increase in nerve filaments. Genetic tests indicated that the conduit activated signaling pathways such as PIK3-Akt, MAPK, and RAS that are associated with the RET pathway, an important pathway in the regeneration of the peripheral nerve system. Gene network analyses showed several upregulated genes linked to growth factor receptor-bound protein-2 (GRB2), a RAS pathway protein whose expression increased after 48 hours of ES.[82]

Chen *et al.*[83] developed piezoelectric scaffolds of PLA with potassium sodium niobate nanowires ($K_{0.5}Na_{0.5}NbO_3$, KNN) coated with polydopamine, which can be wirelessly activated by ultrasound. The peak voltage and current achieved were up to 17.9 V and 2.6 μA, respectively, for composites containing 50 wt% nanowires. The biological properties were evaluated *in vitro* with rat fibroblast (L929), PC12, and neural stem cell (NSC) lines and *in vivo* through a spinal injury model in rats. The scaffold showed improvement in the cellular differentiation of NSCs when exposed to ultrasonic stimulation of 100 kPa for 20 minutes daily, increasing the expression of nestin, neuron-specific microtubule element marker β-Tubulin (Tuj1), and MAP2. *In vivo* tests with ultrasonic stimulation of 100 kPa and a current density of 4.44 μA cm^{-2} (20 minutes every two days for four weeks) showed functional improvement. Piezoelectric scaffolds with ultrasound stimulation exhibited a higher Basso Beattie-Bresnahan (BBB) index, reduced spinal tissue loss, and increased myelination.[83]

Polycrystalline piezoelectric materials present electric dipoles in different orientations, leading to anisotropy and the absence of polarization. However, the alignment of these dipoles is possible through the use of a technique called poling, involving the application of direct or alternating current. As a result, materials can present a more efficient piezoelectric response and surface charges, which can be positive or negative, depending on the process. These properties have been shown to improve the differentiation of specific cell lines, aiding cell development.[84] In SH-SY5Y cell lines, the positive surface charge on a PVDF substrate showed more outstanding cell adhesion, proliferation, and neurite extension.[85] Marques-Almeida *et al.*[85] observed increased cell adhesion and neurite growth when cultured on both a positive and negative surface charge PVDF substrate in primary neurons extracted from newborn rats. These cellular responses resulted from the effects of ES on nerve cells, such as the preferential targeting caused by the inhibition of the PI3K enzyme and the ROCK 1 and ROCK 2 (Rho-associated protein kinase 1 and 2) effectors.[86]

In summary, piezoelectric nanomaterials can be promising candidates for safe and on-demand ES delivery for PNR without invasive procedures. They can be incorporated into biocompatible and biodegradable polymers, covering several applications. However, their toxicities as nanomaterials must be thoroughly investigated before clinical use.

4. Magnetic stimulation

The application of magnetic stimulation (MS) in healthcare has been studied since the '70s as a non-invasive procedure that promotes adhesion, proliferation, and differentiation of several cell types, such as mesenchymal stem cells, dental pulp, and neuron-like cells (PC12).[78,87,88] In addition, MS can improve angiogenesis as it assists endothelial cells in forming capillary-like structures, improving tissue regeneration.[77] Even though experimental results confirm the potential of MS, the physical mechanisms of these effects are not fully understood yet. It is suggested that MS can alter ionic transportation through the cell membrane, modifying channel activity and neural cell physiology.[89]

Several studies focusing on nerve repair showed that regeneration can be improved by applying a pulsed electromagnetic field (PEMF). This procedure is based on a low-frequency electromagnetic field (0.3 to 300 mT) with a determined repetition frequency (2 to 2000 Hz).[90] Byers *et al.*[91] applied a 0.4 mT at 120 Hz in a protocol of 4 hours per day, 5 days per week, for 8 weeks to investigate the *in vivo* regeneration of transected facial nerves in mice. The functional recovery was greater with applying PEMF than in the control group, enhancing earlier regeneration of the transected nerves. Hei *et al.*[90] used different PEMF protocols to evaluate the regeneration of crushed mental nerves in rats: the best results were obtained using 1 mT at 50 Hz once a day. Additionally, it was hypothesized that the improvement in regeneration was due to the SC proliferation and an increase in the expression of BDNF and S100 genes because of the regulation of Ca^{2+} expression. Keyan *et al.*[76] also observed the upregulation of expression of BDNF, along with VEGF, after exposure to PEMF (1 mT at 50 Hz every day), which could explain the improvement in regeneration in the transected sciatic nerve of rats after a 1-month delay (Fig. 3(i)). Fontana *et al.* observed an *in vitro* anti-inflammatory effect in THP-1 monocytes by applying PEMF with 0.3 mT at a 20 Hz protocol.[92]

With the advancement of nanotechnology, magnetic nanoparticles (MNPs) have been studied for tissue engineering applications, given that they interact with subcellular structures and can enhance and amplify the MS effects.[88] One crucial material is based on superparamagnetic iron oxide nanoparticles (SPIONs), which present essential characteristics such as biocompatibility, stability, and magnetic properties. Because of their nanometric size, these particles show superparamagnetic properties, meaning they do not maintain their magnetic behavior without an external magnetic field.[93] SPIONs have already received approval for clinical application in other medical fields, such as ferrofluids for magnetic hyperthermia.[94] The mechanism of interaction between neural cells and MNP is based on the attachment of the particles on the cell membrane or their absorption, in which the application of an external magnetic field leads to a mechanical tension that induces the lengthening of axons and guiding of their growth towards the direction of the magnetic field.[78,95]

