Combinatorial Trends of Tissue Engineering for Peripheral Nerve Regeneration

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Abstract*—Peripheral nerve damage is frequently seen due to injury or illnesses, like diabetes. Despite its prevalence and the fact that many patients with less serious injuries have good clinical outcomes, many patients do not fully recover sensation and in many cases, use of the affected area. For this reason, there has been extensive research into improving or replacing the current treatment options. Many tissue engineering solutions focus on peripheral nerve injury or damage caused specifically by external trauma. The aim of this paper is to list and summarize the primary areas of research for tissue engineering approaches to peripheral nerve regeneration. Moreover, the focus is on the increasing awareness that no single tissue engineering technique is currently capable of providing optimal healing and regeneration for peripheral nerve damage and may never be fully capable of providing complete regeneration. Instead, clinical outcomes may be improved by combining these techniques in multifaceted approaches some of which include combining growth factors and nerve guidance conduits.

***Keywords*—peripheral nerve, regeneration, tissue engineering**

I. Introduction

Every year, 1.5-4% of patients with trauma globally experience peripheral nerve injuries (PNI) as part of their injuries [1]. This correlates to over 5 million patients with PNI globally and a corresponding $1.5 billion market for surgical repair of peripheral nerve injuries in the USA alone [2]. For patients who have PNI, the prognosis is rarely encouraging. Of patients with PNI, 2-5% of patients develop complex regional pain syndrome [3]. While 25% of patients have notable functional recovery [4], the rate of functional recovery is only 40-50% as of 2019 [5].

The peripheral nervous system can be damaged in a number of ways with a number of specific symptoms and injuries. These forms of injuries are communally labeled as peripheral nerve injuries (PNI). While not exhaustive, the primary classification system was proposed by Sunderland [6] and is shown in Table 1 [6, 7, 8]. Since the system's inception, an additional sixth degree has been proposed which covers injuries that are a mix of grades 2 and 4 [9]. This system, most commonly used without the proposed sixth degree [10], can be supplemented or replaced with the Seddon Classification system, which is also shown in Table 1 [6, 7, 8].

TABLE 1: Peripheral Nerve Injury Classifications [6, 7, 8].

Seddon	Sunderland	Description
Neuropraxia	Grade 1	· Caused by ischemia, traction, compression, or non-serious crush · Recovers without long-term damage within weeks · Local myelin is damaged
Axonotmesis	Grade 2	· Caused by crush · Recovers without long-term damage within months · Interneuron axons are damaged without damaging the connective tissue
Axonotmesis	Grade 3	· Caused by crush · Finishes healing within months with variable results · Myelinated axons are damaged as well the endoneurium
Axonotmesis	Grade 4	· Caused by crush · Variable, but incomplete regeneration and recovery time · Least severe degree that is surgically repaired · Damage to myelinated axons, endoneurium and perineurium
Neurotmesis	Grade 5	· Caused by lacerations · Variable, but incomplete regeneration and recovery time · Surgical repair is always performed · Axons, endo-, peri-, epi-neurium, severed

Table 1 also references one of the ways that these classification systems can be used to inform current treatment plans. Surgical intervention is the treatment plan that is primarily indicated in the table, which is almost exclusively done when the nerve is completely transected, creating a gap between the two ends of the nerve. If there is no gap or if this gap is small enough to heal on its own, the treatment plan may consist of physical therapy, medication, or a combination there of. If there is a gap and it is of sufficient size, even surgical repair will not allow for complete recovery. For this reason, there is continuing

exploration of new methods from different fields, including tissue engineering.

Tissue engineering is, by nature, a combinatorial approach [11]. Tissue engineering is an interdisciplinary field that draws from disciplines such as biology, materials science, and nanotechnology to restore, enhance, or replace damaged tissues [12-15]. Historically, tissue engineering approaches can be divided into three separate pillars: scaffold, cells, and growth factors [13]. These three pillars will be examined individually in addition to a fourth pillar, gene therapy, which the author has divided into a separate pillar for clarity and to acknowledge the additional processes required [16]. None of the individual pillars have been able to show satisfactory results in terms of functional recovery [13, 17]. Many studies have noted improved results when the pillars are combined into one treatment, such as scaffolds that have growth factors embedded into them [18-26]. It is likely that, despite the complications that combined approaches incur, they are the most promising way to improve the results of tissue engineering strategies.

II. Current Treatments

A. Medications

One of the oldest treatments for PNI are pharmaceuticals [27]. It is still considered a standard and conservative treatment option because it is a non-surgical option [28]. However, pharmaceuticals can be used in combination with surgery. If surgery is not immediately possible, pharmaceuticals can be used in an attempt to reduce the rate at which motor neurons are lost due to deinnervation [29]. Unfortunately, most medications dilute in the blood, which can reduce their efficacy [28]. The neuropathic pain that is common in peripheral nerve injuries is often treated with antidepressants. These medications have results that are suboptimal, inconsistent between preclinical and clinical trials, and notably dependent upon the state of the brainstem-spinal noradrenergic system [30]. Other medications such as neurotrophic drugs, e.g. B vitamins and neurotrophic factors, can directly improve the regeneration of the axons damaged by the peripheral nerve injury [28]. Unfortunately, growth factors are defined as protein drugs and correspondingly are prone to proteolytic degradation. They are also temperature sensitive and have a short half-life. This, combined with a rapid diffusion in the blood stream, requires a high dosage that is given frequently [31].

