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Materials for Central Nervous System Tissue Engineering

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1. Introduction

Neurodegenerative disorders and traumatic injuries to the central nervous system (CNS) can cause severe and irreversible damage. Nowadays there are few clinical therapies to treat brain damage, and they achieve low functional recovery; most of them are based on drug administration for neurodegenerative diseases with well-defined targets as Parkinson's disease. For traumatic injuries such as traumatic brain injury (TBI), spinal cord injury (SCI), and stroke there is no effective treatment to restore lost functions and the clinical approaches are symptomatic and based on minimizing the damage progression or enhance local plasticity with rehabilitation. On the whole, the limitations of current therapies underline the need to search for novel strategies.

Brain damage has constituted up to now a theoretically unsolvable problem. This was because three of the most important tenets in neurobiology were against the restoration of the brain after damage: 1) there are no new neurons, 2) axons cannot grow within the CNS, and 3) neurons cannot produce new connections between them. Although these tenets have been questioned due to research done during the last decades, there is currently no clinical application useful to produce reconstruction of brain damage. This is probably due to several causes: endogenous regeneration in the adult human brain is, although present, insufficient to recover the lost neuronal population; also, axonal elongation is highly repressed by inhibitory factors, and the lack of the appropriate neurotropic factors and guidance cues. Furthermore, compared to other organs, the brain is a very complex system and it does not suffice to get a high number of new neural cells and connections between them, but these connections between new and old neurons must be exactly organized.

Thus, the problem of neural regeneration includes mainly three processes: the need to have new neurons to replace the lost ones, the need to promote axonal regeneration within the CNS

to the appropriate targets, and the need to make functionally effective connections with the same connectivity of the lost ones.

Despite the existence of neurogenesis in the adult mammalian [1] and human [2] brain, cell replacement cannot be accomplished because of the massive death of new cells. After injury, there is an increased neurogenesis and migration to the damaged area. In an experimental model of stroke, new neurons appeared at the core ischemic area [3]. However, 80% of these neurons disappear from the core 5 weeks after stroke, probably because they die or migrate to other areas. A probable hypothesis for this phenomenon is that the core ischemic zone is not a proper environment for cell survival, since it lacks vascularization, stromal structure or trophic sustain for these cells.

In humans with amyotrophic lateral sclerosis and frontotemporal dementia, an increased proliferation of neural progenitors can be seen which is not able to restore the disease condition [4], probably because these cells die because of the action of a toxic environment produced by the disease.

Biomaterials could constitute a proper environment to facilitate vascularization, provide structural scaffold and improve the milieu in order to facilitate the survival of the newly formed cells [5].

2. Neurogenesis in the adult mammalian brain

It is currently well accepted that most of the neurons in the CNS are produced both in the prenatal as well as in the early postnatal stage. Moreover, during the past decades, neurogenesis in the adult brain of mammals has been reported, humans included. Adult neurogenesis has been shown to occur in two main areas of the adult mammalian brain: the olfactory bulb (OB) and the dentate gyrus (DG) of the hippocampus. While in the hippocampus stem cells and new neurons coexist together, the new neuronal cells in the OB are generated from neural progenitor cells in the anterior part of the subventricular zone (SVZ). The SVZ is a narrow region of tissue within the wall of the lateral ventricle in the forebrain. The neural progenitor cells of the SVZ migrate to the OB where they differentiate into interneurons such as granule and periglomerular cells [6]. It is known that a different migration exists from the SVZ to corpus callosum, where cells differentiate preferentially into oligodendrocytes which could be a target for treatment of neurodegenerative diseases where myelin regeneration is required [7]. There is little evidence of the existence of alternative pathways for cell migration under normal conditions, from neurogenic areas to other regions of the brain. However, it is known that when damage occurs such as in ictus, TBI or SCI it is possible to find migration from neurogenic niches to the injured region [8].

2.1. Structure and organization of subventricular zone and dentate gyrus in mammals

As mentioned above, stem cells responsible of adult neurogenesis in the OB, have their origins in the SVZ, and migrate along a restricted pathway, called the rostral migratory stream (RMS).

The RMS in mice has a length between 5 y 6 mm. Two principal cellular types were observed in the RMS: type A cells (migrating cells) and type B cells (astrocytes). In sagittal sections, type B cells and their processes flanked the chains of type A cells. In frontal sections, type B cells ensheathed the chains of migratory cells [6].

In the SVZ, have been identified at least four different cell types: type A (migrating cells), type B (astrocytes), type C (proliferative precursors or neuroblast) and type E (ependymal cells), these cells were defined by their immunological markers and morphology. Using proliferation markers such as 3HT or BrdU, the most actively dividing cell in the SVZ corresponded to Type C cells [9].

Briefly, the characteristics of the 4 types of the SVZ are: Type A cells (migrating cells), equivalent to those observed in the RMS. Their major characteristics were an elongated cell body with one or two processes, abundant lax chromatin with two to four small nucleoli, and a scant, dark cytoplasm containing many free ribosomes and many microtubules oriented along the long axis of the cells. The nuclei of Type A cells were occasionally invaginated. Furthermore, their membranes showed cell junctions intercalated with large intercellular spaces that allow cell movement. These cells were grouped forming chains and show immunoreactivity to PSA-NCAM, *Dlx-2* and *TuJ1*.

Type B cells (astrocytes) formed a wide network adjacent to ependymal cells surrounding the chains of migrating cells (Type A cell). The cytoplasm of Type B cells was light and contained few free ribosomes. One of the most important characteristic of type B cells was the presence of extensive intermediate filaments in their cytoplasm, and they have also irregular contours that profusely filled the spaces between neighbouring cells. These cells had irregular nuclei that frequently contained invaginations.

Currently type B cells are divided into two subtypes: B1 and B2. Type B1 cells make contact with the ventricular cavity while B2 cells do not. B1 cells show one short cilium to the ventricular cavity, named primary cilium. The number of B1 cells in contact with the ventricle increases drastically when growth factors such as Epidermal Growth Factor (EGF) or ephrins are injected [10,11], possibly the necessary signals for activation are in the cerebrospinal fluid where, probably, the primary cilia could play an important role. Both B1 and B2 cells are immunoreactive against GFAP, vimentin and nestin and no molecular marker can differentiate them. Type B2 was similar to those described in the RMS.

Type C cells (precursor cells) were larger, more spherical (less elongated), and more electron-lucent than Type A cells but more electron-dense than Type B cells. Their nuclei contained deep invaginations and mostly lax chromatin, although sometimes the chromatin was clumped. Type C cells had a typical large reticulated nucleolus. Their cytoplasm contained fewer ribosomes than Type A cells, and no bundles of intermediate filaments of Type B cells. Groups of C cells (2-3 cells) are associated with the chains of A cells. C cells are immunoreactive against *Dlx-2*, a transcription factor, although this marker is not exclusive to this type of cell.

Type E (ependymal cells) formed an epithelial monolayer separating the SVZ from the ventricular cavity. Their main distinguishing characteristics were as follow: the lateral processes of adjacent ependymal cells were heavily interdigitated and contained apical

junctional complexes. The surface exposed to the ventricular cavity contained microvilli and is ciliated. The cytoplasm was electron-lucent and contained many mitochondria and basal bodies located in the apical cytoplasm. Their nuclei were spherical, and the chromatin was non-clumped. These cells are immunoreactive against vimentin, S-100 and CD-24. In addition, microglia, a few pyknotic cells, mitoses, and large neurons were observed sporadically in the SVZ. Furthermore the presence of a blood vessel network plays an important role in the SVZ and there are evidences that the activation of the neurogenic niches is directly related with this network [12,13].

The DG is the other region within the brain where neurogenesis takes place in mammals [14,15]. The function of neurogenesis in the hippocampus has been correlated with memory and learning. The DG is composed of small neurons called granular cells that are typically packed in a V-shaped band. Stem cells are located in the inner of this band and these are responsible for generating new neurons, which will be placed at the cell body layer. Moreover, astrocytes have been identified in the DG as neuronal stem cells [16]. These cells are identified as radial cells, crossing the entire thickness of the cell layer. It is also known that before immature cells transform into mature neurons, they are partially isolated from the environment by astrocytes, and it is thought that probably astrocytes will allow the differentiation to mature and functional neurons. At ultrastructural level, these stem cells are similar to the astrocytes in the SVZ. Before the transformation to mature neurons, stem cells within the DG go through an intermediate stage of indifferentiation, named Type D cells. These cells are characterized by lax chromatin, a crescent number of ribosomes and mitochondria in the cytoplasm.

Although OB and hippocampus are the most studied and widely believed neurogenic regions, it is thought they are not the only ones. Probably more cells exist and keep their proliferative capacity within the brain, maybe in the surroundings of the ventricular cavities. Recently neurospheres have been obtained from the channel spinal cord of rodents, confirming the existence of stem cells in the channel, although the nature of the cell responsible for such proliferation is unknown [17].

2.2. Ventricular zone in humans: Organization and structure

After demonstrating the neurogenesis exist in the human [18], the new step has been the identification and location of the stem cells responsible for adult neurogenesis in humans. They have been located in the walls of the lateral ventricles displaying markers of astrocytary type cells with GFAP. These cells proliferate *in vivo* and *in vitro* and give rise to neurospheres that, in certain conditions of culture, differentiate in neurons, astrocytes and oligodendrocytes proving the existence of stem cells [19].

The organization of the SVZ in the human brain is very different from the one in rodents. Basically, three layers have been identified which have been denominated: 1) ependymal cell layer, 2) Gap layer and 3) Ribbon layer. The ependymal cells layer is formed by a monolayer of cubic cells in contact with the ventricular light that show long radial expansions into the neuropil, containing intermediate filaments and some mitochondria and constituting the

second or Gap layer. In the ventricular wall, facing the light, numerous cilia and microvilli can be seen as well as the unions between them [20].