The latest trend in MS of peripheral nerves consists of MNPs combined with a polymeric matrix to be delivered to the injured tissue. In general, composite scaffolds are developed to reconnect the separated nerve stumps and deliver the MNPs in a synergic approach. Studied with a scaffold composed of chitosan–glycerophosphate and magnetic nanoparticles loaded with SCs demonstrated that the combination of functional materials and MS significantly improved regeneration in rats with a 15 mm sciatic nerve gap. Daily MF stimulation (50 Hz, 2 mT) enhanced SC functional recovery and vascularize-

tion and prevented atrophy, with results comparable to autografts (Fig. 3(ii)).[77] Liu *et al.*[78] developed a laminin-derived peptide (IKVAV)-functionalized polycaprolactone/Fe_3O_4 scaffold to optimize neurite extension and growth factor expression. Under a static magnetic field, the extension and orientation of axon fibers from DRG cultures increased, with the treated group exhibiting better results than the control of only morphological cues. This behavior could be related to the upregulation of nerve growth factor expression, as an increase in NGF production was observed for the MS group, as shown in Fig. 3 (iii). Shlapakova *et al.* developed a magnetoactive composite conduit based on poly(3-hydroxybutyrate) (PHB) and magnetite Fe_3O_4 nanoparticles for peripheral nerve repair. The *in vitro* results on SH-SY5Y cells showed that with the MF application, the morphological and proliferation parameters were better on the PHB/Fe_3O_4 scaffolds. The *in vivo* assay consisted of mice sciatic nerve transection, and biocompatibility results showed that the conduits did not cause acute inflammation and could be successfully applied in nerve regeneration. However, other parameters, such as SFI or myelination, were not evaluated by the authors.[96]

MNPs can also be functionalized, resulting in a multi-strategy approach. Liu *et al.*[79] demonstrated the influence of SPIONs on the phenotype of SCs using *in vitro* assays. A functionalized SPION was developed (Fe_3O_4·Rhodamine 6G@polydopamine) and exposed to a static magnetic field. These MNPs affected cells due to mechanical forces sensed by SCs and transduced into intracellular biochemical signals that promoted nerve regeneration by inducing and maintaining the repair phenotypes of SCs (Fig. 3(iv)). The *in vivo* assays using the sciatic nerve crush model showed that the MS induced the upregulation of growth factors, remyelination of nerve fibers, and improved functional recovery.

The MNPs can be functionalized with growth factors (GF), thus improving the stability of these molecules and promoting a controlled delivery.[97] Ziv-Polat *et al.*[98] prepared dextran- and gelatin-coated nanoparticles and covalently conjugated different growth factors on the IONP surface, including β-nerve growth factor (βNGF), glial cell line-derived neurotrophic factor (GDNF), or fibroblast growth factor 2 (FGF-2). The authors observed a more prominent increase in the stability in dissociated DRG cell culture of GDNF-conjugated samples compared to the others. Additionally, the βNGF-, GDNF-, and FGF-2-conjugated MNPs induced earlier nerve fiber regeneration compared to the corresponding free neurotrophic factors. In another study, Yuan *et al.*[99] developed a core–shell iron oxide and gold nanoparticle functionalized with NGF. These functionalized MNPs showed higher NGF cellular uptake *in vitro* with PC12 cells. Moreover, they compared neurite outgrowth using static or dynamic magnetic field applications and obtained better results using a dynamic approach. Additional pertinent research was summarized in Table 1, where the scaffold material, MNP, and main results are presented.

Future *in vivo* and preclinical studies should optimize the synergistic effects of MS and MNPs to enhance PNR. The following steps involve systematically evaluating the long-term biocompatibility and biodegradation kinetics of MNP-functionalized conduits in relevant large-animal models, such as porcine or primate sciatic nerve injury models, to bridge the

Table 1 Multidimensional approach using scaffolds, MNPs, MS, and the main obtained results

Material	Magnetic material	Results	Ref.
Multi-layered PCL NGC with melatonin and magnetic nanoparticles	Iron oxide nanoparticles	The scaffold could sequentially release bioactive agents, creating a conducive microenvironment for nerve regeneration. The scaffold effectively promoted nerve regeneration in a 15 mm rat sciatic nerve defect model, as shown by improved nerve structure and function. Oxidative stress and inflammation were inhibited in *in vivo* tests.	100
Commercial collagen NGC (NeraGen) filled with magnetic collagen-genipin hydrogel	PEG and NGF/PEG-coated iron oxide nanoparticles	The method showed enhanced recovery in an 8 mm rat sciatic nerve injury model compared to the commonly used hollow NGCs, with improved gain of function for NGF-containing filler.	101
Electrospun poly-L-lactic acid (PLLA) fiber scaffold	SPION	The results for DRG cell cultures indicated that neurite length increased by 40% when stimulated by an alternating magnetic field compared to a static field. SPION-grafted fibers combined with an alternating magnetic field led to a 30% increase in neurite length and a 62% increase in the neurite area, outperforming SPIONs dispersed in the culture medium.	102
Chitosan-based NGCs with GDNF or GDNF-IONP	GDNF-conjugated iron oxide nanoparticles	Results for a 10 mm gap in the rat sciatic nerve model indicated that GDNF-IONP delivered through the chitosan tube significantly improved the regrowth of sciatic nerve fibers, enhancing both functional and morphological recovery compared to hollow chitosan NGC and autograft. Functional assessments showed accelerated motor and sensory recovery in the GDNF-IONP group.	103
Poly-lactic acid (PLLA) microfibers	NGF-releasing iron oxide nanoparticles	When placed in DRG culture, NGF-loaded nanoparticles acted as a targeted release system, causing neurite growth towards them. When combined with aligned PLLA electrospun microfibers, the composite effectively directed neurite growth along the fibers, demonstrating the potential for magnetic guidance to influence neurite alignment and promote nerve regeneration.	104

