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Neural Differentiation of Stem Cells in Biodegradable Three-Dimensional Scaffolds – A Novel Strategy for Nerve Regeneration

Haigang Gu, Zhilian Yue and Qi Zhang

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1. Introduction

The nervous system consists of the peripheral nervous system (PNS) and the central nervous system (CNS). Most functions of the nervous system are performed by neurons, such as movement and sense. But neurons lose the proliferation ability after maturation. Under pathological conditions, injured neurons will degenerate and die. Eventually, patients will lose some of the normal functions [1]. Although astrocytes are required for neurogenesis, synaptic maturation and neuronal activity maintenance, in current opinions, neurological diseases are caused by neurodegeneration or neuronal cell death. How to stimulate the neuroregeneration is still a key challenge in both fundamental and clinical research. Thus far, scientists have made a lot of efforts to develop drugs and devices to stimulate the functional recovery after nerve injury. There are no efficient methods available to stop or reverse neurodegeneration or neuronal cell death [2].

Current strategy for peripheral nerve injuries when the gap is less than 5 mm is to join the distal and proximal stumps of the damaged nerves by microsurgery. When the gap is longer than 5 mm, direct microsurgery will cause the tension of nerve fibers. A nerve graft needs to be used to fill the gap and make the connections between the distal and proximal stumps of the damaged nerves, in order to facilitate the regenerated nerve fibers to find their targets easily during the recovery [3, 4]. Autogenous nerve grafts require a second surgery to isolate the donor nerve tissue, which often leads to second deformities and the morbidities of donor tissues. On the other hand, the quantity for nerve autografting is quite limited. Although allografts and xenografts could serve as a possible alternatives to autografts, systemic immunorejection remains a major concern. Immunosuppression drugs were used to inhibit systemic

immuno-rejection, which will cause adverse side effects [5, 6]. Recent advances in stem cell biology and biomaterials make it possible to develop biodegradable nerve grafts for neural tissue engineering, nerve repair and regeneration. Here, we focus on stem cell-derived neural cells and biodegradable 3D neural scaffolds or conduits for nerve regeneration.

2. Support cells derived from stem cells for nerve repair

For peripheral nerve repair, axon outgrowth needs aligned Schwann cells (called astrocytes in central nervous system) to guide the orientation and to give the support. On the other hand, damaged neurons need to be replaced to perform appropriate functions. Recent progress in stem cell biology and techniques allows us to generate large quantities of functional neurons and transplantable astrocytes from stem cells [7, 8]. Stem cell-derived neural cells have been used for the studies of axon regeneration and for the treatment of neurological diseases, such as spinal cord injury (SCI), Parkinson's disease (PD), Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS), and showed promising functional recovery in the animal models of these neurological diseases [9-12]. Transplanted stem cell-derived neurons could integrate and form functional synaptic connections with host neurons [13]. Furthermore, transplanted stem cell-derived immature astroglial cells could become mature astrocytes by forming connections with blood vessels and transplanted induced oligodendrocyte progenitor cells (iOPCs) could form myelin sheath [14, 15].

2.1. Schwann cells

In the PNS, the majority of glial cells are Schwann cells including myelinating Schwann cells and non-myelinating Schwann cells, which play essential roles for supporting normal neuronal functions and the survival and axonal regeneration of neurons after nerve injury. The myelinating Schwann cells forming myelin sheaths around axons insulate individual axon. Similar functions are performed by in the CNS oligodendrocytes. The non-myelinating cells in the PNS show similar functions with astrocytes in the CNS, which mediate the development, mechanical and metabolic support functions and promoting neuronal survival after injury. Schwann cells are involved in maintaining normal functions of PNS, including secretion and nerve extracellular matrix, nerve development and maturation, and modulation of neuromuscular junction transmission [16]. Schwann cells also produce and secrete different neurotrophic factors, such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophic factor-3 (NT-3), glial derived neurotrophic factor (GDNF) and insulin-like growth factor (IGF) [17, 18]. All these neurotrophic factors are indispensable for neural development and regeneration. Furthermore, Schwann cells present antigens to T-lymphocytes and are involved in the clearing of myelin debris by phagocytosis.

In response to nerve injury, Schwann cells undergo proliferation and their basal lamina forming nerve conduit to support and guide axon regeneration and outgrowth. The DNA and RNA biosynthesis and up-regulation of Schwann cells could be observed as early as 2 h after injury. In 1980, Salzer and Bunge have found that direct mechanical injury is

mitogenic for Schwann cells during Wallerian degeneration and Schwann cells indeed proliferation *in situ* after excision [19]. However, the proliferative ability of Schwann cells is low *in vivo*. In most cases, endogenous Schwann cells proliferation is not enough to support and guide axon regeneration and outgrowth. Extra Schwann cells need to be transplanted after nerve damage. Morrissey et al. developed a culture method that could yield up to 98% pure Schwann cells from adult rat sciatic nerve [20]. Imaizumi et al. transplanted Schwann cells to the rat model of SCI and characterized the functional recovery by electrophysiological recording. They found that Schwann cells transplantation could form new pathway across the transaction site and provide a functional recovery of SCI [21]. Guenard et al. isolated and cultured Schwann cells from rat sciatic nerve and seeded cultured Schwann cells into semipermeable guidance channels. And then, they implanted Schwann cells-loaded nerve guidance channels into 8 mm rat sciatic nerve gap. Interestingly, they found that there was a positive correlation between the number of transplanted cells and the number of myelinated axons. Furthermore, they found that implanted Schwann cells loaded nerve guidance channels could improve the neural regenerative process [22]. Berrocal et al. implanted absorbable collagen conduits in combination with autologous SCs to a critical size defect (13 mm) in the sciatic nerve of male Fischer rats. Their results showed that absorbable collagen conduits loaded with Schwann cells significantly enhanced the regeneration of myelinated axons. The generated axons could grow into the nerve stump into the proximal and middle of the tube 4 weeks after implantation. The regeneration of myelinated axons occupied the entire length of the nerve guide 16 weeks after implantation. Functional recovery was observed in the animals who received implant treatment [23].