The second layer (Gap) is formed by expansions of astrocytes from the layer ribbon and ependymal cells, and is known as hippocellular layer because of its low quantity of cell bodies. The astrocyte expansions contain abundant intermediate filaments and numerous desmosomes between them. Another interesting fact is the existence, in this layer, of ependymal cell aggregates. These cells make up little groups of 5 to 20 cells that have the typical organization of the ependymal cells.

Finally, the third layer, also known as Astrocyte Ribbon, is formed by large astrocytes, myelinated axons, oligodendrocytes and a progressive increase of synapses, as we move away from the ventricular cavity. Astrocytes of this layer have been considered stem cells. Also, there is an interesting fact about the astrocytes that occasionally send a long prolongation, which makes contact with the ventricular light [20]. This fact agrees with what has been seen in the mice SVZ, where presumably, there has been an activation of the neurogenesis by the cells in contact with the ventricular cavity and the appearance of neuronal soma limits the following layer [9]. The main difference with rodents is the lack of precursor or Type C cells and migrating cells.

2.3. The human brain with neurogenic features: Other regions?

The use of trophic factors such as basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) allows us to prove the presence of “sleeping” stem cells in other regions of the brain [21–25] like the third and fourth ventricles [26–28]. But, not only proliferation has been found in these walls, since there are authors that support the existence of neurogenic places distributed in the neurophil [29,30]. Therefore, in these last years there have been studies in which the formation of new neurons in the adult cerebral cortex of mice [29–31] and primates [32] has been proved. These findings have originated controversy between diverse investigation groups because the results cannot be totally duplicated, existing experimental variations to find the “new neurons” [33] and, sometimes, it is even possible that immature cells change to stem cells due to a certain stimulus [34].

Furthermore, the neurogenic term has been questioned since some authors think that, to consider a place as neurogenic, new neurons have to be produced or recruited in normal conditions and without the presence of any stimulus like an injury or an external trophic factor [33,35]. The neocortex, striatum, amygdala, substantia nigra (SN), channel spinal cord, third and fourth ventricle are some of the areas that are being proposed as neurogenic niches in mammals including the human [26,28,33,36–38].

3. The injured central nervous system and the regenerative process

Regeneration in the adult nervous system is limited compared with other tissues. This limitation is due to the lack of proliferation and regeneration of neurons to accomplish correct

connections. The regenerative response is different between the peripheral (PNS) and the CNS. Regeneration in the PNS is more successful due to the presence of Schwann cells (SCs), which provide nutrients, guide and myelinate the peripheral axons and synthesize growth factors. Moreover, a unique structure exists in the PNS: the bands of Büngner, consisting in clusters of oriented SCs and their processes, which remain after axon injury facilitating its regeneration. On the contrary, the predominant glial cells in the CNS are the oligodendrocytes, which provide axon support and myelinate axons, and the astrocytes, which principal function is to provide nutrients to the neurons. Oligodendrocytes express inhibitory proteins for axon growth, such as the myelin associated glycoprotein (MAG) and tenascin R, while SCs lack of these inhibitory proteins. In addition, astrocytes are activated to clustered layers of hypertrophic astrocytes giving place to the glial scar, which constitutes a physical barrier for axon outgrowth. Moreover, hypertrophic astrocytes secrete inhibitory molecules such as sulphated proteoglycans, MAGs, Nogo, etc., constituting a chemical barrier for axon outgrowth [39].

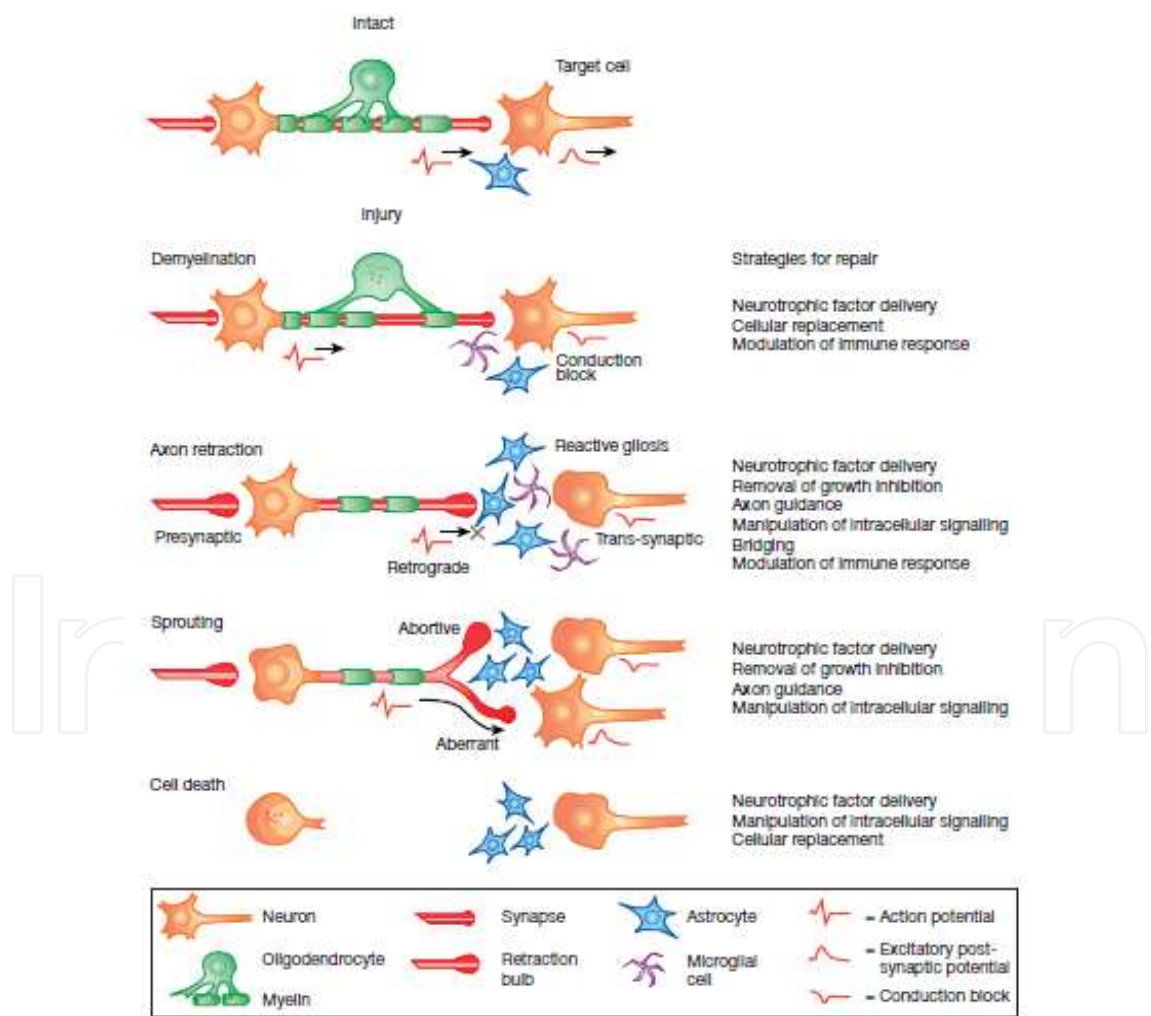


Figure 1. Consequences of neural injury and strategies for repair (from P. J. Horner and F. H. Gage [39], with authorization).

In the case of traumatic injuries as TBI, stroke and SCI the primary damage generates an abrupt loss of cells and may result in the disruption of the blood brain barrier (BBB) and blood spinal cord barrier (BSCB), respectively. The breach in the BBB and BSCB permits infiltration of foreign cells such as macrophages and fibroblasts, and inflammatory molecules activating astrocytes. In the case of neurodegenerative disorders as Parkinson's disease (PD), the loss of neurons is not abrupt but progressive, but the injured site is dominated by a toxic microenvironment as well. The neuronal injury and the inhibitory microenvironment result in degeneration at a cellular level: demyelination, axonal retraction and aberrant sprouting, and cell death. The tissue engineering strategies proposed aiming to repair injured tissues in the CNS must take into account these mechanisms of cell degeneration.

Thus, the main difficulty to regenerate neural tissues in the CNS after an injury is the toxic microenvironment generated and the lack of neuron replacement, although some neurogenesis has been observed after trauma. Indeed, despite the dramatic situation following a CNS injury, several studies have demonstrated that CNS axons could regenerate with an appropriate microenvironment. A favourable environment for CNS regeneration takes into account aspects as the normal function of glial cells, a correct system of nutrient distribution, a permissive physical pathway for axon growth and reconnection and a correct balance and distribution between inhibitory and promoting molecules for axon regeneration.

4. Cell therapy in the central nervous system

Cell therapy aims to supply the damaged tissue with glial cells, neurons, and even extracellular matrix components, adhesion molecules and/or neurotrophic factors. Cells could lead to a neuroprotective effect, help glial and neuronal remodelling, promote angiogenesis and synaptogenesis and modulate the inflammatory response. A wide range of cells has been employed to help regeneration in the injured CNS: glial cells, adult stem cells, embryonic stem cells and pluripotent stem cells [40]; the particular characteristics thereof are discussed next.

SCs are cells that promote regeneration in the PNS because of their ability to generate a permissive environment for axon regeneration and provide neuroprotection. SCs support growth and produce neurotrophic factors, including fibroblast growth factor (FGF), neurotrophin-3 (NT-3), brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF), and secrete proteins such as laminin (LN) to promote axon growth. Moreover, SCs provide pathways for regenerating axons assisting the growth cone extension and guidance, and remove myelin debris following damage and remyelinate the regenerating axons. In some spinal cord lesions, SCs infiltrate into the CNS from the peripheral ganglions and assist the regeneration process. For this reason, some researchers have investigated the implantation of SCs in the damaged spinal cord, and they have demonstrated their ability to promote axonal regeneration and myelination [41]. However, their use to address CNS regeneration is limited because SCs activate astrocytes, which increase the expression of inhibitory chondroitin sulphated proteoglycans (CSPGs) for axon regrowth and overexpress glial fibrillary acidic protein (GFAP) contributing to the formation of the glial scar. Moreover,

the neuroprotective effect of transplanted SCs is limited because they not prevent degeneration and secondary injuries. Other glia (astrocytes, oligodendrocytes, and microglia) has been also studied in connection with CNS regeneration. Implantation of macrophages in the injured spinal cord has shown some degree of regeneration due to their effect clearing myelin debris decreasing the inhibitory MAG levels for axons guidance [42]. However, the regeneration process assisted by macrophages is unclear, because they can inhibit axonal growth after SCI. On the other hand, the use of other glial cells, including astrocytes and oligodendrocytes, is usually addressed by employing precursors limited to differentiate to glial cells. These progenitor cells difficult the formation of gliotic scar than SCs and induce axon regeneration and remyelination [43].