translational gap to clinical applications. Additionally, comprehensive analyses should be conducted to assess axonal remyelination, functional recovery, and potential neuroinflammatory responses induced by MNPs under controlled static and dynamic magnetic fields. Moreover, preclinical trials should compare different functionalization strategies, particularly growth factor-conjugated MNPs, to optimize controlled delivery profiles and minimize off-target effects. These advancements will provide insights into the safety, efficacy, and scalability of functional magnetic NGCs, helping their transition into clinical studies.

5. Light stimulation

The use of light irradiation to treat living tissue, known as phototherapy (PT), has been studied since the '70s and explored in the field of PNI since the late '80s.[105] Perhaps the newest advance in this area, photobiomodulation therapy (PBMT), uses light to modulate cellular behavior through photochemical, photoenergetic, or photoelectric reactions.[106] Different light sources can be used to expose the injured tissue to visible or near-infrared (NIR) wavelengths, such as light-emitting diodes (LED) or low-level lasers (LLL), with protocols consisting in 300–10 600 nm wavelength, power output between 0.001–0.1 W, pulse rate up to 5000 Hz, intensity of 0.01–10 W cm^{-2}, and dose of 0.01–100 J cm^{-2}.[107]

PBMT enhances peripheral nerve regeneration and functional recovery through neuroprotection, reduction in inflammation, and an increase in angiogenesis, expression of neurotrophic factors, outgrowth of neurites, and proliferation of SCs.[107] Such events have been associated with the PBMT effect on mitochondrial activity, in which the absorption of photon energy by cytochrome C oxidase increases ATP formation, thus modulating ROS production, nitric oxide release, and Ca^{2+} homeostasis.[108] Er-Rouassi *et al.*[109] observed an improvement in functional recovery after the section-suture protocol used in mice facial nerves treated with PBMT (820 nm, twice a day for 16 days) compared to the untreated control group, which was associated with the activation of the cytochrome C oxidase. Additionally, PBMT could be used in the denervated muscle after a PNI to treat atrophy, in which the biochemical cascade after increased ATP production enhanced the morphological recovery of the muscle cells.[105]

Li *et al.*[110] also investigated the regeneration of facial nerves, using a crush injury model in rats, with PBMT (980 nm, 30 s/8 h for 12 days), showing upregulation of the phosphatidylinositol-3 kinase/protein kinase B (PI3K/Akt) signaling pathway, which lead to positive effects such as inhibition of apoptosis, increased proliferation of SCs, and improved functional recovery. Even though there are some advances in comprehending the mechanism during the PBMT treatment, the effect on axonal degeneration and regeneration by stimulating mitochondrial activity still needs a more profound investigation.[108] This point and the absence of standard parameters for the technique (such as wavelength, irradiation type, doses, and energy density) are the main limitations of PBMT translation to clinical applications.

Considering a multi-treatment approach, PBMT has been combined with biomaterials and NGCs to achieve better results for PNR. Zhu *et al.*[111] developed a transparent 3D-printed scaffold composed of gelatin methacrylate (GelMA) and polyethylene (glycol) diacrylate (PEGDA). They studied the effect of PBMT using LLL on mouse neural stem cells on the scaffold. Light stimulation (635 nm for 15 s) enhanced the proliferation and differentiation of the cells, indicating that such technology could be applied in neural regeneration. The possible mechanism associated with proliferation was the absorption of light by the mitochondria and accelerated electron transfer, which initiated a cascade of events, such as increased ATP, ROS, and intracellular Ca^{2+}. Those events can affect DNA synthesis, protein expression, and cytoskeletal organization, leading to the observed changes. However, specific assays were not conducted to prove this cascade of events.

Another strategy was developed by Milos *et al.*,[112] where morphological and optical stimulations (green and red LED, 1 s pulses every 1 min for 1 h each day) were used to develop light-sensitive poly(3-hexylthiophene-2,5-diyl) (P3HT) scaffolds with micropillars on their surface. The *in vitro* evaluation of primary cortical neuron cell cultures showed a significant increase in both neurite and axon length of the cells grown on the photo-sensitive material compared to the photo-inert control (Fig. 4(i)). Moreover, the combined action of the topographical and green light stimulation was effective in the optical modulation of neuronal growth and orientation, which can improve the overall nerve regenerative process. The authors hypothesized that the mechanism associated with these changes was the modulation of intracellular Ca^{2+}, but recognized the need for specific studies to demonstrate the link between PBMT and the regulation of neuronal growth.

Advances in nanotechnology allowed the application of non-penetrating wavelengths, such as blue light, to the deep injured tissues using upconversion nanoparticles (UCNPs). These particles are often synthesized with lanthanides and can convert NIR into higher energy lights by energy transfer or photon absorption.[115] Guan *et al.*[116] developed a set of polymeric biomaterials (*i.e.*, poly(ethylene imine) (PEI), polyethylene glycol (PEG), and poly(acrylic acid) (PAA)) decorated with UCNPs ($NaYF_4$:Yb/Er) to promote neurite outgrowth in PC12 cells. The PEI-UCNP had the best results, enhancing neurite outgrowth. NIR irradiation potentiated the UCNP effect, improving cell differentiation *via* the extracellular signal-regulated kinase (ERK) signaling pathway and ROS production.

