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Leading opinion

Neural tissue engineering options for peripheral nerve regeneration

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ABSTRACT

Tissue engineered nerve grafts (TENGs) have emerged as a potential alternative to autologous nerve grafts, the gold standard for peripheral nerve repair. Typically, TENGs are composed of a biomaterial-based template that incorporates biochemical cues. A number of TENGs have been used experimentally to bridge long peripheral nerve gaps in various animal models, where the desired outcome is nerve tissue regeneration and functional recovery. So far, the translation of TENGs to the clinic for use in humans has met with a certain degree of success. In order to optimize the TENG design and further approach the matching of TENGs with autologous nerve grafts, many new cues, beyond the traditional ones, will have to be integrated into TENGs. Furthermore, there is a strong requirement for monitoring the real-time dynamic information related to the construction of TENGs. The aim of this opinion paper is to specifically and critically describe the latest advances in the field of neural tissue engineering for peripheral nerve regeneration. Here we delineate new attempts in the design of template (or scaffold) materials, especially in the context of biocompatibility, the choice and handling of support cells, and growth factor release systems. We further discuss the significance of RNAi for peripheral nerve regeneration, anticipate the potential application of RNAi reagents for TENGs, and speculate on the possible contributions of additional elements, including angiogenesis, electrical stimulation, molecular inflammatory mediators, bioactive peptides, antioxidant reagents, and cultured biological constructs, to TENGs. Finally, we consider that a diverse array of physicochemical and biological cues must be orchestrated within a TENG to create a self-consistent coordinated system with a close proximity to the regenerative microenvironment of the peripheral nervous system.

1. Introduction

Peripheral nerve injury is a common global clinical problem, and it significantly affects the patients' quality of life and causes an enormous socioeconomic burden [1–4]. Following traumatic injury to peripheral nerves, a series of pathophysiological events occurs in the injured nerve, leading to Wallerian degeneration in the distal stump and axon degeneration within a small zone distal to the proximal stump. The macrophages and monocytes migrate into the nerve stumps to remove resulting myelin and axon debris, while Schwann cells proliferate to form bands of Bungner, and produce neurotrophic factors and extracellular matrix (ECM) molecules to stimulate axon regeneration, which begins at the proximal stump and continues toward the distal stump. New axonal sprouts emanate from the nodes of Ranvier, and undergo remyelination by Schwann cells. The regenerating axons extend until reaching their synaptic target to achieve functional reinnervation.

Although the peripheral nervous system (PNS) has a greater capacity for axonal regeneration after injury than the central nervous system (CNS), spontaneous peripheral nerve repair is nearly always incomplete with poor functional recovery. Various types of medical therapy have been undertaken for several hundred years with the intention of improving outcomes [5,6].

When peripheral nerve injury results in a substantial nerve gap where tension-free neurorrhaphy (suturing of the nerve stumps) is impossible, interposition of some form of graft between the nerve stumps is required to bridge the gap and support axonal regrowth. Implantation of an autologous nerve graft [7], which is usually a functionally less important nerve segment self-donated from another site of the body, is accepted as the gold standard therapy for peripheral nerve gap repair. However, there are inherent disadvantages of autologous nerve grafting, including the limited supply of donor nerves, the need for a second surgery, donor site morbidity, and a mismatch between the donor nerve and the recipient site [8,9]; collectively these

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have encouraged the development of alternatives to autologous nerve grafts. With progress in regenerative medicine, and especially in tissue engineering, a subfield of neural tissue engineering has emerged, and various biological and artificial nerve grafts, which are generally placed in the category of tissue engineered nerve grafts (TENGs), have been produced in attempts to supplement, and even substitute for, autologous nerve grafts. As with other tissue engineering constructs, typical TENGs involve both physiochemical and biological cues, which are provided by a biomaterial-based structure, as well as a multitude of cellular and/or molecular components. In recent years many excellent review articles have been published that outline the structure, feature, and nerve regeneration-promoting actions of TENGs, and discuss their clinical applications and future directions [10–20]. Here, we aim to critically discuss the latest advances in neural tissue engineering for peripheral nerve regeneration, focusing on the involvement of new materials, new cues, new techniques, and new concepts.

2. Biomaterials, scaffolds and templates

2.1. Some new principles

We must preface this section with a brief discussion of the development of biomaterials for tissue engineering applications, especially on the basis of biocompatibility considerations. As recently discussed by one of the present authors [21], success in tissue engineering in general has been limited through a lack of understanding of the mechanisms of biocompatibility within a regenerative environment and the consequent difficulty in establishing practical specifications for so-called tissue engineering scaffolds. Tissue engineering is the creation of new tissue for the therapeutic reconstruction of the human body, by the deliberate and controlled stimulation of selected target cells through a systematic combination of molecular and mechanical signals [22]. The delivery of those molecular and mechanical signals does not take place in a vacuum and there will usually have to be a vehicle that controls, with spatiotemporal accuracy, these processes. Such vehicles have usually been described as scaffolds, but this conveys an old fashioned meaning of an inert structure that is temporarily used to assist in the construction of inanimate objects, taking no part in the characteristics of the finished product. A preferred term is 'template' which incorporates the sense of an active structure. In this paper we have to discuss the present cohort of TENGs in the context of traditional concepts of these biomaterials and existing scaffolds, but should bear in mind that future developments will have to be based on new paradigms. As discussed in detail elsewhere [23,24], these paradigms move away from the search for biomaterials and structures that passively allow cells to express new extracellular matrix; instead these materials have to be actively involved in the delivery of cues to cells. Indeed, it should be borne in mind that a tissue engineering template should replicate, as far as possible, the niche of those target cells. We shall return to this matter later in this Opinion Paper.

2.2. Traditional biomaterials selection

As the basic component of TENGs, the neural scaffold guides and protects axonal regrowth in the injured nerve; it should also act as a carrier for the delivery of biochemical cues [25–27]. A wide range of synthetic or natural biomaterials has been used to prepare neural scaffolds. The principal material used in early nerve guides was the non-degradable, biologically inert silicone elastomer. More recently, different classes of biodegradable synthetic polymers, including aliphatic polyesters, poly(phosphoesters), polyurethanes, piezoelectric polymers, and some electrically conducting polymers have served as scaffold biomaterials in neural tissue engineering. Among the US Food and Drug Administration (FDA), or European (CE) regulated commercially available products used for peripheral nerve repair, Neurotube® and Neurolac® are made of polyglycolic acid (PGA) and poly(D,L-lactide-co-ε-caprolactone) (PLC), respectively [28,29].

