Nerves and Nerve Injuries

Volume 2:
Pain, Treatment, Injury, Disease, and Future Directions

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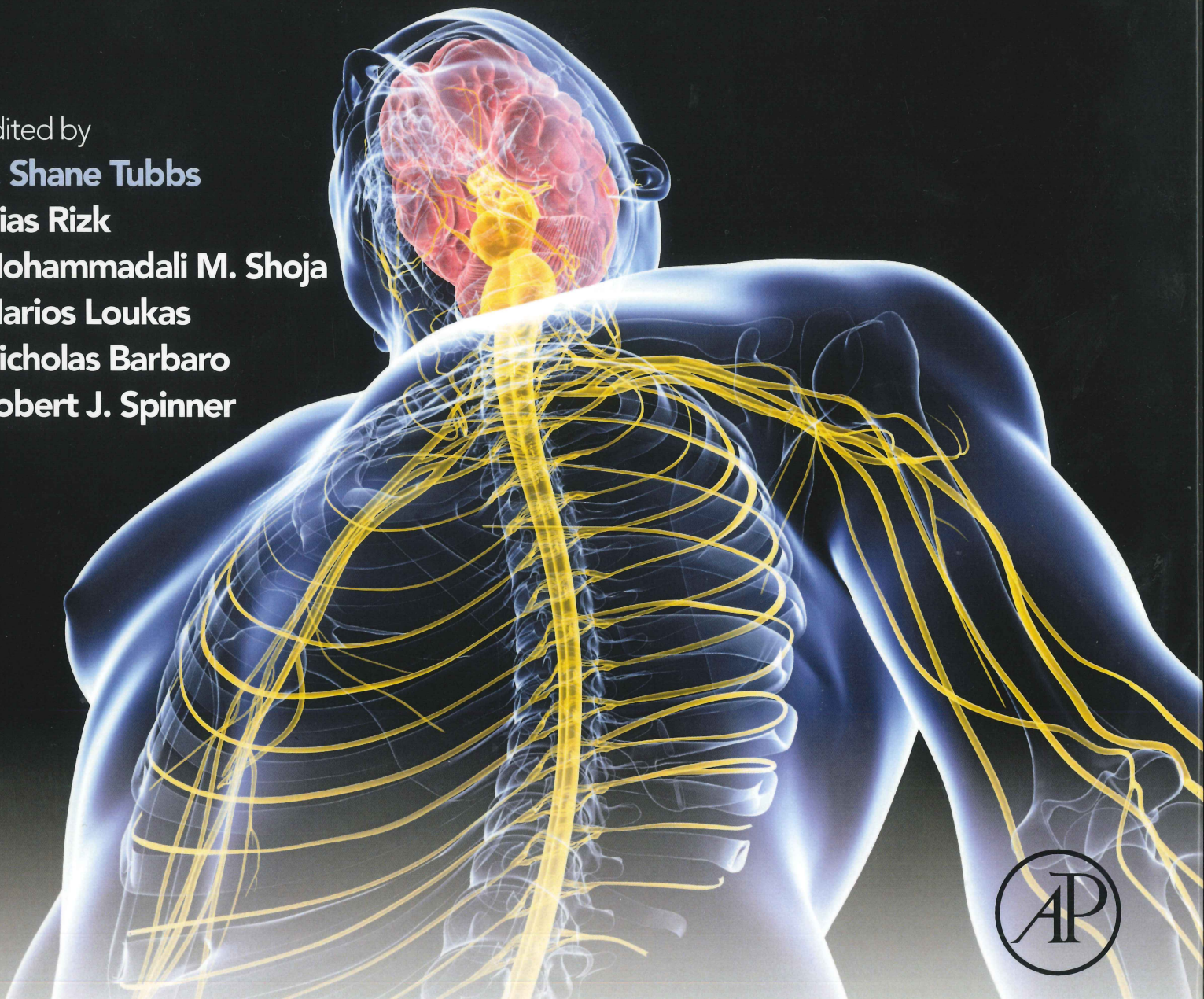

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Chapter 60

Tissue-Engineered Peripheral Nerve Guide Fabrication Techniques

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INTRODUCTION

The current gold standard for the repair of critically sized peripheral nerve defects is the use of autologous nerve grafts but this is associated with many disadvantages, including the need for multiple surgical procedures, limited availability of suitable grafts, loss of function at the donor site, and potential for neuroma formation (Ao et al., 2006; Bender, Bennett, Waddell, Doctor, & Marra, 2004; Flynn, Dalton, & Shoichet, 2003; Guenard, Kleitman, Morrissey, Bunge, & Aebischer, 1992; Jacobs & Fehlings, 2003). To address these challenges, research in the field of tissue engineering has focused on creating a readily available nerve guide to bridge the transected nerve and restore physiological function.

Since the 1880s, different tissue-engineered nerve guides (TENGs) have been optimized in an attempt to produce the most favorable interaction between injured nerves and implanted materials (Angius et al., 2012). As a result of the ease of manufacturing, many early designs were predominantly based on the entubulation model where the proximal and distal stumps of the damaged nerve are inserted into either end of a hollow tube or porous rod (Figure 60.1) (Madison, da Silva, Dikkes, Sidman, & Chiu, 1987). However, elucidation of the native nerve structure has provided insight into the design of increasingly sophisticated and biomimetic architectures for improved tissue function. The purpose of this chapter is to review the important design requirements, available biomaterials, fabrication techniques, and perspectives on future technologies toward the development of clinically viable solutions for the repair of peripheral nerves.

TENG REQUIREMENTS

TENGs serve as temporary three-dimensional templates to direct axonal sprout growth and organization to facilitate the regeneration process (Vats, Tolley, Polak, & Gough, 2003). In addition to providing the framework for tissue development, the ideal TENG should also meet many other necessary requirements to become a clinically viable option. This section briefly discusses the multifaceted requirements of biocompatibility, biodegradability, mechanical compliance, native architectural similarity (biomimetic), and clinical utility that should be considered in the development of an ideal TENG.

Biocompatibility, Biodegradability, and Mechanical Compliance

Biocompatibility is an implanted material's ability to perform with an appropriate host response in a specific application (Williams, 2008). When implanted, a TENG should encourage normal cellular activity without causing any detrimental local and systemic toxic effects to the host tissue. The ideal TENG should also provide a surface-aiding cell adhesion, proliferation, and extracellular matrix (ECM) production without eliciting a chronic inflammatory response, which is a major cause of implant failure (Tang & Eaton, 1995). Implanted TENGs are blood-contacting devices. As such, they must meet the requirements for hemocompatibility; that is, they should not cause hemolysis, destroy blood components, or lead to any blood coagulation or thrombus formation (Mao et al., 2004). Biodegradability is another important requirement for scaffolds in peripheral nerve engineering. Many early TENG designs employed nondegradable materials and required a second surgery to remove the implant to prevent long-term complications from toxicity and chronic inflammatory responses. To avoid the second surgery,

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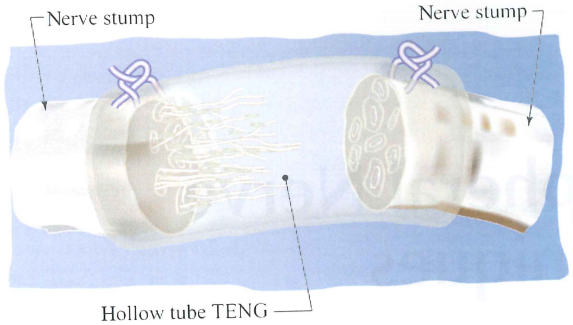

FIGURE 60.1 Peripheral nerve regeneration based on entubulation using hollow tissue-engineered nerve guides (TENGs).

researchers in the field have long favored the use of biodegradable materials that can be degraded *in vivo* at a steady rate to match the rate of neotissue formation. This controlled degradation allows for the gradual creation of void space for the regenerating tissues and the incremental transfer of mechanical loads to the developing nerve (Deng et al., 2008).

In addition to biodegradability, the TENG's mechanical compliance has been recognized as an important parameter in the success of peripheral nerve regeneration. Native nerves are strong and elastic in nature with ultimate tensile stress values of 1.11-3.69 MPa, initial modulus of 0.42-0.73 MPa, and elongations at breaks between 48% and 81% (Borschel, Kia, Kuzon, & Dennis, 2003). Early TENG designs often employed stiff and inelastic biodegradable materials. Unfortunately, the mismatch in mechanical compliance typically leads to increased scar tissue formation at the implant/tissue interface and causes implant failure in mechanically dynamic environments where constant movement, tension, and compression are encountered (Tran, Thevenot, Gyawali, et al., 2010; Tran, Thevenot, Zhang, et al., 2010). To avoid these issues, the biomaterial selection in the design of a TENG is very important because the properties of the material play a dominant role in the biocompatibility, hemocompatibility, degradation rate, and mechanical properties of the resulting nerve guide (Table 60.1).