Olfactory ensheathing cells (OECs) constitute an interesting type of cells in CNS regeneration strategies; they have been employed in animal models of SCI and stroke with several advantages over SCs [44,45]. These cells, in their natural environment, guide axons from the PNS to the CNS, and do not induce unfavourable interactions with the glial scar components. Furthermore, OECs express many of the same neurotrophic factors and proteins as SCs, including NGF, BDNF, GDNF, NT-3 and L1, facilitating axon regeneration and providing neuroprotection. After their transplantation in different SCI models OECs have demonstrated their ability to generate a more permissive microenvironment; they even lead to a limited regeneration, electrophysiological and functional recovery [46–48]. Infusions of OECs have been employed to address stroke, resulting in benefits to repair the damaged cerebral tissue, providing neuroprotection, facilitating neurite outgrowth, activating stem cells, reducing the lesion cavity and decreasing cell apoptosis, even improving the neurological function [44,49]. However, the implantation of OECs alone in the nigrostriatal tract of PD models is not sufficient to promote regeneration and functional recovery, because OECs do not release L-dopa to stimulate dopaminergic neurons [50]. Nonetheless, their role could be to permit the reentry of dopaminergic axons into the striatum once they have grown through a guiding structure, such a peripheral nerve graft [51]. Their transplantation together with other cells, including dopaminergic neurons derived from neural stem cells (NSCs), has demonstrated though ability to provide a slight functional recovery in PD models [52]. Moreover, some studies have employed genetically modified OECs overexpressing some neurotrophic factors in SCI models, resulting in a modest improvement of tissue sparing and functional recovery [53]. Some researchers have transplanted OECs addressing spinal cord repair and observed remyelination of axons, but this could be due to a contamination of the OECs suspension by other cells such as SCs since myelination by OECs has been debated [54]. OECs have been also employed in some controversial clinical studies of stroke and SCI, showing some degree of functional recovery, but these results must be corroborated in further studies [55,56].

Adult stem cells from different origins, including mesenchymal stem cells (MSCs), NSCs and neural progenitor cells (NPCs) have been transplanted in several traumatic injuries and neurodegenerative diseases in preclinical studies, and in clinical trials of stroke and SCI [57]. MSCs and NSCs are multipotent cells; the particular feature exploited in MSCs is its ability to differentiate into cells secreting neurotrophic factors, while NSCs can differentiate in both glial cells and neurons [58,59]. MSCs have been implanted in the spinal cord, showing the formation

of bundles that bridge the lesions, even increase the locomotor function, suggesting that MSCs provide trophic factors and support for host cells and themselves [60]. Meanwhile, NSCs implanted in the injured spinal cord have eventually differentiated into astrocytes [61], thus these cells should be stimulated to favour their differentiation into neurons. The implantation of NSCs in the injured spinal cord may also be combined with growth factors promoting the neural lineage or limiting the effect of the inhibitory molecules in the environment of the damaged spinal cord. This technique has been implemented combining NSCs with agonists of astrocyte differentiation, accomplishing differentiation of NSCs into neurons and functional recovery after SCI [62]. Furthermore, MSCs and NSCs have been transplanted in ischemic brain and stroke rat models showing promising results, including stimulation of endogenous cells and neurogenesis from the SVZ, promotion of angiogenesis, reduction of glial scar, even functional benefits [63–65]. In addition, MSCs have been tested in a clinical trial of stroke to prove their safety in humans, showing promising results [66]. MSCs and NSCs have also been studied to address neurodegenerative diseases, including Huntington's disease (HD) and PD. MSCs have demonstrated migration to the injured striatum, neuroprotection through the release of neurotrophic factors, even functional recovery over months [67–70]. Besides, NSCs implantation into the striatum has resulted in neuron differentiation and some functional improvements [71,72], suggesting that infusions of these cells could constitute a possible therapy for cell replacement in neurodegenerative diseases. In this sense, NSCs have been employed in clinical trials of both PD and HD, resulting in functional improvements over 36 months and 10 months, respectively [72,73]. However, these cells presented problems to engraft the lesion site and showed a poor survival. Other types of adult cells such as the adrenal chromaffin cells and human retinal pigment epithelium cells (hRPEs) have been employed in clinical trials to restore functions in PD, since they secrete dopamine or its precursor L-dopa, and can be employed as suppliers of L-dopa *in situ*. Both adrenal chromaffin cells and hRPEs have demonstrated modest functional improvements [74,75]. However, these cells have a poor survival and only a small fraction is able to secrete L-dopa or dopamine, or is secreted in small amounts.

Embryonic stem cells (ESCs) are derived from the blastocyst and possess interesting properties, including pluripotency and self-renewal, allowing their differentiation into cells from the three germ layers. For this reason, ESCs may constitute a source of different cells, including neuronal ones, with many possibilities in CNS regeneration. ESCs from mouse and human origin have been employed to address experimental studies in different CNS damages. For instance, ESCs have been induced to differentiate, preferably, into oligodendrocytes and implanted in the injured spinal cord of different animal models, including mouse, rats and chick embryos; they have shown to stimulate remyelination and promote locomotor improvements [76–78]. In another work, large amounts of motor neurons have been obtained *in vitro* from human ESCs and have been transplanted into the spinal cord of chick embryos maintaining their phenotype, but the potential benefits of the neurons stemming from ESCs require further characterization [78]. The poor survival of ESCs in the injured spinal cord can be enhanced by the incorporation of neurotrophic factors, including BDNF [79]. Mouse and human ESCs have also been differentiated *in vitro* into dopaminergic neurons, addressing their potential use for PD treatment [80,81]. These findings have led to the use of dopaminergic neurons derived from

ESCs in preclinical PD transplants, avoiding immune response, resulting in slight motor improvements and a reduction of symptoms [82,83]. The use of ESCs is also a promising approach to address several CNS damages; however, using ESCs requires solving many problems, such as poor cell survival and low efficacy of transplanted cells, ethical aspects because of its embryonic origin and the risk of teratoma formation.

As regards induced pluripotent stem cells (iPSs), they do not pose the ethical problems associated with the use of human ESCs and show a lesser degree of immune response. These cells are obtained from somatic cells by overexpressing four reprogramming factors (Oct4, Sox2, Klf4, and Myc), leading to self-regenerative and pluripotent cells like ESCs. These pluripotent cells may be differentiated into the desirable lineage, including glial cells and neurons, by adequate molecular stimuli, and can be transplanted to address several CNS damages. Nevertheless, the use of iPSs gives rise to problems still to be solved, including teratoma formation, aberrant reprogramming, or presence of transgenes before their clinical application.

In summary, preclinical and clinical trials have shown that implantation of cells in the CNS yields transient benefits based on paracrine effects. However, pure cell supply therapy has brought only modest results lagging behind initial expectations, due probably to a variety of factors such as a poor cell survival in an aggressive environment and an inappropriate axon reconnection and guidance.

5. Cues influencing axon guidance and growth

Biomaterials have been studied in a wide range of tissue engineering fields since decades. They are usually manufactured as scaffolds and their general function is to mimic the specific tissue and cell extracellular matrix (ECM) during the regenerative process. Scaffolds directly affect cells behaviour, in terms of their adhesion, proliferation, migration, differentiation, and maintenance of phenotype. Biomaterials in neural tissue engineering can be tailored to trigger these effects in cell behaviour as well. In addition, scaffolds in neural tissue engineering can be useful to guide and stimulate axon outgrowth, provide a neuroprotective effect in the toxic microenvironment of the injured CNS and support glia migration.

In the last decades, researchers have investigated the cues involved in axon growth and guidance by several *in vitro* studies, which are related to cell substrates (structure, composition, mechanical properties, etc.), physical stimuli (electrical signals and mechanical stretches), cellular cues (glial and other cell types) and permissive and inhibitory molecules of axon growth (ECM components, neurotrophic factors, etc.).

5.1. Molecular cues

Cell behaviour including adhesion, differentiation, migration, etc. and particularly, axonal extension, growth and guiding are regulated by different molecules, which become thus crucial in the tasks to address CNS regeneration. Axonal extension and guidance is initiated

by the molecular interaction of microtubules of the growth cone with the surrounding signals (chemical and physical cues). The growth cone development during axonal sprouting is influenced by chemical signals, including ECM and cell adhesion molecules and neurotrophic factors. However, the complex neuronal network within the CNS results from the presence of promoting (growth factors and proteins) and inhibitory molecules of axonal growth (including canonical axon guidance molecules (semaphorins, ephrins, etc.), myelin associated glycoproteins (MAG) and CSPGs). This complex neural network of the CNS begins to form at early stages of embryogenesis and continues through the adulthood. Moreover, some of these molecules may act as promoters or inhibitory ones depending on the stage of the developing CNS.