B. Physical Therapy

Physical therapy is another common and conservative treatment for the effects of peripheral nerve injuries [28, 32]. A common part of peripheral nerve injury is muscle fibrosis and muscle atrophy, both of which are a result denervation [8]. Typically, patients lose sixty to eighty percent of the affected muscle area [8]. This loss of muscle mass can be reversed, to varying extents, if enough of the muscles are reinnervated quickly enough [8, 23]. The exact timeframe required is unknown and may vary depending on the circumstance [8, 23]. Physical therapy can help to restore muscle mass and help patients learn to compensate for any long-term deficits [33].

C. Surgery

The class and severity of the PNI determines much of the treatment plan. In the case of surgical intervention, the main determining factor is the presence and length of a gap. Surgical intervention, as shown in Table 1, is typically reserved for lacerations, which have severed the nerve. The two ends often separate creating a gap. In cases when there is not a gap, the most frequent surgical procedure is an end-to-end suture [10, 34]. The recovery from this procedure has improved over the last several decades, however it remains suboptimal [17].

There are two primary surgical procedures, either a direct tension free neurorrhaphy or an autologous nerve graft. In cases of shorter gaps, direct nerve surgical repair is widely considered to be the gold standard to treat PNI, especially for axonotmesis and neuromesis [7]. The exact definition of what constitutes a short gap and what constitutes a long gap varies throughout the literature. In some cases, a direct tension free neurorrhaphy is recommended for up to 10 mm [12, 33], while in others an autologous nerve grafts are recommended starting at only 5 mm [2, 10, 35].

In the case of a longer gap, an autologous nerve transplantation is considered to be the gold standard [2, 3, 5, 10, 13, 18, 19, 21, 35, 36, 37]. The surgery takes a nerve from the patient that is deemed to be less vital to the patient [2]. This surgery can provide some return of function, but it is rarely complete and is often deemed to be unsatisfying [37]. The surgery also carries several notable consequences. Specifically, there is a limited donor area [18, 19, 22, 36, 37], it requires an additional surgery [18, 19, 22, 36], and the donor site experiences damage that can cause pain, scars, neuromas, and sensory loss [5, 22, 32, 36, 37]. Functionally, a peripheral nerve injury is caused in order to treat the original.

When an autologous nerve graft is not possible or desirable, an alternative substitute can be used. While the different substitutes have their own properties, they are all capable of acting as a scaffold to protect and guide the nerve [13]. An allograft is a similar procedure to an autograft, but it uses a nerve from a cadaver [2]. They are commonly available and reduce concerns of injuring the donor. However, there are common problems of immune rejection and infections [2, 3]. Xenografts, or grafts from animals, have a similar cost benefit profile [2]. There have also been experiments with utilizing vein autografts instead of nerve grafts, which do not provide any distinct advantages over other forms of grafts [38]. Another alternative is an acellular nerve autografts (ANA) [12, 13, 36]. ANA's have the notable benefit of not requiring immunosuppression [36]. For this reason, they are one of the most commonly utilized biomaterials in clinical practice [1]. Unfortunately, they consistently have worse results than autologous nerve grafts [39]. They also do not consist of Schwann cells (SCs) nor do they attract a large quantity of them, which contributes to their poor communication between the matrix and the cells [36, 39]. Due to this limitation, there is significant research into improving them. There are three general avenues that are being explored cellular, biochemical, and physical, though none are implemented clinically [36].

III. Main Pillars of Tissue Engineering Research

A. Scaffolds and Nerve Growth Conduits

Nerve guidance conduits (NGCs) are pre-fabricated structures that seek to emulate a healthy nerve for long enough to support and protect neuronal regeneration [37]. When there is a gap present during PNI, nerve regeneration requires that axons find their way across the gap to reinnervate correctly. Nerve guidance conduits can act as a bridge to help guide and support the axons across longer gaps as well as protecting the axons from the external environment even while permitting the movement of neurotrophic factors and other bioactive agents [13, 37]. NGCs also attempt to reduce the invasion of scar tissue and mimic the native architecture, biochemical interactions, and physical interactions of nerves and the surrounding extracellular matrix (ECM) to help the growth and regeneration of the injured nerves [22, 35, 40].

The mechanical characteristics of scaffolds, especially polymeric scaffolds, need to be carefully chosen and tuned to prevent harm to the patient and improve nerve regeneration. Many mechanical characteristics influence each other, but they can be divided into separate categories in order to fine tune them for certain applications. One such set of divisions are scaffold diameter, wall thickness, porosity, pore size, pore distribution, alignment, and filament size [37]. These factors also influence the biodegradability of the conduits [37]. While not all NGCs are biodegradable, it is preferable as it prevents a second surgery, which can damage the regenerating nerve, from being required. One standard for biodegradability is that the NGC should be able to survive a year in the human body, but begin to biodegrade when axons begin to spread [39]. Generally, biodegradability has to be timed so that it does not degrade before the healing process finishes, but does not take so long that it begins to hinder healing and cause inflammation [37]. The mechanical properties can also influence vascularization, which is important for complete functional recovery [37].