Yan *et al.*[113] used acetylcholine-modified UCNPs (NaYF4: Yb/Tm@NaYF4) for the activation of channelrhodopsin 2 (ChR2), a gene that enables the construction of Ca^{2+} channels, delivered to motor neural cells through adeno-associated virus (AAV) loading. This selective approach could enhance transected tibial nerve regeneration of transgenic mice through

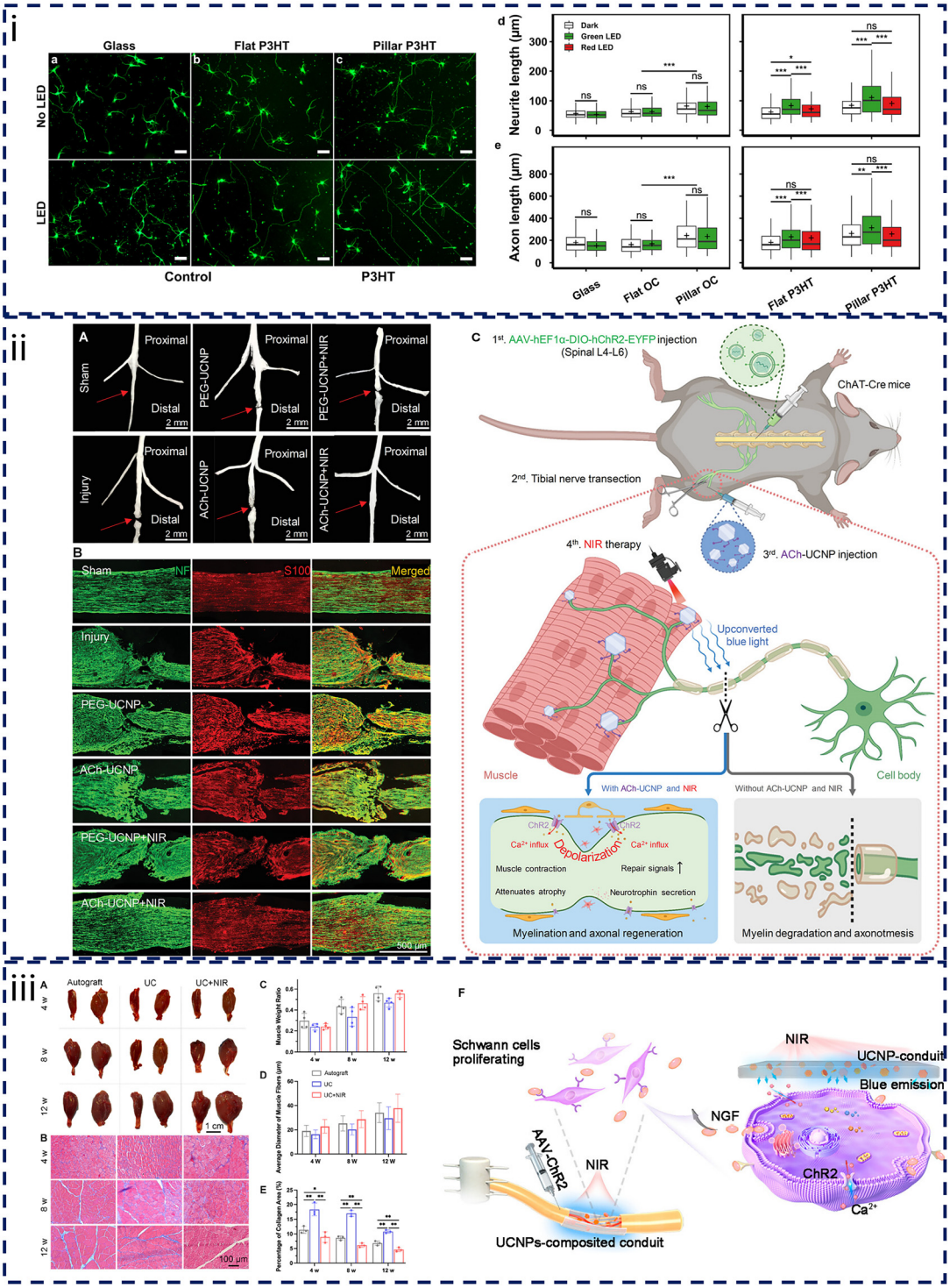

Fig. 4 (i) Photostimulation of neuronal growth on P3HT substrates. Representative images of primary neurons (DIV 3) grown with/without photostimulation on (a) glass, (b) flat P3HT, and (c) P3HT micropillars. (d) Average neurite length. (e) Axon length.[112] Copyright 2024, American Chemical Society. (ii) Regeneration of tibial nerve fibers promoted by UCNP-mediated optogenetics. (A) Anatomical images of the tibial nerves in different groups at week 6 after tibial nerve transection. (B) Immunofluorescence staining of the tibial nerves in different groups at week 6 after tibial nerve transection. (C) Schematic diagram of the tibial nerve completely transected mouse model (the upper part) treated by the NIR-triggered upconversion optogenetic approach, with a zoomed-in view of a cascade of events following ChR2 activation (the lower part).[113] Copyright 2021, American Chemical Society. (iii) Recovery of gastrocnemius atrophy under postoperatively noninvasive optogenetic therapy. (A) Representative photos of bilateral gastrocnemius muscles, with the left displaying the experimental muscles and the right displaying normal muscles. (B) Representative images of Masson-stained gastrocnemius on the experimental side. (C) The muscle weight ratio between the experimental side and the normal side; Quantification analyses of muscle fiber diameter (D) and collagen fiber area percentage (E); (F) promotion mechanism of proliferation and nerve function of SCs.[114] Copyright 2023, John Wiley and Sons.