However, transplantation of Schwann cells is impractical for clinical application. Firstly, nerve tissue that could be used to isolate Schwann cells is limited in the patients. Secondly, Schwann cells need time to grow adequate amounts of cells for transplantation. It will take a couple of weeks, even longer. Thirdly, delayed Schwann cells transplantation often reduces the functional recovery after nerve injury compared with those who received acute application. Scientists began to seek other alternative sources for the treatment after nerve injury. Schwann cells can be generated from adult stem cells, such as mesenchymal stem cells (MSCs) and neural stem/progenitor cells (NSPCs), and pluripotent stem cells including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) *in vitro* and *in vivo*. Stem cells are good alternative source for Schwann cells. Furthermore, stem cells could locally differentiate into glial cells as well as neurons after transplantation.

2.2. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) can almost be found in any adult organ and can be easily harvested from patients. MSCs are capable of self-replication to many passages and can be expanded to enough cell numbers for tissue and organ regeneration. Although MSCs have been firstly harvested from the bone marrow, they actually have different properties from bone marrow stromal cells (BMSCs). BMSCs are a highly heterogeneous cell population, which includes multiple cell types with different potentials for proliferation and differentiation. On the contrary, bone marrow MSCs are a more homogenous subtype of mononu-

clear progenitor cells that have stem cell properties, such as self-renewal capacity and multipotency [24, 25]. Bone marrow MSCs undergo to differentiate into adipocytes, chondrocytes and osteocytes in culture. In addition, bone marrow MSCs express specific cell surface markers, such as positive for CD105, CD166, CD29 and CD44 and negative for CD14, CD34 and CD45. MSCs can also be derived from other non-marrow tissues, such as the liver and adipose, lung, peripheral blood, as well as amniotic fluid, umbilical cord blood and Wharton's jelly of the umbilical cord [26, 27]. MSCs are not only able to differentiate into mesodermal cell phenotypes but also into ectodermal lineage, Schwann cells, astroglial cells, oligodendrocytes and neurons, such as dopaminergic and purkinje neurons and have been used to treat cardiac and neurological disorders [28, 29]. Adipose derived stem cells (ADSCs) are a subtype of MSCs, which isolated from adipose tissue. Like bone marrow MSC, ADSCs are also self-renewal capacity and ability to differentiate into multiple lineages. Compared bone marrow MSC, people found that ADSCs are easier to harvest and culture for longer periods and grow faster [30]. MSCs could be induced to differentiate into neurons and Schwann cells in the regular culture vessels. Furthermore, MSCs have been induced to differentiate into neuronal-like cells expressing neuronal biomarkers, such as Tuj1 and neurofilament, and neural progenitor cells forming neurosphere-like structure in the three dimensional (3D) biodegradable scaffolds [24, 31-33]. All-trans retinoic acid is a most common drug used to initiate Schwann cell differentiation of MSCs. Forskolin, FGF2, Platelet-Derived Growth Factor (PDGF) and Neuregulin NRG1-1 are often used to force the final Schwann cell differentiation [34]. Interestingly, human MSCs can also be induced differentiated into Schwann cells. Human MSCs-derived Schwann cells have Schwann cell morphology and expression Schwann cell-specific proteins, such as p75 neurotrophin factor. Furthermore, Human MSCs-derived Schwann cells secrete several growth factors, such as hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) in vitro and in vivo. Transplantation of human MSCs-derived Schwann cells dramatically enhanced axonal outgrowth in an animal model of spinal cord injury [35].

Although a couple of reports showed that that MSCs can be used to generate neuronal cells, this phenomenon was recently called into question [36, 37]. Firstly, there is no evidence that neural tissues directly generated from MSCs. Secondly, the functional properties of MSCs-derived neurons have not been extensively studied, such as patch clamp recording for neuronal activities and high-performance liquid chromatography (HPLC) for neurotransmitter release. Thirdly, similar culture conditions used to induce MSCs to differentiate into neurons could also induced fibroblasts to neuronal-like cells. However, there is no doubt about clinical improvements demonstrated in animal models and patients after treatment with MSCs. People believe that these clinical improvements are from growth factors and cytokines released from MSCs. MSCs-derived growth factors and cytokines could promote neurogenesis and angiogenesis of damaged brain tissue and inhibit the process of apoptosis. Transplantation of MSCs has shown a significant functional recovery of the animal model of stroke. Nevertheless, MSCs are a good source for cell therapy.

2.3. Neural stem/progenitor cells

Previous studies showed that adult neural stem/progenitor cells (NSPCs) not only locate in neurogenic regions, the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus, but also locate in some non-neurogenic regions, such as cerebral cortex, cerebellum and spinal cord [7]. Multipotent CNS stem-like cells were first cultured from the adult striatum by neurosphere assay. The neurosphere culture system has been widely used to isolate and expand NSPCs under serum-free media conditions as well as with the presence of epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF2). NSPCs are self-renewing, multipotent progenitors in the nervous system that could be induced to differentiate into the three phenotypes in the nervous system under appropriate condition, such as neurons, astrocytes and oligodendrocytes. Functional properties of NSPCs-derived neurons have been characterized by immunostaining and clamp patch recording. NSPCs-derived neurons have all the properties of native neurons. NSPCs have been widely used for the studies of neural development and regeneration. After transplantation, NSPCs-derived neurons could replace the dead neurons in the animal's model of neurological disorders, such as AD, PD and SCI and have shown the functional recovery in these animals' models [38, 39]. In our previous studies, we isolated and cultured NSPCs from mouse and rat brains under DMEM/F-12 medium supplement with B27 (2%) or N2 (1%), EGF (10 ng/ml) and FGF2 (10 ng/ml) by neurospheres assay. After 2-3 passages, most of neurospheres are positive to Nestin, a neural stem cell marker. We found that BrdU could be detected 16-18 h after NSPCs were cultured in the presence of BrdU. 5-bromo-2'-deoxyuridine (BrdU) is a synthetic nucleoside that is an analog of thymidine and is widely used in the detection of proliferating cells in vitro and in vivo. When NSPCs were cultured in the serum containing medium, they are easily induced to differentiate into neurons and astrocytes. These results demonstrate that NSPCs from mouse and rat brain have a high proliferative ability and multipotency. After transplantation to AD rats with fimbria-fornix transection, NSPCs could migrate into adjacent brain tissue and locally differentiate into neurons and astrocytes. Y-maze testing showed that transplanted NSPCs could improve the learning and memory in the rat model of AD [13, 40-45]. NSPCs have been cultured in a 3D bioactive scaffold derived from porcine urinary bladder matrix (UBM) for the treatment of traumatic brain injury (TBI). UBM was able to support extended proliferation and differentiation of NSPCs. After transplantation into rat TBI model, the transplants could reduce neuronal loss and white matter injury, and also significantly ameliorate motor, cognitive and memory impairments [46]. Furthermore, NSPCs-loaded PLGA scaffolds have been used for the treatment of animal model of SCI. NSPCs could differentiate into neurons and glial cells in the PLGA scaffolds after transplantation and make functional synaptic connections with proximal and distal nerve stumps. Retrograde tracking studies showed that the tracer could pass through the nerve gap and be found in the brain [47].