There are several designs of nerve guidance conduit, but the two main categories are hollow conduits and filled conduits. Hollow conduits are older than filled conduits [13]. In gaps up to 10 mm, they can improve reinnervation [13]. However, when they are applied to critical gaps, the axons regenerate inaccurately [39]. In an attempt to improve the results and length of gap that NGCs can be applied to, various fillings and internal nanoscale structures are being explored [2]. In general, an internal structure takes away space for the nerve to grow in, but it can provide support for said growth as well as guidance and targeting [13]. The strategies that are currently being investigated include hydrogels [2], microscope or nanoscale filaments, microscope or nanoscale grooves, fibrils that are magnetically aligned, micro-channels, freeze dried internal structures, and various ways to hold and release growth factors or other bioactive agents [13]. Multichannel fillings are relatively common, due in part to the creation of a larger surface areas [2, 13, 39].

The exterior of the growth conduit is, arguably, equally as relevant as the interior. The surface properties can influence the surrounding micro environment, influencing the regenerative process [14]. Surface coatings and surface topography can influence nerve regeneration [2, 13, 14, 40]. Surface coatings can consist of, among other things, ECM proteins and have been shown to improve not only axon regeneration but overall nerve function [2]. Surface topography, in comparison, does not use bioactive agents but instead consists of the texture and pattern of the NGC's surface. Specifically, topography can be broken down into roughness, the size of any grooves, the orientation of said grooves, and the pore size of the NGC [14]. The exact mechanisms of how cells interact with and are affected by the surface properties of NGCs are still being studied, but the effects of them on the cells and on recovery from PNI have been observed [14]. Some types of topography have been shown to affect the proliferation, differentiation, migration, adhesion, and alignment of cells [14]. It has also been shown to influence neurite guidance and cell-relevant mechanisms like changes in the cytoskeleton [14].

While no optimal material has been found, a number have been researched and explored in order to create a NGC that can endure the stress of the patient's movements [13] and are histocompatible [32]. Polymeric NGC are the most common form of NGC [32] as they fill the same structural role that the original nerve did [37] and can be made out of natural or synthetic materials. Synthetic materials are resilient [32] and can be designed to mimic the nerve and surrounding environment by providing a range of physical, chemical, and mechanical properties [2]. This comes at the cost of low biocompatibility. Some of the more commonly used synthetic materials include polylactic acid, polyglycolic acid, and polycaprolactone [2]. Natural or biologically based materials usually create better surface to cell interactions, which allow for better drug and cell loading [15] as well as bioactivity that can help cell stimulation and migration [2, 15]. They are also usually more biocompatible [32]. Unfortunately, natural materials typically require protracted processes to purify and prepare them [2]. Once they have been prepared, they typically have a lower mechanical strength, higher variations between batches [2], and rates of degradation that are faster than ideal for peripheral nerve injuries [15]. Some of the most common natural materials are collagen, chitosan, gelatin, alginate, and silk fibroin [2, 41]. In an attempt to minimize the downsides and improve the general performance of these materials, there is an increasing amount of research in modifying the structural components of natural materials [2] and combining synthetic and natural materials [37, 42]. One of the more successful combinations was of chitosan and PLA [44]. While natural versus synthetic is the standard division to examine materials, they can also be divided based on the body's ability to absorb them [38]. NGCs can be absorbed through multiple methods including physical dissolution, biochemical degradation, and physical disintegration [27]. The absorption of the scaffold can cause an immunological response. Chitosan can reduce this response to a small degree [34]. A newer strategy are nanomaterials. They can be designed to have an improved chemical stability as well as a more desirable set of electrical, magnetic, or optical characteristics [21].

B. Growth Factors/Bioactive Agents

The application of exogenous growth factor and other bioactive agents is an evolving, but promising therapeutic treatment for peripheral nerve injuries [12, 31]. Growth factors are polypeptides and molecules that are typically released during the normal course of healing [13, 31]. They aid in the

healing process in multiple ways including maintaining and encouraging cell survival, differentiation, proliferation, migration, axon regeneration, remyelination, and the process of reinnervation [31]. Growth factors also assist in the standard processes of molecular signaling [31].

During the standard process of healing and nerve regeneration, growth factors are produced and excreted in a curved trend over time. Just after the injury, growth factors increase from the system's normal levels [31]. Growth factors are especially upregulated in the distal ends of the nerves [31]. This level begins to decrease as time passes, usually within a month, eventually returning to the previous baseline, unless complications are present [31]. Normally, the recovery of motor and sensory function is heavily correlated to the trend of growth factor expression [31]. Additionally, if neurons are without sufficient amounts of growth factors for an extended period of time it can increase the rate of neuron apoptosis, which can prevent or reduce nerve regrowth and healing [31, 44]. Conversely, high enough levels of neurotrophic factors and cytokines can, in some cases, also cause neuron apoptosis. [44]. The application of neurotrophic factors also carries the risk of pathological pain [19]. Because of this, the exogenous application of neurotrophic growth factors during the initial stages of recovery is vital to improve nerve regeneration and functional recovery [22]. Some of the most common growth factors are nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor, neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and fibroblast growth factors (FGF), all of which have slightly different effects [2]. There are also several other biological factors that can be used to help treat peripheral nerve injuries including vascular endothelial growth factor (VEGF) [13, 45] and Hepatocyte growth factor (HGF) [13].