ECM components-based substrates have been employed to obtain scaffolds with binding moieties for direct cell attachment [84]. The most commonly employed ECM components to address *in vitro* studies of axonal growth and guidance include proteins such as collagen, laminin (LN), fibronectin (FN) and their specific peptides: RGD, GRGDS, and IKVAV, etc. The ability of these molecules to generate bindings to a number of membrane-bound receptors has been exploited by several researchers, which have developed ECM components-based scaffolds to favour neuron attachment and neurite outgrowth including collagen porous scaffolds [85] and fibrin multichannel conduits [86]. However, the majority of the synthetic [87] and natural [88] substrates employed usually lack of protein-binding motifs and other permissive molecules of axon growth such as neurotrophic factors, which may be incorporated post-processing by different methods including physical adsorption, encapsulation into substrates, electrostatic interaction, covalent immobilization and cultures of natural or modified cells with the ability to secrete these molecules. The incorporation of molecules for cell adhesion improves growth cone attachment, neurite growth rate, branching and extension compared with the uncoated biomaterials [89–91], even some aspects of neural and glial cells fate can be enhanced, for example proliferation and migration [87]. The incorporation of cell adhesion molecules on substrates can be accomplished by different techniques, including physical entrapment, chemical linking and physical adsorption. These methodologies combine permissive and limiting pathways for axon guidance, to obtain parallel and preferential pathways or microchannels for axon growth. Gradients of permissive molecules on substrates can also be achieved by different techniques including microcontact printing of proteins, selective detachment of immobilized molecules by laser or electron ablation, among others. These techniques enhance neural attachment and increase neurite lengths, and even can favour the orientation of extended axons in the direction of the gradients and/or along the coated substrates and a bipolar morphology [92,93]. The non-permissive molecules of axon outgrowth play an important role on forming the correct axonal pathways; for instance, axons can be grown and guided into channels surrounded by CSPGs, in a similar way than on cell adhesion molecules [94]. Moreover, parallel axons can be grown in multichannels thanks to a combination of permissive molecules forming the axonal pathways and inhibitory ones forming the limiting regions of axon outgrowth [95].

Peptide motifs can be incorporated to the substrates in similar ways as ECM components, and accomplishing comparable improvements in cell attachment, neurite length, glia migration

and orientation of growing axons, since these peptides constitute the functional parts of some relevant proteins implied in cell attachment. Several authors have demonstrated that depleting these functional sites of the cell adhesion proteins leads to an abrupt reduction of neurite lengths and cell migration and the ability of growth cones to turn towards higher peptide densities [96,97]. Some peptides show the ability to form self-assembled nanofibrous gels, with fibres sizes of a few nanometres, by the spontaneous organization of the molecules under physiological conditions, forming non-covalent bonds including hydrogen bonds, Van der Waals forces, electrostatic forces, etc [98]. This self-organization of peptides leads to hydrogels with molecular and topographical cues to enhance differentiation of NSCs into neurons despite astrocytes [98] and neurite extension [99].

Neuronal and glial proliferation differentiation and axonal growth and guidance can be regulated and promoted by growth factors including FGF, insulin-like growth factors (IGF-1, IGF-2), vascular endothelial growth factor (VEGF), and neurotrophic factors, including NGF, BDNF, neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), glial cell-line derived neurotrophic factor (GDNF), CNTF, etc. These molecules are able to promote the differentiation of embryonic and adult NSCs [100–102] and other adult cells, including MSCs [103] and rat pheochromocytoma (PC-12) cells [104] into neuronal fates. Several studies have shown that solubilized- or substrate-immobilized neurotrophic factors produce higher neurite length, cell attachment and neuron survival [105–107]. Besides, some *in vitro* studies have shown the ability of neurotrophic factors to enhance functional connections showing expression of synapses and different neurotransmitters, even the new-formed synapses presented the ability to respond to external stimuli [105]. Immobilization of neurotrophic factors and growth factors can be accomplished by similar methods to those of proteins or peptides, including physical entrapment, chemical linking and physical adsorption for a controlled release during weeks [106]. Their effects can vary depending on the preparation method; for example, the chemical immobilization of NGF onto substrates has shown an increment of differentiation into neurons compared to physical entrapment. Chemical immobilization has resulted in similar neurite lengths and neuron survival to those studies where the growth factor is daily incorporated into culture media [107]; this result is interesting to improve the effect of biomaterials with neurotrophic or/and growth factors for neural implants. Several immobilization techniques of neurotrophic and growth factors permit to perform gradients thereof on substrates, even into scaffolds, with an important effect in neurite and axon guidance [107,108]; they have shown to elicit turning of growth cones and axons outgrowth in the direction of their gradients. The neuronal behaviour, including neurite outgrowth and length, can be even improved combining several neurotrophic and growth factors and modifying the amount of each one. Moreover, this synergistic effect can be also observed by combining factors and cell adhesion molecules. The combination of factors allows NSCs to differentiate into several neural or glial lineages, and particularly some combinations permit to obtain neuronal cells despite astrocytes, suggesting their potential use to avoid the inhibitory glial scar [109].

ECM components, growth and neurotrophic factors are not the unique molecules employed to address neurite outgrowth; other molecules including immunoglobulin-like adhesion molecules (NCAM and L1), receptors (ephrins), neurotransmitters (acetylcholine), etc. have

also been studied to address neural regeneration. Adhesion molecules as L1 and NCAM show similar effects on neurite outgrowth and cell viability than permissive ECM components; however, L1 can induce more the attachment of neurons in detriment of astrocytes compared with LN [89]. Ephrins have an important role in axon growth during CNS development, but in the adulthood, they become inhibitory of axon outgrowth. Curiously enough, on substrates with gradients of ephrins axons grow in the direction of higher gradients but growth stops at higher densities, showing the ambiguous effect of these molecules in axon growth and guidance [110]. Neurotransmitters have an important role in neuron communication and transmission of electrical impulses and, as a consequence, these molecules are vital to obtain a correct synapse formation and function and in neuron survival. Neurotransmitters can be biomimicked by chemical compounds and incorporated to different substrates, showing improvements in neuron survival and neurite extension, even similar to cell adhesion molecules [111].

5.2. Topographical cues

Cell differentiation is a process dependent of external stimuli and is well known that cell-substrate interaction and particularly topographical cues are important in this cellular behaviour. Bellamkonda discussed the anisotropy of both molecular and substrate topography as an important cue to obtain faster and more orientated axon regeneration and to direct growth cones following a unique direction [112]. This idea has resulted in a wide variety of investigations, where researchers have studied axon outgrowth, both *in vitro* and *in vivo*, on anisotropic substrates such as surface patterns, fibres and channels. Different 3D structures with pronounced anisotropy are obtained by different micro and nanofabrication techniques. For example, nanofibres are usually obtained and aligned by electrospinning techniques, while microfibres are fabricated by extrusion with different morphologies [113,114]. Microchannels are principally made by moulding employing fibres templates or other techniques such as laser irradiation, freeze-drying, dipping and ion capillarity [88,115,116]. Nano and micropatterns are fabricated by common techniques such as lithography, photolithography, moulding templates, reactive ion etching, etc. [117], which allow to obtain structures alternating permissive axon pathways (grooves) and high ridges to separate them.

Silicon-based materials such as polydimethyl siloxanes (PDMS) have been commonly employed to develop micropatterns [118]. Researchers have studied exhaustively these systems to optimize their dimensions (groove depth, width and space) to enhance axon growth and guidance. Axon alignment and outgrowth is increased as a response to greater depths of the grooves because cell bodies are restricted to the grooves, avoiding neuronal crossing between grooves. Several studies have stated that an optimal groove width of 20 μm enhances neurite extension and achieves a bipolar structure oriented in the direction of the grooves and with a significant reduction in neurite branching [119]. Other investigations gave similar results using stem cells and neuronal lines, including human neural stem cells [120] and PC-12 cells [121,122]. These studies show a similar behaviour of axons outgrowth in the micropatterned substrates: the channels width must be enough to host the neuronal body but not too much to permit neurite hyperbranching, yielding neuron

bipolar and oriented structure. These findings are conditioned to the sizes of different parts of the cells: neuronal bodies are in the range of 10-20 μm , growth cones about 5 μm , while axons and focal adhesions show sizes about 1-3 μm [123]. However some studies have shown unexpected neuron morphologies on microgrooves, in which neurons can bridge between adjacent channels with no underlying support [124].

Some researchers have exploited the idea of obtaining substrates with topographies that mimic the glial scar environment (combination of permissive and inhibitory cues on axon pathways), for example studying the behaviour of co-cultures of astrocytes and neurons. This idea was addressed by Krsk et al. [125], who fabricated silicon wafers with discrete polyethylene glycol (PEG) grafts by electron-beam, which forms an inhibitory region similar to the glial scar. These authors obtained parallel silicon 3 μm -channels separated with PEG lines and silicon arrays with different distances between PEG spacers (1, 2, 3, 4 and 10 μm). They determined that astrocytes and mouse dorsal root ganglia (DRGs) neurons grew parallel to the PEG lines onto the grooves forming elongated structures and oriented actin filaments and neurites, respectively. Furthermore, both neurites and astrocytes grew only onto above distances of 2 μm between PEG spacers, but astrocyte adhesion and spreading were comparable to the unpatterned silicon when the arrays were 10 μm long between PEG spacers.

Nanopatterned substrates (submicron features) provide differences in neurite growth and alignment with respect to the micropatterns, since the effect of nano-size features can be considered as a roughness phenomenon on cell behaviour. The nanostructured materials increase cell-substrate interactions, promoting cell adhesion and hosting neuronal processes, but not cellular bodies. Nanostructured topographies mimic the structure and dimensions of ECM components and chemicals; they cannot host axons and neural bodies, but their sizes are in the range of the ECM components (organized proteins and polysaccharides) involved in cell attachment, spreading and migration, including fibronectin fibrils (about 100 nm), actin filaments and neurofilaments (10 nm) [123]. Several techniques have been developed to obtain them: electron-beam lithography to obtain roughnesses above 3 nm, chemical and electrochemical etching with motifs above 2 nm and multiphoton excitation to address greater structures with 250 nm. Some studies have found that rough surfaces improve cell adhesion compared to the smoothest ones, in which growth cones reach even half their growth [126]. However, nanogrooves lead to less axon guidance than micropatterns because all somas and axons grow along the plateau and only the smallest axons are suitable to be aligned [127].