In the developing CNS, the initial symmetric cell division of NSPCs occurs to produce more identical stem cells and form neural tube. As development progresses, symmetric cell division is gradually replaced by asymmetric cell division which produce one stem cell and one neural precursor cell. Previous studies have identified neuronal restricted precursors (NRPs) and glial restricted precursors (GRPs) in the brain and spinal cord. These cells are more limited in their

differentiation potential than NSPCs. NRPs have the tendency to differentiate into neurons and GRPs have the tendency to differentiate into glial cells. After transplantation in the model of SCI, transplanted GPCs demonstrated to differentiate into oligodendrocytes and form myelin around axons and increase the locomotor recovery [48]. Furthermore, NSPCs have been used along with Schwann cells to improve axonal regeneration. Olson et al. transplanted NSPCs and Schwann cell-loaded PLGA polymer scaffold into transected spinal cord. They found that NSPCs could differentiate into neuronal cells in the scaffold channels and NSPCs and Schwann cell-loaded PLGA polymer scaffold could facilitate axonal regeneration across the transected spinal cord [49].

The major drawback of NSPCs is that NSPCs locate in the deep of brain and spinal cord. It is almost impossible to harvest autologous NSPCs for nerve repair in clinic. Although NSPCs could be obtained from aborted embryos, ethical issue plagues their clinical application.

2.4. Induced neural cells

In 2012, John B. Gurdon and Shinya Yamanaka shared the Nobel Prize in Physiology or Medicine for their discovery that mature cells can be reprogrammed to become pluripotent. In 1962, Gurdon showed that adult frogs could be generated from the nuclei of single somatic cells by nuclear transfer. In 2006, Yamanaka's group showed that somatic fibroblasts could be induced to ESC-like cells, called iPSCs, by four transcription factors including Oct4, Sox2, Klf4 and c-Myc. These iPSCs have the ability to generate all three lineages, endodermal, mesodermal and ectodermal cells [50]. Later, several groups using similar strategy successfully generated iPSCs from patients. Patient-derived iPSCs have been used to study the pathological mechanisms and drug testing [51, 52]. The principle of differentiated cells regaining pluripotency and conversion of one cell type into another not only let us re-think about the fundamental principles of development but also allow us re-consider autologous cell replacement therapy. Induced PSCs have been used for peripheral nerve repair. Uemura et al. transplanted iPSCs-derived neurospheres-seeded sponge polymer composed of 50% PLA and 50% PCL to 5 mm sciatic nerve gap. The recovery of motor and sensory function can be observed as early 4 weeks. Twelve weeks after transplantation, histological evaluation showed that iPSCs differentiated into GFAP-and S100-positive Schwann cells and Tuj1-and neurofilament-neuronal cells [53]. Wang et al. transplanted iPSCs-derived neural crest stem cells (NCSCs)-loaded electrospinning nanofibrous nerve conduits composed of 70% PLA and 30% PCL to 6 mm sciatic nerve gap. Transplanted NCSCs were able to promote regeneration of peripheral nerves. But they did not observed neuronal differentiation of NCSCs [54].

In 2010, Dr. Wernig's group used a cocktail of transcription factors, Ascl1, Brn2 and Myt1l, successfully convert fibroblasts into functional neurons, named induced neurons [55]. Interestingly, induced neurons have been generated from fibroblasts of patients [56]. Furthermore, induced dopaminergic neurons could integrate into host brain after transplantation. Recently, several group used similar techniques to successfully generate induced NSPCs [57, 58]. Induced NSPCs have similar properties with NSPCs isolated from brain tissue. More interestingly, two groups generated iOPCs from somatic fibroblasts. After transplantation,

iOPCs could differentiate into oligodendrocytes and form myelin sheath. So far, there are no reports showed that iOPCs have been used to treat peripheral nerve injury [14, 15].

3. Biodegradable polymers as nerve guidance conduits for nerve repair and regeneration

The current gold standard for peripheral nerve repair is nerve autografting. However, this approach is associated with a number of clinical complications, in particular, donor site morbidity, limited availability, and nerve site mismatch and neuroma formation at the donor site [59]. Artificial nerve guidance conduits have been developed for bridging the gap. This strategy has been widely accepted for basic research and clinical applications. In general, a nerve guidance conduit for reconnecting the two nerve stumps (i.e., the proximal and distal nerve stumps) contains an appropriate substrate with longitudinal orientation guidance to direct axons to find their targets. To date, the majority of nerve guidance conduits developed is composed of biodegradable polymers. A few of them are FDA-approved and commercially available. These nerve conduits have achieved considerable success in treatment of gap defects with the distances up to 20-25 mm. To further improve on the regeneration capacity of nerve conduits, a number of strategies have been actively pursued, in particular, the combination of nerve conduits with stem cell technologies [60]. In this review, we discuss the key design parameters of nerve conduits, including materials, fabrications methods, and incorporation of bioactive molecules, which play critical roles in governing the cellular fate of the stem cells cultivated in the nerve guidance conduits. Through analyzing and summarizing these experimental results, our goal is to provide insights into the future design of nerve conduits with much improved therapeutic efficacy.

