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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, and 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]

Figure 1. Basic structure of a nerve.

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 extracellular matrix (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 toward 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

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During nerve regeneration, the native ECM must be reconstructed. The major components of the ECM in the nervous system can be divided into four 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, and 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 and 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 biologic characterizations made at the laboratory barely exemplify 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 with 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

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toxicity 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 bone marrow mesenchymal stem cells (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 GF 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 GF.[57]

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 regenerative environment.[29, 58] 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] 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 NTs (e.g., BDNF and 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]

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 toward 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 devel-

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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 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, 69–73] 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, the 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 into two major groups: natural and synthetic.[74] For scaffolds, those that can orchestrate nerve regeneration and are available for use in 3D cell culture are presented in Table I. Mechanical properties of the scaffold can be 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. Cross-linking involves the anchoring of polymer chains to reinforce the final construct, influencing 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.

Table I. Polymeric materials for 3D scaffolds.

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

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

Three-dimensional 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:

Flat (films)

One of the most important reasons to continue developing options for nerve tissue regeneration is to overcome the drawbacks of the 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 due to their simplicity in manufacturing. 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 glycodoxypropyltrimethoxysilane (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 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 with 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 for the rolled 3D conduit showed that, chitosan cross-linked with only DSP induced a 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