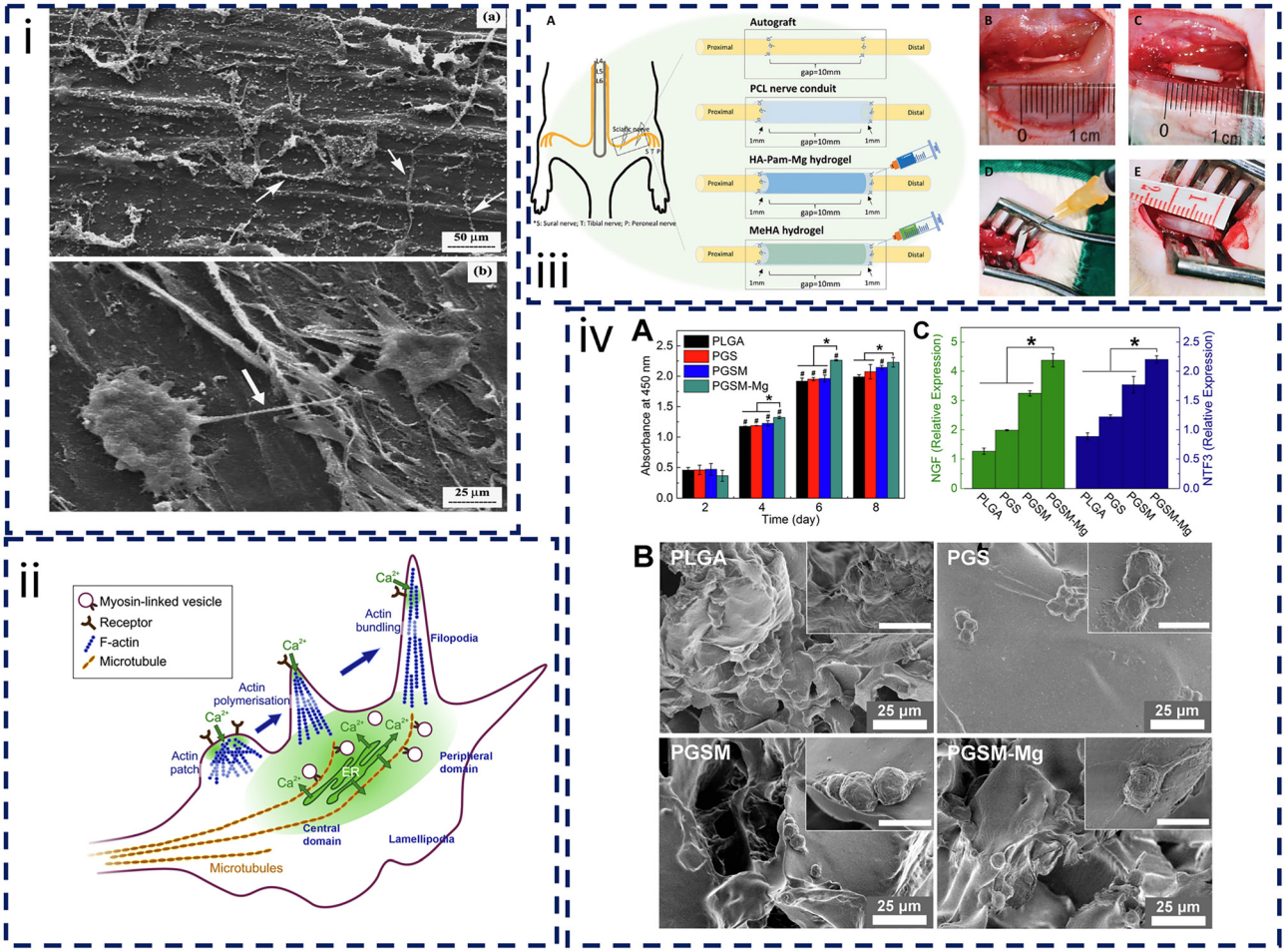

Fig. 5 (i) (a and b) SEM micrographs at two different magnifications of PC12 cells on the PGS/5 wt% $CaTiO_3$ nanocomposite. The arrows indicate cell axons on the membrane.[123] Copyright 2018, John Wiley and Sons. (ii) Schematic illustration of animal surgery. (A) Surgical procedures were performed on SD rats. (B) Repair a nerve defect using autografts. (C) Repair a nerve defect using PCL nerve conduits. (D and E) Surgical procedures in the HA-Pam-Mg hydrogel and MeHA hydrogel groups.[126] Copyright 2022, John Wiley and Sons. (iii) Asymmetric calcium signals drive lamellipodial and filopodial protrusion.[124] Copyright 2017, Elsevier. (iv) (A) CCK-8 assay of SC cultured on PLGA, PGS, PGSM, and PGSM-Mg 3D scaffolds. (B) SEM images of SCs on PLGA, PGS, PGSM, and PGSM-Mg scaffolds after 8 days *in vitro* culture. (C) NGF and NTF3 expression of SCs cultured on PLGA, PGS, PGSM, and PGSM-Mg scaffolds after 6 days.[127] Copyright 2019, Elsevier.