TABLE 60.1 A Summary of the Recent Fabrication Methods, Biomaterials, and Animal Models Used in the Development of TENGs for Peripheral Nerve Regeneration

Fabrication Technique	Biomaterial	Animal Model	Defect Size	Reference
Dip coating	Chitosan	Rat	15 mm	Suzuki et al. (2003)
	Chitosan	N/A	N/A	Zhang et al. (2010)
	Collagen-coated silicone	Rat	10 mm	Itoh et al. (2002)
	Hyaluronan	N/A	N/A	Zavan et al. (2008)
	Polyurethane (PEG-PCL)	Rabbit	12 mm	Yin et al. (2007)
	PLGA	Rat	10 mm	Zhou et al. (2008)
	PLGA	N/A	N/A	Liu et al. (2008)
Mold casting	Chitosan	N/A	N/A	Freier et al. (2005)
	Chitosan	Rat	8 mm	Zheng and Cui (2010)
	PCLF	N/A	N/A	Moroder et al. (2011)
	PLGA	Rat	10 mm	de Boer et al. (2011, 2012)
	Silk	Rabbit	10 mm	Yang et al. (2009)
	Silk	Rat	10 mm	Tang et al. (2012) and Yang et al. (2011)
Sheet-based	Chitosan	Rat	8 mm	Ishikawa et al. (2007, 2009)
	Collagen	Rat	20 mm	Stang et al. (2005)
	Collagen	Rat	10 mm	Ahmed et al. (2005)
	Collagen	N/A	N/A	Goto, Mukozawa, Mori, and Hara (2010)

TABLE 60.1 A Summary of the Recent Fabrication Methods, Biomaterials, and Animal Models Used in the Development of TENGs for Peripheral Nerve Regeneration—cont'd

Fabrication Technique	Biomaterial	Animal Model	Defect Size	Reference
	Heparin/alginate	Rat	10 mm	Ohta et al. (2004)
	PCL	Rat	6 mm	Maturana et al. (2013)
	PLLA	N/A	N/A	Li and Shi (2007)
	PHB	Rat	10 mm	Kalbermatten et al. (2008)
Particulate leaching	Chitosan/PLLA	N/A	N/A	Xu, Yan, and Li (2009)
	PCL	Rat	12 mm	Chung et al. (2011)
	PHBHHx	Rat	10 mm	Bian et al. (2009)
	PLGA	N/A	N/A	Li et al. (2007)
	PLLA	Rat	12 mm	Evans et al. (1999)
	PLLA/PCL	N/A	N/A	Plikk et al. (2009)
Electrospinning	Chitosan	Rat	10 mm	Wang, Itoh, Matsuda, Aizawa, et al. (2008), Wang, Itoh, Matsuda, Ichinose, et al. (2008), and Wang, Itoh, et al. (2009)
	Collagen	N/A	N/A	Timnak et al. (2011)
	PAN-MA	Rat	14 mm	Clements et al. (2009, 2013)
	PCL	N/A	N/A	Cooper et al. (2011), Daud et al. (2012), Ghasemi-Mobarakeh et al. (2008, 2009), and Prabhakaran et al. (2008)
	PCL/collagen	N/A	N/A	Prabhakaran et al. (2009)
	PCLEEP	Rat	15 mm	Chew et al. (2007)
	PLGA	N/A	N/A	Bini et al. (2006)
	PLGA/PCL	Rat	10 mm	Panseri et al. (2008)
	PLGA/silk	N/A	N/A	Wang et al. (2011)
	PLLA	N/A	N/A	Corey et al. (2008), Jin et al. (2012), and Wang, Mullins, et al., 2009
	PLLA/PCL	N/A	N/A	Kijenska et al. (2012)
	Silk	N/A	N/A	Wang et al. (2012)
Gel-based	Agarose	Rat	10 mm	Yu and Bellamkonda (2003)
	Collagen	Rat	6 mm	Oliveira et al. (2005)
	Hyaluronic acid/collagen	Rabbit	5 mm	Zhang et al. (2008)
	Keratin	Mouse	4 mm	Apel et al. (2008)
	Keratin	Rat	10 mm	Pace, Plate, Smith, and Van Dyke (2013)
	Keratin	Rabbit	20 mm	Hill et al. (2011)
	Keratin	Primate	10 mm	Pace, Plate, Mannava, et al. (2013)
	Silk fibroin (SF16) peptide	Rat	10 mm	Wei et al. (2013)

Continued

TABLE 60.1 A Summary of the Recent Fabrication Methods, Biomaterials, and Animal Models Used in the Development of TENGs for Peripheral Nerve Regeneration—cont'd

Fabrication Technique	Biomaterial	Animal Model	Defect Size	Reference
Fiber extrusion	Bioglass	Rat	5 mm	Bunting et al. (2005)
Phase separation	Collagen	Rat	10 mm	Itoh et al. (1999)
	Collagen	Rat	20 mm	Yoshii and Oka (2001)
	Collagen	Rat	30 mm	Yoshii et al. (2002, 2003)
	Gelatin	Rat	10 mm	Gamez et al. (2003, 2004)
	PGA	Canine	30 mm	Nakamura et al. (2004) and Wang et al. (2005)
	PLGA	Canine	25 mm	Shen et al. (2010)
	PLGA	Canine	50 mm	Ding, Wu, et al. (2010)
	PLGA	Canine	60 mm	Xue et al. (2012)
	PLGA	N/A	N/A	Bini et al. (2005) and Yuan et al. (2009)
	PLLA	N/A	N/A	Lim et al. (2012)
	PLLA/PCL	Rat	12 mm	Koshimune et al. (2003)
	PLLA/PGA	Canine	40 mm	Ichihara et al. (2009)
	Polyamide	Rat	10 mm	Terada et al. (1997)
	Polydioxanone	Rat	10 mm	Terada et al. (1997)
	Vicryl	Rat	10 mm	Terada et al. (1997)
	Alginate	Rat	10 mm	Hashimoto et al. (2002)
	Chitosan	N/A	N/A	Ao et al. (2005) and Behrens et al. (2013)
	Chitosan	Rat	12 mm	Ao et al. (2006, 2011)
	Chitosan	Rat	10 mm	Patel et al. (2009) and Amado et al. (2008)
	Chitosan	Rat	15 mm	Huang et al. (2010)
	Chitosan-alginate	N/A	N/A	Francis et al. (2013)
	Chitosan-polypyrrole	Rat	15 mm	Huang et al. (2012)
	Collagen	N/A	N/A	Bozkurt et al. (2007, 2009) and Mollers et al. (2009)
	Collagen	Rat	20 mm	Bozkurt et al. (2012)
	Collagen-chitosan	Rat	15 mm	Hu et al. (2009), Xiao et al. (2013), and Zhang et al. (2013)
	Gelatin-collagen	Rat	10 mm	Ding et al. (2011)
	Gelatin-collagen	Rabbit	10 mm	Ding, Luo, et al. (2010)
	Hyaluronic acid	N/A	N/A	Sakai et al. (2007)
	PHB	N/A	N/A	Khorasani et al. (2011)
	PLGA	Rat	10 mm	Bryan et al. (2000, 2004)
	PLGA	N/A	N/A	Ma and Zhang (2001) and Yang, Qu, et al. (2006)

TABLE 60.1 A Summary of the Recent Fabrication Methods, Biomaterials, and Animal Models Used in the Development of TENGs for Peripheral Nerve Regeneration—cont'd

Fabrication Technique	Biomaterial	Animal Model	Defect Size	Reference
Mandrel-based	PLGA/chitosan	N/A	N/A	Kuo et al. (2009)
	PLLA	N/A	N/A	Khorasani et al. (2009), Yang et al. (2004), and Yang, Qu, et al. (2006)
	PLLA/chitosan	N/A	N/A	Xu et al. (2008)
	Agarose	N/A	N/A	Lynam et al. (2011)
	Chitosan	N/A	N/A	Huang et al. (2005) and Wang et al. (2006)
	CUPE	Rat	10 mm	Tran et al. (2013)
	PCL	N/A	N/A	Bender et al. (2004) and Jeffries and Wang (2012)
	PLGA	N/A	N/A	de Ruiter et al. (2008) and Sundback, Hadlock, Cheney, and Vacanti (2003)
	PLGA	Rat	7 mm	Hadlock, Sundback, Hunter, Cheney, and Vacanti (2000)
	PLLA	N/A	N/A	Li et al. (2009) and Sun et al. (2012)
	pHEMA	N/A	N/A	Flynn et al. (2003)
Rapid prototyping	Polyethersulfone	N/A	N/A	Brayfield et al. (2008)
	PLLA/PCL	N/A	N/A	Radulescu et al. (2007)
	Polyurethane/collagen	N/A	N/A	Cui et al. (2009)

Biomimetics

A TENG should recreate the native tissue anisotropy, nonlinearity, and porosity at the macro-, micro-, and nanoscales to facilitate cell alignment, ECM compartmentalization, nutrient and gas exchange, and the diffusion of neurotrophic factors from the damaged nerve stump to engineer a more functional nerve (Bozkurt et al., 2012; de Ruiter et al., 2008; Sun et al., 2012). Recent years have witnessed the development of biomimetic TENGs based on contact guidance and basement membrane microtube theory, which hypothesizes that axon elongation requires guidance by contact with the appropriate substrate through topographical control (Hoffman-Kim, Mitchel, & Bellamkonda, 2010). For example, longitudinally oriented channels have been introduced into a number of TENGs to better recreate the highly oriented architecture needed for appropriate cellular alignment and promote the body's natural pattern of growth (Figure 60.2).