3.1. Materials consideration for use in nerve guidance conduits

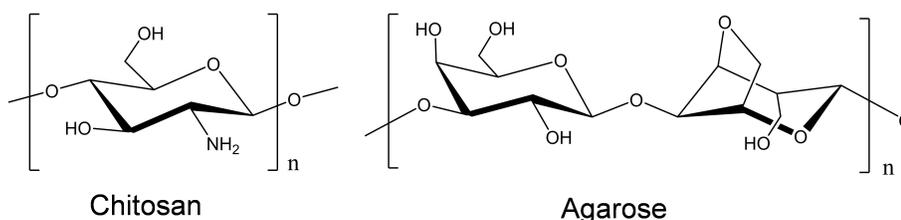
Materials selection is critical to the performance of fabricated nerve guidance conduits. Ideally, materials to be employed for nerve repair need to fulfill the following requirements.

1. They must be biocompatibility for supporting cell growth, differentiation and function.
2. They must be able to provide appropriate surface and mechanical properties that mimic nerve tissue.
3. They must be immunologically inert.
4. They must be biodegradable or bio-absorbable.
5. They must be able to be sterilized.
6. They must be readily fabricated into the desired configurations of conduits.
7. Their production must be amenable to industrial scale-up.

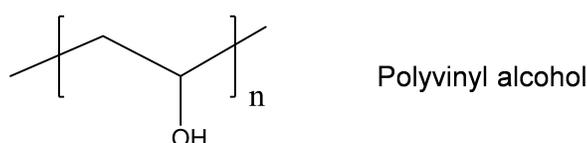
3.2. Materials used in nerve guidance conduits

In the past decades, a broad range of materials have been explored for preparation of nerve guidance conduits for peripheral nerve repair. They can be broadly divided into two categories: naturally-derived biopolymers and synthetic polymers. As can be seen from the following discussion, these materials can address the aforementioned materials considerations to various degrees.

- Naturally-derived polymers



- Non-degradable synthetic polymers



- Biodegradable/bioresorbable synthetic polymers

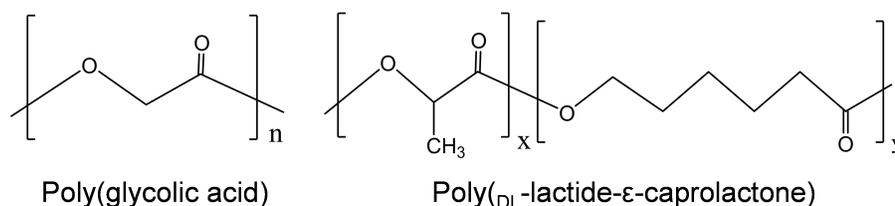


Table 1. Examples of naturally-derived and synthetic polymers used in nerve guidance conduits.

Naturally-derived biopolymers. Examples of the naturally-derived biopolymers for nerve guidance conduits include type I collagen, fibronectin, fibrin glue, gelatin, hyaluronic acid, alginate, chitosan, agarose and silk fibroin *etc.* These materials are typically hydrophilic, and form hydrogel-based matrices by either physical or chemical crosslinking. It is also worth noting two types of multicomponent matrices, such as Matrigel™ and decellularized nerve allografts or xenografts. Matrigel™ is a commercial extracellular matrix (ECM) extract from tissue cultured mouse sarcoma cell lines. It gels *in situ* at room temperature. Matrigel™ contains laminin, heparin sulphate, type IV collagen, entactin, nidogen and growth factors. Decellularized nerve allografts or xenografts are matrices that preserve the inherent structural characteristics of nerve. For instance, Avance® Nerve Graft is a commercially available

decellularized allograft processed from human peripheral nerve tissue, which has been used clinically for reconstruction of periphery nerve gaps with positive results [61].

Naturally-derived biopolymers have demonstrated a number of advantages in the applications of nerve repair. They are in general biocompatible, and biodegradable or bioresorbable. They form hydrogels with structural and mechanical properties similar to nerve tissue. In addition, some of the biopolymers contain cell adhesion moieties, such as type I collagen and fibronectin, which encourages neuronal attachment and outgrowth. However, immunogenicity as a result of animal resources remains a major concern over their practical applications. In the case of Matrigel™, its origin in mouse sarcoma cells plagues its use in clinical applications. Naturally-derived polymers are also known for batch-to-batch variations, and lack of flexibility in terms of structural engineering to modulate the physiochemical properties and degradation kinetics of materials.

Synthetic polymers. Compared to naturally-derived biopolymers, synthetic polymers offer greater flexibility in modulating the physical properties of materials through engineering the polymer composition such as (co)monomer structure and side chain chemistry, and polymer molecular weight. They are also much more amenable to various technologies for materials fabrication. On the other hand, it is noted that the majority of synthetic polymers used in nerve repair lack biocompatibility and bioactivity, which limits cell attachment, growth and differentiation. Viable approaches to overcome the limit involve compositing synthetic polymers with bioactive molecules, and surface functionalization of nerve conduits with bioactive molecules. These approaches will be discussed in detail in Section 3.3.

At the early stage of nerve guide development, several non-biodegradable polymers have been explored to produce nerve guides, including, for example, silicone rubber, polyvinyl alcohol (PVA), polyethylene glycol (PEG), polyN-2-hydroxypropyl-methacrylamide (pHPMA) and poly2-hydroxyethyl methacrylate (pHEMA). A major disadvantage of non-biodegradable nerve conduits is that a second surgery is required to remove the conduits as their chronic presence impede nerves remodeling. In addition, studies have shown that the chronic presence of non-biodegradable conduits led to the inflammatory reactions and scar tissue formation, which ultimately inhibit functional nerve recovery [62, 63].

Amongst the synthetic polymers explored for nerve guidance conduits, a range of biodegradable aliphatic polyesters have attracted most attention. These include, for example, poly(glycolic acid) (PGA), polylactide (PLA), poly(lactic-co-glycolic acid) (PLGA), poly- ϵ -caprolactone (PCL) and poly-3-hydroxybutyrate (PHB) *etc.* These material typically produce relatively rigid scaffolds with hydrophobic surface. Their degradation is predominantly mediated by hydrolysis of their ester linkages in physiological conditions. The degradation rate is dependent on the polymer structure, molecular weight, or its crystallinity. For instance, among PGA, PLA and PCL of similar molecular weights, the degradation rate is $PGA > PLA > PCL$, as a result of increased hydrophobicity [64]. This provides a basis for tailoring the polymer degradation kinetics by varying the structure and ratio of the monomers used for polymerization. A good example is PLGA, whose degradation rate depends on the ratio of lactide to glycolide used for the polymerization, i.e., higher content of glycolide units leads to faster degradation rate, with an exception of 50:50 monomers' ratio that gives rise to the fastest

degradation rate [65]. Synthetic biodegradable polymers have been widely used to fabricate different kinds of films or scaffolds to support cell growth *in vitro* and *in vivo*.