Natural biomaterials for neural scaffolds may fall into two categories: (1) autologous non-neural tissues and allogeneic or xenogeneic neural/non-neural tissues that have been subjected to decellularization [10], and (2) naturally-derived polymers, including extracellular matrix (ECM) molecules (collagen, laminin, fibrin, fibronectin, and hyaluronan), and other polysaccharides (chitosan, alginate, agarose) and proteins (silk fibroin, keratin) [30]. Importantly, many USA/European approved commercially available products are made of Type I collagen, e.g. Neurotube® NeuroGen®, NeuroFlex™, NeuroMax™, NeuroWrap™, and NeuroMend™ [28,29]. Also in China, chitosan-based nerve grafts have been approved by the China's State Food and Drug Administration (CFDA) for clinical trials [31].

Table 1 [32–57] summarizes currently approved, commercially available neural scaffold products for peripheral nerve repair.

It should also be noted that, in order to meet the requirements of preparing optimal neural scaffolds, biomaterials are usually modified or blended with each other.

In addition to natural and synthetic polymers, some ceramic, carbon, and metallic-based materials, have been investigated for use as neural scaffold materials. For example, a biodegradable glass fabric was used to repair the facial or median nerve in the sheep [58,59]; an active piezoelectric nanostructured ZnO ceramic was fabricated into a neural scaffold to support PNS regeneration [60]; carbon nanostructures, including nanotubes, nanofibers and graphene, have been incorporated in some experimental neural prostheses and guides [61,62]; Al/Al_2O_3 nanostructures have been investigated for their biocompatibility with peripheral neural cells [63]; biodegradable magnesium and magnesium alloys have been processed into implants for nerve repair [64,65].

Any biomaterial used to prepare neural scaffolds should possess appropriate physicochemical, biomechanical and biological properties. In the first category are the characteristics of porosity and permeability, while the second involves a balance between flexibility and rigidity. The biological properties, obviously, incorporate biocompatibility and biodegradability as well as the desired surface properties. As noted earlier, these characteristics are poorly understood. Although many biomaterials are essentially non-toxic, non-allergic, non-mutagenic and non-carcinogenic, they are likely to trigger a wide variety of unwanted responses in the human body [66,67]. Moreover, the avoidance of unwanted responses is only part of the story; if the biomaterial is unable to positively influence the performance of the target cell, then functional recovery will be significantly compromised.

The inflammation-inducing property of biomaterials cannot be ignored because the inflammatory response to peripheral nerve injury may induce both positive and negative effects on normal regeneration in the PNS [68]. The inflammatory potential needs to be reflected in the specifications for biomaterials suitable for neural scaffolds [69], and the implanted neural scaffold biomaterial must be elaborately monitored for this inflammatory potential. This was exemplified in a recent study that evaluated the long-term safety of using support cells-containing TENGs to repair a 50 mm long median nerve gap in monkeys in terms of the data from blood test, immunological and tumor marker detection, and histopathological examination of organs and glands [70].

Table 1
Currently government-approved, commercially-available neural scaffold devices.

Product name	Biomaterial	Diameter (d) and length (L)	Degradation time	Company	Animal and preclinical studies (Refs)
Avance® Nerve Graft	Decellularized ECM derived from donated cadaveric nerve	d = 1–5 mm L = 1.5–7 cm	N/A	AxoGen Inc. Alachua, FL	[42,50,54,57]
Salubridge™ Nerve Cuff	Polyvinyl alcohol (PVA)	d = 2–10 mm L = 6.35 cm	Non-degradable	Salumedica, LLC Atlanta, GA	N/A
Neurotube®	Polyglycolic acid (PGA)	d = 2.3–8 mm L = 2–4 cm	6–12 months	Synovis Micro Companies Alliance Birmingham, AL	[33,35,36,40,43,45,51,52,56]
NeuraGen®	Collagen type I	d = 1.5–7 mm L = 2–3 cm	36–48 months	Integra Life Sciences Corp. Plainsboro, NJ	[32,34,44,54,55]
NeuroFlex™	Collagen type I	d = 2–6 mm L = 2.5 cm	4–8 months	Collagen Matrix Inc. Oakland, NJ	N/A
NeuroMax™	Collagen type I	d = 2–6 mm L = 2.5 cm	4–8 months	Collagen Matrix Inc. Oakland, NJ	N/A
AxoGuard™ Nerve Connector	Porcine small intestinal submucosa (SIS)	d = 1.5–7 mm L = 4 cm	N/A	Cook Biotech Products West Lafayette, IN	[53]
Neurolac®	Poly(lactide-caprolactone) (PCL)	d = 1.5–10 mm L = 3 cm	16 months	Polyganics The Netherlands	[37–39,41,46–49]
NeuroWrap™	Collagen type I	d = 3–10 mm L = 2–4 cm	36–48 months	Integra Life Sciences Corp. Plainsboro, NJ	N/A
NeuroMend™	Collagen type I	d = 4–12 mm L = 2.5–5 cm	4–8 months	Collagen Matrix Inc. Oakland, NJ	N/A
SaluTunnel™ Nerve Connector	Polyvinyl alcohol (PVA)	d = 2–10 mm L = 6.35 cm	Non-degradable	Salumedica, LLC Atlanta, GA	N/A

2.3. Neural scaffold configuration

The translation from engineered biomaterials to neural scaffolds involves two interrelated aspects, the scaffold configuration and scaffold fabrication, both of which significantly affect the performance of neural scaffolds.

The scaffold configuration needs to be designed to facilitate support cell distribution and growth of injured nerve tissues in three dimensions. Initially, biomaterials were engineered into the simplest configuration shaped as a cylindrical tube whose single lumen was empty. This scaffold was called a nerve guidance conduit or nerve guidance channel (NGC) with a single hollow lumen. To improve neural scaffolds, more complex configurations have been developed, in which a NGC has either an internal microarchitecture or a multiple-component composition within its lumen. For example, one or more intraluminal channels can be introduced to construct a multichannel NGC, which is designed by mimicking the architecture of nerve fascicles and therefore able to reduce dispersion of regenerating axons within the NGC lumen [71–73]. The question of whether a multichannel NGC is really superior to a single lumen NGC, however, has not yet been conclusively answered because of the difficulty of a meticulous imitation of the *in vivo* situation [74]. Another modification to the simplest configuration of neural scaffolds is incorporation of physical fillers into the NGC lumen to mimic the endoneurial-like structure usually found within autologous nerve grafts. An array of biomaterial-based fillers, in the form of fibers, filaments, gels or sponges, has been included into the lumen of NGCs to provide topological cues for improving nerve regeneration [75–78]. A neural scaffold comprising a chitosan-based NGC and intraluminal filaments of PGA was used to bridge a 30 mm long sciatic nerve gap in dogs [79]. According to the investigators' observation, the NGC was suitable for the ingrowth of blood vessels and to allow diffusion of nutrients and other molecules while preventing cells from entering the interior of the NGC. The PGA filaments served as a directional guide to facilitate proper Schwann cell functioning, and, moreover, the biodegradation products of chitosan and PGA were basic glucosamine and acidic glycolic acid, respectively, which neutralized each other and entered metabolic pathways within the body, maintaining a relatively stable microenvironment without remarkable change in pH [79]. These results, together with data from analogous studies [76,80,81], confirm the importance of luminal fillers in a NGC on the performance of neural scaffolds, which is illustrated by a schematic in Fig. 1.