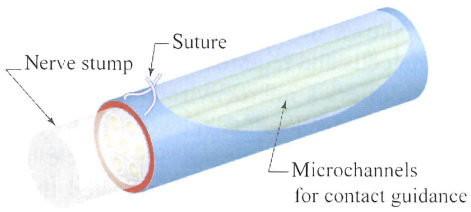

FIGURE 60.2 Peripheral nerve regeneration based on mechanisms of contact guidance through the use of microchanneled tissue-engineered nerve guides.

Clinical Utility

To be clinically viable and ultimately replace the use of nerve autografts, a TENG should also meet strict requirements related to processing, availability, and clinical utility. The resulting nerve guide should be easy to fabricate using cost-efficient methods, sterilizable, and stored without the need of any special equipment. The ideal TENG should also avoid any long-term *in vitro* cell culture needs in order to be considered readily available as an off-the-shelf option in situations when time is of the essence. In terms of clinical use, the engineered guide should be simple and easy to implant in the body using current microsurgical techniques.

BIOMATERIAL CONSIDERATIONS

Naturally Derived Materials

Biomaterials derived from naturally occurring substances are well tolerated *in vivo* because they provide better biocompatibility than synthetic materials, can be degraded by naturally occurring enzymes, have low toxic effects, and enhance the migration of support cells, all desirable features to offer a better environment for tissue regeneration (Hubbell, 1995). Decellularized tissues and organs, which have been successfully used in various tissue-engineering and regenerative medicine applications, are among the first naturally derived strategies for nerve regeneration (Place, Evans, & Stevens, 2009). Although there are numerous approaches to decellularizing tissue, the main goal of the decellularization process is to maximize the removal of cellular material while minimizing ECM loss. The process of removing all the cells from an organ while preserving the native composition and structure of the associated matrix usually involves applications of specialized agents (e.g., chemical, enzymatic, and physical) depending on factors of each tissue, including the tissue's cellularity, density, lipid content, and thickness (Gilbert, Sellaro, & Badylak, 2006). However, every cell removal agent and method inherently alters ECM composition and causes some degree of ultrastructure disruption. Because of this, minimization of undesirable effects instead of complete prevention is the objective of decellularization.

Skeletal muscle tissue has commonly been used as a conduit material in nerve regeneration because of the natural presence of longitudinally oriented basal lamina. Studies have shown that the regenerating nerve does not need Schwann cells and requires only the presence of basal lamina, which offers a microenvironment to promote and direct nerve fiber regeneration (Fawcett & Keynes, 1986; Ide & Kato, 1990). Donor sites for muscle grafts are numerous, and some studies have indicated that skeletal muscle grafts can be effective for repairing short nerve gaps (<1 cm) in laboratory animals (Gulati, 1988). However, the efficiency of skeletal muscle autografts gradually decreases in longer nerve defects. Researchers have also used decellularized arteries and veins as effective conduits to support nerve regeneration (Nectow, Marra, & Kaplan, 2012). From experiments, vein grafts appear to be more abundantly available, induce less donor-site morbidity, and are beneficial for repairs across short gaps for single-function nerves than nerve autografts. They also have thin walls, which allow diffusion of nutrients while being resilient to bar scar ingrowth (Crouzier, McClendon, Tosun, & McFetridge, 2009). However, vein grafts have the tendency to collapse, which then deters regeneration. For this reason, clinical studies suggest that these conduits are practical and reliable for nerve defects between 2.0 and 4.5 cm (Moore et al., 2009). Although the use of decellularized tissues has shown some promise, the methods to remove cellular debris often compromise the resulting ECM, which plays a major role in cellular adhesion, proliferation, and alignment.

To overcome these obstacles, researchers have considered other natural materials for the fabrication of TENGs. Perhaps the most common natural material used in TENG fabrication is collagen, which is a major component of the ECM. Collagen is composed of a family of 28 proteins that share a triple helical structure in the form of an extended rod and has been known to promote cellular proliferation and tissue healing (Miyata, Taira, & Noishiki, 1992). TENGs fabricated from collagen possess excellent biological properties for peripheral nerve regeneration and have exhibited improved regeneration (Li, Archibald, Krarup, & Madison, 1992). However, collagen has relatively high preparation costs, is mechanically weak, and displays very weak antigenic activity. By contrast, gelatin, also a biodegradable polymer, is relatively less expensive and easier to acquire in concentrated solutions, and does not express antigenicity in physiological conditions. Gelatin is created by the thermal denaturation or physical and chemical degradation of collagen and shows outstanding biocompatibility, plasticity, and adhesiveness (Kang, Tabata, & Ikada, 1999). Although gelatin has many benefits, a major drawback of gelatin-based TENGs is that they are soft and prone to rapid clearance from the implantation site.

Another class of naturally derived materials for TENG fabrication consists of polysaccharides such as chitosan, alginate, and agarose. Chitosan is attained from the N-deacetylation of chitin and consists of D-glucosamine and *N*-acetyl-D-glucosamine copolymers (Nettles, Elder, & Gilbert, 2002). Chitosan has gained increasing interest in tissue engineering because of its antitumor and antibacterial activity, biodegradability, and biocompatibility (Khor & Lim, 2003). Chitosan can be obtained from crab tendon after being detached from the calcium phosphates and proteins (Yamaguchi et al., 2003). When used for a TENG, chitosan has been shown to accelerate nerve healing while improving nerve differentiation and growth. Another type of polysaccharide derived mainly from brown seaweed and bacteria is alginate, which is naturally abundant and shows excellent biocompatibility and biodegradation properties. Alginate is a linear polysaccharide copolymer of (1-4)-linked D-mannuronic acid and L-guluronic acid, and its physical properties in gel form generally vary, depending on the overall molecular mass of the polymer and the proportion of guluronic to mannuronic acid residues (Augst, Kong, & Mooney, 2006). A polysaccharide derived from red algae is known as agarose and has been shown to improve cell responses from coupling proteins and glycosaminoglycans to the polymer (Dodla & Bellamkonda, 2006). Although naturally derived structures and materials have shown great potential in peripheral nerve tissue engineering, their applications as constituent materials for TENG fabrication are limited

because of their poor mechanical properties, batch-to-batch variations, high swelling behavior, and their relatively fast *in vivo* biodegradation rate (Kim, Baez, & Atala, 2000).

Synthetic Materials

Before the establishment of biodegradable alternatives, TENGs were made using nondegradable synthetic materials. Silicone tubes are most commonly used for nerve regeneration because they are not permeable to large molecules and provide an isolated environment for the study of different ECM analogue effects for axonal elongation (Chamberlain et al., 1998; Chen et al., 2000; Williams, Longo, Powell, Lundborg, & Varon, 1983). Nondegradable TENGs have also been fabricated using plastic such as acrylic, polyethylene, and expanded polytetrafluoroethylene (Ciardelli & Chiono, 2006). Unfortunately, nondegradable materials remain *in vivo* as foreign bodies after nerve regeneration, causing excessive scar tissue formation, and show inflexibility and a lack of stability, which leads to a chronic foreign body reaction and ultimately device failure (Dahlin & Lundborg, 2001). To address this limitation, biodegradable synthetic materials have become a more attractive option to prepare TENGs. These materials have degradation rates within a reasonable time span, and the properties of these biodegradable synthetic materials can be customized to fit the requirements of a particular application (Hutmacher, Goh, & Teoh, 2001).

For example, polyesters are among the most widely researched biodegradable materials. Many types of polyesters are synthesized by ring-opening polymerization or polycondensation and are degraded back into their monomeric forms *in vivo* by hydrolysis of ester linkages in the polymer backbone (Ikada & Tsuji, 2000). Polyesters have been established to be very biocompatible and safe *in vivo*, leading to their widespread use in FDA-approved devices including sutures, drug delivery systems, and orthopedic screws and plates. Aliphatic polyesters, including poly(L-lactic acid) (PLLA), poly(lactic acid-ε-caprolactone), poly(L-lactide-*co*-glycolide) (PLGA), poly(1,3-trimethylenecarbonate-ε-caprolactone) and poly (caprolactone) (PCL), are in a common class of degradable synthetic polyesters used for a variety of biomedical applications (Jerome & Lecomte, 2008; Vert, 2005). Their material properties in terms of degradation behavior, mechanical performance, thermal properties, and hydrophilicity can be varied to fit a particular application based on the original monomeric ratios. The use of a copolymer or a blend of polymers represents a new research endeavor to prepare neural scaffolds. Unfortunately, many of these degradable materials are not sufficiently compliant for use in many applications involving soft tissues located in mechanically dynamic locations (Yang, Webb, Pickerill, Hageman, & Ameer, 2006). The mechanical irritation resulting from the compliance mismatch between the scaffold and native tissue often leads to inflammation and scar formation, which ultimately prevents the implant from being effectively integrated with the surrounding tissue (Tran, Thevenot, Zhang, et al., 2010). As a result, intense research currently focuses on the development of novel materials with a wide range of biodegradable and elastomeric properties, which can sustain and recover from multiple deformations (Tran, Thevenot, Gyawali, et al., 2010). Among these, citrate-based materials have been shown to offer a wide range of controllable mechanical and degradation profiles along with surface affinities toward many cell types (Yang, Webb, et al., 2006). This new class of biomaterials are all synthesized with nontoxic monomers using simple and cost-effective procedures. These materials all share one common monomer, citric acid, which is a nontoxic metabolic product of the Krebs cycle. Citric acid is a versatile monomer that participates in prepolymer formation through a simple polycondensation reaction while preserving pendant functionality for postpolymerization to produce a cross-linked polyester network with degradable ester bonds. Cross-linking confers elasticity to the polymers similar to the ECM, in which collagen and elastin are all cross-linked polymers. In addition to the multifunctionality and biocompatibility of citric acid, the sodium form of citric acid, sodium citrate, is an anticoagulant currently used in hospitals. Thus, it is hoped that citrate-based materials may also possess suitable hemocompatibility for blood-contacting applications.