Nanofibres constitute an interesting nanostructure to address neural cell attachment and axon outgrowth because most of the ECM proteins assemble naturally into fibres with diameters at the nanometric scale in the physiological environment. Nanofibres technologies permit to obtain 3D structures or scaffolds and not simple superficial topography or roughness, which differentiates these substrates from the above mentioned superficial micro and nanopatterns. Researchers have developed different processes to obtain them, including phase separation, electrospinning and self-assembly of peptide nanofibres (SAPNs).

Phase separation allows obtaining fibres between 50 and 500 nm, depending on the process parameters (temperature, polymer concentration, and solvent-polymer system). This method is achieved by several solvent-removal steps with temperature shifts of a polymeric solution,

obtaining porous or fibrous structures (both of them interesting structures for different neural tissue engineering problems). These nanofibrous scaffolds provide a substrate for cell differentiation of neural lineages, even neurite outgrowth can be accomplished [128]. However, this nanofibre fabrication method shows several disadvantages because it employs large amounts of solvents and prevents to obtain aligned fibres, which limits their potential uses to address the orientation of glial cells and neurites.

Electrospinning is another current and interesting methodology to obtain nanofibres for neural tissue engineering. It is quite simple and permits to achieve nanofibres with diameters at the nanometric and micrometric scale. In addition, their operation parameters allow manufacturing a wide range of fibrous morphologies including random and aligned fibres and more complex and precise structures. The principle of the electrospinning process consists in the extrusion of a polymer solution through a small orifice (with the help of a pump pushing a syringe piston towards its needle) at high voltages, and the evaporation of the solvent as the jet is projected onto a metallic collector yielding a fibre that superposes with time forming a mat. Size and morphology of the fibres can be modified by varying operation parameters such as polymer concentration, viscosity, polymer-solvents interaction, distance from the collector, the applied voltage and external variables (temperature and humidity). The basic electrospinning process can be easily modified to obtain other fibrous disposition and morphologies: rotating drum collectors or two spaced collectors allow obtaining aligned fibrous tubes, crosslinking agents permit to fabricate polymeric networks, salts may be used to obtain porous fibres, and the most advanced electrospinning processes allow to fabricate fibres structures with a nanometric precision. This technique is able to render fibre mats (membranes) of a large amount of polymeric compositions, both synthetic and natural. The most employed synthetic biomaterials in neural tissue engineering are the family of aliphatic polyesters including poly(L-lactic acid) (PLLA), polyglycolic acid (PGA) and poly- ϵ -caprolactone (PCL), which are interesting biopolymers to be used in implants for neural tissue engineering for their biodegradability; furthermore, PLLA and PCL are approved by the Food and Drug Administration (FDA) for uses in some clinical methods. However, a wider range of synthetic polymers has been studied as electrospun substrates for axon outgrowth and guidance, such as polyamides, polyacrylates, polyacrylic acids, polydioxanone (PDS), etc. These synthetic polymers can support well glial and neural cells, in general, but they show different effects over cell behaviour due to their discrepant properties. On another hand, some researchers have developed methodologies to obtain electrospun fibres from natural polymers such as collagen, gelatine, chitosan and hyaluronic acid, because they form part of the tissues and, as a consequence, they have similar mechanical properties and cells recognize their domains easier than in the synthetic ones. However, natural polymers generally present poor mechanical properties and the electrospun fibres obtained from them show the limitation of handling for clinical uses. In addition, these materials present high costs, low reproducibility and may contribute to the immunogenic response. In order to meet the good mechanical properties and reproducibility of the synthetic materials and the major biocompatibility of the natural ones, electrospun mats of composites from synthetic and natural polymers (gelatine/PCL, collagen/PCL, PLLA/laminin, etc.) have recently been developed. Another option is to biofunctionalize synthetic fibres with ECM components (collagen, fibrin, etc.) by covalent binding or physical

adsorption; these biofunctionalized fibres enhance biocompatibility, neurite extension, and glial cell migration and alignment [91,97].

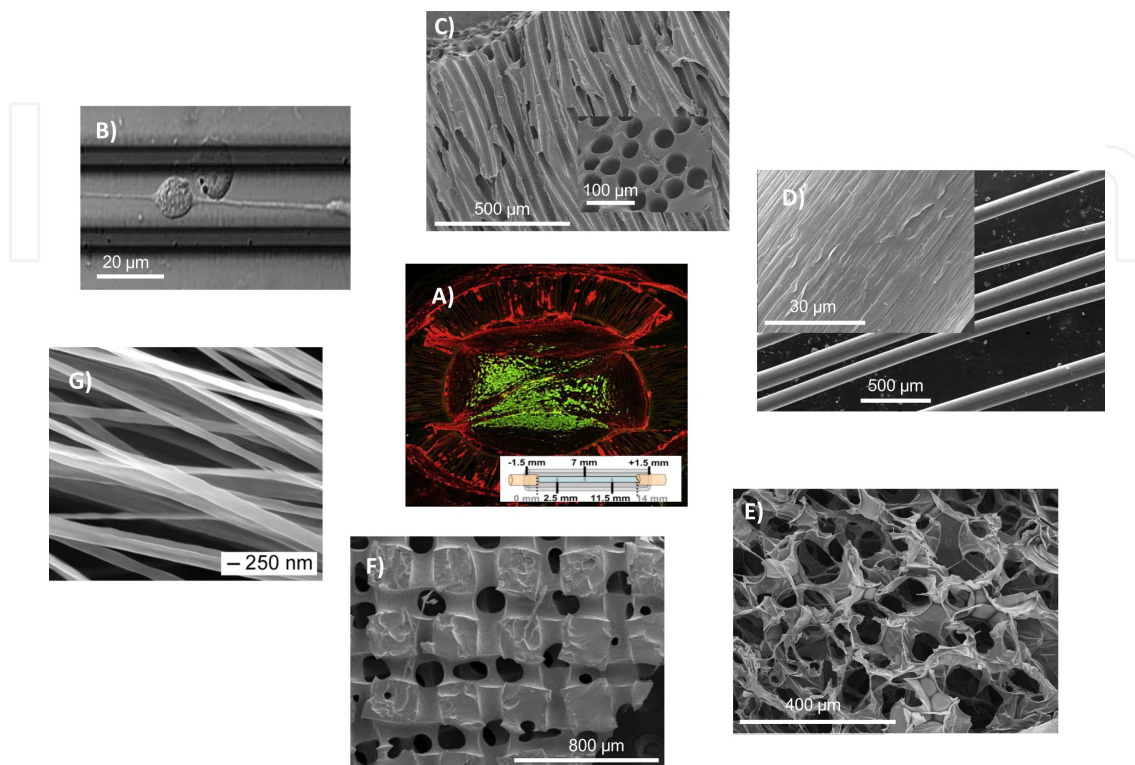


Figure 2. Topographical motifs included in the biomaterials usually employed in CNS regeneration. A) Typical conduits based on an external channel and an inner matrix for peripheral nerve and spinal cord regeneration (from I. P. Clements et al. [116] with authorization). B) PC-12 cell growing along a micropatterned substrate (from M. J. Mahoney et al. [122], with authorization). C) Multichannel poly (ethyl acrylate) scaffold, inset: detail of the multichannel scaffold cross-section (own our group). D) Microfilament of polycaprolactone obtained by micro-extrusion (own our group). E) Crosslinked hyaluronic acid scaffolds with interconnected porous structure (own our group). F) Poly (ethyl-acrylate)-based scaffolds containing interwoven microchannels (own our group). G) Aligned electrospun fibres of polylactic acid (from J. Xie et al. [113], with permission).

Electrospun scaffolds have been employed as artificial matrices for neurons and glial cells. The most common biodegradable synthetic biomaterials, performed as electrospun scaffolds, show good cytocompatibility and they are even more biocompatible than as flat substrates [129]. Some researchers have investigated cell differentiation in different electrospun fibres and have reported differentiation of NSCs and ESCs to neural lineages [130,131]. More in detail, others have concluded that fibre alignment does not influence the NSCs differentiation to a neuronal lineage, which does depend on the electrospun fibres diameter, because fibres in the nanometric scale enhance NSCs to neural lineage whereas the micrometric ones do not [132]. Similar studies with ESCs have demonstrated that both, random and aligned fibres are suitable for cell differentiation to a neural lineage; however, only the aligned fibres inhibit astrocyte differentiation [131]. This interesting result may be useful in order to employ aligned fibres as substrates for ESCs supplying neurons and inhibiting the glial scar formation in some CNS injuries. Other studies have shown that electrospun scaffolds with aligned fibres enhance a

bipolar morphology of glial and neural cells and an extension of axons, even DRG ganglia may be oriented in the direction of electrospun fibres. In addition, neurites grow faster and longer in the aligned fibres than in the random ones [91,133].

5.3. Cellular cues

The role of glial cells in the regeneration of the injured CNS is crucial because they exert several functions over neurons in the healthy and developing CNS. Glial cells can be permissive or inhibitory of axon outgrowth; even they change their roles in the developing, adult or injured CNS. The most studied glial cells to address axon guidance include SCs, astrocytes and olfactory ensheathing cells/olfactory nerve fibroblasts (OECs/ONFs).

SCs promote the axonal cone growth and show a neuroprotective effect in the regenerating axons by the release of some neurotrophic factors in the injured PNS. This property in the PNS environment has been widely explored to address axon outgrowth in the CNS. The principal role of SCs involves myelination and ensheathing the peripheral axons, but they produce MAGs, which are inhibitory of neurite outgrowth and hyperbranching. Some authors have studied the influence of SCs in axonal extension *in vitro*, showing that aligned monolayers of SCs directly affect neurite outgrowth and alignment without the help of other topographical cues [134].