a) Delivery Methods

Growth factors and bioactive agents need to be delivered to the injury site in the appropriate dosage and, ideally, with as little waste as possible [31]. The factors can be given to the patient as pharmaceutics, though it comes with several disadvantages, as outlined earlier [28]. The spatial and temporal distribution of the growth factors caused by the initial high concentration of injections, pharmaceuticals, and other similar methods combined with the short half-lives, pleiotropic effects, and level of biological activity in growth factors that requires small doses creates a difficult situation to tailor any delivery system to [13]. Additionally, the delivery systems, notably injections, can damage the delivery site due to the current requirement of multiple deliveries [44]. Studies have shown that, regardless of the delivery system, the use of a single type of growth factor is insufficient to support all stages of healing for all of the types of cells in the peripheral nervous system and surrounding tissue [31]. The combined use of multiple growth factors that are delivered with spatial and temporal control showed improved results [18, 31]. This begins to move the administration of growth factors to combined approaches, even within the pillar.

b) Combinations with Scaffolds and Nerve Guidance Conduits

Nerve guidance conduits are increasingly used in combination with growth factors. The results of NGCs, in terms of the microenvironment, axonal survival, and reinnervation, can be greatly improved with some implementations of growth factors and other bioactive agents [2, 22, 31]. Having growth factors and other bioactive agents in scaffolds, usually biodegradable ones, can allow for a more controlled release [31]. This can reduce issues with the immune response and allows for a more precise dosage and duration to be distributed [31]. Additionally, containing growth factors within the tube can ensure that the factors are available to the nerve for longer periods of time without reapplication [39]. Unfortunately, this strategy must be carefully executed to ensure that the nerve is not oversaturated [2].

A variety of materials have been tested in conjunction with growth factors with various results. It is generally agreed that natural, biodegradable materials are preferable, to allow aid in the release of growth factors and prevent secondary injury [27, 31]. There is also some materials science research into tailoring the materials used in NGCs to improve results when combining NGCs and growth factors [27].

There is no optimal method to load growth factors or other bioactive agents into NGCs, though several methods have been studied with some success. Two of the simplest methods are direct incorporation or layering the growth factors [31]. Multiphase loading, which allows for some control over the location and order of the factors' release is also common [31]. Nerve guidance conduits can also utilize the axon's natural ability to find a path for reinnervation by loading the factors so that the nerve's natural growth allows it to interact with the correct amount of the factor at the right time [13]. Hydrogels and microspheres have been studied as integration strategies [20]. There was also a study that showed that self-assembled protein-inorganic nanoflowers showed promise as the structure provides an increased amount of space for cells to attach [39].

The promise of improved results with the use of multiple growth factors in combination could be supported by the use of NGCs as a delivery method. The NGC ensures that the growth factors remain local to the site of injury while allowing for more precise control of the timing, dosage, and location of release [31]. Unfortunately, the mechanisms of this control are still being researched. This leaves the risk of flooding the injury site with too much of too many growth factors, which can impair the healing of the nerve [2]. Despite the complexity of attempting to use multiple growth factors together, this is a promising line of research that is garnering increased attention [2]. Future areas of research also include increasing the number of bioactive agents that are incorporated into NGCs [21].

C. Cell Therapy

Cell therapy, or the application of cells to treat damage and disease, has been prominently researched for the central nervous system [27, 45, 46, 47], but many of these same tactics can be modified or used directly for the peripheral nervous system [27].

a) Cell Types

Mesenchymal stem cells (MSCs) are commonly explored for cell therapy in peripheral nerve injuries. MSCs are multipotent cells from the mesoderm [34]. They exhibit high self-renewal, potential for multi-directional differentiation, and low immunogenicity [34]. These properties, in addition to being relatively easily to isolate, make them attractive targets for research and treatment [34]. One of the primary roles of interest is the secretion of growth factors [12, 19, 28]. This can influence vascularization, metabolic activity, and signal responsiveness in the damaged tissues [38]. They are also capable of influencing what SCs produce and how macrophages assemble near the site of injury [38]. Unfortunately, MSCs have some challenges for use. One of which is that during long term research, MSCs will begin to age. The exact effects of this on the efficacy and side effects of cell therapy are unknown and being researched, but it is believed to cause issues [34].

Another commonly used cell type are Schwann cells. These are a form of glial cells, which, among other roles, help to remove debris from the site of the PNI [34] and utilize their ability to convert to a pro-myelinating phenotype to assist in regeneration [2]. Their application has proven beneficial in some circumstances. There is research into altering SCs in various ways, such as with adhesion molecules, to improve the results [9]. Some studies have also found that combining them with other types of cells, such as olfactory ensheathing cells can improve the results of cell therapy [9]. Regardless of whether the cells are used in combination or not, Schwann cells create an immune response in the body which must be taken into consideration and requires immune suppression [13, 34]. Some studies have used autologous SCs, which some consider to be the gold standard of cell-based therapies [13]. However, these studies must instead deal with the secondary procedure required to acquire the cells from the patient and the complications that arise from it, such as the small amount of cells available and injuries to the donor site [2, 24]. The process of culturing poses issues regardless of the origin. The cells are difficult to isolate, have long culture times, a slow rate of division, and prone to fibroblast infiltration [2, 36].