2.4. Neural scaffold fabrication

Neural scaffolds, especially with the simplest configuration, can be fabricated by many well-defined techniques, including immersion precipitation particulate leaching [82,83], extrusion [84,85], injection molding [86,87], non-woven or woven mesh rolling [45,88,89], centrifugal casting [90], spinning mandrel technology [91], film casting plus rolling [92,93], and molding plus freeze-drying [94]. Some advanced fabrication techniques have now been developed for preparing scaffolds with more complex configurations, such as a multichannel NGC [73,95], or a NGC containing longitudinally aligned fibers [96,97], micro-grooves [98,99] or hydrogels [100] within their lumens.

After implantation, a neural scaffold should act as a substrate for adhesion, proliferation, migration, and function of neural cells. The *in vivo* interactions between scaffolds and host cells/tissues are complex and bi-directional: not only will the scaffold elicit cell and tissue responses, but host cells/tissues will change the local environment provided by the scaffold through deposition of ECM molecules [101]. To better adjust these interactions, nanotechnological techniques have been extensively introduced to the fabrication of neural scaffolds having a nanoscale topography, which closely resembles the architecture of natural ECM [102] and induces cell contact guidance in nerve regeneration [103]. With the emergence of nanomaterial-based scaffolds, nanostructures are largely incorporated into the common polymer-based scaffolds in the process of fabrication, aiming to improve their bulk and surface properties. Diverse manufacturing methods, such as electrospinning [104,105], phase separation [106,107], and self-assembly [108,109], as well as computer-aided design-based fabrication techniques [19], have been used for preparing nanostructured scaffolds, intended to enhance axonal regrowth. Promising results have been reported concerning peripheral nerve repair with nanostructured neural scaffolds [78,96,110–112].

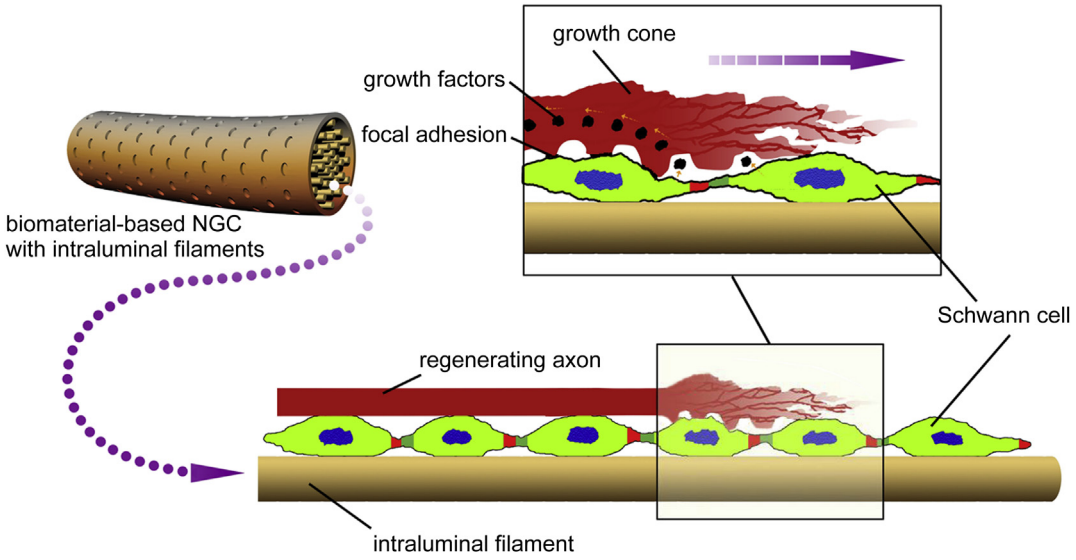

Fig. 1. A schematic diagram showing how a biomaterial-based NGC with intraluminal fillers supports cell migration of Schwann cells and guides axonal growth after implantation to bridge a peripheral nerve gap. Also shown (the boxed area) is the local magnification.

3. New attempts with support cells and growth factors

To enhance the outcome of peripheral nerve regeneration through the use of scaffolds alone, efforts have concentrated on the optimal incorporation within a TENG of biochemical cues, including support cells, growth factors, and/or cytokines.

3.1. Cellular components of TENGS

Schwann cells, neural stem cells, embryonic stem cells, and marrow stromal cells have been the most studied support cells [17] although several others have been reported.

Olfactory ensheathing cells (OECs) develop from a peripheral origin, the olfactory placode, and retain the ability to self-renew and differentiate, and are considered as peripheral nerve progenitor cells [113,114]. OECs-containing silicone tubes were noted to support an improved axonal regeneration in 50 or 79% of rats with a 15- or 12-mm sciatic nerve injury gap [115]. Although the therapeutic potential of OECs in peripheral nerve repair is yet far from conclusive, there have been later studies reporting on the treatment of peripheral nerve injury by direct injection of OECs to the injured site [116–119].

The application of stem cells from different sources in the field of neural tissue engineering has attracted much interest, and the bone marrow mesenchymal stem cells (also named bone marrow stromal cells, BMSCs) are one of the most important stem cells. They localize in the stromal compartment of the bone marrow, where they support hematopoiesis and differentiate into mesenchymal lineages [120–125]. Because they are easily obtained through the aspiration of the bone marrow and expanded in a large scale by *in vitro* culture, BMSCs have found increasing applications in cell-based therapies for various diseases, including neural injury and disorders [126–132]. Despite the indispensable value of Schwann cells for the construction of TENGs, autologous Schwann cells are difficult to obtain in large number, and allogeneic Schwann cells are involved in immunological rejections. Therefore, BMSCs have become a promising alternative to Schwann cells for use as support cells within TENGs, showing considerable success in experimental studies [70,133–135].

Stem cells from sources other than the bone marrow are now getting more attention. The gliogenic secondary neurospheres derived from induced pluripotent stem (iPS) cells have an ability to differentiate into Schwann cells. iPS cells were added to a PLC-based NGC, followed by implantation across a sciatic nerve gap in mice, showing regeneration of peripheral nerves and functional recovery [136].