CNS glial cells regulate several functions of neurons; for this reason, these cells have been studied to address their effect in neurite growth. The most widely studied CNS glial cells are the astrocytes, which main function is to provide nutrients to neurons. Astrocytes have been reported to be appropriate glial cells to differentiate neural progenitor cells (NPCs) [135]. Some authors have cultured neurons on monolayers of previously aligned astrocytes by several techniques such as electrical fields [136] or topographical cues [137], demonstrating that the bipolar morphology of astrocytes is able to enhance neurite outgrowth and alignment, even neurite outgrowth can be compared to what happens onto micropatterned proteins. However, co-cultures of neurons and random astrocytes have not demonstrated important changes in neurite outgrowth, because the astrocytes are in a multipolar shape characteristic of the hypertrophied ones, which constitute the glial scar [138].

OECs constitute one of the most promising adult cell types in order to address CNS injuries. These cells present some similarities with SCs and astrocytes (molecular expression, morphology and functions). However, they are the unique glial cells with the ability to cross the PNS-CNS transition zone helping the olfactory axons outgrowth from the nasal epithelium to the olfactory bulb. Their ability to interact with both nervous systems has given rise to the idea that OECs may be able to regenerate axons in the presence of gliotic components. In this sense, authors have demonstrated how OECs enhance neurite extension and increase the amount of axons in gliotic-based substrates such as myelin, even extension and neurite density resulted similar than using permissive substrates for axon growth such as LN [139]. Co-cultures of cortical neurons with aligned OECs have shown higher neuronal lengths and similar alignment of neurites than with astrocytes, but OECs lead to a more bipolar morphology of neurons [140].

Glial cells act on neurite outgrowth and neuron alignment by means of different cues, which include their own topography and molecular cues related with the released inhibitory and permissive molecules. The effect of molecular components has been largely investigated as commented above, but the influence of cell topography is still a rather unexplored phenomenon. A few investigations have addressed the effect of cell topography as an independent factor in neurite alignment and outgrowth by original methods. The methodology employed consists in obtaining reliefs of a substrate that mimic the shape of different cells, employing different techniques such as polymerization and lithography of masks of cellular features obtained by CAD software or impression of cell replicas. Normally, the biomimetic reliefs are formed by bipolar and oriented morphologies to enhance neurite outgrowth and alignment and the most common cells-masks include SCs, astrocytes and endothelial cells morphologies. Aligned cellular motifs of SCs have an influence on neurite alignment and increase their length compared to flat substrates and random motifs, even perpendicular reliefs have an inhibitory effect in axon outgrowth [141]. Although astrocytes and endothelial cells can enhance neurite alignment too, SCs have improved the most these aspects of neurites, higher degrees of alignment and sizes of their dimensions (weight, length and height), in comparison to astrocytes and endothelial cells [142].

5.4. Electrical cues

As is well known, electrical voltages play an important role in cell behaviour. Particularly, the transmission of information between neurons is accompanied by electrical changes in their environment; this phenomenon is known as action potential (AP). Electrical stimuli are produced by the existing voltage gradient across the cell membrane and the complex equilibrium of environmental and cellular ions and ionic channels across the membrane. The steps of the process include depolarization of the neural membrane as a consequence of the stimulus received by dendrites of a neighboring neuron, subsequently the Na^+ channels open and if there are sufficient opened channels the entering flux of Na^+ modifies the cell membrane potential. The following step includes the repolarization of the membrane by the opening of the K^+ channels, which act slowly and restore the original action potential. However, before reaching the normal membrane potential, neuron is hyperpolarized preventing it from receiving another stimulus and neuron cannot generate another AP during a brief time, ensuring that signals travel always in a unique direction.

Electrical stimulation of neural cells has led to the development of new frontiers for neural tissue engineering in the recent decades, including investigations of new conductive biomaterials and fabrication of electrodes to address several damages in the CNS. Actually, the efforts have focused on the study of conductive polymers for their electrical conductivity combined with their characteristics as organic polymers. The electrical properties of these polymers are due to the presence of loosely held electrons in their backbones that form conjugated π bonds, and with the help of doping chemicals the polymer can be negatively or positively charged. Traditionally, dopant agents employed have been inorganic compounds such as ionic species (chloride, perchlorate, iodine, etc.). However, the introduction of active biological dopants is more attractive from the point of view of biomedical applications [143]. The most commonly

studied conductive polymers include polypyrroles (PPy), polyaniline (PANI) and polythiophene derivatives, specially, the poly (3, 4-ethylenedioxythiophene) (PEDOT), but other emergent materials as piezoelectric polymers and carbon nanotubes and nanofibres (CNTs and CNFs) as fillers are promising to develop conductive polymers for neural tissue engineering. Several studies have shown that PPy are biocompatible with different cell types [144] and, particularly, with neural cells [145]. PANI and PEDOT as conductive polymers for general tissue engineering applications have been less studied than PPy ones, but in the recent years they are more investigated for these purposes. Recent studies have demonstrated the absence of cytotoxicity of PANI and PEDOT by *in vitro* studies, showing the biocompatibility of these alternative biomaterials [146,147]. Moreover, the tissue response of these three conductive biomaterials has been studied preliminary in some *in vivo* studies, resulting in non-toxic effects and with an acceptable inflammatory response [148–150].

External electrical stimuli can be applied to cells or tissues to produce an artificial AP leading to electrical changes in cells; this phenomenon is termed as electrical stimulation. Electrical stimulation can influence cellular activities such as proliferation, migration, differentiation, extension of processes and protein expression [151–153]. Just as other cells, neuron activity can be affected by electrical fields, both direct and alternate currents (DC and AC, respectively). Some works have shown how an electrical stimulation influences the direction and extension of neurite outgrowth [148,153] and the alignment of other cellular types such as astrocytes as well [154]. In other studies, the electrical stimulation has been found to increase neurite extension in differentiated PC-12 cells cultured on PPy films [155]. Most recent studies have corroborated the enhancement of neurite outgrowth with electrical stimulation in other cell types and different conductive biomaterials, for example NSCs on PANI [156], cochlear neural explants on PPy [157] and DRG explants on PPy [158]. In addition, these works have demonstrated, as Patel et al. [159] suggested, that one explanation for the increment in neurites length observed is the electrophoretic accumulation of proteins on the surface of the substrate.

As stated previously, CNTs and CNFs are an emergent and promising group of conducting materials for neural tissue engineering. CNTs are composed of rolled structures of sheets of graphene, either single-(SWCNTs) or multi-walled (MWCNTs). The most relevant properties of these materials are their ability to conduct electrical stimuli, form structures with a very large exposed surface per volume unit, and good mechanical properties (strength, flexibility, ductility, etc.). The first experiments with CNTs showed that they are cytotoxic when they are in suspension in a cell culture; nevertheless, posterior studies did not show any cytotoxicity when they are immobilized in the culture [160]. This fact meant the beginning of their use in tissue engineering, and for this purpose, it was necessary to obtain CNTs with higher purity and fabricate more soluble CNTs by functionalization of their surfaces. Other novel conducting polymers are the piezoelectric ones, which have the ability to generate electrical stimuli by the help of small mechanical deformations, being the application of external stimuli unnecessary. The most common piezoelectric polymer is the poly (vinylidene fluoride) (PVDF). This material has demonstrated good attachment and proliferation of neural cells when it is combined with l-lysine [161]; even neurite outgrowth can be enhanced compared with non-piezoelectric materials [162]. Unfortunately, piezoelectric biomaterials have the drawback of

not allowing the external control of the electrical stimulation, for what it seems preferable to employ conducting polymers in neural tissue engineering.

Conducting polymers possess a high degree of conjugated bonds providing them rigidity, insolubility and difficult processability, for what these polymers may be transformed or employed in composites with others. Besides, they are usually non-biodegradable and usually offer poor biocompatibility; for these reasons some researchers have investigated the possibility of obtaining conducting biodegradable biomaterials by different techniques including polymerizations that form degradable linkages, blending conductive polymeric segments with biodegradable polymers, and fabrication of copolymers of biodegradable monomers and conductive biostable ones [163,164]. With the aim of improving neurite outgrowth and alignment of cells, bioactive molecules such as neurotrophic factors have been immobilized on conducting polymers [165], their topography has been modified by surface treatments to generate microchannels [166] or they have been electrospun to obtain nanofibrous matrices [158].

5.5. Mechanical cues

The CNS is one of the softest tissues in the body, with elastic modulus between 0.1 and 10 kPa [167]; others, more rigid as bone and connective tissues, present higher elastic moduli ranging 15-30 MPa and 100-1000 kPa, respectively [168]. Rigidity has an important role at early stages of embryogenesis and development of tissues, and particularly in the CNS, since cells regulate the formation of ECM components and their migration by mechanical cues. The rigidity of the brain increases with aging, and the highest elastic modulus of the nervous system tissues corresponds to the spinal cord [169]. This accounts for the modulation of the differentiation of neural precursors into glia or neurons by changing the substrate's rigidity; for example, ESCs differentiate into glia in soft substrates whereas they do to neurons in more rigid ones [170], and NPCs and NSCs show a similar behaviour, differentiating preferably into neurons on softer substrates with moduli between those of the neonatal and the adult brain [169,171].

In general, axon extension increases in softer substrates, so elastic biomaterials are more suitable to address axon regeneration. The most commonly employed substrates with modulated stiffness in neural regeneration are gels as hyaluronic acid, fibrin, polyacrylamide and PEG due to their mechanical properties similar to the CNS tissue. Similar trends in axonal extension and branching have been observed in different types of neurons: the softer or more elastic substrates generally increasing neurite length, neuron attachment and branching compared to harder ones [170,172,173]. However, axonal extension decreases in very soft substrates, when they present elastic moduli much lower than that of the CNS tissue [174,175]. Co-cultures of astrocytes and neurons on hydrogels with tuneable mechanical properties have shown that, unlike neurons, astrocytes attachment decreases on soft substrates. This interesting result has highlighted the beneficial uses of soft substrates to address CNS injuries with presence of glial scar [170]. However, neurons have shown good attachment and large neurite extension and branching cultured on substrates, such as PDMS, which possesses higher elastic modulus than CNS tissue, suggesting that neuronal attachment depends on substrate chemistry too [176].

