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Polymeric scaffolds for three-dimensional culture of nerve cells: a model of peripheral nerve regeneration

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Abstract

Understanding peripheral nerve repair requires the evaluation of 3D structures that serve as platforms for 3D cell culture. Multiple platforms for 3D cell culture have been developed, mimicking peripheral nerve growth and function, in order to study tissue repair or diseases. To recreate an appropriate 3D environment for peripheral nerve cells, key factors are to be considered including: selection of cells, polymeric biomaterials to be used, and fabrication techniques to shape and form the 3D scaffolds for cellular culture. This review focuses on polymeric 3D platforms used for the development of 3D peripheral nerve cell cultures.

Introduction

All species experience a reaction to a given action; be it physical, chemical, and/or physiological. The reaction could be a visible change, or something occurring at the atomic level. The nervous system is the platform that communicates, transfers, and integrates the

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Conflict of Interest

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details for regulation and control of each of their biological actions, reactions, and the events happening in the middle. This occurs via a dedicated set of cells (e.g., neurons, glial cells) that together constitute the neuron fibers, fascicles, nerve ramifications, and overall nervous system which interconnects everything in the body. A nerve may be damaged due to severe nerve injury (neurotmesis or axonotmesis)^[1] or neurodegenerative diseases (peripheral neuropathy),^[2] both requiring medical intervention. Currently, healing and recovery of the nerves' fundamental performance remains limited: even with a successful nerve reconstruction resulting from an autologous nerve transplantation. Autologous nerve transplantation is the first line therapy (the "gold standard") as it is ready to address complicated nerve gaps resulting from a serious wound. It is non-immunogenic, and provides an immediate nerve bridge that contains viable Schwann cells (SCs) with the correct growth factors for axonal renewal. As part of the current challenges and opportunities that bound this approach there is: the potential need for a second surgery, the scar and/or neuroma formation, the inferiority against the original nerve, and functional loss (only 51.6% may gain a motor recovery, and 42.6% may obtain sensory healing).^{[3]–[5]}

In United States, damage to the nervous system is impacting approximately 20 million people,^[4] and implicates around \$150 billion in annual health-care expenses.^[5] Therefore, significant efforts and resources for the studies of nerve regeneration strategies have been invested, including a deep fundamental understanding of the regeneration process using cellular models.

Platforms for 3D cell culture have been developed in order to mimic the native peripheral nerve system (PNS) cell growth and function, in order to study tissue repair or diseases of the nervous system (e.g., Parkinson, Alzheimer).^{[6]–[8]} There is a need to introduce better approaches for therapeutic nerve regeneration. 3D cell culture models provide a practical and empirical way of observing cell activity and enable the quantification of the possible outcomes under different controlled conditions. The properties and functions of every cell in a distinct body are already encoded within the cells, at a level of detail that organizes and supports the complete sequence of biological events for the development of cells to their mature stage with a specific role.^[9] In the case of a damaged cell and/or tissue, it is expected that with the appropriate conditions the cells will activate and respond to specific signals to perform their role in the reconstruction and regeneration of damaged tissue.^{[10],[11]} 3D cell culture provides models to study the underlying biology of peripheral nerve remodeling, and gives insight on the response to novel therapies or strategies for tissue repair. Although the focus is 3D cellular model for peripheral nerve regeneration, the study of 2D models can be advantageous as they are mostly composed of flat and rigid surfaces and are more manageable for cell observation thus facilitating the study of topographical cues.^{[12]–[14]} The main drawback of 2D models is that they do not represent the naturally 3D environment of cells, leading to nonpredictive data for in vivo experiments.^{[12],[13]}

Experimental strategies are focused on modeling the ideal settings to provide the appropriate environment for neurons in an effort to restore a level of performance comparable prior to any injury. Research in regenerative medicine and tissue engineering uses a variety of polymeric devices to offer a suitable 3D cellular arrangement that supports neuronal growth and maturation to produce modern healthcare alternatives.^[15] 3D cell culture models will

precede in vivo developments. The general approaches for 3D cellular models are relevant as they are closer to in vivo systems, and enable a more profound perspective of the cell development and fate characteristics (e.g., via imaging, molecular biology and genomics/transcriptomics techniques, and other analytical techniques); thus, ensuring the proposed designs, treatments, and potential benefits are well understood before any test subject is exposed to measured risks, or otherwise unwanted effects.^[8]

For example, many authors choose the sciatic nerve as the model test tissue after in vitro testing using 3D cell culture model. Sciatic nerve is the biggest nerve in the human body,^[16] which facilitates management and visibility of the studies. Sciatic nerve is associated to multiple pain related diseases, such as sciatica, which is caused by irritation of the nerves in the lower spine in consequence of lumbar spinal stenosis (narrowing of the spinal canal in the lower back), degenerative disc disease, etc.^{[17]–[21]} Also, the fact that it extends from the spinal nerve in the lower back via many ramifications into peroneal and tibial nerves all the way to the toes, makes it ideal to model different types of nerve tissue regeneration. Rat serve as the primary animal model to study the sciatic nerve.^[22]

Designing 3D cellular models used for peripheral nerve regeneration is challenging due to the complexity of the nervous system.^[23] The role of the nervous system cannot be underestimated because it interconnects many different tissues throughout the body (as the sciatic nerve example). Neurons contain critical signals that will affect further systems, sensory and motor functions, and overall health.^[24] Growth factors (GFs) are key elements on the cascade of events to promote axonal advancement because of the interactions with surrounding cells and extracellular matrix components.^{[19],[25],[26]} Neurotrophic factors (NTs), promote nerve cell proliferation, neurite outgrowth, or neuronal cell differentiation.^[27] NTs are: nerve growth factor (NGF), brain-derived growth factor (BDNF), glial cell-derived neurotrophic factor (GDNF), NT-3 and NT-4/5.^[28]

In this prospective article, we reviewed recent works on 3D cell culture models for nerve regeneration, and present some of the knowledge gained – including materials, fabrication techniques, and analytical methods. We will begin this review with an overview of neuronal biology, followed by a literature review of recent works on 3D cell culture models.

Peripheral Nerve Biology

There are two (2) areas defining the nervous system, the central nervous system (CNS) consisting of the brain and the spinal cord; and the peripheral nervous system (PNS), which is distributed along a large network of neurons connected to the tissues, organs, and anatomical systems in the body that sustain life. The nervous system is the channel for inputs and outputs (stimulus and a response) which enables organisms to become aware of their environment and strive about it. The nervous system manages all the actions in a body, either voluntary or involuntarily. For this, different types of neurons are continuously active: the afferent neurons, which obtains information from the five senses, and pass it to the CNS; the multipolar interneurons, which relay messages between other neurons within the CNS; and the efferent neurons, which drive our motor skills via the PNS. If a patient suffers an efferent fiber injury, they may not be able to move the innervated tissue because the brain

impulse would not relay to the muscle. Likewise, if the impairment happens in an afferent fiber, the brain will not sense stimulus within the environment. As nerves carry both types of neuron fibers, a combination of efferent and afferent fibers are affected by injury.

There are many levels of complexity among voluntary and involuntary reactions that are made possible by the nervous system. Identifying a situation, deciding what to do next, and performing an action is an example of the communication of the brain and the body, and the velocity of this process will vary on other factors not in the scope of this discussion. However, there are some “closed loops” where afferent fibers are directly connected to efferent fibers, and an impulse does not necessarily reach the brain (e.g., reflexes, equilibrium, and other involuntary actions).

Nerves are formed as a cascade of conduits inside other conduits.^[29] The nerve is surrounded by a protective tissue known as the epineurium; inside of it are the blood vessels and neuron bundles along with conduits called fascicles. As illustrated in Fig. 1, the neurons can carry impulses or action potentials to the soma, then propagate the signals along the axon and axon terminals. The axons are protected by a myelin sheath produced by SCs located within the endoneurium. Early regeneration of the axon is what prompts the restitution of the nerve function.^[30] There is some regenerative ability in the nervous system,^[31] and this is true specifically in the PNS as it is exposed to macrophages that clear damaged tissue; and later assisted by SCs, which guide axon regeneration and provide the myelin sheath to support the re-connection between any gap.^[32]

The PNS nerves branches out of the CNS to sense and collect input signals, and it is also responsible for transmitting the commands that controls muscle movement. The biochemical explanation of neuronal impulses, also known as potential actions, is described as an electrochemical wave propagated within the surface membrane of a neuron. The initial stimulation of neuron, opens channels leading to sodium (Na⁺) influx that depolarizes the membrane. The signal propagates as the adjacent section of membrane allows Na⁺ influx and depolarization. This wave of depolarization continues at a rapid rate down the neuron, resulting in a nerve impulse traveling inbound (afferent, sensory) and outbound (efferent, motor) to or from the brain in milliseconds.^[29]

After a severe nerve injury, the axon separates from the soma and, cytokines/GFs surrounding the injury will recruit and activate macrophages which then infiltrate the site of injury to clean-up axon and myelin debris – this is known as Wallerian degeneration.^{[16],[33]} Wallerian degeneration is a controlled phase that extends to the following node of Ranvier, which is a gap in the myelin sheath that is exposed to the ECM. Wallerian degeneration stops the synapse and communication between the neural networks leading to atrophy of the associated muscle or gland, which could become permanently deteriorated if the axon fails to re-establish connectivity.

The neuron gap is defined as two parts: the proximal stump closest to the soma and distal stump that will degenerate. After macrophages remove the degenerated debris, the soma grows as the nucleus migrates towards the cell boundaries, and the local non-myelinating SCs at the endoneurium align to guide axon regeneration. A regenerated axon sprouts from

the proximal end, and grows to join the two ends to reestablish the network connection. To ensure the proper reconnection, surgery helps to establish a guide for these sprouts leading to eventual re-innervation, either by using autologous nerve transplant or an engineered scaffold.