The skin dermis contains neural crest–related precursor cells, and the skin-derived precursor cells (SKPCs) can be cultured to differentiate into neural crest cell types with the characteristics of neurons and Schwann cells in the PNS [137,138]. The SKPCs with neurotropic function show a full capacity of differentiating into Schwann cells and promoting axon regeneration *in vivo* [139]. In one study, SKPCs were injected into neural scaffolds (NGCs) that had been prepared with L-lactide-trimethylene carbonate (PLA-TMC) copolymer or type I collagen, respectively, to generate a TENG, which was then used to bridge a 16-mm sciatic nerve gap in rats. The results of the study confirmed the beneficial effects of SKPCs on nerve regeneration [140]. In another study, porcine SKPCs were found to induce prominent nerve regeneration in porcine peripheral nerve injury sites after SKPCs were added to a collagen/fibrin NGC for bridging a 10-mm femoral nerve gap in pigs [141]. More studies demonstrate further evidence for the effectiveness of SKPCs as support cells within TENGs [139,142].

Adipose tissue has also been identified as a niche for multipotent stem cells that have a phenotypic profile comparable to that of BMSCs and can differentiate into a myelinating Schwann cell phenotype in culture with lineage-specific stimuli [143,144]. In consequence, adipose-derived mesenchymal stem cells (AMSCs), also named adipose-derived stem cells (ADSCs), are potentially valuable because of their capability of multilineage differentiation in a manner resembling that of BMSCs. Importantly, AMSCs are superior to BMSCs in some aspects, such as the convenient harvesting of AMSCs through liposuction, a much less invasive method than bone marrow aspiration, and the greater availability of adipose tissue than bone marrow [145]. To apply AMSCs for neural tissue engineering, many experimental studies in diverse animal models have been accomplished, in which different neural scaffolds containing either undifferentiated or differentiated AMSCs have bridged peripheral nerve gaps of different lengths [146–160]. All these studies indicate the favorable effects of AMSCs on peripheral nerve reconstruction and open a new approach to the use of support cells for constructing TENGs.

In addition, gene modified stem cells are used for new options of neural tissue engineering. BMSCs were genetically engineered to express nerve growth factor (NGF) using an adenoviral vector, and they were then plated onto PLGA substrate, which could be used for preparing neural scaffolds [161]. GDNF-modified human amniotic fluid-derived mesenchymal stem cells (AFMSCs) were embedded in Matrigel, which was implanted to the injured nerve in a rat model of sciatic nerve crush, and GDNF expression by modified AFMSCs was sustained up to 4 weeks to promote nerve regeneration [162]. GDNF- or BDNF-modified neural stem cells were incorporated into a poly(D,L-lactide) NGC, which was able to promote nerve regeneration and functional recovery in a rat model of sciatic nerve transaction [163].

These attempts are also regarded as some examples of cell-based delivery of growth factors in neural tissue engineering (as described in Section 3.2.).

3.2. Improvements in growth factor delivery

Upon injury to peripheral nerves, the local presence of growth factors at the injury sites plays a vital and complex role in modulating phenotypic changes of a variety of neural and non-neural cells. Although the endogenous growth factors secreted by neural cells in the distal nerve stump can support axon regeneration, the supportive action may not be sustained indefinitely due to an obvious decline with time in cellular production of growth factors, and hence the continuous supply of growth factors is critically required, which is mainly dependent on the addition of exogenous growth factors.

To date the most commonly used growth factors belong to two classes: (1) neurotrophins, including NGF, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3); and (2) growth factors with neurotrophic actions, including glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) and fibroblast growth factors (FGFs). The details about their application for neural tissue engineering are available in review papers [16,17].

It has become clear that different delivery systems and profiles induce different positive impacts on nerve reconstruction [164], giving rise to efforts to improve these systems. Several classical strategies are available for continuous release of growth factors from TENGs, such as adsorption of growth factors to the surface and/or bulk of a scaffold, incorporation of growth factors into the scaffold materials during the scaffold fabrication, entrapment of growth factor-loaded microspheres into a scaffold, covalent immobilization of factors onto the scaffold, and installation of an osmotic minipump or injection device.

For example, fibrin gels were loaded with GDNF-containing, poly (D, L-lactide-co-glycolide)(PLGA)-based microspheres for treating a delayed injury to rat common fibular nerves. The GDNF-containing microspheres improved the regeneration of common fibular nerves and the reinnervation of extensor digitorum longus muscles, suggesting the effectiveness of a microsphere-based technology to encapsulate growth factors in the delayed nerve repair [165]. As another example, a double-walled microsphere delivery system was created for the sustained release of GDNF and showed a sustained release of GDNF *in vitro* (longer than 50 days), and the double-walled microsphere was also incorporated into a PCL-based NGC for being implanted across a 15 mm rat sciatic nerve gap, in which an improved nerve regeneration and localization of Schwann cells around double-walled microspheres were observed, confirming the dynamic release of bioactive GDNF from the delivery system [166]. The same authors' subsequent study [167] further showed the long-term effect of GDNF-loaded, double-walled microspheres on sciatic nerve regeneration.

The short-/long-term effects of microsphere-delivered NGF or GDNF on nerve regeneration were evaluated in a rat 10 mm long sciatic nerve gap model. The initial beneficial effect of NGF- or GDNF-containing microspheres in a PLGA-based NGC was observable, reaching the maximum value by 6 weeks without significant enhancement in the ensuing period [168].

The cell-based delivery of growth factors has been developed. A silicone-based NGC seeded with genetically modified Schwann cells over-expressing fibroblast growth factor-2 (FGF-2) was used to bridge a 15 mm sciatic nerve gap in adult rats. Different FGF isoforms overexpressed by implanted Schwann cells improved both lengths and number of regenerating myelinated axons over different time periods post grafting [169–171]. Likewise, the design of GDNF-transduced Schwann cell grafts for enhancing regeneration of erectile nerves represents the same attempt of cell-based delivery of neurotrophic factors [172,173]. In addition, a combination of iPSc-derived neurospheres and basic fibroblast growth factor (bFGF)-containing gelatin microspheres was incorporated into a neural scaffold (a synthetic polymer-based, double-layered NGC), and the constructed TENG was used to bridge 5 mm long sciatic nerve gaps in mice, achieving regenerative outcomes to some degree [174]. The above-mentioned are typical examples of the combined use of support cells and growth factors as biochemical cues within TENGs.

Of note, it is very important to maintain the activity of growth factors during prolonged release from their cell environment. Accordingly, modification of growth factors for immobilization on, or for high-affinity binding to, cells or scaffold biomaterials has attracted much attention. Fibrin-binding NGF mixed with fibrin gel was noted to enhance neurite extension from cultured dorsal root ganglia by 50% relative to native NGF and by 350% relative to the fibrin gel alone despite the reduced activity of the fusion construct [175]. Similarly, subcutaneous implantation of a collagen membrane loaded with collagen binding domain fused NGF could enhance nerve growth into the membrane under the influence of controlled NGF delivery [176].