neural regrowth across the gap region, recruitment of glial and SC, enhancement of BDNF expression in acetylcholinergic motor neurons, and remodeling of the neuromotor junction from muscle atrophy (Fig. 4(ii)). Li *et al.*[114] combined several strategies to develop a composite NGC, based on chitosan-graphene oxide-UCNPs, to mediate light stimulation of a transected sciatic nerve loaded with AAV-ChR2. The light stimulation on SCs was monitored *in vitro* with RSC96 cells, and it was observed that increased intracellular Ca^{2+} enhances cellular activity, proliferation, and expression of NGF (Fig. 4(iii)). Additionally, the *in vivo* assay using the NGC to bridge a 10 mm defect on a rat sciatic nerve indicated that optogenetic stimulation promotes myelination and axonal sprouting and improves functional recovery, electrophysiological reconstruction, and re-innervation after nerve injury.

Phototherapy has been applied in regenerative medicine since the middle '80s, with undeniable advantages in PNR. However, standardization of key variables, such as wavelength, pulse duration, and intensity, remains a challenge, as well as systematic dose–response studies. A deeper investigation of the interplay between PBMT-induced mitochondrial activation, ROS modulation, and Ca^{2+} homeostasis is needed to clarify its effect on axonal regeneration and SC dynamics. To help the clinical translation of PBMT, future *in vivo* studies should focus on refining irradiation parameters, elucidating molecular mechanisms, and integrating PBMT with advanced biomaterials. Those present specific challenges that need to be considered, such as the physiological effect of long-term stimulation on the tissue to avoid protein denaturation or cell apoptosis, and the limited penetration range of the material's stimulation. The synergistic potential of PBMT with next-generation biomaterials, including optically responsive NGCs and UCNP-functionalized scaffolds, can be further explored with the aim of a complete recovery.

6. Ion-release mediated cell signaling

Cell signaling mediated by ion release has recently emerged as a crucial mechanism in promoting PNR. Understanding ion release and its effects on the body is essential for the development of effective biomaterials that promote the proper repair of injured peripheral nerves.[117–121] Ions released from implantable materials play a vital role in cellular signaling, influencing cell adhesion, proliferation, and differentiation.[122] Several studies have explored materials that can release specific ions and their impact on PNR.

Calcium (Ca^{2+}) ions have been shown to significantly affect PNR. The regulation of intracellular Ca^{2+} levels promotes normal neurite elongation and growth cone motility, directly influencing axonal growth. Zargar Kharazi *et al.*[123] studied the use of NGC, composed of a poly(glycerol sebacate) (PGS) elastomeric matrix and calcium titanate ($CaTiO_3$) nanoparticles that enabled controlled Ca^{2+} release, enhancing PC12 cell adhesion and proliferation while promoting axon extension (Fig. 5(i)). Possible mechanisms related to the ion effect relate to extracellular Ca^{2+} entry into growth cones, mediated by voltage-gated calcium channels (VGCCs) and transient receptor potential channels (TRPCs), which is essential for growth cone motility in response to guidance cues such as netrin-1 and BDNF. These ions modulate the cytoskeleton by inducing the reorganization of actin filaments and microtubules, promoting filopodia protrusion and axonal growth, as shown in Fig. 5 (ii).[124] Ardhani *et al.*[125] developed a gelatin hydrogel membrane containing carbonate hydroxyapatite and studied the *in vitro* effect of the calcium release on PC12 cells. They observed an initial burst release followed by a gradual, sustained release after 24 h. This calcium availability enhanced neuronal differentiation and neurite outgrowth, as evidenced by higher neurogenic differentiation percentages, increased acetylcholinesterase (AChE) activity, and longer neurites than CHA-free controls. The authors suggest that extracellular calcium supplied by the scaffold activates VGCCs, increases intracellular Ca^{2+} levels, and triggers neurogenic signaling pathways such as Src-Ras activation and NGF1A gene expression, supporting neuronal growth.

Mg^{2+} ions are often employed in their free form in other biomaterials in PNR.[128] This ion inhibits secondary injury after nerve damage by antagonizing the *N*-methyl-D-aspartate (NMDA) receptor, thus avoiding cellular apoptosis and preserving nerve function. Magnesium also suppresses the inflammatory response by reducing macrophage activation and the expression of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α, helping to prevent the apoptosis of SCs and supporting their proliferation. These cells secrete neurotrophic factors, including NGF, which promote axonal growth and nerve repair by activating pathways such as PI3K/Akt.[129] Metallic glasses based on $Mg_{70}Zn_{26}Ca_4$ coated with a layer of tannic acid and poly(*N*-vinylpyrrolidone) (TA/PVPON) produced through a layer-by-layer (LbL) technique demonstrate a significant reduction in corrosion rate and a marked increase in SC viability compared to uncoated magnesium.[130] However, while magnesium promotes nerve tissue regeneration, controlled ion release is necessary, as high local concentrations of this element can cause neurotoxicity, leading to inhibitory effects on neuronal growth.[126,129]