The key component of neural communication in the body is the action potential generated at the synapse, which implies that an ideal biomaterial for TENG fabrication should also possess a degree of electrical conductivity to enhance nerve regeneration (Schmidt, Shastri, Vacanti, & Langer, 1997). Polypyrrole (PPy), a well-known conducting polymer, has been used in nerve tissue-engineering applications to enhance nerve regeneration by electrical stimulation (Zhang et al., 2007). Moreover, the antioxidant properties of PPy and polyaniline are advantageous in preventing free radical damage at the site of injury and minimizing scar formation (Chepelev et al., 2006). Past evidence has surfaced to suggest that exposure to electrical charge might enhance nerve regeneration (Kotwal & Schmidt, 2001). Piezoelectric materials generate transient charge in response to mechanical strain; however, similar to electrically conductive materials, their nondegradable nature and questions about their safety in biological systems have delayed their widespread use in TENG fabrication (Schmidt & Leach, 2003).

FABRICATION TECHNIQUES

During the field's infancy, TENGs were fabricated as hollow tubes using nondegradable materials to connect the severed nerve stumps and facilitate neurotrophic communication between the nerve ends. As technology and

our understanding of nerve regeneration have evolved, an increasing number of more complex and sophisticated scaffold fabrication techniques have been introduced to improve on the traditional entubulation models in the repair of peripheral nerve defects. This section provides a review of TENG fabrication techniques.

Dip Coating

Dip coating is a relatively simple fabrication technique used to produce hollow TENGs for tissue regeneration based on the entubulation model. In this technique, the polymer of choice is first dissolved into a solvent to create a relatively dilute polymer bath. Next, cylindrical rods made of materials that cannot be dissolved by the solvent are immersed into and removed from the bath at a constant speed. The solvent is allowed to evaporate, and the process is repeated allowing a series of thin films to build up until the desired thickness is achieved. Solid rods of various shapes and dimensions can be used to dictate the resulting TENG lumen geometry, and the total number of coats and polymer concentration controls the guide wall thickness. To achieve a uniform wall thickness, a variety of devices have also been developed to control the rods' dipping speed and rotation. Because of the simplicity of the technique, nearly all biomaterials can be used in this process, which facilitates the manufacturing process and delivers great flexibility when designing a TENG to fit a particular application.

Polyurethane hollow tubes based on PCL, poly(ethylene glycol) (PEG), and 1,6-hexamethylene diisocyanate have been fabricated by the dip-coating technique to produce TENGs which were able to produce myelinated axon regeneration after 4 weeks of implantation in a rabbit sciatic nerve defect model (Yin, Wang, Yan, & Zhang, 2007). The dip-coating technique has also been used to coat collagen onto a silicone tube to improve the biocompatibility and tissue regeneration through a relatively inert and nonbioactive material (Itoh et al., 2002). Three different collagen cross-linking methods, ultraviolet irradiation, heating, and glutaraldehyde, were compared, and, interestingly, only ultraviolet irradiation cross-linked collagen coatings showed nerve regeneration comparable to isograft controls. Other natural materials including hyaluronan, chitosan, and maggot homogenate products have also been used as a coating material to improve biocompatibility and enhance nerve regeneration (Suzuki et al., 2003; Zavan et al., 2008; Zhang et al., 2010). A drug-releasing TENG has also been fabricated by incorporating collagen microspheres loaded with bovine serum albumin into a PLGA bath solution. It is envisioned that nerve growth factors (NGFs) can be loaded to provide a controlled and sustained released for up to 2 months over the course of TENG degradation (Liu et al., 2008; Zhou, Liu, & Liu, 2008).

Mold Casting

To improve uniformity, wall thickness, and overall processing time, injection molding fabrication techniques have been borrowed from industrial manufacturing processes in the development of hollow tubes for peripheral nerve regeneration. In this fabrication technique, a polymer is dissolved in an organic solvent and subsequently injected into a custom mold, which dictates the resulting shape of the construct. Molds are typically made of glass, steel, or Teflon because of their availability, machinability, and inertness to allow for easy removal, respectively. Following solvent evaporation, the construct is removed from the mold or subjected to various conditions for polymer cross-linking. Similar to dip coating, a wide variety of biomaterials can be used in the mold-casting fabrication process to offer flexibility during the design process.

Chitin hollow tubes have been fabricated from chitosan solutions using acylation chemistry and mold-casting techniques (Freier, Montenegro, Shan Koh, & Shoichet, 2005). Dilute chitosan solutions were injected into a sealed cylindrical glass mold, which contained a fixed central cylindrical glass core. Although these TENGs showed the ability to support the adhesion and differentiation of primary chick dorsal root ganglion neurons *in vitro*, the compressive properties showed room for improvement. In the same study, chitin tubes reinforced with polymer coils were also fabricated by the same technique after mounting a PLGA coil onto the cylindrical glass core to create a chitin/PLGA coil hybrid material, which significantly improved the TENG's compressive mechanical properties. Studies led by Gu have shown that silk can be formed into a hollow tube reinforced with silk fibers through mold casting to support the growth of mesenchymal stem cells (MSCs) and improve the outcome of nerve regeneration comparable to nerve autografts after 12 weeks of implantation in a 10 mm rat sciatic nerve defect (Tang et al., 2012; Yang et al., 2009, 2011). Electrically conductive hybrid TENGs have also been recently reported using the mold-casting technique. Novel electrically conductive hollow tubes were fabricated by injecting a solution of poly (caprolactone fumarate) containing polypyrrole (PCLF-PPy) inside custom glass molds (Moroder et al., 2011). Although these TENGs were not evaluated *in vivo*, it is envisioned that neural cells can be seeded onto the hollow tubes and conditioned to enhance nerve regeneration from electrical stimulation.

Synthetic polymers such as PLGA have also been used in mold-casting fabrication strategies. In studies by de Boer et al., PLGA solutions were injected into Teflon molds, resulting in a cylindrical nerve conduit with an inner and outer diameter of 1.6 and 2.2 mm, respectively (de Boer et al., 2011, 2012). The hollow PLGA tubes were evaluated *in vivo* for their ability to contain and deliver a neurotrophic

factor in the repair of a 10 mm rat sciatic nerve defect. Before completion of the procedure, the hollow PLGA tubes were filled with various solutions containing either saline, saline and NGF, or saline with NGF-loaded microspheres. However, no significant difference was observed between all study groups in terms of ankle angle, retrograde tracing, and electrophysiology. Hollow chitosan tubes have also been filled with bone marrow mesenchymal stem cells (BMSCs) and used to repair an 8 mm rat sciatic nerve defect. After 16 weeks, the implanted BMSCs differentiated into neural stem cells and animals treated with stem cell-filled conduits showed improved sciatic function index scores comparable to autograft controls (Zheng & Cui, 2010).

Sheet-Based

In sheet-based fabrication techniques, thin biomaterial sheets are either rolled into a tube or simply sandwiched on top of and below the nerve defect. Ohta et al. have demonstrated that two thin heparin/alginate gel sheets loaded with basic fibroblast growth factor can be used to "sandwich" a rat sciatic nerve defect to enhance the vascularization of the defect area when compared with alginate gels alone (Ohta et al., 2004). Other materials such as chitosan gels have also been used to sandwich nerve stumps and were found to heavily recruit activated macrophages, which aid in phagocytosing myelin debris and secrete neurotrophic factors to promote nerve regeneration (Ishikawa et al., 2007). The same chitosan gels were also seeded with bone marrow stromal cell (BMSC)-derived Schwann cells and used to sandwich an 8 mm rat sciatic nerve gap. Immunohistochemistry and electron microscope analysis revealed the presence of regenerating axons after 7 days of implantation with Schwann cells forming myelin sheaths on the regenerating axons after 1 month of transplantation, respectively (Ishikawa et al., 2009).

Thin sheets have also been used to fabricate tubular TENGs by rolling a single sheet around a cylindrical rod further subdividing the lumen of the guide into concentrically smaller guidance tubes to imitate the fascicular nerve structure (Maturana et al., 2013). Thin collagen sheets have been rolled into tubes and later cross-linked by glutaraldehyde or microwave irradiation to fix the rolled sheet into a tubular structure; however, the *in vivo* results showed inferior nerve regeneration for both groups when compared with nerve autografts (Ahmed, Vairamuthu, Shafiuzama, Basha, & Jayakumar, 2005; Stang, Fansa, Wolf, Reppin, & Keilhoff, 2005). In a separate study, poly-3-hydroxybutyrate (PHB) sheets were rolled around a 16-gauge needle and seeded with rat Schwann cells, which were fixed with fibrin glue onto the scaffold before implantation. The results showed superior nerve regeneration distances and Schwann cell intrusion into the PHB strip scaffolds when compared with hollow PHB tubes (Bian, Wang, Aibaidoula, Chen, & Wu, 2009). Novel TENGs with the potential to guide cell orientation through topographical control have also been fabricated using holographic diffraction gratings to imprint micron and nanoscale topographies onto thin PLLA thin films, which were subsequently rolled into a tubular construct (Li & Shi, 2007). The patterned PLLA films are hypothesized to facilitate axon path finding, accelerate neurite growth, and induce glial cell alignment through contact guidance.