Exosomes are biovesicles released by dying cells [34] that are 30-120 nm in diameter, also referred to as nanoscale, and are derived from the endosomal membrane [15, 48]. They have a lipid bilayer and surface proteins, which enable them to attach and ferry their internal contents into specific cells [15]. The internal contents can include proteins; lipids; and genetic materials, such as messenger ribonucleic acid (RNA), microRNA, and noncoding RNA [15, 34, 48]. The ability to connect and carry to cells allows them to assist in and be components of multiple biological functions and processes, such as cell to cell signaling [34, 44], the proliferation of tumor cells, angiogenesis, and parts of the immune response [15]. Depending on the circumstances, exosomes can target cells locally or use circulatory pathways to move to cells in other locations in the body [34].

In cell therapy for peripheral nerve injuries, exosomes are valued for the fact that, in comparison to SCs and MSCs, they are less influenced by the surrounding microenvironment, less difficult to store, and less tumorigenic [15]. The sources of exosomes have been shown to vary the resulting effects of their applications. This has been shown to be true even between different types of cells that are reprogrammed to become mesenchymal stem cells [44]. When derived from MSCs, they have been shown to have some success combatting glutamine-induced injury to the nervous system [34]. When derived from SCs, they have been shown to increase the regenerative ability of axons in in vitro and in vivo studies [44]. There is continuing research into the effects of cell type on the exosomes as well as ways to modify the exosomes to influence specific cell activities and parts of nerve regeneration [44].

b) Delivery Methods

Cell therapy can be delivered to the injured nerve in several ways. Cells can be injected directly into or near the site of injury [23, 34]. Injections of differentiated adipose stem cells have been used to supplement autografts with positive results in rats [23]. There have also been encouraging results when MSCs are directly transplanted into the severed nerve, though there have been reports that the injected cells can spread into other tissues and impair the nerve regeneration [34]. The possibility of cells moving to undesired regions of the body as well as of damage to the nerves and local microenvironment has encouraged other avenues of exploration [38]. This includes exploration of intravenous injections [38]. While this method may avoid the damage of injections to the site of injury, it could have the same issues of cells moving to undesired regions [38]. It would also focus on utilizing MSCs in response to inflammation [38].

c) Combination with Scaffolds and Nerve Guidance Conduits

One of the largest avenues of research is the combination of cell therapy and nerve guidance conduits. The theoretical goal is to use biological materials and implanted cells to create a three-dimensional conduit that can replace the damaged tissues [15, 34]. Both MSCs and SCs have been explored in combination with NGCs. In many ways the integration of these cells is similar, though their differing roles and properties alters the results and mechanisms. The use of MSCs laden NGCs has been proposed to assist nerve regeneration and influence the local microenvironment with a lower possibility of the cells migrating to adjacent tissues or damage to the local tissue than injections have [34]. The use of SCs has been proposed to assist with remyelination [2].

Cells can be loaded into nerve guidance conduits in several ways. It is common to simply fill a hollow NGC with cells. Unfortunately, this method leads to a high percentage of cells being lost [9]. This problem can be somewhat mitigated if the cells are first put into a hydrogel then injected, cocultured with, or otherwise placed into the NGC's lumen [9, 13, 38]. This improvement in cell retention comes with a higher cost and manufacturing complexity [9]. Variations of this method include the use a different form of medium to suspend the cells before injection [38] and intraluminal hydrogels [13]. Alternatively, a hydrogel filled with cells can be placed into the NGC's scaffolding [9].

Integrating the cell therapy with NGCs allows for synergistic approaches. Nerve guidance conduits used with cell therapies must be carefully tailored to ensure that their properties support the attachment, survival, efficacy, and, if necessary, differentiation of the cells [13]. This can entail mixing materials or choosing ones that have inherently favorable properties. For example, when chitosan is one of the materials used it can help the growth of SCs as well as reduce fibroblast infiltration [43]. Many NGCs are attempting to better emulate ECM [2, 14] which, among other roles, affects cell migration [40]. Some materials and methods have better results with one type of cell than another. For example, SCs work better than BMSCs in conjunction with fibrin tubes [9]. To an extent, growth factors are used in cell loaded scaffolds as well, as cell's ability to create and release growth factors is expected and relied upon when they are used [13, 15].

Currently, all methods to integrate cells and NGCs are affected by a few common problems. First, only a low quantity of cells typically survives implantation. This is theorized to be due to immune rejection [20]. This leads to a requirement for a large quantity of cells, which can be difficult to obtain, maintain, and load. There are also the unfortunate possibilities of fibroblasts infiltrating the cells and of the surgical insertion of the NGC damaging the nerves [9].

In addition to stem cells, MSCs, and SCs, exosomes have been tested with biological scaffolds or bio-scaffolds. Once the scaffolds have been implanted, the exosomes can move to aid in regeneration based on the porosity of the scaffold and the cross-linking characteristics. The two most common methods to attach the exosomes to the scaffolds are implanting the exosomes and utilizing diffusion. In general, these combined scaffolds are capable of maintaining the exosomes at the site of the injury and the properties of the scaffold, encouraging cells to migrate into the scaffold by working synchronously with the surrounding tissue and microenvironment, and modifying the phenotype of local cells by releasing exosomes into the ECM. This comes at the cost of decreased mechanical stability, increased thermal sensitivity, accelerated disintegration, and potential contamination. These integrated scaffolds also require a complex manufacturing process and a correspondingly higher production cost. The exact capabilities and drawbacks of these scaffolds depend on the parameters used to create them. Initial tests indicate that alginate hydrogels are the most promising material [15].