During nerve regeneration, the native ECM must be reconstructed. The major components of the ECM in the nervous system can be divided in four (4) groups: collagens and related molecules as structural parts (e.g., Types I, II, III, IV, and V); non-collagenous glycoproteins as binding agents of cells to mediate their effects or induce cell binding to other molecules (e.g., laminin (LN), fibronectin, entactin, and vitronectin.); glycosaminoglycans (GAGs) to facilitate cell migration, adhesion, and organization, as well as support the formation of proteoglycans by providing the chains of several functional groups (e.g., hyaluronic acid, dermatan, chondroitin, keratan, heparan); and finally proteoglycans, which have been closely associated with fundamental cellular processes, cell attachment and growth, and cell receptor signaling/binding (e.g., chondroitin sulfate, heparin sulfate).^{[34],[35]}

Selection of Cells

The use of in vitro cellular models have advantageous features over in vivo models, particularly those aspects regarding technical complexity, costs, and ethical concerns.^[36] The appropriate selection of cells for the in vitro cellular models will support projections and expectations for potential in vivo models. Moreover, in vitro cellular models are not necessarily subjected to regulations as with animal or human models. The goal is to obtain representation of the subject tissues and translate the research towards clinical developments. Yet, the biological characterizations made at the laboratory barely exemplifies what is to be expected in normal conditions, regardless of the source. For this, researchers can use primary cells (cells isolated directly from human or animal tissue), or cell lines (cells that have been continually passaged over a long period of time). Cell lines are cost-effective option, but may be less physiologically representative of native cells because cell lines have been immortalized. Primary cells may be more representative of the native tissue, however, they need to be isolated from animals leading to increased oversight. Within the PNS, there is increased accessibility to obtain primary cells, such as SCs from sciatic nerve and neurons or mixed cultures from dissociated or whole dorsal root ganglia (DRG), compared to CNS cells that typically need to undergo several cell-specific purification steps. Researchers should select their test subjects strategically considering the resources available, and in a step wise approach per the progress obtained, and future developments envisioned.

As an actual cellular model for neurons, whole DRG or dissociated DRG neurons are commonly used. The DRG is obtained from a cluster of nerve cell bodies located in the dorsal root of the spinal cord, constituting afferent fibers. Their outgrowth have been demonstrated both alone or when co-cultured with SCs.^{[36]–[38]} DRGs are a pivotal test subject for experiments using growth and/or regulatory factors.^[39]

Other neural cells used for nerve regeneration studies include primary neurons sourced from the CNS, including neurons from the hippocampus, and cerebral cortex. For research purposes, these cells are mainly derived from rodents and, are very effective to profile the

basic physiological properties and behavior of neurons at the experimental conditions, including those for the PNS. Some of the physiological properties include cell reactions to scaffold composition and stiffness.^{[40]–[42]} These cells are commonly used for the evaluation of the neurotoxicity of pharmacologic compounds, since the brain areas sources for these cells are susceptible for neural toxins.^{[43],[44]}

Alternatively, back in 1976, Greene and Tischlert established the PC12 cell line (not a neural cell line, but derived from a neuroendocrine tumor of the adrenal medulla) as it has a reversible response to NGF. PC12 are a commonly used cell line for investigating cell/material interactions as well as pharmacologic studies since there is ample knowledge regarding their behavior, proliferation, and differentiation. Also, PC12 cells have a high throughput as a first level assessment tool, they are robust, and substantially easier to culture than primary or stem cells. Nowadays, the PC12 cells are a versatile resource that are continuously used for the research of polymeric scaffolds and improved 3D cell culture strategies.^{[45]–[48]}

Glial cells are the main neuronal support cells of the nervous system with the SCs (e.g., S42 cell line, or primary SC isolated from the sciatic nerve) being the most widely studied for PNS regeneration.^{[19],[21],[49]–[52]} These cells contribute to neuron pathfinding by guiding their axons between the proximal to distal stumps, and by producing myelin sheath to protect them. SCs are so important to nerve regeneration that further alternatives for them have been explored by using differentiated stem cells (ADSC or iPSC) to SC-like cells^{[14],[20],[53],[54]} or BMSC to effect nerve regeneration, either by direct and/or indirect paracrine signaling to express glial markers.^{[55],[56]} SCs can be induced from ADSCs cells by addition of glial growth factor and co-culture with SCs. A key factor when working with cell differentiation and proliferation is cell density, since it has been reported that higher cell density equals an increase of cell-cell interactions, which is also related to the influence of glial growth factor.^[57]

Alternatively to SCs, olfactory ensheathing cells (OECs) are a distinct type of glial cells found in the CNS exclusively supporting the olfactory tract nervous system. Their function is to provide myelin sheath to the unmyelinated axons of the olfactory neurosensory cells, as SCs does for PNS. Important facts about the OEC uniqueness and properties for using them in nerve repair studies is because, in nature, they continuously sustain a regeneration environment.^{[29],[58]} The olfactory neurons are regularly replaced from a population of precursor stem cells (about every 7 weeks), and OECs become more actively phagocytic to discard axonal debris and dead cells (in cell culture studies, they are capable of phagocytose bacteria).^{[59],[60]} Then OECs, together with other glial cells available at the olfactory bulb, encourage the corresponding axonal growth. During these cycles, OECs can move through glial scar tissue (important feature for damaged nerve reconnection) producing neurotropic factors (e.g., BDNF, NGF) and expressing key markers (e.g., S100, and p75),^[61] allowing researchers multiple perspectives to understand nerve regeneration via the glial cells interactions.^[62]

Now, some researchers have concentrated their attention into macrophages,^{[63],[64]} which precede the accomplishments of SCs. It has been documented that the extent of macrophage

presence at the site of injury (M2, anti-inflammatory phenotype), grants a favorable influence in nerve regeneration.^{[65]–[67]} In the native nerve regeneration process, macrophages together with fibroblasts provide the initial platform and route for the undifferentiated SCs to form clusters that eventually guide the growing axons across the gap, all the way back towards their target.^{[66],[67]} For 3D cell culture models, such types of platforms may be provided by polymeric scaffolds.

Among the preferred cells selected to investigate nerve regeneration as previously discussed, recent development uses cellular co-culture systems. Briefly, a cellular co-culture system allows further visibility of a more complex system whether to study specific interactions (synergetic or antagonist), or to evaluate multiple factors controlling cellular behavior. Co-culture can be comprised of heterogeneous cells to cells; cells with specific GFs or other pharmacologic agents; and cells to drive the behavior of other cells.^{[19],[20],[50],[51],[68]–[73]} Any use of these cells as models are possible if some type of suitable platform is available that will enable them to attach and perform their function. Therefore, 3D cell culture success depends on the matrix to be seeded, such as the polymeric scaffolds that offer the 3D structures.

Polymeric Materials in use

Polymeric materials can be divided in two (2) major groups: natural and synthetic.^[74] For scaffolds, those can orchestrate nerve regeneration and are available for use in 3D cell culture are presented in Table I. Mechanical properties can be designed and customized per intended application depending on the molecular weight and polymerization reaction (and/or copolymerization ratio) for a given polymer. Another key element for the constitution of polymers to serve their intended purpose is their cross-linking. Once polymerization is completed, the cross-linking involves the anchoring of polymer chains to attain certain properties that reinforce the final construct and influences cellular behavior. Cross-linking sets a controlled way to inhibit degradation of the polymer by enzymatic activity, or cellular remodeling, and offers a longer duration to investigate other aspects of research interest, or as long as needed for biomedical applications. For example, polymeric scaffolds (within an animal or patient) would be designed to degrade over time. Its purpose is to facilitate tissue ingrowth, and timely degradation will allow the cells to replace the entire scaffold.

Common cross-linking methods include chemical (e.g., aldehydes) or via radiation (e.g., UV, heat) enabling different levels of polymer bonding, therefore affecting cells development as their microenvironment changes.

3D Cell Culture Techniques

An appropriate environment is attained when the platform provided mimics the native ECM attributes as much as possible to support cell migration, proliferation and differentiation. It is known that the ECM regulates cell development via biochemical cues and signaling. A polymeric scaffold entails the mechanical properties to support cell attachment and its progress in a 3D space. Various preparation techniques are described:

1. Flat (Films)

One of the most important reasons to continue developing options for nerve tissue regeneration is to overcome the drawbacks of current gold standard techniques such as the autograft, for example donor site morbidity or the need for multiple surgeries. Polymeric structures may replace the use of autografts, and thus serve as ideal platforms for 3D cell culture. Flat films are often employed to study PNS regeneration because it is the most basic geometry to manage. Raimondo and colleagues,^[118] developed a flat chitosan cross-linked scaffold that later was rolled and glued with biomedical cyanoacrylate glue, obtaining a 3D structure that served as a conduit for *in vivo* test as a bridge between two nerve stumps with a 10 mm gap of the median nerve. The main component is the polysaccharide chitosan, which is very attractive for its unique physicochemical properties along with good biocompatibility, biodegradability, and antibacterial features.^{[118],[119]} Given its poor mechanical strength,^[95] a cross-linking technique, which supported glial cell proliferation, was applied using dibasic sodium phosphate (DSP) and glycidoxypolytrimethoxysilane (GPTMS). To validate this, the tests were divided into two main groups: an *in vitro* test by culturing the RT4-D6P2T cell line (a schwannoma cell line) and dorsal root ganglia (DRG) explants over the flat surface, and an *in vivo* test using 3D scaffold rolled as a conduit on long rat median nerve defect. In the case of cultured cells, 84% of the population took an elongated form and DRG showed a high neurite growth and expression of Bcl2 gene was observed after four days of culture. The adaptation of the cells to the substrate was demonstrated by expression of Bcl2 (which regulates cell survival) that was lower for 3 days, compared to the control, and after day 6 it reached a similar value to control (glass). This proves the successful combination of chitosan and cross-linking techniques to develop a competent substitute for nerve autografts. *In vivo* results, which were done with 3D scaffold, rolled as conduits, for the chitosan cross-linked with only DSP showed positive results with functional recovery at week 6 and almost comparable results with autografts at week 9, oppose to chitosan cross-linked with DSP and GPTMS that detached from suturing site due to the fragility of the scaffold.^[118]