Particulate Leaching

Particulate leaching is one of the first and simplest fabrication techniques used to introduce porosity into the resulting scaffold. This method dissolved a polymer in an organic solvent system. Next, particulates or porogens, which cannot be dissolved in the organic solvent, of a specific size range are added to the polymer solution, and the mixture is then shaped into the desired geometry. Following solvent evaporation, a polymer-particulate composite is formed, which is then immersed in a bath to leach or dissolve the particles leaving behind a porous structure. This fabrication method is very advantageous in that a wide variety of polymers can be used, and the resulting pore size and porosity can be controlled by the size and amount of the particulate, respectively. In addition a variety of porogens such as salt, sugar, macromolecules, and microparticles can be used as a porogen to fit a specific application (Li et al., 2007; Liao et al., 2002).

A number of reports have described the use of the particulate-leaching fabrication technique in conjunction with previously described fabrication techniques to introduce porosity into the walls of a hollow tube for peripheral nerve regeneration. A study by Plikk et al. used a hybrid dip-coating and particulate-leaching method to coat glass rods with an aliphatic polyesters copolymer/solvent/salt solution (Plikk, Malberg, & Albertsson, 2009). This method is highly adaptable in that various phases of materials, porosities, and pore sizes can be fabricated by dipping the glass rod into different solutions in a layer-by-layer approach. Using the same fabrication method, porous poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx) bilayered hollow tubes were fabricated by dip coating a stainless steel wire into two separate solutions to create an internal layer with 10 μm pores encapsulated by an external layer with pores sizes ranging from 30 to 50 μm (Bian et al., 2009). It is believed that the internal layer of smaller pore sizes can prevent connective and scar tissue formation within the lumen of the TENG. After 1 month of implantation to repair a rat sciatic nerve defect, increased compound muscle action potentials (CMAPs) and the absence of connective tissue ingrowth

penetration were clearly observed in the bilayered TENG when compared with scaffold of uniform porosity.

Highly porous PLGA hollow tubes have also been fabricated using a novel particulate-leaching and mold-casting technique (Li et al., 2007). Gelatin microballoons were combined with PLGA solutions and inserted into a custom mold. Following removal of the microballoon porogen, it has been hypothesized that the hydroxyproline residue from the gelatin inside the pore walls can improve cell adhesion onto the PLGA scaffold. Sheet-based techniques have also been used with particulate leaching to produce porous hollow tubes of concentrically smaller dimensions. Porous PCL sheets have been fabricated using PEG macromolecules as the porogen (Chung et al., 2011). The porous PCL sheets were grafted with NGF and Tirofiban (TF), a nonpeptide RGD-mimetic molecule, and rolled into a tube. The rats that were implanted with PCL-NGF/TF conduits regenerated nerves expressed beta-III tubulin (TB), growth association protein-43, and myelin basic protein along their longitudinal axis.

Electrospinning Fibers

Fiber-based strategies for peripheral nerve regeneration have gained increasing attention because of their ability to promote axonal regeneration by providing the necessary topographical cues for cell alignment. By producing fibers in the micro- and nanoscale range, electrospinning fabrication techniques are able to recreate the native fibrous tissue architecture, which may facilitate cell and ECM compartmentalization to engineer a more native-like and functional nerve. In addition, electrospun fibrous scaffolds show remarkably high porosity and surface-to-volume ratios, increasing the area available for cell attachment, which results in higher cell densities when compared with other structures.

In the electrospinning process, polymer fibers are drawn from a polymer solution using an electrical charge. A very high voltage is applied to a polymer solution-filled syringe, which induces an electrical field to create charge repulsions strong enough to overcome the polymer surface tension. As the intensity is increased, the ejected polymer solution forms a jet, and the solvent evaporates to form fibers as the jet travels to a collector. By controlling the polymer viscosity, conductivity, elution rate, electrical field strength, and the distance between the syringe needle tip and collector, fibers ranging from micrometers to nanometers in size can be created. However, the electrospinning technique places certain constraints on the biomaterial such as high molecular weight, limiting the available biodegradable materials that can be electrospun. Polymers that have been electrospun for use in peripheral nerve regeneration are collagen (Timnak et al., 2011), silk (Wang et al., 2012), PLLA (Corey et al., 2008; Kijenska, Prabhakaran, Swieszkowski, Kurzydlowski, & Ramakrishna, 2012; Wang, Mullins, et al., 2009), PLGA (Bini, Gao, Wang, & Ramakrishna, 2006; Wang, Hu, Lin, Dong, & Wu, 2011), and PCL (Cooper, Bhattarai, & Zhang, 2011; Daud, Pawar, Claeyssens, Ryan, & Haycock, 2012; Ghasemi-Mobarakeh, Prabhakaran, Morshed, Nasr-Esfahani, & Ramakrishna, 2008, 2009; Prabhakaran, Venugopal, Chan, & Ramakrishna, 2008, 2009).

By collecting the polymer fibers around a cylindrical rod, electrospun hollow tubes can be fabricated for peripheral nerve engineering. A polymer blend of PLGA and PCL was used to electrospin fibers 2.5-8.0 μm in diameter, which were collected around a 1.2-mm-diameter copper wire (Panseri et al., 2008). Four months after implantation in a 10 mm rat sciatic nerve defect, myelination and collagen IV deposition were detected in concurrence with regenerated fibers, neural tracers revealed the reestablishment of functional neuronal connections and evoked potential results showing the reinnervation of the target muscles in the majority of the treated animals. Aligned fiber orientations can also be fabricated by collecting the expelled polymer fibers onto a rotating mandrel. Aligned biodegradable, fiber-based tubes encapsulating human glial cell-derived neurotrophic factor (GDNF) were fabricated via electrospinning a copolymer of caprolactone and ethyl ethylene phosphate (PCLEEP) (Chew, Mi, Hoke, & Leong, 2007). Implantation to repair a 15 mm rat sciatic defect showed electrophysiological recoveries in 20%, 33%, and 44% of the rats with the circumferentially aligned tubes, longitudinally aligned tubes, and longitudinally aligned GDNF-loaded groups, respectively, showing the synergistic effect of an encapsulated growth factor to facilitate a more significant recovery.

Wang et al. have published a series of reports on a novel bilayered scaffold (Wang, Itoh, Matsuda, Aizawa, et al., 2008; Wang, Itoh, Matsuda, Ichinose, et al., 2008; Wang, Itoh, et al., 2009). Aligned chitosan fibers were collected onto a rotating mandrel, which were later inserted into a hollow chitosan tube fabricated by dip coating (Wang, Itoh, Matsuda, Ichinose, et al., 2008). By introducing aligned fibers into the walls of a solid tube, the tensile strength along the axis of the tube was increased and provided a suitable topography for cellular alignment. This design was later improved with the incorporation of glycine spacers into the CYIGSR sequence resulting in the amino acid sequences CGGYIGSR and CGGGGGGYIGSR, which were covalently bound to the electrospun chitosan mesh surface to examine the effects of peptide mobility on nerve regeneration (Wang, Itoh, Matsuda, Aizawa, et al., 2008). Nerve regeneration into chitosan tubes, on which the CGGGGGGYIGSR peptide was immobilized, exhibited efficacy similar to that of the autografts because of the presence of laminin-1 (LN-1), which has been shown to enhance Schwann cell migration, attachment, and neural

outgrowth. Electrospun-aligned poly(acrylonitrile-*co*-methyl acrylate) (PAN-MA) fiber films have also been fabricated into thin sheets (<10 μm thick) and inserted into polysulfone hollow tubes to determine the effects of film number (1 vs. 3) on nerve regeneration in a 14 mm rat nerve defect (Clements et al., 2009, 2013). After 13 weeks, both the 1-film and the 3-film channels supported nerve regeneration resulting in functional muscular reinnervation; however, the 1-film polysulfone tubes supported enhanced regeneration compared with the 3-film channels in terms of regenerated axon profile counts and measures of nerve conduction velocity (NCV).

Gel-Based

Many of the peripheral nerve regeneration strategies discussed thus far aimed at repairing transected peripheral nerves using hollow tube designs. Although some hollow tubes have been approved for clinical use, functional recovery is rarely achieved. To improve on the entubulation model of peripheral nerve regeneration, numerous studies have suggested that conduit fillers may improve nerve regeneration by providing a physical substrate to better guide the regenerating axons. In addition, hollow tubes have been shown to collapse *in vivo* because of the thin walls and lack of internal support. Frequent movement in a dynamic environment coupled with muscle contraction, body weight, and surrounding scar tissue formation have all been a cause for hollow tube collapse. From this realization, preclinical focus shifted from the development of novel hollow conduits to the development of bioactive luminal fillers to place inside existing hollow tubes.