Research into combining scaffolds and cell therapy is ongoing. There is continuing research into exploring cells in combination with different methods of manufacture as well as ways to reduce the common problems of these methods. There is also exploration of other ways to combine pillars of tissue engineering research. One such avenue of research has been the injection of cell therapy in conjunction with the use of scaffolds that have not been loaded with cells [23]. This has had good results, however, has the unfortunate effect of keeping the same disadvantages as injections. The removal of which is usually considered to be one of the advantages of NGC delivery [34].

D. Gene Therapy

While far from being clinically approved, gene therapy is being researched as method to improve sensory and motor nerve regeneration [29]. Gene therapy is, in most contexts, a collection of tools and processes that can be used to alter the deoxyribonucleic acid (DNA) of or introduce new genetic information to cells in order to modify the cell's genetic material [34]. In order to make this a feasible treatment, the delivery mechanism need to be improved in terms of spatial and temporal precision [29]. This pillar is unique in that it is primarily done in deliberate combination with one or more of the other pillars, which is why it is often categorized as aspects of other pillars of tissue engineering rather than its own pillar. Genetic therapy has focused on modifying cell therapy or delivering growth factors. Additionally, genetically altered cells can be used with NGCs and other scaffolds to increase their efficacy [17].

Gene therapy is delivered by some form of vector. One of the most commonly used vectors to treat PNI are adeno-associated viral vectors (AAV) [16, 17]. There are a minimum of twelve variations, all of which can be produced at high concentrations at clinical grade and do not produce viral genes, have high possibilities of insertional mutagenesis, or of causing immunogenic reactions [17]. Another frequently researched form of vector, especially for SCs, is lentivirals [17]. Lentivirals also have a low risk of mutagenesis, but are associated with possible risk for the altered cells [17].

Gene therapy has been researched in combination with cell therapy in attempt to improve its effects and reduce its side effects and limitations. In general, cells are genetically engineered to reduce or eliminate unwanted genetic features of cells or to increase the production of growth factors or molecules for migration and adhesion [13, 34]. Standard MSC cell therapy suffers from, among other issues, decreased life spans of the cells and decreased cell concentrations near the damaged nerves [34]. Genetically altered cells can solve or reduce these problems [34]. There is also evolving research into utilizing miRNA to encourage the differentiation of MSCs into certain types of nerve cells [34]. However, as of 2019, arguably the majority of genetic therapy focuses on altering SCs [13]. Much of this research focuses on the expression or modification of growth factors, such as dealing with the "candy store effect" and increasing the production of c-Jun [13]. However, all genetic research on cell modification focusing on or considers how to

improve the control and reliability of modifications to cells and how the can be most effectively delivered [34].

Genetic alterations can force a gene to create more of a natural or artificial gene, protein, or molecule than it normally would, which can be used to help treat peripheral nerve injuries [34]. This overexpression can not only influence the surrounding tissue, but it can make them more susceptible to other types of stimuli [34]. Genetic therapies that have focused on neurotrophic factors have been shown to improve remyelination, the healing of axons, and action potentials in motor nerves [17]. A commonly explored growth factor is GDNF [12, 29, 44]. In addition to the standard results of neurotrophic factors listed above, it has been shown to be a particularly influential method for controlling SC proliferation and migration [29]. In one study, it was favorably compared to NGF and BDNF [44]. Unfortunately, it has to be carefully controlled for dosage and timing to prevent coil formation and axonal trapping [29].

The emerging nature of this pillar means that the future work required is significant. The primary concern of a large portion of research is to determine which of the existing vectors functions best in various situations [17]. The most significant step towards clinical acceptance, though, is ensuring that previously created or creating new vectors and therapies are safe for human use [12, 17]. This entails not only ensuring that the vector itself does not cause harmful side effects, but ensuring that the therapy is reliable, controllable, and free from harmful effects. For example, one set of criteria for the safe activation of growth factors is that the gene therapy uses a molecule that can be introduced without side effects, the therapy halts when the molecule stops being applied, and the transactivator protein that is used does not activate the immune system or cause side effects [17]. One of the reasons that this is a difficult undertaking is because the current understanding of how cellular and molecular factors influence, interact with, and behave during nerve regeneration is incomplete. This does not only apply to the manipulation of growth factors. For example, the mechanisms that cause the pro-regenerative capabilities of SCs to decrease over time are still unknown. Understanding these mechanisms would be a significant stride for genetic therapy [17].

IV. Other Avenues of Research

While not strictly within the scope of a review of tissue engineering methods, non-tissue engineering methods are relevant to the continuing theme of increasing combinations within the field. The primary focus of these methods within the review is how they can be used in combination with tissue engineering methods to increase their efficacy without interfering with the mechanisms of the tissue engineering method. However, these methods also have benefit to treat less severe cases of peripheral nerve injury that do not necessarily warrant tissue engineering intervention and to treat adjacent issues with the peripheral nervous system, such as peripheral neuropathy. Figure 1 shows a summary of the non-tissue engineering methods reviewed by this paper.