Yao *et al.*[126] developed an injectable hydrogel based on hyaluronic acid (HA) and pamidronate (Pam), encapsulated with magnesium ions (Mg^{2+}) and combined with a polycaprolactone (PCL) conduit, which facilitates continuous magnesium ion release. Evaluating the effect on DRG cultures, Mg^{2+} promoted neurite outgrowth in a concentration-dependent manner, with optimal results at 10–20 mM, attributed to the activation of the PI3K/Akt signaling pathway, which promotes neurite outgrowth and axonal regeneration, and the upregulation of Sema5b, a key axon guidance molecule. *In vivo*, assays on mice transected sciatic nerve with a 10 mm gap showed enhanced myelination and SC proliferation in the NGC group, although autografts had better function recovery (Fig. 5(iii)). Sun *et al.*[127] have shown that biodegradable elastic scaffolds based on poly(glycerol-sebacate-maleate) and magnesium ions (PGSM-Mg) can significantly enhance SC adhesion and proliferation over eight days, inducing the expression of specific neural genes such as NGF and neurotrophic factor 3 (NTF3) by ensuring controlled magnesium ion release (Fig. 5(iv)).

Zinc is another therapeutic ion that can enhance regeneration. Zn^{2+} ions inhibit bacterial growth, aid neural development, and improve wound healing. This element can also be associated with SC proliferation and myelination processes.[18,131] Zn^{2+} ions are additionally linked to neurotransmitter and hormone secretion, playing an essential role in motor coordination development and sensory processing. Ekram *et al.*[132] developed an electrospun PCL/$ZnCl_2$ conduit. They observed that, besides increasing hydrophilicity and mechanical properties of the scaffold, zinc chloride addition improved adhesion and proliferation of mesenchymal stem cells in *in vitro* culture. Another study developed a self-healing hydrogel based on bisphosphonate-modified alginate and Zn^{2+} interpenetrating polymer networks of silk fibroin. The material allowed for a controlled release of zinc, which modulated inflammation and improved functional recovery, including motor and sensory function, in a 2 mm rat SCI model.[133] The therapeutic effect of Zn^{2+} was linked to its anti-inflammatory role *via* inhibition of the NF-κB pathway, upregulating the anti-inflammatory molecule A20. This modulation reduced microglial activation and supported a regenerative microenvironment.

Bioactive silicate-based glass nanoparticles have also been considered potential aids in PNR.[134] Composed of an interconnected SiO_2 network, these glasses are designed to release therapeutic ions, *i.e.*, ions present in the glass matrix that stimulate specific cellular responses, thus promoting tissue regeneration.[135] These therapeutic ions can play critical roles in cellular signaling, proliferation, and differentiation, aiding nerve repair.[128,131] Zhang *et al.* studied the release profile of different ions present in bioactive glasses, namely Si^{4+}, Ca^{2+}, Na^{+}, Zn^{2+}, and Ce^{4+},[17] and the effect of their incorporation into poly (lactide-*co*-glycolide) (PLGA) and Pluronic F127 composites.[128] The results indicate suitable ion concentrations and

release profiles for PNR. However, *in vitro* and *in vivo* studies are needed to confirm biological effects.

Rare earth elements (REEs), particularly lanthanides, have also gained attention in the development of novel biomaterials.[136,137] Although traditionally known for their optical and nuclear properties with magnetic resonance imaging and luminescence applications, REEs have also shown promise as therapeutic ions. When incorporated in biomaterials, elements such as cerium (Ce), yttrium (Y), and gadolinium (Gd) add innovative functionalities, including antioxidant, antimicrobial, anti-inflammatory, and neuroprotective properties, which can positively influence neuronal regeneration and the growth control of support cells.[138] Schubert *et al.*[139] suggested that cerium and yttrium oxide nanoparticles act as neuroprotective agents, mitigating oxidative stress-induced cell death and improving neuronal viability in HT22 hippocampal nerve cells. In addition, Y- and Zn-doped borate bioactive glasses have shown promising results by significantly enhancing neurite growth in dorsal root ganglion explants.[140]

Cerium oxide nanoparticles (CeONPs) have been explored, showing promising results in mitigating oxidative stress and reducing inflammatory responses in nerve injuries. Qian *et al.* developed a collagen/CeONPs /PCL conduit using asymmetrical 3D manufacturing, reducing oxidative stress and inflammation in a rat with a 15 mm sciatic nerve defect, thus restoring functional and morphological nerves[141]. Rahimi *et al.* developed gelatin-PCL scaffolds with CeONPs for spinal cord injury repair, finding improved motor function, decreased pain, increased expression of regeneration-associated markers, and reduced inflammation.[142] Behroozi *et al.* combined CeONPs-loaded PCL scaffolds with PBMT in a spinal cord injury rat model. Their results demonstrated enhanced motor function recovery, reduced neuropathic pain, and mitigated inflammation, as evidenced by reduced glial fibrillary acidic protein (GFAP) and connexin 43 (Con43) expression.[143]

These findings suggest that applying REEs as therapeutic ions can improve neuronal regeneration and offer new possibilities for neural tissue engineering applications. However, long-term comparative studies are needed despite the regenerative potential of REEs-containing materials. They will help elucidate the mechanisms of action of these materials and assess their efficacy in clinical applications so far.[140]

In summary, ion release focusing on PNR application has emerged as an alternative to growth factors, being successfully incorporated into NGCs for enhanced nerve repair. Different ions act on distinctive stages of the regeneration process, and their timely release can be adjusted depending on the application. The current research is primarily *in vitro*, focusing on material science rather than biological effects. Extensive research, such as the one conducted for CeONPs and magnesium, should be broadened to other promising elements. Additionally, combining ion release with other biophysical stimuli, such as PMBT or ES, must be investigated.