In the latest stage of embryogenesis and during the maturation up to the adult CNS, axons are submitted to great strains resulting in abrupt changes in their extension. The knowledge of this natural phenomenon has led to investigate how strain can influence axon extension. As an example, axon outgrowth has been studied in engineered bioreactors with the ability to stretch axons on permissive and flexible substrates such as collagen. The normal axon outgrowth is limited to 1 mm/day by the synthesis and transport of proteins and structural components to permit the extension of growth cones. However, cultures of DRG on collagen in a stretch-grown bioreactor and applying a constant strain of 1 cm/day have demonstrated that axons can grow up to 5 cm in only 8 days, which is a much longer extension than that reached by conventional therapies in the regeneration of CNS tracts (2-3 cm). Moreover, the stretched axons maintained their typical cytoskeletal proteins along them and an appropriate structure in their cross section [177], even the electrical signals showed normal action potentials due to the increment of the K^+ and Na^+ channels density [178]. Cyclic strains can influence neurite outgrowth just as constant ones. Cultures of neural cells with cyclic strains have demonstrated a synergistic effect of applied strain and cycles frequencies. This effect has been studied in cultures of PC-12 cells on PDMS substrates with cyclic stretch and strain levels of 4, 8 and 16% and strain rates of 0.1, 0.5 and 1 Hz, showing that only two operation conditions enhance neurite density, length and orientation [179]. In contrast, other operation conditions seem to inhibit neurite length, density and orientation compared to static substrates.

5.6. Physical and chemical cues

Some investigations are related to the study of the effect on neural cells of surface properties of biomaterials such as charge, functional groups density and wettability. Surface wettability can be modified by several techniques such as plasma polymerization, even surfaces with gradients of wettability can be obtained. In works on the effect of surfaces with uniform or graded wettability on hippocampal neurons it can be observed that graded substrates and particularly their most hydrophilic regions show an increased cell density, longer and faster development of the processes than uniform substrates and hydrophobic regions [180]. The surface gradients seem to promote interactions between neighbouring cells and the development of neuronal networks. Similar results were found by culturing PC-12 cells onto polyethylene surfaces with wettability gradients prepared by a corona discharge treatment [181]: cell density was the highest at intermediate wettability of 55° but the length of neurite processes increased on more hydrophilic regions, suggesting an important role of surface hydrophilicity on the differentiation of cells into neurons. Different gradients of wettability on the surfaces of a substrate can be obtained by self-assembled monolayers (SAMs), by the addition of different siloxanes with methyl-terminated molecules. On these substrates, PC-12 cells can differentiate to neurons with neurotrophic factor-free medium, and the differentiation is enhanced on substrates with higher surface wettability. These results suggest the importance of mechanical, chemical and spatial cues in neural fate [182].

6. Biomaterials for traumatic brain injury and similar neuronal damages

Traumatic brain injury and similar brain damages including hypoxia or stroke produce an abrupt loss of neuronal and associated glial population at a primary stage. This initial damage is followed by inflammatory processes leading to a secondary loss of neurons and glial cells and the astrocyte activation forming the inhibitory glial scar for axon outgrowth. Brain tissue damage constitutes, thus, a multistep process leading to the formation of a lesion cavity and necrosis, surrounded by glial scar avoiding axonal regrowth and, as a consequence, the successful healing of the damaged tissue. The cavity generates a loss of brain tissue forming a hostile region to support cells that, together with the inhibitory glial scar, avoid its regeneration leading to psychiatric and neurological symptoms. The inhibitory nature of the CNS due to the presence of astrocytes and oligodendrocytes, the lack of enough neurogenesis niches and the toxic microenvironment after brain injury avoid the regeneration in the brain.

The justification for employing biomaterials after brain damages is principally this lack of cell support, and biomaterial scaffolds should be able to mimic the natural environment of cells promoting their attachment and/or entrapment aiding the regenerative process. Scaffolds can lodge cells in a tridimensional context, allowing diffusion of nutrients and acting as substrates to enhance cell survival, neurite extension, axon regeneration and cell infiltration. The selection of the biomaterials in brain regeneration is based on the principles to achieve neuroprotection of the surrounding native tissue avoiding the secondary cell death, and neuroregeneration of the damaged tissue to restore functions.

Normally, the use of natural polymers is preferable rather than synthetic ones because most of these biomaterials are present in the tissues and their effects are well known; however, many synthetic biomaterials have though a good integration with brain tissue and present some advantages respect to the natural ones, including lower cost, enhancement of mechanical properties, handling, etc. The functional groups within the employed biomaterials can affect cell attachment, since surface charge, hydrophilicity and hydrophobicity are determinant on the biocompatibility of the biomaterials. Cells prefer to attach on positive surfaces because their membrane contains residues of sialic acid, which provide a net negative charge. On the other hand, substrate hydrophilicity and hydrophobicity govern the formation of non-specific protein layers, which can cause inflammation, and the conformation of proteins involved in cell attachment.

The device size is an important parameter in order to cause the minimal damage due to the invasive surgery. Besides, the biomaterial morphology is crucial to ensure nutrient transport, vascularization, cell attachment and colonization in the inner structure. Moreover, ideal structures should provide high surface/volume proportions (interconnected porous scaffolds, microspheres, nanofibres, etc). The surface of substrates plays also an important role in cell attachment and behaviour (view previous section). Mechanical properties such as elasticity influence cell spreading; thus, softer substrates (mimicking the mechanical properties of the brain) can favour the differentiation of neural precursors and NSCs into neural lineages. From the biological point of view, a basic requirement of any material used is obviously its biocompatibility, avoiding adverse cell response, which leads to the formation of gliotic scar and/or

acute inflammation. Thus, researchers look for materials not inducing astrocyte reaction and showing a modest inflammatory process. Besides, the biomaterial should preferably biodegrade without leaving any foreign residue as the regenerating tissue replaces it.

Preclinical strategies using biomaterials to address brain damages should satisfy these requirements and provide brain neuroprotection and neuroregeneration. Most of the studied biomaterials for brain regeneration include *in situ* formed gels [183] and porous interconnected scaffolds [184]. In addition, other types of structures such as fibres [185], aligned microchannels and interwoven microchannels have been also proposed [186].

6.1. Natural biomaterials

Natural polymers are similar to some substances present in tissues (polysaccharides, proteins and glycosaminoglycans), even many natural biomaterials form part of the ECM compounds such as collagen and hyaluronic acid. Therefore, implant devices based on natural polymers have in general high biocompatibility. Moreover, in some cases, they may provide signals to cells improving cell-substrates interactions and modeling cell behaviour. They are usually biodegradable by enzymatic degradation, making them good candidates for implantable devices in brain.

A commonly employed biomaterial for tissue engineering is collagen, which is a fibrillar protein forming part of the ECM compounds. Collagen is a natural biodegradable polymer and shows good biocompatibility when it is employed for brain regeneration purposes. This natural polymer has been implanted in the brain forming interconnected porous scaffolds or *in situ* gels. Several authors have employed commercial scaffolds from decellularized and dried tissues from human donors, maintaining the natural structure of collagen tissues [187–189]. Moreover, collagen may be tailored by different processes, such as freeze-drying, in order to obtain porous scaffolds, which are more suitable for cell ingrowth [190]. However, this biomaterial alone does not reduce the size of the lesion cavity nor produces significant functional recovery [187]. The implantation of crosslinked scaffolds based on mixtures of collagen and glycosaminoglycans (GAGs) such as hyaluronan, chondroitin sulphate and heparan sulphate seems to lead to some functional recovery [188]. Freeze-dried collagen-GAG scaffolds with 140 µm-pore diameters and porosity around 96% remained in rat brains 28 days after implantation, reduced the lesion volume, increased the amount of cells in the surrounding tissue, promoted the secretion of neurotrophic factors such as GDNF and BDNF by the host cells and stimulated neurogenesis of neural precursors from the SVZ. Consequently, the inflammatory response of brain tissue was not excessive, promoted cell proliferation and differentiation of migratory neuroblasts, suggesting certain degree of regeneration. Moreover, the results from different tests regarding behaviour, motor and reflex response in animals (adhesive-removal tests and neurological severity scores-NSS-) suggest that animals with biomaterial implants show less neurological deficit than the untreated groups; therefore, collagen-GAG scaffolds achieve some functional recovery without the need to combine with other therapies including drug release or cell entrapment. Some brain functional recovery can be accomplished combining collagen-based interconnected porous scaffolds with seeded and expanded MSCs. MSCs have the ability to release neurotrophic factors and migrate into the

lesion boundary zone, remodelling the injured brain tissue and reducing the lesion cavity [188–190]. The implantation of these hybrids in the brain of rats and mice promoted angiogenesis by the formation of new vessels, and increased the neural fibre length in the scaffolds. Some neuroregeneration was assessed by sensomotor functional recovery and improvements in spatial learning, which are higher compared with separated treatments, due to the beneficial effects of the collagen substrates to support and localize cells and the molecular signals produced by MSCs [187]. The use of this biohybrid system implies an important limitation that is the need for open-surgery to implant it in the brain. To avoid such invasive process, an *in situ* gelation can be followed, since collagen is able to form gels at physiological temperatures. For example, mixtures of collagen with NSCs and proteins (fibronectin and laminin) have been gelled *in situ* and employed in the brain. This post-implantation gelation of collagen with NSCs allows cells to distribute widely in the lesion site, oppositely to what happens when NSCs are implanted alone. The incorporation of proteins improves transplanted cell survival during weeks, and the use of laminin shows the highest levels of NSCs survival, which is related with the improvements of cognitive functions suggesting a higher grade of neuroregeneration [183].