Axonal regeneration is highly desired,^[46] and work contributes through the bioengineered LN-coated chitosan multi-walled conduit containing BMSCs was tested *in vivo* to serve as a bridge between a 10 mm long gap in the sciatic nerve of Sprague-Dawley rats. The chitosan component has been very attractive due to its biocompatible and biodegradable properties.^{[119],[120]} An overview of the chitosan film preparation consisted of lyophilized chitosan, re-dissolved on 2% acetic acid and placed on coverslip to allow the solvent to evaporate. The chitosan film was modified with plasma treatment to increase the hydrophilicity, followed by LN covalently bonded by surface loading. Three groups were tested: empty silicone conduit, LN-modified chitosan scaffolds in silicone conduit (multi-walled) and LN-modified chitosan scaffold with BMSCs in silicone conduit (multi-walled plus BMSC). The BMSC resulted to be key element during *in vivo* experiment to down-regulated the extent of inflammation, reducing hyperplasia, and for proper neuronal growth, Fig. 2 (A–C). This is also corroborated with recovery index for $74.31 \pm 26.51\%$ for the conduit of BMSC, $17.31 \pm 5.89\%$ for the chitosan group and $31.52 \pm 10.43\%$ for the empty group.^[46]

Chitosan was also the focus of the work by Meyer and colleagues,^[121] in which the main concern was the capacity of artificial nerve guides to reconstruct critical length of sciatic nerve defects (15 mm) in vivo using healthy Wistar and diabetic Goto-Kakizaki rats. The diabetic rats were included to evaluate if the effect is the same under a relevant increase of blood glucose level that resembles type 2 diabetes patients, the above is done as a control because diabetic animals are less sensitive to nerve injury and they have a slow regeneration capacity.^[122] The innovation is the introduction of the chitosan film to chitosan nerve tubes in order to achieve recovery of a critical defect length of 15 mm, since it was proven that the hollow chitosan tubes were capable to repair defects of 10 mm.^[123] The chitosan film promoted SC attachment and supported DRG neurite outgrowth in vitro,^[124] and this motivated combining the fine-tuned chitosan nerve guides (CNGs) tubes with the chitosan film. A novel outcome in diabetic rats is the presence of substantial regenerative matrix after 56 days of surgery, due to an increased number of activated SC which attracts re-growing axons. Improvement on functional and morphological results of nerve regeneration, compared to hollow CNG, states that the role of chitosan of modifying an existing product introduce a new generation of medical devices for peripheral nerve reconstruction.

PLLA has also been used to develop multifunctional films due to its biodegradability, low toxicity, and functional end groups.^{[125],[126]} The word multifunctional applies since the film possess surface micropatterns for topographical cues along with surface gradients of NGF, which it is released in a controlled manner using nanoparticles. The multifunctional films were tested in vitro using PC-12 cells as a model of peripheral nerve regeneration. An overview for the engineering of the multifunctional films consisted on a dry-phase inversion technique to develop the porous topography that allowed the distribution within the film of the micro particles CPEG:CPH [composed of random copolymers: CPTEG, CPH] that controlled the release of the growth factors. The surface gradients were developed using longitudinal micropatterns through ester-amine interactions and physical self-assembly. Fig. 3 shows a SEM image of the topography of the multifunctional films (A); and the bioactive release of β -NGF promoting neurite extension (B). Neurite extension was quantified through the device outgrowth module software used for the collection of cellular images in two different β -NGF conditions: β -NGF released from microparticles and soluble β -NGF directly added to PC-12 cells in culture media. The film promoted guided neurite extension of cells of 10 μ m total per cell in 2 days.^[119]

Similarly, Torigie et al., used a film model to study the regenerative effects of hyaluronan tetrasaccharide (HA4) on axons applied to common peroneal nerves cut from mice. Although HA is considered as a regenerative inhibitory agent in the CNS, HA can be depolymerized to specific forms of oligosaccharides containing different properties. For example, HA4, has been shown to decrease the death of K562 bone marrow cells and, increase the growth of PC-12 cells.^[127] The proximal end of the nerve was maintained in vivo between two 3×4 mm sheet of fluorine resin films sewn together and accompanied by the addition of a few drops of Ringer's Solution, thus observing the growth of regenerating axons. For the purposes of this investigation, instead of the Ringer's solution, HA4 drops were applied in the surface of the film at concentrations of 10, 100, or 1000 μ g/mL and the neurite behavior was evaluated at 6 hours, 2, 3 and 4 days later. In all groups (HA4 10, 100, 1000, and control) after six hours, terminal bulbs could be seen at the proximal tip of the

injured nerve, but only in the HA4 100 and HA4 1000 groups were extensions of terminal sprouts observed, and on day 2 the regenerating axons were spread over the film. HA4 100 was cited as promoting increased axon length with a growth rate of 268 $\mu\text{m}/\text{day}$, approximately 3 times faster than controls. From day 3, the appearance of SCs occurs which accelerate the growth of regenerating axons. At this stage, the growth of HA4 100 group was equal to that of the control and it was possible to determine that the SCs were distributed along the axons. This improvement in regeneration using HA4 is due to the reduction of the time of onset of terminal sprouting and the accelerated growth of axons, which manifests itself in response to an increase in the metabolism of neurons.^[127]

2. 3D Printing

3D printing is a novel technique that is transforming science and education and has emerged since 1980s as an additive manufacturing technology with fundamentals based on stereolithography and is also known as Rapid Prototyping (RP) or Additive Manufacturing (AM).^{[128]–[130]} 3D bio-printing is the use of 3D printing equipment combined with printing processing of biocompatible materials and cells through layer-by-layer. This technology was introduced in 1993 by Langer and Vancanti, with broad applications on tissue engineering and regenerative medicine fields.^[131] In general, the process consists of: imaging, design, selection of materials and cells, and printing of the product.^[129] The printing materials are what assigns the classification of the developed scaffolds as: solid, soft, and free scaffold.^[128] In the case of 3D scaffolds for peripheral nerve regeneration related with soft tissues and organs, these are classified as soft and free scaffolds.^[128]

One of the main advantages of 3D bio-printing technology is the capacity to develop complex structures of custom-tailored shapes and sizes as well as internal shapes, and pores that can be controlled to adjust the diffusion of oxygen, nutrients, and waste of cells.^{[132],[133]} 3D bio-printing technology promises to elaborate structures that foment cellular function, provide mechanical support and generates patient specific products.^{[128],[131]} As part of current challenges, 3D bio-printing technologies involves high working temperatures to achieve melting of materials that compromises scaffolds bioactivity, this is specifically seen on nozzle-based 3D printing technologies as well as Fused Deposition Modeling (FDM) and its variants.^{[128],[129]} Additional drawbacks are present when the equipment involves nozzle technologies which only accepts fluids with low viscosity and low cell density because of the high shear force and have issues with clogging. These challenges and limitations are overcome with laser printing, a nozzle free system, which has a wide range of working viscosity values (1–300 mPa/s) along with high cell density solutions with negligible effect on cell viability during the process.^[134] However, laser technologies also have its drawback such as: time consuming because of the necessary low overall flow rate needed for the rapid gelation, and high cost. Upon the many 3D-bioprinting strategies the most common are inkjet, micro-extrusion, and laser.^[129] 3D-bioprinting represents a risen technology for the development of patient specific products.

As mention above 3D bioprinting uses 3D printing equipment, an example is the use of a commercially inexpensive desktop 3D printer to tailored custom architecture of bio-conduits for peripheral nerve regeneration.^[73] The choice of material is crucial on 3D printing

technology specially on inkjet bioprinting, since it needs to be printed liquid and then form a solid 3D structure. 3D structure is achieved by the use of materials that can be cross-linked after the deposition by chemical, pH or ultraviolet mechanisms.^[129] This specific material requirement is the reason for the modification of gelatin which adds properties such as photocross-linking and radical polymerization. Gelatin methacryloyl (GelMA), is one of the chosen material for the desktop 3D printer application.^[73] The developed cryoGelMA, named after the conduit preparation via cryopolymerization, is turned into a bio-conduit by the introduction of multipotent adipose-derived stem cells (ADSCs) to generate local environment to foment the regeneration of axons, which is possible with the use of 3D printing. The versatility of 3D printing is taken as an advantage to engineer conduits of different geometries such as multichannel or bifurcating and even personalized structures.^{[25],[131]} To validate the conduits, an in vitro test was performed using ADSCs, and in vivo testing was performed using a rat model with a 10 mm sciatic nerve gap. The positive respond of the ASCs to the bio conduits is assured through the deposition and accumulation of extra cellular components along with the expression of neurotrophic factors such as BDNF and GDNF, all to assure axonal regeneration. It is important to highlight that 3D bioprinting does not alter the properties of the materials. The cryoGelMA conduits meet the standards of an average degradation time frame which are, complete degradation at 2–4 months, along with an inflammation time line of no more than a three-week period of acute/inflammatory, which will lead to infection.^[135] This approach is an example of the potential of 3D printing for the fabrication of a precise structure control. The use of a desktop 3D printer using “lock and key” molds, emphasizes the simplicity, flexibility and low-cost application with projections to reconstruct complex personalized nerve conduits to mimic physiological conditions and fomenting cells function to achieve an axonal regeneration.