The injection of a biodegradable hydrogel into the lumen of a hollow TENG is a simple method to provide a physical substrate for the regenerating nerve. Keratin gels have been used to fill silicone hollow tubes for the repair of 4 mm sciatic nerve defects in mice, and results showed that in keratin-filled conduits, nerves had lower conduction delays, greater amplitudes, more myelinated axons, and larger axons than nerves regenerated through empty conduits after 6 months of implantation (Apel et al., 2008). Motivated by these results, the same group evaluated the regenerative capabilities of keratin-filled collagen tubes in the repair of a 2 cm rabbit sciatic nerve defect (Hill et al., 2011). Similar to previous studies, the use of keratin as a filler resulted in a significant improvement in conduction delay, amplitude recovery, and myelin thickness when compared with empty conduits but was not as successful as autograft controls. Keratin-filled collagen conduits were also evaluated in the repair of a 1 cm nerve gap in nonhuman primates (Pace, Plate, Mannava, et al., 2013; Pace, Plate, Smith, & Van Dyke, 2013). The results showed that keratin-filled collagen tubes improved the return of CMAP latency and baseline NCV when compared with saline-treated nerves. Larger nerve areas and higher myofiber densities were also reported for the keratin-filled conduits when compared with saline controls.

In addition to keratin, silk fibroin peptide (SF16) hydrogels have been used to fill silicone tubes and studied in the repair of 10 mm rat sciatic nerve defects. Animals treated with SF16-filled conduits showed significant improvements in amplitude recovery, axon density, average axon diameters, and thicker myelin when compared with animals treated with saline-filled silicone conduits (Wei et al., 2013). Hyaluronic acid and collagen composite gels have also been used to fill collagen conduits in the repair of rabbit facial nerves (Zhang et al., 2008). The hyaluronic acid/collagen composites were first seeded with neural stem cells and cultured for 3 days in the presence of neurotrophin-3 before implantation. After 12 weeks, the composite conduits showed comparable nerve fiber arrangements to uninjured rabbits with improved electromyography and electrophysiology results. A study by Oliveira et al. compared the benefits of bovine tendon collagen and rat-tail collagen as a filler for polyethylene conduits (Oliveira, Vidal, & Langone, 2005). Six weeks after implantation to repair a 6 mm sciatic defect rat model, the bovine collagen-treated animals showed that bovine tendon collagen significantly increased axon numbers in the conduit because of the ability of the collagen to self-organize. Yu and Bellamkonda (2003) have evaluated whether a polysulfone tube filled with an agarose gel, which was modified to present LN-1 and NGF, could enhance nerve regeneration in a 10 mm rat sciatic nerve gap. Two months after implantation, the gross morphology of the regenerated nerve, success rate of regeneration, and the total number and density of myelinated axons in the modified agarose-filled conduit matched those of rats treated with nerve autograft. Functional measurements such as relative gastrocnemius muscle weight and sciatic functional index in the agarose-modified conduits were also comparable to autograft controls, indicating that hydrogels modified with neurotrophic factors can be used to fill hollow TENGs to mediate neurite attachment and growth.

Fiber Extrusion

Although filling the inside of a hollow tube with biodegradable gels has shown promise, these strategies still do not provide the necessary internal surface area for a dense population of nerve fibers and Schwann cells to occupy the conduit. To increase the surface area of the luminal filler for hollow TENGs, many groups have considered textile manufacturing methods to fabricate longitudinally aligned microfibers in attempts to replicate the basal lamina structure of peripheral nerves. These fiber-filled tube designs enhance axonal growth by inducing the formation of bands of Büngner, which have ultimately allowed researchers to

increase the repairable gap length. Fiber extrusion is a fabrication technique based on the extrusion of a polymer melt or solution and can be divided into three techniques: (1) Melt spinning is a process in which a polymer is heated to its melting point, extruded through a spinneret under high pressure, and collected onto a rotating drum to form continuous fiber strands. (2) Dry spinning involves dissolving a polymer in an organic solvent to form a polymer solution, which is then extruded through a spinneret and immediately heated to remove the solvent. (3) Wet spinning is similar to dry spinning except that the polymer solution is extruded in a coagulation bath to remove the solvent. In all three methods, the spinneret controls the ultimate shape and size of the resulting fibers. Although micron-sized fibers can be produced at high speeds and volumes, the limited number of polymers that can be extruded restricts this method.

Medical sutures are extruded fibers that have been studied as filler for hollow tubes. In this study, 10-mm gaps in rat sciatic nerves were bridged using a TENG consisting of a hollow silicone containing seven longitudinally placed sutures to compare the regenerative effects of polyamide, polydioxanone, or polyglactin sutures with a diameter of 250 μm (Terada, Bjursten, Papaloizos, & Lundborg, 1997). After 6 months, the resorbable sutures showed significantly higher axon numbers when compared with the nondegradable polyamide sutures. Using the same strategy, a nondegradable silastic tube was filled with extruded bioactive Bioglass 45S5 fibers (Bunting, Di Silvio, Deb, & Hall, 2005). The resulting TENG was evaluated *in vivo* across a 0.5-cm gap in the sciatic nerves of adult rats. After 4 weeks of implantation, the authors report that axonal regrowth was indistinguishable from that which occurs across an autograft.

Until now, studies have focused on the use of nondegradable tubing to contain the extruded fibers. To improve these designs, porous hollow PLLA tubes have also been fabricated by extruding a polymer/salt solution, which showed comparable nerve fiber densities to isografts after 16 weeks of implantation to repair a 12 mm sciatic nerve defect (Evans et al., 1999). Yoshii et al. have reported on the effects of the total number of extruded collagen fibers on the repair of rat sciatic nerve defects up to 30 mm in length (Yoshii & Oka, 2001; Yoshii, Oka, Shima, Taniguchi, & Akagi, 2002, 2003). Two thousand or four thousand collagen filaments 20 μm in diameter were inserted into the lumen of a collagen tube and stabilized with poly(ethylene glycol diglycidyl ether) and ultraviolet radiation. After 12 weeks of implantation, no significant difference in the average mean diameter of regenerated axons was seen, but the number of regenerated myelinated axons was much higher when more fibers were used to fill the conduit. Poly(glycolic acid) (PGA) fibers have also been used to fill the lumen of chitosan tubes (Wang et al., 2005). After 6 months of implantation to repair a 30 mm canine sciatic nerve defect, the nerve trunk was reconstructed with restoration of nerve continuity and functional recovery. In addition, the target skeletal muscle was reinnervated to improve the locomotion activities of the operated limb.

Extruded gelatin fibers have also found use as filler for hollow TENGs (Gamez et al., 2004; Gamez, Ikezaki, Fukui, & Matsuda, 2003). Hollow photocross-linked gelatin tubes filled with neurotrophic factors in combination with extruded gelatin fibers showed the highest regenerative potential in terms of functional recovery, electrophysiological responses, and tissue morphological regeneration in the repair of a 10 mm rat sciatic nerve defect after 1 year of implantation. Although significant improvements in nerve regeneration with larger gap sizes have been reported, the previous strategies have mainly been designed without the use of cells. Studies led by Gu set out to determine whether the regenerative potential of a fiber-based TENG could be improved with the incorporation of MSCs (Ding, Wu, et al., 2010; Xue et al., 2012). PLGA-extruded fibers were used to fill chitosan tubes, seeded with MSCs, and implanted in a 60 mm sciatic defect in canines. At 12 months, behavioral analysis, electrophysiology, retrograde fluorogold tracing, and histological examination showed that regeneration and functional recovery in TENGs seeded with MSCs were similar to those of autografts and better than those of scaffolds alone. From these studies, it can be seen that an improvement in regenerative effects can be obtained when using fiber-based strategies for peripheral nerve regeneration due to the increased surface area for cell growth and their ability to direct longitudinal cell movement through contact guidance.

During the course of regeneration, the dynamic mechanical forces acting on the TENG and fibrous tissues surrounding the implanted guide can exert compressive forces on the guide causing either collapse or kinks, which can hinder nerve growth in the lumen of the conduit. To address this concern, many groups have designed TENGs composed of multiple fibers braided or knitted into a tubular design resulting in conduits of a highly flexible nature and resistant to kinks during movement. Many biodegradable fibers including PLLA, PGA, PLGA, and PET have been extruded and subsequently braided together (Bini, Gao, Wang, & Ramakrishna, 2005; Lim, Kim, & Park, 2012; Yuan et al., 2009). For example, PLLA and PGA fibers have been braided together into a tubular shape and coated with collagen to improve biocompatibility. The braided copolymer tube was evaluated in a 30 mm sciatic canine defect, and after 12 months of implantation, the braided tube successfully maintained luminal space, contained a higher number of axons in the distal end, and had significantly higher muscle action potentials when compared with solid PGA/collagen tubes.

Phase Separation

Of all the discussed fabrication techniques, *phase separation* is a relatively easy, quick, and very popular fabrication method to increase surface area and provide the optimal structural and cellular framework for cell migration across the nerve gap. Phase separation is a technique in which a highly porous polymer foam structure is produced when a homogeneous polymer solvent solution becomes thermodynamically unstable under a temperature reduction, which causes the mixture to separate into a polymer-rich phase and a polymer-lean phase. The polymer-rich phase solidifies to form a honeycomb-like matrix while the polymer-lean phase forms pockets of ice crystals resulting in pores after solvent removal. The phase separation technique is very advantageous because highly porous structures with up to 98% porosity can be produced with nanofibrous features within a relatively short time period, thus circumventing the long fabrication times associated with fiber-based techniques. In addition, high temperatures are not required in many instances; this allows for the incorporation of temperature-sensitive bioactive substances. However, disadvantages with phase separation are the difficulties in controlling the pore size and the necessary high polymer molecular weights, which limit the available biomaterials for fabrication.