Numerous studies report on how diverse techniques may be used to immobilize NGF onto neural scaffolds. Among various newly-developed procedures, crosslinking is commonly used for immobilization, in which the choice of cross-linkers is an essential step. Compared with many traditional cross-linkers (e.g. glutaraldehyde and carbodiimide), genipin, a natural agent with low toxicity, has many advantages. Genipin was used to crosslink the biomaterial chitosan and then immobilize NGF onto the modified chitosan, which was further processed into a NGC. A series of *in vitro* tests suggested that continuous release of NGF from such systems may be applicable for peripheral nerve repair [177]. An *in vivo* study was subsequently reported, in which this type of NGC was used to bridge a 10 mm long sciatic nerve gap in rats and the release system of NGF aided peripheral nerve repair [178]. Similar reports have also tested the use of genipin in NGF crosslinking [179,180].

Photochemical reactions are used to immobilize NGF and tirofiban (a nonpeptide glycoprotein IIb/IIIa antagonist) onto the surface of PCL-based NGC, which effectively promoted the regeneration of injured nerves in a rat long nerve gap injury model [181]. Photochemical techniques were also adopted to immobilize NGF and another activator onto neural scaffolds, which showed the promoting effects on the growth and neuron-like differentiations of PC12 cells *in vitro* [182].

Coaxial electrospinning was used to immobilize NGF onto the aligned core–shell nanofibers, which were then inserted to a PLGA-based NGC to construct a TENG for bridging a 13 mm long sciatic nerve gap in rats, and peripheral nerve regeneration was promoted by controlled release of NGF [183]. Coaxial electrospinning was also

adopted to prepare NGF-containing, PLC-based neural scaffolds for bridging a 10 mm long sciatic nerve gap in rats, achieving favorable outcomes of peripheral nerve repair [184]. Also, differential adsorption was able to introduce NGF gradients into neural scaffolds, and NGF gradient-immobilized TENGs produced improvements in morphological and functional restoration in a rat 14 mm long sciatic nerve gap model [185].

4. Potential use of RNA interference

RNA interference (RNAi) refers to the silencing of a particular gene by using a double-stranded RNA (dsRNA) with homologous sequences to that of the target mRNA [186]. Since it was first discovered in 1990s [187], RNAi has attracted much attention as a research tool to control the expression of specific genes in living cells. Also there is interest in its use as a concomitant therapeutic strategy to inhibit target gene expression in many devastating diseases and injuries because of its high sequence specificity and capability of inducing robust and potent knockdown of target genes [188,189]. Here we are interested in the question of whether RNAi strategy is also applicable for the understanding and treatment of peripheral nerve injury.

To trigger RNAi, the long dsRNA molecule (100–700 nucleotide long) can be cleaved into smaller dsRNA molecules (~21 nucleotide long with 2 nucleotide 3′ overhangs), called short interfering RNAs (siRNAs), with the help of Dicer, an RNAse III enzyme. Then siRNAs are incorporated into a multiprotein RNA-induced silencing complex (RISC), and the activated RISC further recognizes and cleaves the mRNA that is complementary to the siRNA. On the other hand, RNAi can also be triggered by microRNAs (miRNAs), which regulate gene expression at the post-transcriptional level in cells.

4.1. siRNA

The siRNA-based method has been used to show functional RNAi in axons and to clarify an approach to spatially regulate mRNA transcripts at a subcellular level in neurons [190]. The method has also been adopted to confirm that the RNAi machinery may exist in peripheral nerve axons and function independently from the neuronal cell body or Schwann cells [191]. In order to elucidate the cytoskeletal remodeling process within injured axons after peripheral nerve injury, siRNA-induced RNAi of a NudE-like protein (Ndel1, viewed as an integrator of the cytoskeleton) was performed in transected axons, and the results showed that local silencing of Ndel1 by siRNA dramatically reduced axonal regeneration *in vivo* [192]. In order to identify intracellular inhibition of neuronal growth signals and look for intrinsic regeneration pathways within axons, it was found that either pharmacological inhibition of PTEN (phosphatase and tensin homolog deleted on chromosome 10) or its mRNA knockdown using siRNA could induce a robust increase in the plasticity of neurite outgrowth *in vitro* and *in vivo* [193]. Down-regulation of Sprouty2 by siRNAs was found to promote elongative axon growth by activation of the Ras/Raf/ERK pathway [194]. In addition, it has been reported that knockdown of erythropoietin-producing hepatocellular receptor A4 (EphA4) protein by 2 independent siRNAs increases Schwann cell migration and peripheral nerve regeneration [195], and that knockdown of nectin-like 4 (Necl-4) by short hairpin RNA inhibits Schwann cell differentiation and subsequent myelination in cocultures [196].

4.2. miRNAs

miRNAs are a class of ~22 nucleotide non-coding RNA molecules that negatively regulate the expression of a wide variety of genes, mainly through direct interaction with the 3′-untranslated regions (3′-UTR) of their target mRNAs [197]. It is estimated that miRNAs regulate up to 60% of the total human genes at the post-transcriptional level [198]. This fact highlights the pivotal role of miRNAs in a diverse array of physiological and pathological processes. The importance of miRNAs for neural development and degeneration has been delineated [199,200], and their involvement in peripheral nerve injury and regeneration is now been actively studied [201,202].

A recent study [199] showed that the deletion of Dicer (a key molecule in biogenesis of miRNA) disrupted the production of Dicer-dependent miRNAs, impeded peripheral nerve regeneration according to behavioral, functional, and histological examination *in vivo*, and inhibited axonal growth from neurons *in vitro*, thus confirming the significance of Dicer-dependent miRNA pathway for successful repair of peripheral nerve injury. The same authors indicated in another study that not only was miRNAs-triggered RNAi observed in transfected peripheral nerves, but RISC, as a RNAi effector complex, was identified in transected axons treated by miRNAs, suggesting a miRNA machinery in response to peripheral nerve lesion [203]. Some newly published studies have investigated the influences of miRNAs on neurite outgrowth from adult dorsal root ganglia (DRGs) neurons following sciatic nerve transection injury [204–207]. These showed that miRNAs could regulate neurite growth from adult DRG neurons in distinct ways: miRNA-21 promoted neurite outgrowth by directly down-regulating Sprouty2 (SPRY2) expression [204]; miRNA-222 targeting PTEN promoted neurite outgrowth [206]; while miRNA-145 inhibited neurite outgrowth by inhibiting Robo2 expression [205]. In order to determine the necessity of Dicer and miRNAs for nerve myelination, recent studies showed that the ablation of Dicer1/miRNA from Schwann cells led to glial overproliferation and aberrant myelination, although the specific molecular approaches for gene silencing varied among different studies [208–211].