An example of microextrusion, one of the most used techniques of 3D printing, is through the development of a silicone 3D scaffold that under the same printing process offers control over the geometry, physical and biochemical cues.^[21] The geometry is showcased by the development of a two-branched device to mimic bifurcating nerve, the physical cues via printing patterns (microgrooves), and biochemical cues through the printing of growth factors gradient, nerve growth factor (NGF) for sensory path and glial cell derived neurotrophic factor (GDNF) for motor path, on gelatin methacrylate hydrogel droplets that encapsulated the growth factors. As one of the advantages of 3D printing, this work uses the original tissue structure as template for the pathway geometry with the use of structured light scanning (SLS) technique that eventually leads to the 3D nerve model scaffold. The choice of material for the 3D neuro scaffold is silicone mainly because is a neuro compatible material, very stable with promising biodurability, and biocompatible, ideal for 3D printing microextrusion technique.^[136] The 3D conduit developed through microextrusion shows a multifunction conduit with physical, biochemical, and doubled branched device. To showcase the 3D printing capacity implementing physical and biochemical cues an in vitro testing showed that primary superior cervical ganglion (SCG) neurons followed the physical cues, measured with fast Fourier transform (FFT). In addition, it is shown that the NGF gradient serve as chemoattractant for sensory axons and the GDNF gradient increased migratory velocity of SCs from 5.1 to 12.6 $\mu\text{m}/\text{h}$. The end product consisted of a silicone hollow conduit with gradient patterns of growth factor hydrogel droplets that serve as

biochemical cues, and also physical cues by the addition of microgrooves, which mimics the 3D structure of a real bifurcating nerve, all possible with the use of 3D printing technology which elevates the versatility of this technique.

As mentioned above, the materials used to perform 3D printing are the ones that classify the type of scaffold, and an example of a free scaffold is one in which is only composed of fibroblast multicellular spheroids developing a biological tubular structure.^[137] Bio 3D printing technique is characterized by developing a complete biological tubular structure, in this case using homogenous fibroblast multicellular spheroids. A pre-designed 3D tube-like structure represents the position of the spheroids that are robotically placed into skewers of a 9×9 needle array Fig. 4 (A–B).^[137] This “needle –array” arrangement promotes multicellular spheroids to fuse and after one week are removed and cultured on bioreactor to obtained a structurally scaffold -free construct. The Bio 3D conduit was evaluated in vivo on rat right sciatic nerve, with a 5-mm gap that was bridged using an 8 mm Bio 3D conduit and control group with silicone tube. After eight weeks post-surgery, the Bio 3D conduits promoted peripheral nerve regeneration confirmed with successfully bridged through the nerve gap, in the case of silicone conduit very thin regenerated nerve was observed, Fig.4 (C–D). This is a novel example of how a three-dimensional structure for peripheral nerve regeneration is developed without the use of a foreign material with the use of 3D bioprinting.

As part of the 3D printing emerging technologies to offer more custom made specialized scaffolds, the digital micro-mirror array devices microfabrication technique (DMD) provides an important space for innovation. DMD is a technique that takes advantage of free radical polymerization of photo-curable compounds to form/print solid 3D structures. Instead of doing so via a point-by-point scanning, the DMD system does it in a layer-wise mode. In fact, Pateman et al. combined together a microstereolithography setup including a DMD system.^[50] The DMD system implements a chip that puts together micro-mirrors (1024 × 768) to reflect UV light shapes into a pre-polymer preparation and make entire layers at once (Fig. 5). These layers have high precision and resolution; achieve accurate designs (pore sizes, contours) and can be made to fit any possible model.^[138]

Not only does this technique allow freeform fabrication with unlimited alternatives to construct and mimic the nerve structure tissue, but some authors have found other breakthrough uses. For example, S. Suri and colleagues^[77] used DMD to build a gradient of fluorescent microparticles in a HA scaffold (Fig. 6), this as a proof-of-concept showed that DMD can be applied to create gradients of biomolecules. A gradient of biomolecules like NGF favors an organized and controlled nerve regeneration.^[139] Also Fig. 7 presents the succesful culture of SCs inside the 3D scaffold composed of HA, developed using the DMD technique. Cells were seeded in the scaffold lumen, to demonstrate the viability of the cells in the custom design shapes. The cells were able to spread inside the geometries and expressed S100 proteins, which indicates that the adhered cells expanded over the 3D polymeric scaffold surfaces.^[77]

It is important to make the reader aware that the virtue of the photo curable polymer demands special considerations. The main challenge confronted with the DMD techniques is

related to UV light penetration and scattering. UV light induces the free radical polymerization; however, its sensitivity is enough to make the 3D scaffold structure to change. If light goes to unintended areas, the final product will not meet expectations. As a countermeasure to aberrant UV-induced photo polymerization, Suri and colleagues controlled the exposure of UV at each layer, and used a free-radical quenching agent to limit the activation of polymer chains.^[77]

The work of Soman and colleagues demonstrated that DMD facilitated the cell culturing of neurons derived from human embryonic stem cells, and promoted the successful differentiation and guided growth of neurites to reach its target at the distal stump. This was made possible because DMD enabled the custom geometries that help the 3D interconnectivity via the scaffolds' pores of a PEG compound, which supported the alignment of neurite connections (Fig. 8). The studies were done with soft and stiff non-biodegradable PEGDA and biodegradable gelatin-based biomaterials scaffolds.^[140]

3. Freeze-drying/Porous

Freeze-drying, or lyophilization is a useful technique to transform biomaterial solutions, which in many cases is composed of delicate proteins, into a dry solid form. The main underlying principle of this technique is sublimation: the polymer to be casted into a 3D structure is dissolved into an emulsion, then the removal of water and volatile substances is done by developing ice crystals at a very low temperature - depending on the solutions' characteristic eutectic point the target temperature will vary. Then, by exposing the frozen solution to a vacuum enables the formed crystals to pass from its solid state to a vapor state directly resulting in a porous 3D polymeric structure. In this manner, drying happens without an excessive heating that could affect the involved biomolecule. The pore size of the 3D scaffold will depend on the crystals formed while the water/solvent within the polymer solution freezes. If the freezing rate is too fast, then crystals will be smaller and after the sublimation, the resulting pores will be smaller. Otherwise, a slow freezing rate will enlarge the ice crystals, and consequently bigger pores.^[141]

Some of the polymeric materials and cell lines that have been recently studied using the freeze drying fabrication technique include works with collagen and stem cells.^{[142]–[145]} Here, the researchers reported the influence that stiffness, pore sizes architecture, and composition of GAG types (e.g., HA, chondroitin sulphate) had over stem cell differentiation. This was performed to confirm the importance of the ECM components to increase or reduce the biomolecular signals in the cellular activity. Different approaches were used to enhance control stiffness for medical applications.

There are many biomaterials that usually would be a challenge to combine together, and obtain the desired biomolecular effects by design; ideally, systems of various macromolecules are thought to complement each other.^{[146],[147]} However, there are practical issues such as incompatible solvents, and/or conflicting mixing conditions (temperature) that will not facilitate their merging. For these reasons, Niu et al. were able to demonstrate that one of the main advantages of the freeze drying technique is to blend in a homogenous emulsion of natural and synthetic polymers forming CS-PLLA, and ultimately create a suitable method to develop a 3D scaffold with novel features to successfully perform

cell culture. Using the MC3T3-E1 osteoblast precursor cell line Niu et al. was able to demonstrate the cell viability, survival, and proliferation on the freeze dried CS-PLLA scaffold. Other researchers have used the same polymeric combined CS-PLLA scaffold loaded with recipient BMSCs to successfully promote nerve regeneration and functional recovery of the sciatic nerve in animal models; however, in this instance the scaffold was not freeze dried.^[148] The work of Z. Zhang and H. Cui showed that chitosan with lactic acid (PCLA) combined via the freeze drying technique resulted in a scaffold with better histocompatibility properties for BMSC than each material by itself.^[149]

Other practical issues of a co-polymeric combination prepared by freeze-drying are: biodegradation, swelling, and loss in mechanical properties. In vivo degradation is expected as part of the scaffold design; furthermore, it must be considered as part of the potential medical device application; that once its objective is achieved (after the tissue is regenerated) the body continues its natural lifecycle. Polymeric degradation can be induced by pH change, hydrolysis, and immune biocompatibility, among others. Combined co-polymers may have different degradation rates, and 3D cell culture models will enable researchers to understand and improve materials and conditions for the different healthcare needs. For that, the advanced controls that the freeze-drying technique permits regarding pore size and its interconnectivity within the scaffold matrix are favorable for studying cross-linking alternatives.^[150]

Another technique that integrates freeze drying, is the freeze casting. Freeze casting benefits from the ice crystallization phenomena, and its freezing gradient/direction to develop a microstructure architecture for the scaffolds, whether porous, layered, or custom oriented. This happens as ice grows, carries the polymer dispersion along, and form separate clusters of the polymer particulates in the space between the ice crystals, therefore forming lamellar shapes. Later, via freeze drying, the ice sublimates leaving the designed scaffold. Preparation method, solvent used, polymer concentration, and freeze drying cycle will determine the mechanical properties of the layered material. For example, particles locked in the ice crystals will convey and arrange connections between lamellae formations. The speed at which the ice grows in the freeze drying cycle, (particularly at the freezing stage) will determine the pore sizes of the future scaffold.^{[151],[152]} Francis et al investigated how to enhance axonal regeneration after spinal cord injury. They fabricated a porous chitosan-alginate scaffolds with longitudinally oriented channels using the freeze casting method, with the objective of enabling a guidance/path for the regenerating axons towards proximal and distal stump. They used DRG explants, these neural cells adhered and extended neurites through the scaffold in parallel alignment with the channel direction. Additional molecules were added (e.g., LN) to enhance the NGC properties, and characterize its potential applications.^[153]

4. Electrospun Fibers

Several authors have worked on demonstrating that nanofibers can guide neurite outgrowth along the direction of fiber alignment.^{[154],[155]} Electrospinning technique is a good alternative for this objective because nanofibers of several polymers can be prepared with an aligned structure that can be used to guide tissue regeneration.^[156] Basically, a polymer

solution is pumped through a needle that is exposed to a high voltage (Fig. 9). The solution must be received in a metallic collector, which is connected to the other end of the power supply and this generates an electric field that conducts the fiber for its deposition.^[157] The main advantage of this method is that other elements such as fiber diameter, elastic modulus, morphology and other mechanical properties can be adjusted thanks to the versatility of the technique by addition or variation of factors in the process and also, these characteristics can be determined through various techniques.^[158] One of the main method limitations is the strict humidity control inside the chamber (which also leads to temperature control). High humidity implies the fiber formation with very diverse and unstable properties or may even prevent their formation. Another important point is the viscosity of solutions prepared for electrospinning: a very viscous solution may block the needle or the induced electromagnetic field may fail to break the surface tension of the solution. If the solution is not very viscous, it flows very quickly and leaves the tip of the needle like a “spray”, which implies that the solution flow must be decreased and therefore, the elaboration of the membrane is made very slowly. The use of strong and toxic solvents becomes the main disadvantage of the method, since it is subjected to strict regulations and procedures for their disposition and waste management. Also, it is important to emphasize that electrospinning uses high voltages, which puts at risk the health of the operator and must have high safety measures like the use of protection elements (in many cases increase the cost of the process).