7. Clinical trials

There are clinical trials regarding different strategies for nerve reconstruction after injuries. Considering the application of external stimuli, ES is the most explored technique. Wong *et al.* conducted a double-blinded trial (n = 36 patients) for treating transected digital nerves. After nerve coaptation, fine electrodes were used for 1 hour of 20 Hz continuous ES or sham stimulation.[42] The results showed earlier sensory factors improvement, such as cold and warmth detection threshold and static 2-point discrimination. Although a trend of improvement in the motor function assay was observed, it was not statistically relevant. Other trials aiming to investigate the effect of ES on nerve regeneration include: (i) nerve regeneration and smile outcomes following two-stage cross-face nerve graft facial reanimation surgery with 10 minutes of ES, called brief electrical stimulation therapy (BEST);[144] (ii) nerve healing and improvement functional recovery following surgical intervention with BEST for peripheral nerve injury in the arm;[145] (iii) the application of 1 h of low-frequency ES during surgery aiming improvement of hand function and nerve regeneration after repair for nerve injury in the arm;[146] the use of 30 min ES applied to the distal end of the damaged nerve in the arm to improve functional regeneration.[147] Using autografts and other microsurgical treatments makes it possible to understand the ES regeneration mechanism, protocols, and efficacy in humans. The following steps involve using ES combined with conductive NGCs, aiming for tuned piezoelectric materials for extended treatment.

MS is also applied in clinical trials regarding PNR. A randomized clinical trial evaluating the safety and effectiveness of magnetic peripheral nerve stimulation obtained positive results regarding neuropathic pain treatment, showing reduced pain and improved quality of life for the treated group.[148] The evaluation of the effect of repetitive transcranial and peripheral magnetic stimulation on the transected median nerve was recently proposed as a pioneering clinical study.[149] To expand the MS application, other trials on axonotmeses are desired, followed by their combination with functional NGCs.

Other techniques, such as light stimulation, have been studied for nerve regeneration. A randomized, double-blinded, sham-controlled trial indicated that PMBT reduced the effects of chemotherapy-induced peripheral neuropathy.[150] A randomized, double-anonymized clinical trial showed that LLL therapy improved outcomes in pain scores, function assessed through the QuickDASH questionnaire (Disabilities of the Arm, Shoulder, and Hand), and sensory nerve conduction velocity in the treatment of ulnar nerve entrapment.[151] However, understanding the effect of light stimulation on transected nerve injuries and graft applications is still challenging. As the light application is limited by beam penetration, biomaterials and NPs could help with deeper tissue stimulation.

Considering the ion release to improve PNR, the research is based on pre-clinical evaluations. Additionally, no current trials on biophysical stimulation combined with functional

biomaterials exist. This could be related to the need for further safety evaluation and validation of proposed NGCs, as there are few clinical trials regarding new biomaterial applications on PNR. Both functional NGCs and biophysical stimulation techniques must be investigated with the aim of PNR.

8. Conclusions and perspectives

Achieving peripheral nerve regeneration with complete functional recovery of nerves represents a grand challenge in tissue engineering, considering the high prevalence of injuries, costs, and consequences for patients. Currently, NGCs are already being used in clinical practices. However, they do not achieve better results than autologous nerve grafts. Alternatives to improve the success of these implantable devices include combining the NGCs with external stimulation and tuning them using nanotechnology to develop functional scaffolds. These strategies rely on specific mechanisms of nervous system regeneration and can improve growth factor production, cell proliferation, and cell modulation, as well as affect tissue inflammation and angiogenesis.

This review showed the great potential of functional biomaterials combined with several stimulatory strategies, highlighting the effect of each stimulus – electrical, magnetic, light, and ion release – on the nervous tissue and its repair process. Developing biomimetic and multifunctional materials for NGCs, based on a solid understanding of the regenerative process and cellular behavior, can enhance nerve regeneration. A multifunctional approach combining materials, stimuli, and nanotechnology seems promising to achieve complete functional recovery of PNI.

The main challenges to be overcome are: (i) guaranteeing the safe application of nanotechnology in the human body, with studies that encompass the long-term effect of these materials; (ii) further understanding of the biological responses elicited by the different stimulations and how they genuinely contribute in improving nerve repair; (iii) applying such strategies in the frame of an ambitious translational approach in clinical trials, to bring the most suitable and even "personalized" options to the patients suffering from peripheral nerve injuries. With special regard to the third challenge, it is worth pointing out that internationally recognized clinical protocols for ES, MS, or LS application must be developed to guarantee the translation of these therapies to clinical practice under safe conditions. Even so, the future of nerve repair is hopeful; with a solid understanding of the regenerative process and cellular behavior, we can explore the development of biomimetic and multifunctional materials for the construction of NGCs that will modify and improve nerve regeneration.

Author contributions

L. R. Lourenço: conceptualization, data curation, formal analysis, investigation, methodology, writing – original draft. E. F. dos Santos: formal analysis, investigation, methodology, writing – original draft. L. C. T. de Jesus: formal analysis, investigation, methodology, writing – original draft. C. A. Ezegbe: formal analysis, investigation, methodology, writing – original draft. F. Baino: formal analysis, investigation, writing – review and editing. R. Borges: conceptualization, formal analysis, investigation, methodology, writing – review and editing. J. Marchi: conceptualization, formal analysis, investigation, funding acquisition, project administration, methodology, supervision, writing – review and editing.

Conflicts of interest

The authors state that there are no conflicts of interest regarding this work.

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

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