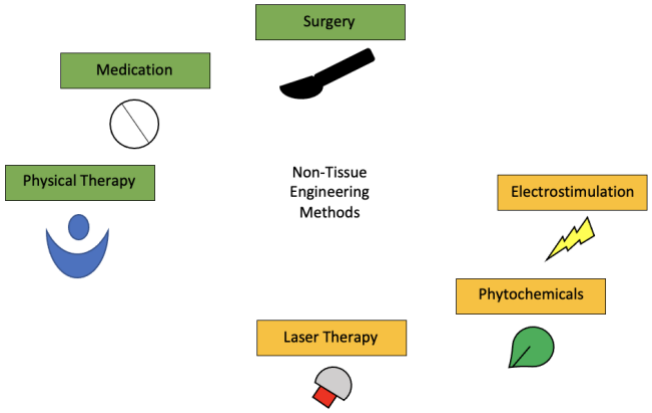

Fig. 1: A summary of the non-tissue engineering methods to treat peripheral nerve injuries reviewed by this paper. Methods shown in green are ones that are currently in clinical use, ones that are shown in yellow are ones that are still being researched.

A. *Electrostimulation*

Studies have shown that electrical stimulation or electrostimulation (ES) can influence neural cells [35]. In general, electrostimulation can promote regeneration of axons, functional rehabilitation for motor and sensory functions, and alleviate neuropathic pain, all of which are points of concern for patients with PNI [12, 28]. While not in use clinically due to the necessity of more research, electrostimulation is becoming one the most popular non-surgical forms of treatment [28, 49].

Electrical stimulation can be performed in multiple ways; the most common difference is the location that the electrostimulation is applied, which alters the effects. The majority of studies focus on electrostimulation at the injury site [28]. This form of ES has been shown to decrease staggered regeneration of nerves and increase the rate of regeneration of axons with only an hour of stimulation at 20 Hz [28, 49]. Unfortunately, this method required surgery and can cause damage to the implantation site [28]. This risk can be slightly mitigated by implanting them under ultrasound guidance or by attaching the electrodes during the surgery to repair the PNI with direct suturing or autografts [28, 49]. The ES can also be applied to the spinal cord. When stimulation of the spinal cord and the injury site are done in conjunction, the results can be promising. Some feel that this is the most promising avenue of ES [28]. Alternatively, the electrostimulation can be applied to the skeletal muscles [28]. This form of ES has been shown to improve the recovery of afferent nerves [28]. Notably, this therapy has also been shown to spike the activation of insulin. Therefore, this therapy is proposed to be best approximately three days after the initial injury [28].

There has been research into designing scaffolds and materials that could be used in combination with electrostimulation to increase its efficacy. This would primarily be focused on integrating with electrostimulation at the site of injury, though materials science could improve

electrostimulation at other sites as well [27]. There has been an interest in conductive scaffolds due to the fact that they can increase cell affinity and assist in propagating neuronal signals [35]. While not designed for this application, this research could eventually be integrated into these systems. Additionally, certain classes of material can assist in moving the charge at the meeting point between tissue and the electrodes, which has been used in cases of low voltage on the sciatic nerves of mice [27]. There has also been interest in designing biodegradable electronic systems for the central nervous system, the mechanics of which could theoretically be adapted for PNI, though they may be cost prohibitive [27].

Another practice that has been studied is electroacupuncture [10, 28]. This involves the practice of running the electrostimulation through acupuncture needles that are inserted into specific locations that have been determined by Chinese medicine. The needles enter not only the skin, but the subcutaneous nerve tissue and the skeletal muscle, which combines the effect of electrostimulation on all these areas [28].

Specific applications of a ketogenic diet have been studied in conjunction with electrostimulation [50]. The ketogenic diet was reintroduced in the 1920s and is primarily used to treat epileptic children [51]. It has since been shown to be neuroprotective and effective in some forms of neurodegenerative diseases [52]. The diet can alter the metabolic state of the patient, which has been shown to help after spinal cord injuries and can help in the treatment of PNI [50]. More specifically, a 3:1 fat to carbohydrates ketogenic diet coupled with electrostimulation decreased the likelihood of developing hypersensitivity and increased axon density, axon diameter, myelin thickness, and muscle force in certain muscle groups after a crush injury to rats [50].

B. Other

There is emerging research in plant-derived compounds, referred to as phytochemicals. These methods appear to have less side effects than similar methods, such as pharmaceuticals. Many phytochemicals have been shown to be effective in treating neurodegenerative diseases. The individual phytochemicals function differently, ranging from anti-inflammatories to improving remyelination to immune-modulatory effects [7].

Laser therapy is another emerging therapy that can be used in conjunction with other forms of repair. Cell therapy, specifically MSCs, have been shown to have synergistic improvements in results with low level laser therapy [26]. Laser phototherapy has also been shown to work on its own [10, 42].

V. Future Research

A. Combinations

There is no standard term for a method to treat peripheral nerve regeneration that uses multiple pillars. They are referred to as “advanced”, “enhanced” [36], “complex”, “combined”, and “multifunctional”, among others. These terms are sometimes even interchanged in the same paper [2]. This makes tracking their development and proliferation more difficult. A review of these combinations is shown in Figure 2.