Linked to tissue regeneration, many authors have used electrospun nanofibrous scaffolds as structures for 3D cell cultures for a wide range of nerve cell lines, e.g., highly aligned PLLA fibers have been studied with primary neurons (DRG explants), human neuroblastoma cells (SH-EP and SH-SY5Y Cells), Schwann Cells, PC-12 cells, among others. PLLA is a material widely used for its known biocompatibility, degradability, and aligned structures to support neuronal growth. Han et al., obtained aligned electrospun fibers made from PLLA which allowed the proliferation of neurites and the communication of SCs. Electrospun scaffolds were applied in the culture of embryonic stage nine (E9) chick DRGs and rat SCs. With respect to the cells, they could perform the quantification of the density and length of the neurite outgrowth. E9 chicken DRGs cells were seeded into high and low density PLLA fibers to evaluate the effect on neurite outgrowth (cells were stained for neurofilaments). They observed the aligned fibers guided growth in a directed way, and by increasing fiber density, it increases growth by approximately 83% compared to 65% for low density fiber. It has been shown that the neurites grow once the DRG explants are attached to the fibers, and upon contact with the aligned configuration of the fiber, the neurites change their direction to grow parallel to them. Even in experiments of more than 10 days, when cells bodies have been dissociated from the fiber, neurite continues to grow in the direction of fiber. Also, highly aligned fibers favor increased neurite growth compared to less aligned fibers (20% above to randomly aligned fibers). For the SCs, they attach to the fibers and grow in a directed manner for both high and low-density fibers but higher growth rates were observed in high density fibers and their morphology becomes elongated and narrow. In the case of explants, SCs move away from them to join behind the neurites. Neuroblastoma cells were also shown to be elongated and narrow in aligned fibers oriented parallel to the fibers.^{[147],[159]} Many literatures suggest aligned fibers are important for peripheral nerve studies since it enhances the regeneration and maturation of SCs.^{[87]–[89]}

The influence of electrospun fiber size on SCs behavior has been studied through the manufacture of cross-linked gelatin electrospun fibres having different diameters in the range of 300 to 1300 nm in which SCs were cultured. Gelatin is highly biocompatible, biodegradable, not immunogenic, and maintains the collagen properties that favor cell growth. GPTMS or γ -glycidoxypolytrimethoxysilane was used as cross-linker agent. By increasing the gelatin concentration and flow rate during electrospinning, fibers with greater diameter (340 nm to 1306 nm) and porosity were obtained. The increase in fiber diameter did not affect cell adhesion but lead to a decrease in cell extension area so that the cells became more elongated. Cells are highly influenced by physical stimuli, and it is known that fiber diameter alter cellular morphology and proliferation (diameter can be controlled through modification of the electrospinning parameters). In general terms, the increase in fiber diameter affects other aspects, such as less organization of the actin cytoskeleton, increased filopodia formation (and cell migration), reduced proliferation, and even improvement in axonal growth.^{[50],[160]} The cells lines NG108-15 neuronal cell and primary rat SCs were evaluated under various diameter of PCL aligned electrospun fibers, individually, and as co-culture and with rat dorsal ganglion cultures for 3D neuronal –glial co-cultures. It was shown that cell length on narrow fiber (1 μ m) results on better neural outgrowth specially when evaluate as co-culture, meaning that this is a promising model since it mimics neural tissue properties. Although it was not measured, the positive response of SCs on the neuronal cell culture extension is attribute to the excretion of neurotrophic factors such as NGF and BDNF and also expression of ECM proteins.^[51]

Yao and colleagues, made an electrospun cylindrical nerve guide conduit using polycaprolactone (PCL) and designed it so that in the inner part of the scaffold the fibers would be aligned and thus determine which is the optimal diameter to favor the directional growth and neurite extension. PCL is characterized by being a slow degrading polymer and its application to a slow process such as nerve regeneration is favorable. The cylindrical scaffolds were fabricated on 1.4 mm diameter stainless steel wire to simulate the mouse sciatic nerve and it was possible to obtain random fibers on the outer side and aligned fibers on the inner surface. Alternatively, fiber films were also made with aligned or random orientation to analyze the guided neurite growth and images were obtained using SEM and FFT function were used to determine the fiber orientation (Fig. 10). In this study, PC-12 cells were seeded with NGF as supplement. After 5 days of cell culture, the neurite grew parallel in an oriented way in aligned fibers with growth close to 190 μ m compared to a 155 μ m and randomly growth to random fibers thus showing that the NGCs provide a means to facilitate regeneration of axons.^[161]

A novel type of silk electrospun fiber with Gold Nanoparticles (GNP), shows the versatility of the method for different applications. Degummed silk fibroin fibers were immersed in a GNP solution and then subjected to freeze dried. The resulting powder was dissolved in formic acid (98% pure) at a concentration of 100 mg/ml and mixed with 1 mg of polyethylene oxide (PEO) per milliliter of solution. This final solution was electrospun for development of a nerve guide conduit (NGC) by rolling the fiber onto a stainless-steel needle. This NGC was pretreated with SCTM 41 (rat Schwann cell) for 5 days before being tested for sciatic nerve regeneration in vivo. SCTM 41 showed a favorable response when there was cell adhesion and growth on the internal and external conduit surfaces and NGC

with GNP showed very similar results to those exhibited by a normal nerve after 9 and 18 months of implementation. At structural level, all NGCs were intact and maintained adhesion to the sutured ends.^[162] In a similar manner, growth factor-containing particles can be designed and incorporated into the general process of manufacturing the nanofibers.^[163]

Fiber alignment seems to promote axonal regeneration as shown in the research of Sperling et al.,^[164] where mESCs interacting with 3D scaffolds composed of PLGA were studied.^[139] The target was to evaluate the response of cell survival, morphology and differentiation over factors such as surface acceptance of polymer PLGA and fiber orientation, using random and aligned fibers. Immunocytochemistry was used to corroborate the expression of neural markers cultured on the PLGA scaffold. The marker for neural precursor cells, nestin, was highly expressed on aligned oriented fiber when compared to random. Expression of beta 3-tubulin helped to demonstrate the typical neuronal morphology including neurite branching and outgrowth processes. Moreover, expression of these markers was higher on 3D PLGA scaffolds as compared to flat scaffolds, highlighting the importance of 3D models. In a more recent work, Schuh et al., obtained homogeneous electrospun fibrin-PLGA fibers under established conditions in which they seeded cells and their results were favorable for proliferation along the fiber. MSCs were isolated from rat adipose tissue, which were in vitro differentiated to SCLs and SCs were isolated from rat sciatic nerves. Proliferation and cell viability assays were used to evaluate cell-scaffold interaction. Electrospun fibrous scaffolds were made with a parallel orientation and the seeded cells showed a positive response to the biomaterial as it grew along the fiber after 3 and 7 days of culture. They also propose the use of their nanofibers for in vivo studies by seeding cells inside the scaffold, modifying it to a self-shaping fibrin tubular structure with the incorporation of a hydrogel in the electrospun fiber.^{[140],[165]}

5. 3D Cell Encapsulation

In this section, the focus is not only the technique for the development of the 3D structures but rather to showcase how these 3D arrangements are incorporated with cells monoculture and co-culture to mimic the cell microenvironment, via 3D cell encapsulation. Cell encapsulation is an approach in which hydrogel plays an important role along with scaffolds both serving as platforms for monoculture and co-cultures. Hydrogel is a technique applied to a variety of polymers such as: poly (vinyl alcohol) (PVA), poly (glycolic acid) (PGA), poly (lactic acid) (PLA), and naturally derived biological components such as cellulose, collagen, chitosan, hyaluronic acid, and basement membrane extract, having applications on tissue engineering and drug delivery fields, to name a few.^{[166]–[168]}

Once the proper techniques are performed to develop hydrogels, they turned into platforms with ideal conditions to mimic the microenvironment that surround cells. Hydrogel unique features are attractive for biomedical applications which are: high water content, rubbery structure, biocompatibility and high capability to incorporate significant amounts of functional groups, all combining to mimic living tissue.^{[168],[169]}

A challenge with hydrogels is the control of matrix stiffness and biological cues, which is not completely achieved with the use of natural hydrogels, mainly because of permanent protein structure and product variation, since they are derived from a natural source. These

challenges are overcome with the modification of functional groups to form synthetic and hybrid hydrogels, being capable to control gel formation and degradation, supporting the fact that hydrogel techniques have a wide range of applications.