Based on the importance of Schwann cells for peripheral nerve regeneration, more recently, the miRNA-mediated regulation of Schwann cells' responses to peripheral nerve injury has been investigated: miR-34a interacted with positive regulators (Notch1 and cyclin D1) of dedifferentiation and proliferation to control cell cycle dynamics in Schwann cells, while miR-140 targeted the transcription factor Egr2, a master regulator of myelination, and modulated myelination in DRG/Schwann cell co-cultures. In addition, miR-140 was reported to target the transcription factor Egr2, a master regulator of myelination, for modulating myelination in DRG/Schwann cell co-cultures [212]; miR-182 inhibited proliferation and migration of Schwann cells by targeting fibroblast growth factor 9 (FGF9) and neurotrimin (NTM) at an early stage following sciatic nerve injury [213]; miR-221 and miR-222 promoted proliferation and migration of Schwann cells by targeting longevity assurance homolog 2 (LASS2) after sciatic nerve injury [214]. Fig. 2 illustrates how the aforementioned siRNAs/miRNAs affect peripheral nerve injury and regeneration.

In terms of their composition, miRNAs belong to a class of small noncoding RNAs, while another class of long noncoding RNAs (lncRNAs), each of which contain nucleotides ranging from 200 to more than 100,000 [215], are found to be highly expressed in the brain [216,217], serving as mediators of mRNA decay, as scaffolds for nuclear substructures, as host genes for miRNAs, and as regulators of chromatin remodeling [218,219]. A recent study explored the temporal regulation of lncRNA expression in DRGs during peripheral nerve regeneration, and indicated that down-regulated lncRNA BC089918 could promote neurite outgrowth of DRG neurons [220]. This interesting finding reveals another layer of lncRNA regulation of the intrinsic growth capacity of neurons.

Finally, it should be pointed out that the application of RNAi for neural tissue engineering, and for other diseases and injuries, faces

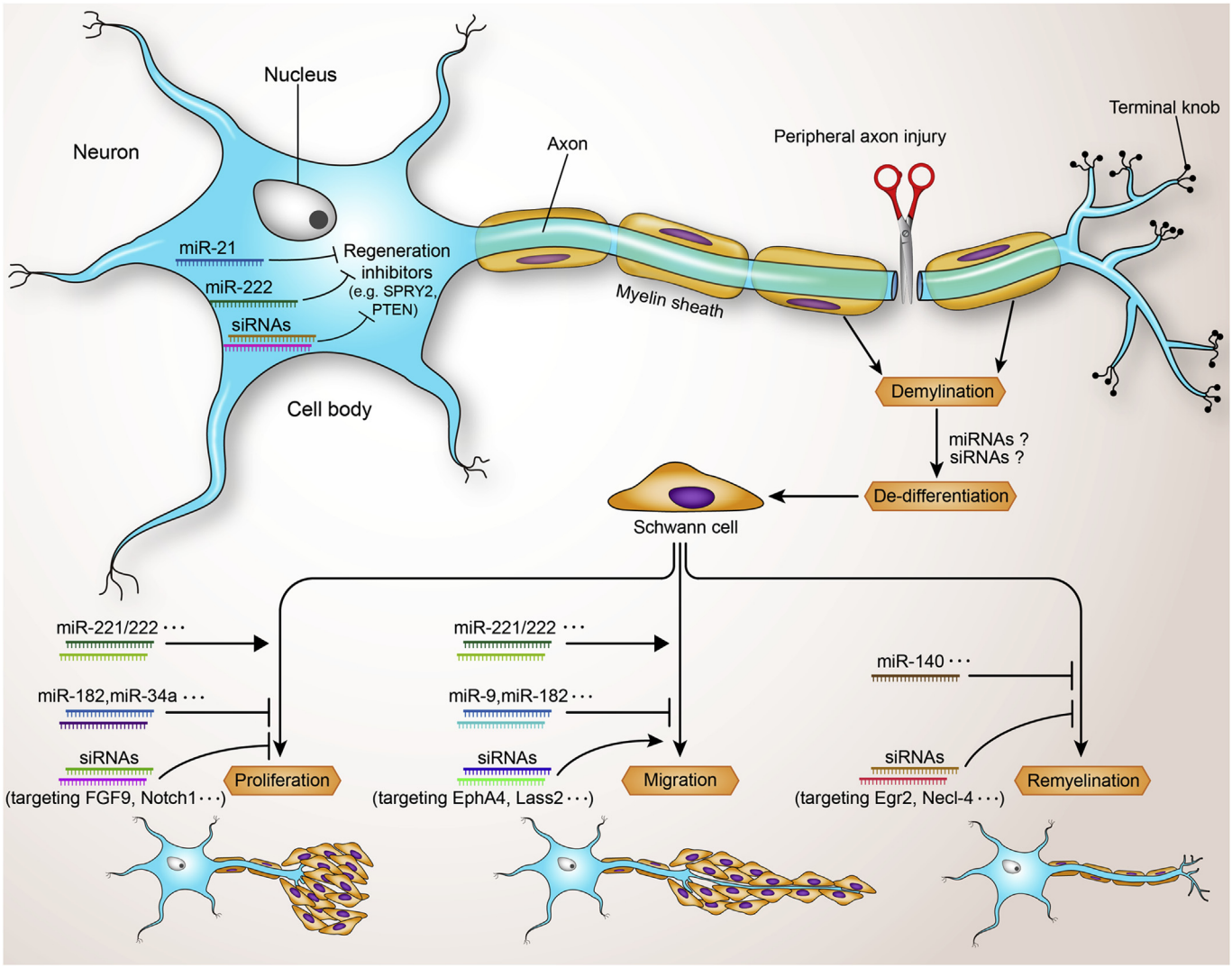

Fig. 2. A schematic diagram showing that after peripheral nerve injury, miRNAs and siRNAs regulate intrinsic neurite growth capacities of neurons and modulate phenotypic changes of Schwann cells through inhibition of their respective targets, and suggesting the impacts of RNAi on peripheral nerve injury and regeneration.

significant challenges from the development of a potent delivery system with high specificity, low immune stimulation, and little cytotoxicity [221]. Various nanocarrier systems, including liposomes, nanoparticles, dendrimers and carbon nanotubes, have been designed as siRNA delivery vectors to overcome the common biological barriers, especially biodegradation in the bloodstream, renal clearance and inadequate entry into cells [222,223]. For example, electrospun PCL nanofibers were successfully functionalized with RE-1 silencing transcription factor (REST) siRNAs, and this new design of nanofibrous scaffold-mediated REST knockdown enhanced neuronal differentiation of stem cells [224]. As another example, a non-viral delivery approach has been developed by joint use of siRNA-containing nanoparticles with a polymer-based, microstructured scaffold, in which the local delivery of RhoA-specific siRNA caused a significant reduction in the target mRNA level and allowed neurite outgrowth from PC12 cells even in an inhibitory environment [225]. In addition, a polymer-based, microstructured neuronal prosthesis loaded with siRNA-containing nanoparticles was prepared to undergo a variety of *in vitro* tests for side effects of this formulation [226]. Obviously, a comprehensive evaluation of both therapeutic and side effects is necessary for *in vivo* use of the siRNA nanoparticle formulation in CNS and PNS repair.