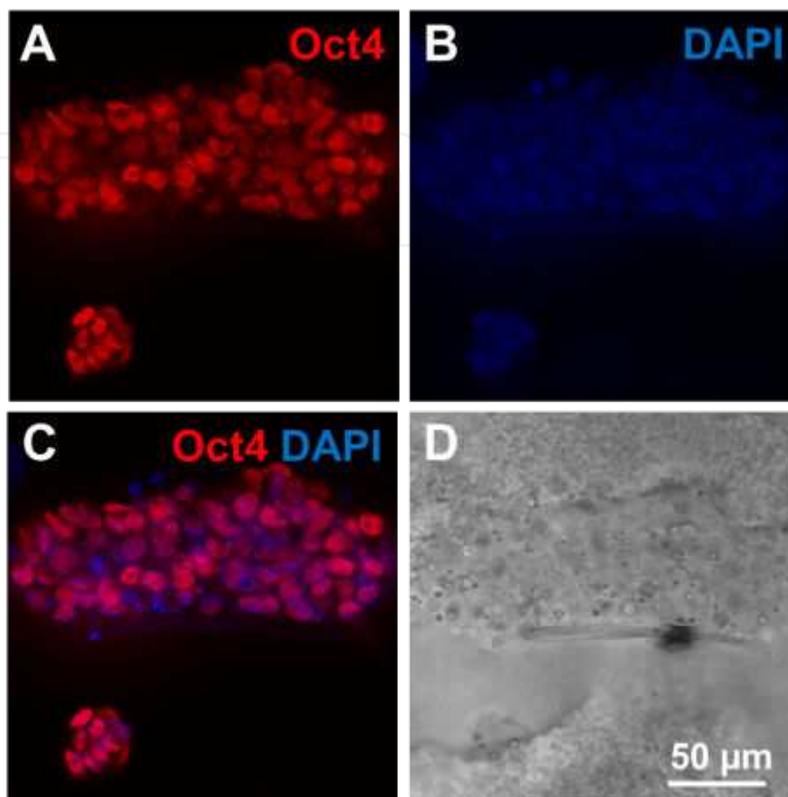


Figure 1. Immunocytochemistry assay showed proliferation of embryonic stem cells in 3D cellulosic hydrogel scaffolds after 2 days plating. A. Oct4 showed the pluripotency of embryonic stem cells. B. The total cells were showed by DAPI. C. A merges with B. D. DIC imaging showed proliferation of embryonic stem cells in 3D hydrogel scaffolds.

Despite the substantial research activities in engineering of polymer biodegradability, development of neural matrices with desired degradation kinetics in the course of nerve regeneration still remains a key challenge. This is due to the inherent complexity of *in vivo*-degradation, which necessitates multidisciplinary efforts to bring together materials scientists, biologists and clinicians to tackle this challenge. Ideally, a nerve conduit should provide adequate mechanical support and protection to facilitate axonal regeneration across the nerve gap, while undergoing degradation with the kinetics matching the rate of nerve regeneration, in order to make way for the regenerating nerve. Development of appropriate *in vitro*-models that can simulate *in vivo* degradation of nerve conduits may help to speed up the problem-solving process and facilitate the delivery of nerve conduits with desired *in vivo*-degradation profiles to meet specific needs in clinical applications.

3.3. Key considerations in nerve conduit design

In its simplest form, a nerve conduit takes the form of a hollow tube for bridging nerve gap defects. A nerve conduit implant needs to satisfy a set of basic requirements in both material

and technical aspects, including biocompatibility, non-immunogenicity, biodegradability/bioabsorbability, mechanical integrity with nerve tissue, and ease of sterilization and fabrication into the desired dimensions. These considerations underpin the development of a variety of single lumen hollow conduits that have shown positive effects in treatment of short defects (<20-25 mm). These conduits differed mostly in the structure and composition of the polymers used for conduit fabrication. Some of them have already gained approval in clinical applications, which will be discussed in detail in Section 3.4.

To improve the therapeutic efficacy in treatment of large nerve defects, a number of biomimetic strategies have recently been adopted to modify the existing design of nerve conduits, by providing bio-regulative cues to better mimic nerve tissue at various levels (anatomic, physical or structural). The ongoing research activities in this area have been catalyzed by our increasing understanding of *in vivo*-behavior of nerve conduits, as well as the advances in material fabrication and in tissue engineering. Some of the key strategies will be briefly reviewed here.

Topographic guidance. Longitudinally-oriented topographic cues have been introduced to nerve conduit design, with a view to promoting the growth and orientation of regenerating axons. This can be microfibers, multichannels, or 3D matrix fillers with longitudinally-oriented architectures, as is illustrated in Figure 2. For instance, Kim *et al* reported on polysulfone conduits containing uniaxially electrospun nanofibers of poly(acrylonitrile-co-methylacrylate) [66]. Incorporation of such aligned sub-micron topographic cues was shown to significantly promote both sensory and motor nerve regeneration across a 17 mm peripheral nerve gap in a rodent model, without the delivery of any exogenous neuro-simulative agents (e.g., neurotrophic factors and extracellular matrix proteins). Nerve conduits with aligned multichannels can be prepared using an injection-molding technique, the same technique as used for fabrication of single lumen nerve conduits.