In many applications, hydrogels are combined with other techniques such as wet spinning to produce complex structures, for example a macroporous alginate hydrogel fibrous scaffold for nerve repair.^[169] In brief, this structure was produced by wet spinning a solution composed of gelatin particles, alginate solution, and dorsal root ganglion cells (DRGs) into 0.5 M CaCl₂ cross-linking solution. This approach highlights the use of hydrogels to develop 3D structures with cell encapsulation, more specifically a co-culture since DRGs are a mix of SCs and neurons. Crosslinking of hydrogels is one of the most important factors to produce a 3D, hydrophilic, insoluble polymeric network, it can be physical or chemical mechanism and gives hydrogels stability.^[170] Also, alginate was used since it is a polysaccharide that has a positive effect promoting axonal development, nerve repair, and ease of gelation property.^{[19],[171]}

A scaffold's porosity is key for the diffusion of nutrients and oxygen, waste elimination, and is directly related to cell proliferation and tissue development. An example of how to control the porosity in a hydrogel is by the introduction of gelatin particles as porogens, with a particle size range <90 µm, to the 1.5 % w/v alginate solution, and then followed by extraction using distilled water at 37 °C, leaving a 3D assembly composed of alginate fibers with pores. The target variable to prove the effectiveness of the 3D macroporous alginate scaffold, was neurite outgrowth from the encapsulated DRGs. The results showed neurite outgrowth over a distance of 150 µm on day 11 on 3D macroporous structure, compared with no significant outgrowth over 15 days on non-macroporous. The reason for no significant neurite outgrowth is because of absence of pores and channel structure needed for the neurite extension. The combination of wet spinning with hydrogel principles, along with gelatin particles represent a prominent example of a 3D macroporous complex that serves as a structure to support neurite extension.

As mentioned above, the versatility of hydrogels can be seen by the combination of nerve conduits with hydrogel filling making a more stable and 3D arrangement that had a positive effect to promote cell adhesion and proliferation.^[172] The hydrogel composition consisted of agar-gelatin at a 20:80 weight ratio (wt/wt) blend, crosslinked by the addition of genipin and the conduit was a porous PCL guide of 1.3 mm diameter and 15 mm length. A total of 20 µL of hydrogel was inserted using a syringe. The performance of the composite conduit was tested in vitro to prove the capacity of SCs to adhere, proliferate, and migrate. Also, a three – dimensional migration assay, using neonatal olfactory bulb ensheathing cells (NOBECs), proves no obstruction through the hydrogel solution, Fig. 11 (A–B). Hydrogel presence on NGC supported cell, migration, adhesion, and proliferation. Hydrogels have the potential to continue enhancing conduits function for peripheral nerve regeneration.

Cell Encapsulation of co-cultures offers to study cell-cell and natural ecosystem interactions.^{[68],[173]} The immediate advantage of this technique is a more detailed representation of physiological environment compared to monoculture, by the control of several factors like, type of medium, cell concentration, time scale, and the addition of degree of contact

between cell populations. The degree of separation can be achieved through semi-permeable membranes, gels, microfluidic devices, and others.^{[68],[69],[174]} The way in which the cell-culture degree of separation is arranged defines the experimental set-up, for this case three-dimensional arrangements with scaffolds and hydrogels will be highlighted. Challenges that bound this technique is the high level of complexity mainly because of the multiple cell interactions generating unpredictability and instability, which leads to another challenge of poor knowledge of data acquisition tools since it involves multiple interactions and even ones that are unknown.^{[68],[173],[174]}

Co-culture experimental set up depends upon the separation and suspension techniques or materials in which the cell populations will be exposed.^[68] It is important to highlight that the applied technique defines whether it will be a two or three-dimensional structure. An example of a co-culture technique that generates a three dimensional structure is spheroidal co-culture, also known as multicellular spheroid (MCS) which are developed by a number of techniques including: hanging drops culture, single-cell culture on nonadhesive surface, micromolding techniques, spinner flask culture, rotary cell systems, porous 3D scaffolds, and centrifugation pellet culture.^[174] The co-culture spheroids were suspended in a 3D collagen matrix and together represented a three dimensional structure for peripheral nerve regeneration.^{[69],[175]} The co-cultured consisted of Schwann cells (SC, 500 cells/spheroid) and NG108-15 a hybrid cell line (NG, 50 cells/spheroid). The cell ratios indicates that each spheroids contains ten times more quantity of SCs cells than NG cells, this could be attributed to enhance the spheroid environment with growth factors secreted by SCs cells. The reason for the choice of cells, is that SCs and NG108-15 cells (which are neuroblastoma cross glioma cell line) complement well based upon the abilities of SCs to provide physical guidance axons and NG108-15 cells' ability to differentiate toward mature motor neuron phenotype.^[176] The technique to developed the spheroids was hanging drops. The process starts with cell being centrifuged on non-adhesive wells, containing cell culture medium and 20% of methocel stock solution which prevents adhesion of cells and acts as an inert viscosity modulation substance. Methocel concentration is a parameter used to control de average size of the spheroids.^[176] A clear collagen gel was develop and serve as the matrix in which the pellets containing the spheroids were introduce, finally obtaining the 3D co-culture as hanging drops on the collagen matrix, as shown on Fig. 12.

Neurite length was the response variable showed that in 2D culture is around 65 μm and the 3D model ranged from 115.56 μm to 176.6 μm , for 2.3X longer neurites for 3D model when compared with 2D.^[69] The main reason for this behavior is that the model was able to increase the interactions between both cell types. In order to minimized cultivation differences between 2D and 3D cell culture both had a collagen layer. It is clear that no neurite growth into the collagen layer was noticed on 2D cell culture, which confirms that 3D setup was capable to increase cell-cell contacts and promote neurite growth.

A co-culture of SCs and adipose-derived stem cells (ADSCs) seeded on silk fibrin/ collagen scaffold is an approach to develop a tissue-engineered nerve conduit (TENC).^[20] The explanation for the choice of cells is that ADSCs are adult stem cells that can be induced to neurospheres and neuronal-like cells when exposed to neurothrophic media, and this specific media is created by the presence of SCs. Schwann cells have the capacity to secrete a variety

of neurotrophic factors, NGF, BDNF, GDNF and others that help differentiate stem cells, making an ideal complement to promote and accelerated axonal growth. The three dimensional structure was developed by blending a solution of silk fibroin and collagen, at a ratio of 4:2, followed by cast molding and finished by lyophilization. It is important to mention that the conduit structure, stability, and biocompatibility, is dependent upon the choice of materials, for this case silk fibroin is added because of its excellent mechanical properties which complement well with biocompatible properties of collagen. The silk fibroin/collagen structure is seeded with the co-culture obtaining a tissue-engineered nerve conduit. Tissue regeneration was achieved on 1 cm long sciatic nerve defect in rats which had results similar to autologous nerve grafts, but superior when compared with plain (non-co-culture scaffold). The conduit seeded with the co-culture mimics the native microenvironment, not only promoting the nerve regeneration but also accelerating axonal growth as confirmed by the transdifferentiation of ADSCs when co-cultured with SCs. 3D models such as spheroidal co-culture and seeded co-culture on three dimensional scaffolds, projects to fill the gap between the monoculture assays and in vivo studies in order to represent cell-cell and cell-ECM complex interactions, offering a more detailed representation of physiological structures and conditions.^{[37][95]}

DRG cells are a mixed of SCs and neurons, and are categorized as a co-culture, and many experiments have been made with this type of cells. An example, is the use of a single walled carbon nanotube-composite hydrogel with electric stimulation, in which the neurite extension of the encapsulated DRG cells was evaluated. The hydrogel solution was composed of collagen type I and growth factor-reduced Matrigel®, in order to support SCs spreading, viability and migration. It was proved that neurite extension was induced with the presence of the hydrogel and also by the electric stimulation, however it's known that cells within the DRG, such as glia cells may also influence in the results.^[177] Another approach using DRG cells is with the combination of technologies of aligned PLLA microfibers with nerve growth factor releasing composite nanoparticles, both technique induced neurite extension with the presence of growth factor on nanoparticles and aligned microfibers.^[163] Overall DRG cells are a co-culture very representative of the response and performance necessary to reflect neurite extension.^{[163],[177]} Hydrogels and scaffolds are platforms that offer 3D structures and co-culture simulate nerve cells models. Both applications filled the gap between in vitro and in vivo experiments by being capable to simulate the microenvironment.

Validation of peripheral nerve 3D cell culture models

Engineered polymeric scaffolds serve as highly tunable materials for 3D in vitro models of peripheral nerve. The 3D environment can more accurately recapitulate the native environment compared to 2D substrates, providing fundamental knowledge of neural tissue repair. 3D cell culture models are first validated by measuring adhesion of both neuron and glial cells, and by measuring neurite extension and glial cell proliferation. Additionally, protein and gene expression must be considered and can be evaluated on the 3D scaffolds. For example, Tuj1 is typically evaluated as an early marker for neuron development.^{[41],[54],[140],[164],[178]} GAP43 and synapsin I, are both known to be up-regulated throughout neuronal development.^[179] For glia, during the transition of immature SCs towards

myelinating, PMP22 and EGR2 are upregulated while a down-regulation of NCAM and low affinity NGF receptor (LNGFR) occurs.^[180] Also, adhesion of SCs to scaffolds may be validated by an overexpression of S100.^{[14],[19]–[21],[51],[53],[54],[56],[63],[66],[77]}

However, evaluating a handful of expressed proteins and/or genes gives a limited picture of how the proposed 3D models are behaving in comparison to native tissue. A complete transcriptomic and/or proteomics profile of the in vitro 3D cell culture is crucial for the development of proper models that closely resemble native tissues. For instance, transcriptomic analysis allowed for the confirmation that engineered GF induced neural progenitor cells closely resembled brain derived neural progenitor cells.^[181] Other work has used microarray analysis to identify new genes involved during peripheral nerve regeneration.^[182] Recently, a proteomics analysis was performed to evaluate MSCs cultured in three similar ECM mimetic materials from different sources.^[183] They observed that each material induced a unique matrisome signature, but they all shared a common set of proteins. Very little work has been published on validating 3D cell culture models using an omics approach. Carefully designed 3D environments must be validated via transcriptomic or proteomics analysis by comparing them to native cells or tissues to provide high fidelity to the 3D cell culture model. Lastly, high-throughput biomaterial screening platforms have emerged as useful strategies for the design and testing of biomaterials to serve as 3D environments for cell culture.^[184]

Conclusions

The baseline for 3D cell culture platforms continues to expand. Many options are currently available to researchers. It is important to continue efforts and integrated strategies that not only can be executed at research laboratories but expedite the introduction of more effective therapies and sustainable treatments. There is still work to be done in the nerve regeneration field, especially to gain knowledge about improvements to overhaul many of the practical limitations, and what are the explicit points that remain departing from the nerve biology. 3D cell culture models may provide answers to these fundamental questions in neural tissue regeneration.