It is worth mentioning that in a study [193], PTEN siRNA was added to a silicone NGC, which was used to investigate the *in vivo* effect of PTEN inhibition on peripheral nerve regeneration. This study may be considered the first attempt to apply RNAi in the field of neural tissue engineering. Another latest study demonstrated that miRNA-9 inhibited Schwann cell migration by directly targeting collagen triple helix repeat containing protein 1 (CTHRC1) *in vitro*, and that a silicone conduit containing steroid-conjugated miRNA-9 mixed with Matrigel was implanted to bridge a rat sciatic nerve gap, suggesting the *in vivo* inhibitory effect of miRNA-9 on Schwann cell migration [227].

5. Additional cues

5.1. Angiogenesis

The importance of angiogenesis, the growth of new blood vessels, for tissue engineered constructs is well known because the survival of cells and tissue in the body depends largely on the supply of oxygen and nutrients and the removal of metabolites by a branched blood vessel system with an optimal distance of $<200\ \mu m$ between small capillaries in the tissue [228–230]. Similarly, the relationship between angiogenesis and neural tissue engineering

was noted as early as in 1990s [231], when some attempts were tried to augment the angiogenesis within neural scaffolds, for example, the insertion of blood vessels into a silicone NGC for bridging 10 mm or 25 mm sciatic nerve gaps in rats, respectively [232,233]. In comparison to these earlier attempts, the currently preferred approaches are based on the use of signaling molecules within TENGs.

Nitric oxide (NO) is a short-lived signaling molecule with multiple functions in different systems, including the modulation of vascular growth [234]. NO is synthesized from L-arginine by activation of nitric oxide synthase (NOS). Three isoforms of NOS are identified in peripheral nerves: the neuronal isoform (nNOS) in discrete neuronal populations, the endothelial isoform (eNOS) in vascular endothelium; and the inducible isoform (iNOS) in various cell types, including macrophages and glial cells [235]. Intriguingly, nerve regeneration and vascularization are delayed in eNOS knockout animals versus wild type controls [236], and specific knockdown of eNOS inhibits vascular remodeling through reducing endothelial cell migration [237]. These findings suggest that the manipulation of NO supply within TENGs could contribute to simultaneous formation of new capillaries and regenerating axons.

Vascular endothelial growth factor (VEGF), a signaling molecule produced by hypertrophic chondrocytes, is also a fundamental regulator of both normal and abnormal angiogenesis [238]. VEGF not only has neurotrophic activity to stimulate axonal outgrowth and to enhance survival and proliferation of Schwann cells [239], but also improves intraneural angiogenesis by promoting endothelial sprouting during peripheral nerve regeneration [240–242]. Therefore, concentration gradients of VEGF in 3D hydrogels were able to guide the movement of endothelial cells, thus offering an approach to enhancing blood vessel growth in tissue engineered scaffolds [243]. A VEGF-containing silicone NGC was used to bridge 10 mm long sciatic nerve gaps in rats, and the long-term observation demonstrated that VEGF significantly increased vascular and axonal regeneration and enhanced target muscle reinnervation in a dose-dependent manner [244]. Bio-printing was used to incorporate VEGF-releasing fibrin gel and neural stem cells into a collagen hydrogel scaffold, in which the VEGF-releasing fibrin gel together with neural stem cells caused better morphological changes as well as migratory responses, suggesting roles of VEGF beyond angiogenesis in the process of nerve regeneration [245].

5.2. Electrical stimulation

Electrical charges stimulate cellular differentiation in various tissues types [246], and neurite extension can be enhanced on the substrates that are based on electrically conducting polymers, such as polyaniline, polypyrrole, polythiophene, and polyacetylene [247–249], or based on piezoelectric materials such as poly(-vinylidene fluoride) [250]. The interaction between neural cells and electrically conductive biomaterials may arise from an increased adsorption of positively charged matrix proteins onto the negatively charged surface of biomaterials. When a direct electric current passes through the substrate derived from electrically active biomaterials, enhanced effects on neurite outgrowth are observed. The conductivity and antioxidant property of polyaniline and polypyrrole make them attractive candidates for neural scaffold materials, but the use of these substances is limited by their non-biodegradability. A compensatory method was proposed by blending them with other biodegradable biomaterials and using the composite material to prepare neural scaffolds [251].

Direct current electric fields are present in all developing and regenerating animal tissues, and electric field treatment of damaged tissues in the nervous system may have clinical potential [252]. Studies further show that the axis of neural cell division, the establishment of neuronal polarity, the polarization of intracellular structures, and the direction of neuronal migration are all regulated by an extracellular electrical cue [251–255]. A brief stimulation protocol was designed to deliver electrical signals after nerve injury, which improved the amount and accuracy of motor and sensory reinnervation [256–259]. The mechanism for this improvement may involve an accelerated response of peripheral neurons to injury stimuli, which is mediated by BDNF signaling [259], and a clinical trial in carpal tunnel syndrome patients provided an evidence that brief low frequency electrical stimulation accelerated axonal regeneration and target reinnervation in humans [260]. Similarly, an electric circuit was implanted in rats with a sciatic nerve crush injury, and the repair outcome showed that low intensity direct electric stimulation enhanced nerve regeneration and augmented blood supply by increasing the number and diameter of the vasa nervorum [261]. From this study, we suspect that the improved angiogenesis may be a second beneficial outcome of electrical stimulation.

There remain some unsolved problems involved in the joint use of electrical cues with TENGs. For example, adult rats with transected sciatic nerves were subjected to the bridging of a 10 mm nerve gap with a silicone rubber NGC, followed by electrical stimulation, and the regenerated myelinated fibers and target reinnervation were significantly better if the regular electrical stimulation was initiated 1 day after bridging than if the stimulation was initiated 2 weeks after bridging [262]. The results suggest that the time course of electrical stimulation is important, and a rapid onset of electrical stimulation may accelerate axonal regrowth across the nerve gap. Moreover, electrical stimulation may have either a positive or negative impact on peripheral nerve regeneration depending on the pattern, strength, and/or timing of the stimulation [263].

6. Conclusions and perspectives

Despite the gold standard for peripheral nerve gap repair, autologous nerve grafts fail to achieve an entirely satisfactory restoration of function after they are implanted. Intended to supplement and replace autologous nerve grafts, TENGs should be able to compete with or even surpass autologous nerve grafts in the outcomes of nerve regeneration and functional recovery. Therefore, although the past several decades have witnessed great advance from the earliest nerve tube to the state-of-the art TENG, neural tissue engineering needs further significant progress towards the development of ideal TENGs and their translation to clinical applications.