ECM proteins, such as laminin, fibronectin, and collagen, are good candidates of intraluminal fillers for nerve conduits as they promote axonal extension. Technologies are required in order to produce aligned intraluminal structures of these proteins. An early study by Dubey *et al* employed magnetic fields to align the collagen gels in Teflon tubes, which gave rise to enhanced neurite elongation from dorsal root ganglia explants [67]. Matsumoto *et al* developed nerve conduits of PGA that were further coated with collagen and internally filled with longitudinally-oriented, laminin-coated collagen microfibers [68]. Axonal regeneration over an 80 mm gap of canine peroneal nerves was demonstrated. Alternatively, using a special freezing process, 3D matrixes collagen with longitudinally oriented pores can be prepared, which may find applications as aligned intraluminal fillers [69].

Mechanical compliance. Mechanical compliance of nerve conduits is an important consideration in conduit design. It plays a key role in directing cellular and tissue response to implanted nerve conduits, which could affect the performance of the nerve conduits. In addition, studies have shown a critical role of material mechanical properties in directing stem cell differentiation [70, 71]. Nerve tissues are soft and highly hydrated, while nerve conduits prepared from synthetic polymers, such as polyesters, are often rigid and hydrophobic. Strategies have been sought to develop hydride conduits that integrate synthetic polymer with naturally-derived biopolymers into various configurations to unify the advantages of both types of materials.

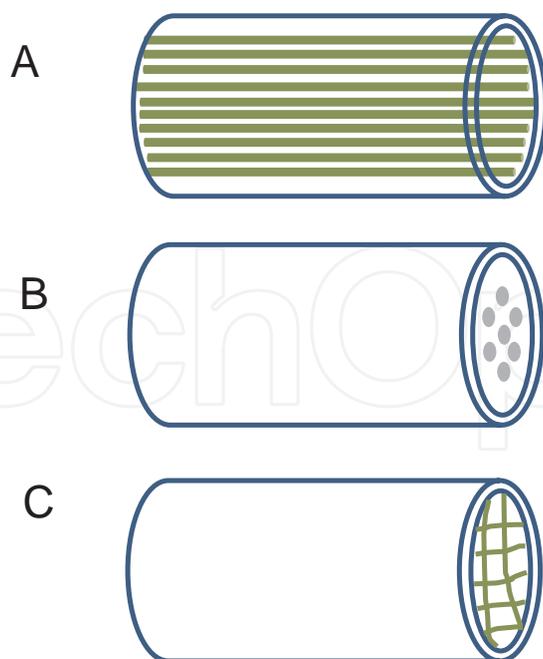


Figure 2. Schematic illustration of the nerve conduits modified with longitudinally oriented topographic cues. (S) nerve conduit with aligned nanofibres or microfibers; (B) nerve conduit containing multichannels; (C) nerve conduit containing 3D scaffolds with longitudinally oriented pores.

Examples of hydride conduits are those made of composites of naturally-derived biopolymers and synthetic polymers, or those incorporated with a soft hydrogels of ECM proteins to provide the matrix for axonal growth and regeneration [72, 73]. As previously discussed, those ECM hydrogel filaments need to be aligned to enable optimal nerve regeneration.

Surface bioengineering. This strategy involves coating or surface modification of nerve conduits with neurostimulatory molecules, in particular cell adhesion molecules, to promote cell adhesion, proliferation and differentiation, thereby improving the regeneration capacity of nerve conduits. The cell adhesion motifs that have been explored for nerve conduits include collagen, laminin, lamnin fragment peptides, fibronectin and Arg-Gly-Asp (RGD). For instance, laminin has often been used for coating of nerve conduits, and has been shown to enhance Schwann proliferation and migration, and neurite outgrowth. RGD-modified PCL nanofibers using a polyether diisocyanate was shown to promote faster Schwann cell migration and axonal growth [74].

Growth factor delivery. As discussed previously, neurotrophic factors play a critical role in promoting neuronal survival and differentiation. The capability to *in situ* deliver neurotrophic factors is now becoming an essential feature of next generation of nerve conduits. A popular approach is embedding growth factors in a hydrogel matrix that serves as intraluminal filler. Release of the entrapped growth factors can be diffusion-controlled and/or degradation-controlled, which is subject to the nature of the matrix-growth factor interactions. Increasing the crosslinking density of the hydrogel matrix can produce stiffer matrix, which may lead to more retarded release of the entrapped growth factor. Howev-

er, this approach may have limitations as the level of stiffness of a hydrogel matrix should not pose any hindrances to the axonal growth across the lumen. Inclusion of heparin as a component of hydrogel matrix has shown a viable approach to modulate heparin-binding growth factors delivery. For instance, a heparin-containing hydrogel matrix was reported, comprising of fibrin with a high excess of immobilized heparin-binding peptides, heparin and neurotrophins, such as beta-nerve growth factor (β -NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) [75]. The heparin bound to both the immobilized peptides, and neurotrophins, which was responsible for slow diffusion release of β -NGF, BDNF or NT-3. Enhanced neurite extension was demonstrated in these heparin-containing matrices, but not in the matrices containing only fibrin and neurotrophins. A recent study also showed that the heparin-containing fibrin matrices with NGF resulted in a high level of sciatic nerve regeneration as compared to the control groups [76].

Alternatively, growth factors can be introduced into nerve conduits either as a component of coating or being embedded directly in the wall. For example, NGF-containing microspheres of PLGA were formulated with an aqueous solution of poly(2-hydroxyethyl methacrylate), and coated on the inside of pre-formed nerve conduits prepared from poly(2-hydroxyethyl methacrylate-co-methyl methacrylate) [77]. The microsphere-coated nerve conduits showed more sustained release of NGF for > 28 days, compared to those coated with poly(2-hydroxyethyl methacrylate) and NGF, though no *in vivo* studies were reported. Nerve conduits with the capacity of co-delivery of synergistically acting glial cell-line derived neurotrophic factors (GDNF) and NGF were reported by Madduri et al [78-80]. GDNF and NGF were loaded into the nerve conduits of collagen, and dried and coated with PLGA in ethyl acetate. *In vitro* studies showed that the combination of GDNF and NGF exerted a synergistic effect on the axonal elongation, axonal branching and growth kinetics. Compared to the conduits releasing GDNF alone, enhanced early nerve regeneration in a 10 mm rat sciatic nerve gap model was also demonstrated for the conduits with co-delivery of GDNF and NGF.