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Abbreviations

CPH

1,6-bis (p-carboxy phenoxy) hexane)

CPTEG

1,8-bis (p-carboxyphenoxy)-3,6-dioxaoctane

ADSC

Adipose-derived stem cells

AM

Additive Manufacturing

BMSC

Bone marrow mesenchymal stem cells

BDNF

Brain-derive neurotrophic factor

Cams

Cell adhesion molecules

CNS

Central nervous system

CS-PLLA

Chitosan and poly lactic –based co-polymers

CNGs

Chitosan nerve guides

cryoGelMA

Cryopolymerized gelatin methacryloyl

DSP

Dibasic sodium phosphate

DMD

Digital micro-mirror arrays devices

DRGs

Dorsal root ganglia

EGR 2

Early growth response 2

ECM

Extracellular matrix

FFT

Fast Fourier Transform

FDA

Food and Drug Administration

FDM

Fused Deposition Modeling

GelMA

Gelatin methacryloyl

A/GL_GP

Genipin

GDNF

Glial cell derived neurotrophic factor

GPTMS

Glycodoxypropyltrimethoxysilane

GAGs

Glycosaminoglycans

GNP

Gold Nanoparticles

GFs

Growth factors

GAP43

Growth-associated protein

HA4

Hyaluronan Tetrasaccharide

HA

Hyaluronic acid/hyaluronan

iPSC

Induced pluripotent Stem Cells

LN

Laminin

LNGFR

low affinity NGF receptor

MSCs

Mesenchymal stem cells

mESCs

Mouse embryonic stem cells

MCS

Multicellular spheroids

NOBECs

Neonatal Olfactory Bulb Ensheating Cells

NGF

Nerve growth factor

NGCs

Nerve guide conduits / Nerve guide channels

NCAM

Neuronal cellular adhesion molecules

NT

Neurotrophin

NT-4/5

Neurotrophin 4/5

NT-3

Neurotrophin-3

OECs

Olfactory Ensheathing Cells

PNS

Peripheral nervous system

PCLA

Poly(chitosan-g-lactic acid)

PCL

Poly-ε-caprolactone

PEG

Poly-ethylene glycol

PEGDA

Poly (ethylene glycol) diacrylate

PEO

Polyethylene Oxide

PLGA

Poly-lactic-co-glycolic acid

PLLA

Poly-L-lactic acid

Ppy

Polypyrrole

PVA

Polyvinyl alcohol

RP

Rapid Prototyping

SEM

Scanning Electron Microscopy

SC

Schwann Cells

PMP 22

Schwann Cells peripheral myelin protein 22

SCLs

Schwann-like cells

SH-EP and/or SH-SY5Y Cells

Human Neuroblastoma Cells

SLS

Structured Light Scanning

SCG

Primary Superior Cervical Ganglion Neurons

TENC

Tissue engineered nerve conduit

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Figure 1.
Basic Structure of a Nerve

Figure 2.

Multi-walled chitosan channel inner silicone conduit (A); the connective tissue –like surrounding the outer tube of the laminin conjugated chitosan scaffolds and nerve regeneration along with the outer wall of the conduit (B); nerve grow back through the inner channels of the BMSC-treated chitosan scaffold conduit (C). The arrow indicated a regenerated nerve. "Reprinted from New nerve regeneration strategy combining laminin-coated chitosan conduits and stem cell therapy, *Acta Biomaterialia* 9 (2013), S. H. Hsu, W. C. Kuo, Y. T. Chen, C. T. Yen, Y. F. Chen, K. S. Chen, W. C. Huang, and H. Cheng, New nerve regeneration strategy combining laminin-coated chitosan conduits and stem cell therapy, 6606-6615, Copyright (2013), with permission from Elsevier".

Figure 3.

(A) SEM images of porous, OVA encapsulated 50-50: CPTEG-CPH particles incorporated in patterned PLLA films; (B) Bioactivity of β -NGF released from 50:50 CPTEG: CPH microparticles (125 μ g/ml) on the neurite extension of PC-12 cells. PC12 cell density: 2×10^4 cells per cm^2 . Incubation time: 2 days. Neurites of the cells in every condition were immunostained with β III-tubulin along with Cy3 secondary (shown in red), and DAPI for nuclei (shown in blue). ("Reprinted from Development of multifunctional films for peripheral nerve regeneration, 2016, M. Uz, A. D. Sharma, P. Adhikari, D. S. Sakaguchi, and S. K. Mallapragada, Development of multifunctional films for peripheral nerve regeneration, Copyright (2016), with permission from Elsevier")

Figure 4.

(A): The pre-designed 3D tube-like structure. The green spheres represent homogeneous multicellular spheroids that were developed using only human normal dermal fibroblasts. (B): The Bio 3D conduit according to the pre-designed 3D model. (C): Regenerative nerve after eight weeks of surgery in the Bio 3D, (D): Regenerative nerve after eight weeks of surgery in the silicone tube. [Reprinted from: Yurie H, Ikeguchi R, Aoyama T, Kaizawa Y, Tajino J, Ito A, et al. (2017) The efficacy of a scaffold-free Bio 3D conduit developed from human fibroblasts on peripheral nerve regeneration in a rat sciatic nerve model. PLoS ONE 12(2): e0171448. doi:10.1371/journal.pone.0171448]

Figure 5. Schematic of the DMD process (adapted from Ref. [140])

Figure 6. Scaffold with two gradients running in opposite directions. Red and green fluorescent micro particles were mixed in a prepolymer solution and the scaffold was fabricated. The graph indicates the intensity profile of the fluorescence and thus the micro particle concentration. (Scale bar, 1 mm). "Biomedical Micro devices, Solid freeform fabrication of designer scaffolds of hyaluronic acid for nerve tissue engineering, 13, 2011, 991, Shalu Suri, © Springer Science+Business Media, LLC 2011. With permission of Springer"

Figure 7. Fluorescence micro- graphs of the fabricated scaffolds. (a) and (b) show the SCs seeded inside the scaffold. The cells remained viable after 24 h when stained with calcein. Red fluorescent particles were added in the prepolymer solution to better visualize the scaffolds and the internal structure post- fabrication. (Scale bar, 1 mm). "Biomedical Micro devices, Solid freeform fabrication of designer scaffolds of hyaluronic acid for nerve tissue engineering, 13, 2011, p. 993, Shalu Suri, © Springer Science+Business Media, LLC 2011. With permission of Springer"

Figure 8.

SEM images of PEG scaffolds with (a, b) log-pile and (c–f) hexagonal internal architecture support survival and transition of neurospheres to neuronal morphology, spreading from adherent cell clusters, and forming 2D and 3D intercellular projections. Neurospheres evenly penetrate and adhere to hexagonal PEGDA scaffolds. Larger neurospheres are largely excluded from the interior of the scaffold. (g–i) Neurons form 3D lattices on log-pile and hexagonal PEGDA scaffolds labeled for Tuj1. White arrow in (h) denotes a cell cluster adhered to the side of the log-pile scaffold with neural connections along the struts. Scale: 100 μm . "Biomedical Micro devices, Three-dimensional scaffolding to investigate neuronal derivatives of human embryonic stem cells, 14, 2012, p. 13, Pranav Soman, © Springer Science+Business Media, LLC 2012. With permission of Springer"

Figure 9.
Schematic of the electrospinning equipment and process at our home lab.

Figure 10.

Analysis of the fiber alignment. (A) Sample of electro spun PCL conduit. (B) Cross-section image of the conduit. (C–D) SEM images of Aligned fibers with varying fiber diameters. (E) Randomly oriented fibers. (F) Neurites of PC12 cells on randomly oriented and (G) Aligned neurite growth on aligned PCL fibers. From “Orienting neurite growth in electrospun fibrous neural conduits”, 90B, 2009, p. 483, Li Yao, Norah O'Brien, Anthony Windebank and Abhay Pandit. Copyright © 2009 by John Wiley & Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.

Figure 11.

Three-dimensional migration assay. Representative images of migrated neonatal olfactory bulb ensheathing cells (NOBECs) 0 (A), and 72h (B) after incorporation in A/GL-GP (agar and gelatin cross-linked with genipin) hydrogel. Cells were stained with cell tracker green (CTG) and visualized in green; biomaterial autofluorescence is visualized in red. [Reprinted from: Tonda-Turo, C., et al. "Development and characterization of novel agar and gelatin injectable hydrogel as filler for peripheral nerve guidance channels." *Journal of tissue engineering and regenerative medicine* 11.1 (2014): 197–208]

Figure 12.

3D co-culture, model of experimental setup. Spheroidal formation hanging drops and transfer in 3D collagen matrix. "Reprinted from The Neuro-spheroid-A novel 3D in vitro model for peripheral nerve regeneration, 246, D. Kraus, V. Boyle, N. Leibig, G. B. Stark, and V. Penna, Journal of Neuroscience Methods, 97–105, Copyright (2015), with permission from Elsevier".