This opinion paper has highlighted the latest advances in neural tissue engineering, with an aim to help capture the real-time dynamic information in the field. We have to place the current peripheral nerve regeneration scenario, and prospects for the future, into the context of general developments of all components of tissue engineering, including the biomaterials and their biocompatibility, the structural characteristics of scaffolds/templates, the performance of the source cells and the delivery of the various cues or signals. Fig. 3 provides a concise overview of the compositions of TENGs.

It has to be recognized that many materials have been tested and used for TENGs without clear resolution of the optimal structure, a fact by itself which indicates that there is much to learn about their performance. We have delineated here the experimental use of various inorganic and indeed metallic materials, which go beyond the framework of traditional biomaterials, for neural template fabrication. We have also addressed the favorable features of some nanostructured neural scaffolds due to their topographical resemblance of natural ECM architecture. This is a

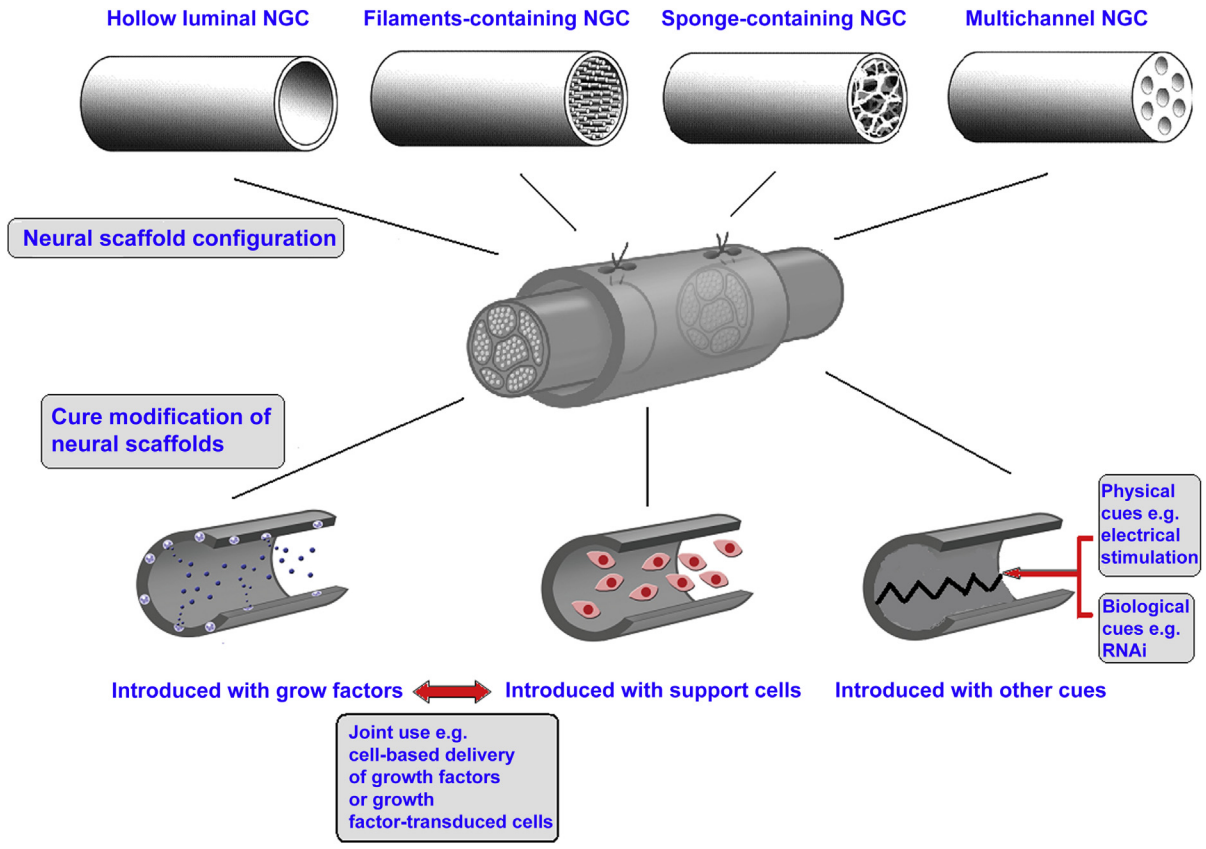

Fig. 3. A schematic diagram showing how an ideal tissue engineered nerve graft (TENG) is constructed by incorporating a diverse array of physical and biological cues to a neural scaffold with different configurations.

key issue in the light of the need for the template to replicate the niche of the target cells. It is unlikely that conventional materials, including most synthetic polymers, will meet the strict requirements of this cell niche concept; the use of decellularized natural tissues and various forms of biopolymers, including hydrogel forms of both proteins and polysaccharides are clearly very important here.

We have described the incorporation of SKPCs and AMSCs (as support cells) into neural templates in the construction of TENGs, and illustrate newly-developed delivery systems for growth factors within them. We emphasize the significance of RNAi for peripheral nerve regeneration, and anticipate the application of RNAi reagents for TENGs. We also speculate on the possible contributions of additional cues, such as angiogenesis and electrical stimulation, to the improvements of TENGs. Certainly, there are still other prospective cues that can be integrated within TENGs, such as molecular inflammatory mediators [264–267], bioactive peptides [268,269], antioxidant reagents [270,271], or even biological constructs, for example, an *in vitro*-formed nerve equivalent through co-culture of DRGs and Schwann cells [272]. These extra cues are ready to demonstrate their promising applications in neural tissue engineering.

At this time, the construction of an effective TENG should be considered as a complex scientific and engineering problem that involves multifaceted interactions between a diverse array of physicochemical and biological cues, which have been and are still being elucidated within the constantly updated knowledge of peripheral nerve injury and regeneration. The various cues have distinctive effects on the performance of TENGs, but it is necessary to understand and implement the orchestration of the different cue-induced effects. A considerable number of comparative studies must be conducted to decipher: which are more prominent cues, whether and how different cues are interrelated to and interfere with each other. Obviously, the research on these issues will benefit from an improved insight into the molecular events and mechanisms that underlie peripheral nerve injury and regeneration [273–276].

So far, not only in animal models have TENGs achieved good results, but clinical trials with TENGs to treat human patients with peripheral nerve injury have also met with a certain degree of success [28,29,277–279]. Especially, many commercial available products of neural scaffolds have been used in the clinic with promising outcomes (see previous review articles [17,28,29] and Table 1). Nowadays, TENGs used in the clinic, however, are limited to those composed of a neural scaffold alone without any biochemical components due to the presence of various barriers. To push the translation of neural tissue engineering strategies into the clinic, we anticipate that a TENG with a close proximity to the regenerative microenvironment of the PNS will be developed.

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