3.4. Commercially available nerve guidance conduits

Table 2 summarizes the nerve guidance conduits that have been approved by the US Food and Drug Administration (FDA), and/or the European Union with a Conformité Européenne certification (CE) for clinical applications. They are all in the configuration of single-lumen tube. NeuraGen™, NeuroMatrix™ and NeuroFlex™ are derived from type I collagen, which is a major component of ECM. AxoGuard™ is made of ECM materials derived from porcine small intestine. It contains almost intact ECM, including cell adhesion proteins, growth factors, glycosaminoglycans and proteoglycans *etc.* SaluBridge™ and SaluTunnel™ are non-resorbable conduits, and are prepared from PVA hydrogel. Neurotube® is a PGA-based, woven tubular device, with high porosity to provide an oxygen-rich environment for the regenerating nerve [60]. Neurolac® is the only FDA approved transparent conduit based on synthetic biodegradable polyesters. It is noted that the fabrication of these nerve guides do not involve any biofunctionalization with or incorporation of bioactive molecules. These nerve conduits thus meet only the basic requirement of conduits, by providing physical guidance cues via conduit morphology to direct axonal regeneration.

Product name	Materials	Degradation	Some key issues
SaluBridge™, SaluTunnel™ (from SaluMedica)	PVA	Non-resorbable	Non-resorbability
Neuroflex™, NeuroMatrix™ (from Collagen Matrix Inc.)	Type I bovine collagen	4-8 months	Risk of adverse immune response
NeuraGen® (from Integra life Science)	Type I bovine collagen	36-48 months	Risk of adverse immune response
AxoGuard™ (from Cook Biotech)	Porcine small intestinal submucosa (SIS)	3 months	Risk of adverse immune response; Risk of infectious disease transmission
Neurotube® (from Synovis® Micro)	PGA	3 months	Rapid loss of mechanical properties; acid degradation products
Neurolac® (Polyganics B.V.)	Poly(DL-lactide-ε-caprolactone)	16 months	Rigidity and inflexibility; foreign body reactions; polymer fragments

Table 2. Clinically-approved nerve guidance conduits (adapt with permission from [60, 81-83]).

3.5. Next generation of nerve guidance conduits in tandem with stem cell delivery

Stem cells have been used with nerve conduits or 3D scaffolds to obtain the maximum efficient therapeutic effects of nerve repair. Stem cells-loaded nerve conduits and 3D scaffolds have much higher potential for nerve repair compared with nerve conduits or 3D scaffolds alone. For example, Park et al. cultured NSCs in PGA scaffolds for 4 days before transplantation. And then NSC-PGA complexes were transplanted into the infarction cavity of the brains in the mouse model of hypoxia ischemic injury by glass micropipettes. The results showed that PGA provides a good support the survival and neuronal differentiation of transplanted NSCs. Transplanted NSCs differentiates into neurons in the infarct area. Antegrade and retrograde tract tracing showed that transplanted projects the axons to internal and external capsule and the contralateral hemisphere through corpus callosum. Animal behavioral function has not been tested in this report [84]. Liu et al. transplanted ADSCs-loaded biodegradable GGT nerve conduits containing genipin crosslinked gelatin annexed with tricalcium phosphate (TCP) ceramic particles into 10 mm gap in the sciatic nerve after injury. They found that ADSCs could differentiate into neuron-like cells in the GGT nerve conduits and ADSCs-loaded biodegradable GGT nerve conduits significantly increased sciatic function index and functional recovery [85]. Furthermore, BDNF or GDNF-transfected NSCs-seeded PLA microporous nerve conduits were transplanted into sciatic nerve gap after injury. The conduits seeded with GDNF-and BDNF-transfected NSCs significantly increased the degree of myelination and the size of regenerated tissue compared with those seeded with the nontransfected NSCs. The greatest number of blood vessels was found in the animals transplanted with GDNF-transfected NSCs-

seeded PLA microporous nerve conduits. The functional recovery was significantly improved for BDNF or GDNF-transfected NSCs-seeded conduits assessed by the functional gait and electrophysiology [86].

PCL conduits filled with bone marrow-derived MSCs were tested for repair of transected sciatic nerves in mice [87]. Use of the MSCs grafted conduits was shown to significantly improve the survival of sensory neurons and motor function, and restore gastrocnemius muscle function in mice. In a separate study, MSCs were loaded to silk fibroin-based conduits that were filled with oriented silk fibroin filaments, which were tested for bridging a 10 mm-long gap in rat sciatic nerve [88]. At the early weeks after nerve grafting, the grafted MSCs were shown to enhance the gene expression of several growth factors, such as BDNF, bFGF, and ciliary neurotrophic factor, and S100, a marker of Schwann cells. These were arguably responsible for accelerated axonal elongation at 4 weeks, and an improved outcome in sciatic nerve regeneration and functional recovery at weeks 12 in the groups treated with MSC-grafted conduits, when compared to those treated with acellular conduits. The nerve regeneration efficacy of the MSC-grafted conduits was shown to approach that of autologous nerve grafts.

4. Nerve conduits in clinical applications

In the past decades, autogenous and polymer-based nerve conduits have been used for nerve repair in clinic. Both of them have showed positive clinical outcomes in the patients.

4.1. Biological autogenous nerve conduits in clinical applications

Previous clinical studies show that autogenous vein conduits are able to repair nerve injury in patients. A retrospective clinical study evaluated 22 digital nerve repairs in the finger using autogenous vein conduits, and reported that two-point discrimination for 11 acute digital nerve repairs with vein grafts and poor results for delayed digital nerve repair [89]. In 1990, Chiu and Strauch compared autogenous vein grafts with conventional nerve graft in 22 patients. They demonstrated that autogenous vein grafts were as efficient as conventional nerve grafts to bridge a small nerve gap (≤ 3 cm). But this study did show how long these patients were operated after nerve injury [90]. Similar clinical study was performed by Laveaux et al., and reported that vein grafts is less efficient than nerve grafts in delayed nerve repair and vein grafts produce similar good results in emergency cases [91]. A long-term sensory evaluation of nerve repair was performed by Lee and Shieh, and reported that vein conduit grafts could produce excellent sensory recovery [92]. In 2001, Pogrel and Maghen used autogenous vein grafts to repair continuity defects, ranged from 2 to 14 mm, of the inferior alveolar nerves (n=6) and lingual nerves (n=10). All the patients received grafts between 4 and 10 months after injury. They found that vein grafts can form a physiological conduit for nerve regeneration and are more successful with short gaps [93]. More recent interesting study implanted male vein grafts to femoral nerve injury of female rats and found that male vein cells could integrate into female injured nerve and participate in remyelination and nerve

regeneration [94]. In 2012, Liard et al. evaluated that adult neural stem cells-loaded autogenous vein grafts to reconstruct nerve gaps in pig model and demonstrated that neural stem cells transplantation increased 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) expression and promoted functional recovery of stimulo-detection [95].