Table I

Polymeric Materials for 3D Scaffolds

Polymeric Type	Material	Basis	Bioactive properties	Ability to be modified	Other features	References
Natural Polymers	Collagen	Exist as fibrils in the endoneurium or as the non-fibrillar component of the basal lamina. Major components of the native ECM. It accounts for approximately 30% of the mammalian musculoskeletal tissues.	Provides the structural baseline of the ECM. It Possess a higher biocompatibility, and provokes low to none immune response.	A Young's modulus up to 11.5 GPa have been reported for collagen fibers. Collagenous cellular scaffolds can be made as fibrous scaffolds, hydrogels, sponges, foams, porous scaffolds.	One of the oldest biomaterials in use. Several commercial products approved by medical regulatory bodies (e.g., FDA) already on the market.	[72],[75],[76]
	Hyaluronic Acid (HA)	Polysaccharide globally present in human body; in almost all body fluids (e.g., synovia) and tissues (e.g., vitreous humor, hyaline cartilage). It is a major ECM component, and offers important biological properties.	It is involved in the regulation of cell adhesion and cell motility, cell differentiation and proliferation.	Provides mechanical properties of tissues. Superior flexibility to mold accurate 3D platforms for the development multiple applications. HA cellular scaffolds can be made as fibrous scaffolds, hydrogels, viscoelastic solution, macroporous and fibrillar sponges, nanoparticles, nonwoven mesh matrix, and flexible sheets.	HA presence, combination, derivatives, and alternatives with other materials has shown promising results as an ideal substrate for in vitro peripheral nerve cell culture and in vivo nerve regeneration.	[77]–[83]
	Silk	Fibrous proteins produced in fiber form by silkworms and spiders.	Biocompatible for in vitro and in vivo studies.	Silk cellular scaffolds can be formed in a variety of biomaterials, such as gels, sponges and films, for medical applications.	Silk scaffolds have been successfully used in wound healing and in tissue engineering of bone, cartilage, tendon and ligament tissues.	[20],[75],[84]
	Gelatin	It is a denatured form of collagen. Specifically, it is a	Same as collagen, however its main characteristic is that	Gelatin hydrogel mechanical properties are poor an unstable,	Due to its low cost, it is used very often in the	[85]–[90]

Polymeric Type	Material	Basis	Bioactive properties	Ability to be modified	Other features	References
		hydrolysis of the collagen fibrils turned into smaller peptides, with a broad range of molecular weights that will vary and settle its conformation based on the hydrolysis approach (e.g., mixing, temperatures, times, additives, etc.).	it does not present an antigenic potential.	especially under physiological conditions, therefore it must be combined with other components and/or cross-linked. When not lose its properties and can be prepared, molded and used as an ECM mimetic. Gelatin cellular scaffolds can be made as, hydrogels, nanoparticles, viscoelastic solution, that can be molded and dried to form conduits.	pharmaceutical and cosmetic industry.	
	Chitosan	Polysaccharide derived from Chitin, which is obtained from the outer shell of crustaceans, and body parts of some insects via an alkali treatment.	Chitosan is nontoxic. However, it offers antimicrobial activity. Is understood that the polycationic nature of Chitosan put across by its positively charge glucosamine groups interact with negatively charged microbial cell surface, producing weakening bacterial activities.	Chitosan cellular scaffolds can be made with a wide range of porous sponges, fibrous scaffolds. Yet, it has a high rate of degradation, and low mechanical stability.	It is the second most abundant polysaccharide on earth, second to starch. Because of it's characteristically charge density, it is a potential option of the transport of polar drugs.	[91]-[95]
	Acellular tissue grafts	Biological tissue, derived from animal or human, with all cells removed. These tissue scaffolds provide a collagen arrangement for cellular and tissue remodeling. The cleaning of its initial cells intends	As it is non-immunogenic, the risks for incompatibility are minimized. The use of this material has the advantage that formerly it sheltered cells, and consists of areas ready to host cells with less	Such polymeric scaffolds are prepared to sustain the appropriate structures for the physiological nerve regeneration and axon bridging between injured nerves gaps. Acellular scaffolds can be made as wound dressings, 2D sheets,	In this type of natural material, there is no assurance on the quantity of preloaded molecules (e.g., neurotropic factors) to stand within, and must consider the innate variation that	[47],[96]-[98]

Polymeric Type	Material	Basis	Bioactive properties	Ability to be modified	Other features	References
		to make it non-immunogenic.	resistance to growth. Also, it contains specialized molecules (e.g., LN, fibronectin) crucial in cellular development.	or shaped (e.g., folded, and attached) into desired form.	will exist from graft to graft.	
Synthetic Polymers	PEG - Polyethylene glycol	PEG is frequently used for implantation medical devices. In vitro, it is known for its capacity of gathering cells. It tends to fuse the membranes of adjacent cells due to its molecular composition, and its end hydroxyl groups.	It is reported that modifications of PEG are favorable to a rapid and permanent restoration of axons in PNS (and CNS as well). It interferes with the stable dissolution of injured peripheral nerve fibers and immediately acts to restores physiological functions.	PEG molecules can be easily modified with carboxyl, and acrylate, and easily attached to other molecules or bioactive agents. PEG cellular scaffolds can be made as fibrous scaffolds, hydrogels, sponges, foams, porous scaffolds.	Some researchers prefer the use of PEG technologies since it is a biomaterial readily available and approved by medical regulatory bodies (e.g., FDA).	[99]–[101]
	PLLA - Poly-L-lactic acid	PLLA is a biodegradable polymer produced from natural resources (e.g., starches). It is classified as a “thermoplastic” polyester, therefore respond to heat and reheated without significant degradation; easily malleable to desired shapes.	It possesses a very slow degradation rate, and a glass transition from 55 -80 °C; therefore, it is normally in a crystalline state when implanted. Because of this, it is immunogenic, and must be combined with other materials to help its rapid degradation.	A tensile strength up to 66 MPa, and a flexural strength up to 110 MPa. It is a versatile material with an ample range of properties (biocompatibility, mechanical, biochemical, etc.). PLLA cellular scaffolds can be made as fibrous scaffolds, hydrogels, sponges, foams, porous scaffolds.	One of the major benefits is related to its eco-friendly renewable production from organic matter and proven nontoxicity. It is a nontoxic bio-absorbable material approved by medical regulatory bodies (e.g., FDA), which is considered a suitable alternative for multiple tissue engineering applications.	[102]–[104]
	PLGA – Poly-lactic-co-glycolic acid	It has been studied and developed, mostly for drug delivery applications. In regards, tissue	PLGA is biocompatible and biodegradable, holding a wide range of custom degradation times.	Its versatile to many mechanical properties. PLGA cellular scaffolds can be made as scaffolds, fibers, hydrogels or	Greatest application in the pharmaceutical industry for the manufacture of devices that possess	[105]–[107]

Polymeric Type	Material	Basis	Bioactive properties	Ability to be modified	Other features	References
		engineering, its capabilities for controlled release are important for the use of growth factors, and/or other molecules that supports cellular migration and growth.	Hydrolysis, breaks down PLGA to its basic monomers: lactic acid and glycolic acid; which are part of the human body metabolism, therefore, nontoxic under normal physiological conditions.	injectable microspheres.	controlled release properties of drugs. Approved by medical regulatory bodies (e.g.,FDA) for clinical use.	
	PCL – Poly-ε-caprolactone	PCL is biodegradable polyester with high solubility in organic solvents.	It possesses a melting point of 60 °C and a glass transition temperature of about –60 °C; therefore, normally in a viscoelastic liquid state, and often is used together with other materials as a stabilizer to improve their processing characteristics.	The tensile strength of bulk PCL spans from 10.5 to 16.1 MPa, its modulus ranged from 343.9 to 364.3 MPa, and the tensile yield strength ranged from 8.2 to 10.1 MPa. These properties significantly decrease as porosity in a scaffold increases. PCL cellular scaffolds can be made as fibrous scaffolds, hydrogels, viscoelastic solution, macroporous and fibrillar sponges, nanoparticles, nonwoven mesh matrix, flexible sheets, etc.	PCL blends with other materials (e.g., resins) convey a hydrophobic tendency of the compound. Also becomes resistant to other fluids.	[52],[108]–[110]
	PPy- Polypyrrole (Conducting polymers)	Conductive biomaterials which are able to enhance neurite extension with low electrical stimulation.	Recent developments used this material to study mechano-transduction inducing cell behavior.	PPy can be made as coating of actual scaffolds, and/or to prepare films. It is an insulator, but can be modified to be an electrical conductor (up to 90 S/cm).	Demonstrated that a properly conducted electrical stimulation will increase the average displacement of SCs throughout nerve gaps and facilitate its functional regeneration.	[15],[48],[111],[112]
	Silicone	Silicone is a nontoxic inert polymer with low	It is available and approved by health regulatory	It is inert, versatile, and can be shaped	This material was one of the first ones to be used in nerve	[42],[66],[70],[73]

Polymeric Type	Material	Basis	Bioactive properties	Ability to be modified	Other features	References
		(and stable) thermal conductivity, and low chemical reactivity. It is capable of repealing water and serve to seal.	authorities in numerous biomedical presentations.	easily to form required scaffolds.	repair applications. Although there have been some clinical disadvantages in practices (irritation in some patients), today it is an option to perform cellular culture studies.	
	Polyvinyl alcohol (PVA)	PVA is a water-soluble, nontoxic, biocompatible, and nondegradable synthetic polymer.	PVA by itself does not support cellular development as good as when combined with other polymers. It was demonstrated that dimensional and porosity changes in the PVA scaffold will affect the possibilities of the cell cultured.	It has high tensile strength and flexibility. PVA has a melting point of 230 °C, and can be used as film forming, emulsifying and adhesive. It is very resistance to oils, and organic solvents.	The higher water absorption, the lesser its tensile strength.	[113]-[115]

Many other polymeric biomaterials could be explored, as presented by relevant reviews preceding this one. [116],[117]