Numerous studies have been reported using phase separation fabrication methods for peripheral nerve engineering. For example, nanoporous PLLA scaffolds have been developed for nerve tissue engineering and used to differentiate nerve stem cells. PLGA/chitosan mixtures have also been phase separated into highly porous constructs to differentiate BMSCs with the aid of NGF (Kuo, Yeh, & Yang, 2009; Yang et al., 2004). Porous chitosan rods have also been fabricated using phase separation and later encapsulated by an electrospun PCL mesh to improve on the compressive strengths of the TENG (Behrens, Glasmacher, Duda, & Haastert-Talini, 2013). Cylindrical-shaped scaffolds have been cut from alginate phase-separated sponges and compared with collagen sponges in a 10 mm rat sciatic nerve defect (Hashimoto et al., 2002). Eight weeks after implantation, very few myelinated axons were present in any of the groups, but myelinated axons similar to those of a normal nerve were present after 21 months. Much better nerve regeneration was also found in the alginate sponge in comparison to collagen sponges.

Phase separation has also been used in conjunction with other fabrication techniques such as mold casting to produce highly porous cylindrical-shaped scaffolds. Photocross-linkable hyaluronic acid tubular conduits were fabricated by pouring a hyaluronic acid solution into custom molds and phase separating to create porosity within the tube walls (Sakai et al., 2007). Novel bioactive TENGs have also been fabricated by phase separation of a PDLLA solution inside a custom mold followed by the electrostatic self-assembly of chondroitin sulfate and chitosan by dip coating to enhance the bioactivity of PDLLA (Xu, Yan, Wan, & Li, 2008). GDNF and laminin have also been incorporated into the tubular chitosan phase-separated scaffolds (Patel, Mao, Wu, & VandeVord, 2009). A rat sciatic nerve injury model was used to test these nerve guides, and histologically after 6 weeks, the axon area and myelination were significantly higher in the GDNF group compared with the controls but were not significantly different at later time points, indicating that the bioactive scaffolds can enhance the nerve regeneration process in early stages. GDNF has also been incorporated into PLGA phase-separated scaffolds produced in a custom mold, which were subsequently seeded with primary rat Schwann cells (Bryan et al., 2000). The resulting TENGs were used to repair a 1 cm rat sciatic nerve defect, and after 12 weeks of implantation, histological studies revealed a reduction in the total axon count and the number of myelinated axons in TENGs seeded with exogenous Schwann cells when compared with saline controls. In contrast, the addition of GDNF alone enhanced the total number of axons and significantly increased the number of blood vessels present within the TENG. Although combining GDNF with Schwann cells negated the enhanced numbers of axons and blood vessels seen with glial growth factor alone, this combination resulted in the highest myelination index and the fastest conduction velocities.

In a similar study, highly porous chitosan tubes were created using phase separation and seeded with BMSC-derived Schwann cells and compared with constructs seeded with sciatic nerve-derived Schwann cells in the repair of a 12 mm sciatic defect in rats (Ao et al., 2006, 2011). After 3 months, the mid-shank circumference, NCV, average regenerated myelin area, and myelinated axon count in TENGs seeded with BMSC-derived Schwann cells were similar to those treated with sciatic nerve-derived Schwann cells but significantly higher than those bridged with phosphate buffered saline-filled conduits. Phase-separated scaffolds have also been fabricated by Bryan et al. to study the effects of electrical poling on neurite outgrowth and nerve regeneration (Bryan et al., 2004). Neuro-2a cells were seeded and poled on PLGA phase-separated TENGs for various time periods and then implanted to bridge a 1 cm gap in the rat sciatic nerve. After 4 weeks, nerves regenerated through poled guides displayed a significant increase in conduction velocity and number of axons across the guides when compared with nerves regenerated through unpoled TENGs, showing that electrical poling can promote neurite growth, axon regeneration, and the conduction rate of the repaired nerve.

A very advantageous aspect of phase separation in peripheral nerve engineering is the ability to create

longitudinally oriented channels throughout the length of the guide by gradually lowering the temperature of the polymer solution using a defined temperature gradient and constant cooling rates. By controlling the freezing process, one can control the direction of polymer-lean ice crystal growth into columnar shaped structures termed *unidirectional phase separation*. Following removal of the polymer-lean phase, a large number of longitudinally oriented channels are created throughout the TENG, which mimic the native bands of Büngner to guide axonal growth and enhance target reinnervation. A number of studies have been published using unidirectional phase separation with a variety of materials including collagen (Mollers et al., 2009), chitosan (Ao et al., 2005; Huang et al., 2010), alginate (Francis et al., 2013), PHB (Khorasani, Mirmohammadi, & Irani, 2011), PLLA, and PLGA (Khorasani, Mirzadeh, Talebi, Irani, & Daliri, 2009; Ma & Zhang, 2001; Yang, Qu, et al., 2006). Bozkurt et al. have published a series of reports using collagen unidirectional phase-separated scaffolds to show that primary dorsal root ganglion and Schwann cells can infiltrate the TENG in an oriented fashion (Bozkurt et al., 2007, 2009). Because of these results, the group then set out to evaluate the TENGs in the repair of 2 cm rat sciatic nerve defects. The results showed that unidirectional phase-separated TENGs seeded with Schwann cells promoted the alignment of Schwann cells in a columnar fashion within the longitudinally oriented microchannels. This cellular arrangement was not only observed prior to implantation but at 1 week and 6 weeks after implantation (Bozkurt et al., 2012).

To improve the protein attachment onto synthetic TENGs, Ding et al. added a mixture of collagen, gelatin, and nanosilver into silicone tubes for unidirectional phase separation (Ding et al., 2011; Ding, Luo, Zheng, Hu, & Ye, 2010). The resulting TENGs, which showed enhanced laminin and fibronectin adhesion, were implanted into rabbits to repair a 10 mm injury of the sciatic nerve. After 30 days, the nanosilver-containing TENGs showed a higher rate of laminin adsorption, thicker myelin sheath formation, and improved conduction velocity and nerve potential amplitude when compared with TENGs without nanosilver. A series of publications led by Luo has reported on the development of a collagen/chitosan TENG fabricated using unidirectional phase separation. The group was able to show that the collagen/chitosan blends could repair a 15-mm-long sciatic nerve defect in rats without the exogenous delivery of regenerative agents or cells (Hu et al., 2009). The scaffolds were then seeded with Schwann cells and evaluated in the same animal model, which showed that axonal regeneration and functional recovery in scaffolds seeded with Schwann cells was superior to bare scaffolds (Zhang et al., 2013). In a separate study, the same group incorporated a peptide, which carried RGD sequences, into the TENG surface by a chemical method. The coated scaffolds were used to repair a 15 mm sciatic nerve defect in rats. Four weeks after implantation, a linear growth of axons in the longitudinal structure was observed, and the number of regenerated axons remarkably increased. Two months later, the scaffold was partly absorbed and replaced by large quantity of regenerated axons (Xiao et al., 2013). To determine whether electrical stimulation regimens could enhance nerve regeneration in these TENGs, PPy nanoparticles were incorporated during the unidirectional phase separation process (Huang et al., 2012). Intermittent electrical stimulation was applied to the conductive scaffolds and later used to bridge a 15 mm sciatic nerve defect in rats, which showed that axonal regeneration and myelination of the regenerated axons was significantly enhanced by electrical stimulation. In addition, both motor and sensory functional recovery were significantly improved and muscle atrophy was partially reversed by electrical stimulation.

Mandrel-Based

Microfabricated TENGs have shown great potential for improved function based on the mechanisms of contact guidance and basement membrane microtube theory, which hypothesize that axon elongation requires guidance by contact with the appropriate substrate through topographical control. However, previous design strategies such as fiber extrusion, electrospinning, and phase separation place strict requirements on the biomaterial, limiting the available materials that can be used for fabrication. To overcome this issue, novel fabrication methods that have been introduced provide highly porous internal matrices and allow for cellular control through contact guidance without limiting the available biomaterials. Temporary mandrels have been introduced in combination with mold-casting techniques to produce longitudinally oriented channels, which better recreate the highly oriented architecture needed for appropriate cellular alignment and promote the body's natural pattern of growth (Brayfield, Marra, Leonard, Tracy Cui, & Gerlach, 2008; Flynn et al., 2003; Lu, Simionescu, & Vyavahare, 2005; Zhang & Yannas, 2005). In addition to being architecturally biomimetic, these multichanneled TENG designs are also advantageous in providing improved nerve target reinnervation, a larger surface area for cell growth allowing for denser populations, and internal support for improved mechanical strength (Bender et al., 2004; Flynn et al., 2003; Hu et al., 2009; Huang, Huang, Huang, & Liu, 2005; Krych et al., 2009; Li, Rickett, & Shi, 2009).