Autogenous muscle grafts are another option to provide a scaffold for the nerve fiber to grow. In 1988, Norris et al. used frozen and thawed to denature skeletal muscle and transplanted skeletal muscle to injured digital nerves in 8 patients. 7 out of 8 showed an excellent level of recovery, MRC sensory clinical score S3+[96]. In 2008, Pereira et al. treated 38 patients with leprosy by skeletal muscle autografts ranging between 2.5 cm and 14 cm length. The clinical results showed that sensory recovery was noted in 89% patients and 80 % of ulcers caused by posterior tibial nerve damage were healed [97]. Furthermore, to increase clinical effects, the vein conduits filled with muscle are also used to bridge peripheral nerve gaps. The basic idea is that vein could provide regeneration guidance and muscle serves supporter to avoid vein collapse. In 1993, Brunelli et al. reported that vein plus muscle grafts could have similar functional recovery to those found in traditional nerve grafts. More interestingly, axon number in vein plus muscle grafts group is significantly higher than that of traditional nerve grafts group [98]. In 2000, similar work was done by Battiston et al. 21 patients suffered nerve defects of 5-60 mm were treated with vein filled with skeletal muscle. 85% of patients showed good clinical results [99].

Although vein and muscle grafts are more available than nerve grafts, isolating vein and muscle grafts also need second operation. To overcome this critical clinical problem, scientists have developed different synthetic polymer-based nerve conduits for nerve repair.

4.2. Synthetic polymer-based nerve conduits in clinical applications

In 1998, Sanda Stanec and Zdenko Stanec used non-absorbable polytetrafluoroethylene (ePTFE) tube to reconstruct nerve defects between 1.5 to 6 cm length, and demonstrated that 78.6% patients suffered 1.5 to 4 cm length nerve defects had functional motor and sensory recovery, but only 13.3% patients suffered 4.1 to 6 cm length defects had similar recovery [100]. Due to non-absorbable nerve conduits need secondary surgery to remove them, most of synthetic nerve conduits are made of biodegradable materials. In 2009, Rosson et al. evaluated 6 patients with short-gap motor nerve injuries treated with bioabsorbable conduit, the Neurotube™, and observed that all patients had some return of motor function. It demonstrated that motor nerves with short-gap injuries could regenerate cross this conduit [101]. In 2011, Rinker and Liao compared the clinical output of woven polyglycolic acid and autogenous vein conduits for reconstruction of digital nerve gaps and reported that sensory recovery after digital nerve reconstruction with autogenous vein conduit was similar to that using polyglycolic acid conduit and similar cost profile and less postoperative complications were observed in both of them [102]. Taras et al. reconstructed 22 isolated digital nerve lacerations in 19 patients with a bioabsorbable collagen conduit, and showed that 13 out of 22 achieved excellent results, 3 of 22 obtained good results, and there were no poor results [103]. A retrospective study of 10 cases was performed by Thomsen et al. in 2010. All patients were operated on for painful

neuroma and underwent repair with collagen conduits (Revolvner[®], Orthomed). Fifty percent patients had excellent or good results at static two-point discrimination testing [104].

Although nerve conduits are commercially available, their clinical application is far satisfied. Limitations of nerve conduits in peripheral nerve repairs were reported by Moore et al, in 2009. In this 4 cases report, 3 patients were treated with type I collagen nerve conduit (NeuraGen, Integra NeuroSciences) and 1 patient was treated with polyglycolic acid nerve conduit (GEM Neurotube, Synovis, Birmingham, AL, USA). There were no clinical effects in these patients. Side effects were reported by some patients [105]. In 2010, Wangenstein and Kalliainen reviewed 96 patients' clinical data, who received type I collagen nerve conduit (NeuraGen, Integra NeuroSciences) for nerve repair. Only 35-45% patients had sensory recovery [106].

5. Perspectives

Recent progress of biodegradable materials and stem cells provides more options for nerve regeneration. Neural tissue engineering is a new thing but has been widely used for nerve regeneration in basic research and clinical application. Although the research of peripheral nerve repair has started many years ago, functional recovery is still unsatisfied. The functional recovery largely depends on nerve gap, the location of injured nerve, patients' age and methods of treatment chosen. From the literatures, there are limited choices for nerve regeneration: (1) For tiny nerve gap, microsurgery joining the distal and proximal stumps of the damaged nerves should be first choice; (2) For small nerve gap ($\leq 2-3$ cm), autogenous nerve or vein grafts and acellular nerve conduits can be used for nerve repair; (3) For larger nerve gap (≥ 3 cm), just nerve conduits are not enough to support nerve regeneration. The studies of animal trials showed that combination of nerve conduits with supporting cells could be best choice to obtain maximum extent functional recovery. However, most popular animal model for studying peripheral nerve regeneration is rat sciatic nerve injury model. Rat is a small animal compared with human. It is impossible to expect that the similar functional recovery would be obtained in the peripheral nerve injury patients with similar treatment done in rat animal model. More works need to be done with large animals, such as monkey, to optimize the approaches. Furthermore, personal medicine for cell therapy needs patients-derived cells. Experience with induced pluripotent stem cells, induced neural stem cells, and induced neurons make it possible to generate large quantity of patients-derived cells for clinical application. Practically, multiple-disciplinary approaches should be combined together to generate optimal clinical recovery for patients with peripheral nerve injury.

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Author details

Haigang Gu¹, Zhilian Yue² and Qi Zhang¹

1 Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, USA

2 Intelligent Polymer Research Institute, AIIM Facility, Innovation Campus, University of Wollongong, Australia

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