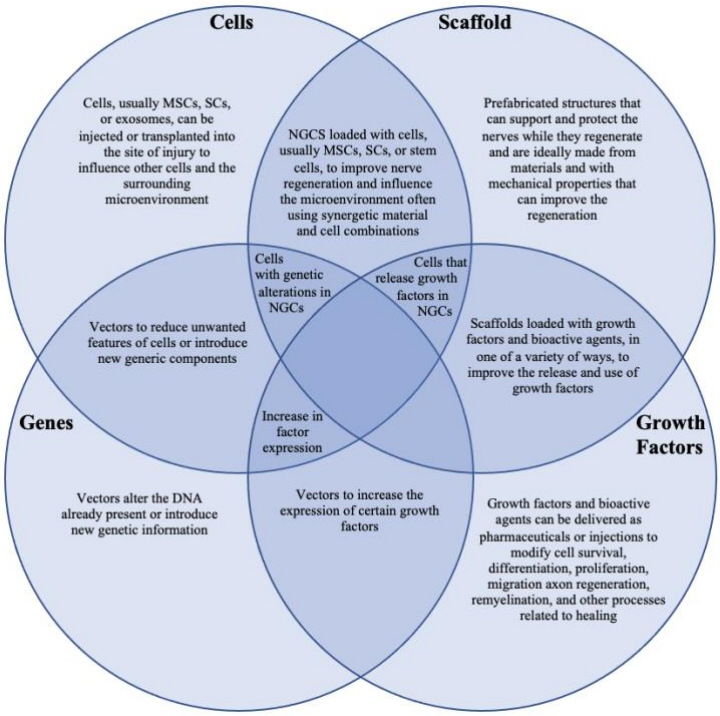

Fig. 2: A review of the tissue engineering pillars and how they can be combined. The sections left blank are, to the knowledge of this paper, currently theoretical.

Most studies combine only two methods, but in some rare cases three methods have been combined. This is likely due to the complexity that would come from additional methods and the difficulty associated with determining the influence of any specific factor or combination of factors. Despite the complications, continuing research on how to combine standard pillars and methods has potential to improve nerve regenerative outcomes. Additionally, sufficient research may eventually reduce the difficulty of combined methods.

B. Evolving Understanding of Physiology

Tissue engineering approaches to treat peripheral nerve injuries attempt to emulate, modify, and manipulate the natural physiology of the peripheral nerves. This is heavily influenced by the field's understanding of and ability to mimic the existing physiology of the nerves and surrounding microenvironment. As the field's understanding of the physiology continues to change and evolve, so too will any future tissue engineering approaches.

Currently, there is exploration into the possibility that microglia responsible for the maintenance of neuropathic pain play different roles depending on the sex of the patient. There have been some demonstrated differences in rodent models [52]. This sex difference in the peripheral nervous system demonstrated [52] is, to a degree, supported by current literature. To the knowledge of this paper, there are no studies comparing the responses of the peripheral nervous system in human men and women after peripheral nerve injuries. However, it has been well documented that there are sex-based differences in the incidence, presentation, and treatment of illness that affect the peripheral nervous system [53]. Additionally there is evidence that how sensation, especially pain, is processed and interpreted is sex dependent [54]. It is possible, and even likely, that these differences are not solely contingent upon the peripheral nervous system, but they do support the theory that there are sex-based differences in the peripheral nervous system and that they can influence the treatment of peripheral nerve injuries.

C. Machine Learning and Artificial Intelligence

Machine Learning and artificial intelligence are being developed to offload computational work and support large-scale data processing. Artificial intelligence can be used in the treatment process as early as the initial visualization. One of the most common diagnostic tools for peripheral nerve injuries is ultrasound. Artificial intelligence is being developed to clean the images and then classify them to determine what level of damage is present at what location in order to make this technique more useful and reduce inaccurate readings [55]. The development of pharmaceuticals is another early area that can utilize artificial learning. Networks can be designed to find possible medications or combinations of medications to treat certain issues. These results are then used to narrow the number of treatments that are put through preclinical trials, theoretically making the process of drug development more efficient and cost effective [56, 57]. Artificial intelligence may be able to assist later in the treatment processes as well, depending on treatment method. For the creation of scaffolds in particular, artificial intelligence and machine learning are an attractive option to help design them and to print them, both ex and in vivo [32, 58]. Artificial intelligence can ideally be developed to the point where it can tailor conduits to the tissue and patient's requirements [58].

VI. Conclusion

The advancement of treatments for peripheral nerve regeneration is moving in multiple directions, each of which have their own costs and benefits. The combination of these methods creates added complications to design, testing, manufacturing, and, though it is beyond the scope of this paper, affordability and regulation. Despite these complications, the current limitations of traditional therapies and the improved results of experiments that utilize combined methodologies indicate that combined therapies are the avenue with the most potential.

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Acronyms and Abbreviations

PNI	Peripheral Nerve Injury
ANA	Acellular Nerve Autograft
SC	Schwann Cell
NGC	Nerve Growth Conduit
ECM	Extracellular Matrix
NGF	Nerve Growth Factor
GDNF	Glial Cell Line-Derived Neurotrophic Factor
BDNF	Brain-Derived Neurotrophic Factor
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
VEGF	Vascular Endothelial Growth Factor
HGF	Hepatocyte Growth Factor
MSC	Mesenchymal Stem Cell
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
AAV	Adeno-Associated Viral Vectors
ES	Electrical stimulation

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