A wide variety of materials has been used as mandrels. For example, heat granulated sucrose can be caramelized into fibers and dip coated into a polymer solution. After soaking in water to dissolve the sugar fibers, longitudinally oriented microchannels 8-100 μm in diameter were produced (Li et al., 2009). Extruded PCL fibers, which were embedded into a poly(2-hydroxyethyl methacrylate)

solution and subsequently photocross-linked, have also been used as a mandrel (Flynn et al., 2003). After sonication in acetone, the PCL fibers were dissolved, leaving behind 100-400-μm-diameter channels. In a similar technique, polystyrene and poly(methyl methacrylate) fibers have been dip coated in agarose and then dissolved in tetrahydrofuran (Lynam et al., 2011). Poly(vinyl alcohol) extruded fibers were similarly used as a mandrel to create PCL multichanneled TENGs (Bender et al., 2004). Sutures have also been used to create microchanneled PCL scaffolds using a mandrel, electrospinning, sheet-based fabrication technique. PCL fibers were electrospun onto sutures to make a thin scaffold sheet and rolled into a tube. The sutures were then removed to create multichannels 33-176 μm in diameter.

Acupuncture needles have also been used as a mandrel in multichanneled TENG design because of their resistance to corrosion, sterility, and wide range of available sizes. Multichanneled and highly porous chitosan and PLLA TENGs have been recently developed using a combination of phase separation and acupuncture needle mandrel-based techniques (Sun et al., 2012; Wang et al., 2006) but have not yet been evaluated *in vivo*. Jian Yang's lab has recently reported on the development of a mechanically compliant multichanneled TENG based on cross-linked urethane-doped polyesters (CUPE), which are a biodegradable family of strong, soft, highly elastic, and biocompatible materials (Dey et al., 2008; Tran et al., 2013). The multichanneled TENGs were fabricated using a combination of mold-casting, particulate-leaching, mandrel-based, and dip-coating techniques in which a CUPE/salt solution was cast between acupuncture needles held in place with micromachined titanium shims (Figure 60.3). Next, the entire construct was dip coated with a CUPE solution to create a nonporous outer sheath, which provides a suitable surface for surgical implantation via suturing and the necessary scaffold mechanical strength for dynamic environments *in vivo*. One major advantage of this approach is that the number, diameter, and spatial distribution of the channels can be finely controlled through computer-aided design of the titanium shims. The CUPE nerve guides were evaluated *in vivo* for the repair of a 1 cm rat sciatic nerve defect. After 8 weeks of implantation, the CUPE TENGs displayed fiber populations and densities comparable with nerve autograft controls.

Rapid Prototyping

Rapid prototyping is a group of techniques used to generate highly precise and intricate scaffold structures directly from computer-aided design data. These techniques are considered to be a bottom-up approach in which the resulting scaffold is constructed in a layer-by-layer manner. A wide variety of rapid prototyping techniques has been developed for other areas of tissue engineering (Landers, Hubner, Schmelzeisen, & Mulhaupt, 2002; Yang, Leong, Du, & Chua, 2002; Yeong, Chua, Leong, & Chandrasekaran, 2004); however; only a few TENGs have been produced using these methods. Cui et al. developed a double-layer polyurethane-collagen hollow conduit for peripheral nerve regeneration using a novel double-nozzle, low-temperature deposition manufacturing (DLDM) system (Cui et al., 2009). The DLDM system is based on a digital prototyping approach and phase separation. What is unique about this

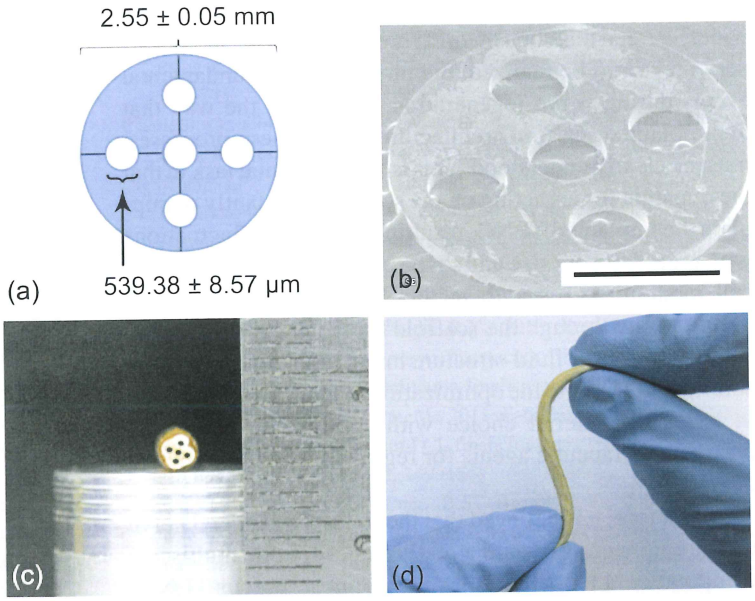

FIGURE 60.3 (a) Design and geometry of titanium shims and (b) overall scanning electron microscope (SEM) image (scale bar 1 mm). (c) Photograph of a multichanneled cross-linked urethane-doped polyester (CUPE) TENG cross section and (d) multidirectional bend without kinks to show the material's soft and elastic nature. *Reprinted from Journal of Biomedical Materials Research. Part A DOI: 10.1002/jbm.a.34952, Tran RT. et al., Fabrication and characterization of biomimetic multichanneled cross-linked-urethane-doped polyester tissue-engineered nerve guides, 2013, with permission from John Wiley and Sons.*

system is that two different biomaterials with dissimilar properties can be simultaneously combined into a single construct. For example, a bilayered hollow tube can be fabricated with collagen in the luminal layer to improve biocompatibility and with a polyurethane outer layer for improved mechanical strength. The DLDM system has high forming precision and has the ability to control the resulting wall thickness while maintaining a tight and seamless integration between the two layers of differing materials.

Nerve regeneration scaffolds have also been fabricated using ink-jet technology. Ink-jet fabrication methods are an attractive rapid prototyping option because of the incorporation of data-driven, noncontact approaches, which enable precise volumes of materials to be deposited with high speed and accuracy at the target site. PLLA and PCL copolymer hollow tubes were fabricated for nerve regeneration using an ink-jet system that digitally controlled the various manufacturing parameters (Radulescu et al., 2007). Brayfield et al. developed a scaffold for controlling specific neuronal cell body and axonal process outgrowth by using excimer laser ablation modifications of microporous polyethersulfone (PES) hollow fibers (Brayfield et al., 2008). The excimer laser ablation was used to generate 5-μm-diameter channels within the walls of PES hollow fibers to compartmentalize the growth of neuronal cell bodies from their axonal processes. It is hypothesized that the compartmentalization of neuronal cell bodies from their axons can lead to a more controlled synaptic network between cell bodies within the scaffold.

FUTURE DIRECTIONS AND CONCLUSION

Although the past decades have witnessed an accelerated development in peripheral nerve regeneration strategies, the limited functional outcomes of these tissue-engineered approaches have delayed their clinical application. It is widely agreed that for a medical breakthrough for peripheral nerve repair to be realized, the molecular and fundamental events during nerve regeneration, as well as the way that the implanted scaffold architectures affect these processes, must be clearly understood. This is a monumental task in that the dynamic forces acting on the scaffold constantly manipulate the implanted TENG, resulting in periodic fluctuations with regard to pore size and shape. As TENGs are filled with cell medium and growth factors, the mechanical loading induces flow through the scaffold's porous microstructure. This is a complex fluid-structure interaction problem whose solution is the key to the optimization of the scaffold's design in terms of material choice with coordinated control of porosity, therapeutic agents for regrowth, and programmed degradation.

Therefore, it is believed that the field of peripheral nerve tissue engineering would greatly benefit from multiscale computational tools to design and optimize porous TENGs for peripheral nerve regenerative therapies that are capable of assisting in material and microfabrication process selection, as well as in fine-tuning material microarchitecture. Predicting how a scaffold's microarchitecture and its degradation over time determine diffusion, transport, and mechanical properties is the key to the rational design and optimization of TENGs. That is, knowing what initial microstructure yields desired macroscopic properties and how these change over time can help determine the selection of a material and its fabrication processes. The computational techniques required to achieve this capability are multiscale going from molecular dynamics up to the continuum level. Although pieces of this technology exist, its full-scale realization and validation are years into the future.

In addition to developing these computational models, challenges also remain in designing scaffolds capable of supporting and delivering multiple growth factors. Whereas previous studies have shown that growth factors have pleiotropic effects on cells, the appropriate dosing, timing, and combination of growth factor release and ability to accomplish this in an *in vivo* setting remain a significant challenge to the success of a TENG. To address this, embryonic stem cells and pluripotent cells will likely have a major impact on advancing the field from its current position by using the cells to secrete their own coordinated cascade of growth factor release. Although promising, these strategies do not circumvent the long *in vitro* culture times needed, thus limiting their off-the-shelf availability. Exciting new research in chemotactic strategies that use chemical signals to recruit pluripotent cells from the surrounding defect area have been reported. These strategies are believed to offer great potential in addressing these concerns (Chamberlain, Fox, Ashton, & Middleton, 2007; Schantz, Chim, & Whiteman, 2007). In any event, similar to other developments in different fields of tissue engineering, we are headed for a future filled with exciting new developments.

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