Hyaluronic acid (HA) is a negatively charged GAG with a high molecular weight and an abundant ECM component found in the CNS tissue. As biomaterial, it presents interesting properties for neuroregeneration purposes, including biocompatibility, biodegradability and a high degree of swelling. HA products from the degradation process stimulate endothelial cell proliferation and migration promoting angiogenesis. The high degree of swelling permits to accommodate and cover entirely the lesion cavity, conferring it mechanical properties similar to those of the brain tissue and allowing nutrients, ions and solutes transport. Besides, HA of high molecular weight has an anti-inflammatory effect, allowing the control over microglia activation and avoiding foreign body reaction. The ability of HA to absorb large amounts of water allows it to be cross-linked under diluted solutions, obtaining hydrogels. HA cross-linked hydrogels and a successive step of freeze-drying allow obtaining porous structures, which can be from nanoporous to interconnected macroporous (scaffolds) structures. The formation of each structure depends on the crosslinking conditions and is due to the freeze-drying of the water absorbed by the molecule. Thus, the principal advantage of porous scaffolds is to increase the surface area for cell attachment and ingrowth. HA-based hydrogels containing pores up 60 μm implanted in the rat brain have demonstrated their angiogenic activity, inhibition of formation of on the one hand a glial scar and on the other of a secondary cavity or an envelope by hyperplasia or connective tissue and cell aggregation [187]. The major limitation of HA is its poor cell adhesion; subsequently, it is usually employed with adhesion molecules or in combination with other polymers. For example, HA-based scaffolds with immobilized LN have demonstrated improvements in the astrocyte response and the stimulation of neurite ingrowth, suggesting that HA-based scaffolds with these proteins promote neuroregeneration [191]. HA-based scaffolds with peptides such as IKVAV show similar effects than with laminin immobilization, that is, a limited and localized inflammatory response, angiogenesis, neurite ingrowth and the complete integration of the scaffold within the host tissue [184]. In order to obtain interconnected porous scaffolds, HA may be copolymerized with adhesive molecules. Therefore, scaffolds based on copolymers of HA and poly-d-lysine enhance cell migration and attachment without increasing inflammatory

response when they are implanted in rat brains [192]. Poly-d-lysine in combination with HA grants scaffolds with a more positive net charge, which improves cell attachment as mentioned above. The limitation to implant HA scaffolds is again the invasive surgery required, but HA can be combined with other polymers to form gels *in situ*. For example, combining methylcellulose with HA thermoresponsive gels can be obtained, which, similarly to HA scaffolds, reduce the lesion cavity and attenuate microglia activation [108]. The combination of HA hydrogels with neuroprotective molecules, such as erythropoietin (EPO), promotes migration and proliferation of NSCs and NPCs from the SVZ, reduces apoptosis and increases the amount of neurites, suggesting some neuroregeneration in the injured cortex [193]. The neuroregenerative effect of HA biomaterials can also be promoted by attaching to the hydrogel receptor antibodies of inhibitory molecules for neural growth, such as NoGo [194].

Chitosan is derived from the alkaline deacetylation of chitin, a natural polysaccharide found in crab shells and many shellfish. The degree of deacetylation produces a positive charge and, consequently, increases cell attachment, reduces inflammation and enhances biocompatibility. Chitosan is naturally degraded by the effect of lysozyme and its biodegradability can be controlled by the degree of deacetylation. It has shown good biocompatibility with neurons and glial cells *in vitro*, although the viability of neural cells should be improved [195]. Chitosan becomes a thermally responsive gel with glycerolphosphate salt at physiological temperatures, and the gelation process forms a hydrogel able to attach cells and transport nutrients, even the degradation process allows cell infiltration. This property has been exploited to obtain gels *in situ* for brain repair avoiding invasive surgeries. However, the implantation of chitosan gels into rat brain has been found to produce a high inflammatory response by activation of macrophages, leading to the complete phagocytation of the biomaterial in a few days [196]. The activation of macrophages is due the receptor-mediated binding of N-acetylglucosamine, and this effect should be controlled for its future application in the human brain.

Methylcellulose is a cellulose derivative obtained by methyl substitution, forming a water-soluble compound and a thermoresponsive gel. The gelation of this compound can be performed at physiological temperature by altering salt concentration and composition, allowing it to be injected into brain lesions by a minimal invasive surgery. Moreover, its potential use for brain regeneration comes motivated by the approbation of a methylcellulose system (Methocel A®) in nerve repair by the FDA. Methylcellulose gels injected in rat brain have demonstrated reduction of cavity area and glial scar thickness and a similar inflammation area compared with control groups (lesion without gel injection), concluding that methylcellulose is an appropriate biocompatible biomaterial for brain regeneration [197]. However, the principal limitation of methylcellulose gels is that they are not biodegradable; for this reason they have been mixed with biodegradable polymers such as HA to obtain semi-biodegradable gels for their use in the brain [193].

Other ECM compounds, including LN and fibrin, have been employed as permissive substrates for brain regeneration; they are usually employed as injectable *in situ* forming gels or by mixing with other polymers to improve cell attachment. These substrates are able to immobilize neurotrophic factors such as BDNF yielding a permissive substrate for neurite growth and a neuroprotective effect by the incorporation of this molecule. The use of these

systems in ischemic models has resulted in a reduction of the infarct volume and in the stimulation of neurogenesis in the dentate gyrus of hippocampus of rats [197]. Hybrid systems employing fibrin gels and MSCs are improved by the incorporation of a growth factor (bFGF), attaining advances in cell survival by the reduction of the apoptotic activity. These systems implanted into rat brain provided more significant improvements in morphological and functional recovery, including reduction of the cavity lesion and neurite and astrocyte infiltration within the gels [198]. The use of SAPNs in brain regeneration has resulted promising due to the ability of these peptides to form nanofibres percolating in a continuous network under physiological conditions. The peptide solution can be injected with a non-invasive surgery and the self-assembled peptides form nanofibrous gels by the presence of ions, filling entirely the cavity lesion and yielding a natural substrate with molecular and topographical cues for axon ingrowth. The gelled SAPNs are integrated within the host tissue and reduce the secondary injury that causes further cell loss, decreasing the lesion volume. Moreover, the astrocyte activation is lower, which reduces the thickness of the glial scar, and the microglia activation decreases during the acute inflammation, suggesting that SAPNs can be successful employed in brain regeneration [199].

6.2. Synthetic biomaterials

Unlike natural polymers, synthetic ones show lower costs, may be tailored in multiple fashions with different properties, are easier to be obtained and the batch processes are more repetitive than natural ones. The fabrication methodologies and modifications of synthetic polymers allow obtaining a wide range of properties and structures for the purposes of brain regeneration. Thus, substrates with properties similar to those of neural tissue can be produced, either biodegradable or bioestable in the shape of structures mimicking the natural ECM.

Acrylate-based biomaterials have been widely proposed for peripheral nerve repair. Poly (2-hydroxyethyl methacrylate) (PHEMA) and poly (hydroxypropyl methacrylate) (PHPMA) are the most common acrylate substrates employed in brain regeneration, for its ability to form hydrogels, yielding substrates with similar properties to the natural tissue. Both polymers show a good biocompatibility and integration with the host tissue adhering firmly to the surrounding brain tissue [200]. The implantation of crosslinked PHEMA and PHPMA materials owning interconnected porous structures led to a dense glial scar and CSPGs deposition surrounding the interface material-host tissue. However, both polymers allowed the infiltration of astrocytes, tissue compounds (fibroblasts, collagen and blood vessels) and neurites within the porous structure. PHPMA scaffolds showed improvements towards brain regeneration in terms of neurite infiltration and presence of larger amounts of neurites and connective tissue within their pores. However, these scaffolds presented a low cell adhesion, which can be enhanced by the incorporation of adhesive peptides. Thus, the immobilization of IKVAV and RGD peptides on PHPMA led to the structural continuity across the cavity, resulting in a more adequate substrate for axonal ingrowth and reduction of glial scar [201,202]. PHPMA has been combined with RGD motifs and engineered fibroblasts to express CNTF and/or BDNF, resulting in larger amounts of regrowing axons, less astrocyte prolifera-

tion and similar inflammatory response than hydrogels containing only engineered fibroblasts [202].

Aliphatic polyesters are a family of synthetic polymers widely employed for CNS regeneration; they are easily obtained by ring opening polymerization. The most common aliphatic polyesters are PCL, PLLA, PGA and PLLA-PGA co-polymers (PLGA). The FDA has approved the use of these polymers for several tissue engineering purposes, which makes them attractive in the regeneration of brain damages. A large variety of structures can be achieved with these polymers due to their easy processing (porous scaffolds, electrospun fibres, etc.). They show different mechanical properties and biodegradation rates, the development of a wide range of intermediate ones by the possibility of forming co-polymers. PCL and PLGA have been designed as porous scaffolds with a spongy structure for TBI models and have demonstrated a good biocompatibility with rat brain tissue [203]. Both polymers have shown the ability to maintain the volume of the lesion cavity rather than control groups, preserving the healthy tissue of the primary injury and attenuating the secondary tissue loss. PCL and PLGA reduce cell death, induce neurite ingrowth and do not cause severe inflammation. However, PCL has some advantages over PLGA scaffolds because it provokes a lower microglia and astrocyte activation, and promotes higher levels of neurite ingrowth [203]. PCL with different architectures has been tested for brain regeneration. As an example, PCL nanofibres, both random and aligned, implanted in rat brain have shown a good biocompatibility through the absence of encapsulation and foreign body response, and they have revealed unexpected results. The random nanofibrous scaffolds allow neurite infiltration in the fibrous mesh, whereas the aligned ones show no neurite ingrowth and they are preferentially localized on the surface of the mesh after 7 days [185]. This phenomenon reveals that aligned fibres constitute a good substrate for neurite alignment and growth on its surface by the phenomenon of perpendicular contact guidance without infiltration into the inner network. PCL channel-based architectures with different internal structure (single channel, parallel channels and perpendicular channels) have been employed to address brain damages and guide axon growth in rats. All channel-based structures have demonstrated to be useful for neural ingrowth and they reduce glial scar formation. Particularly, orthogonal channels resulted in the optimal structures for neurite alignment constituting a more permissive environment for cell ingrowth and tissue integration, while parallel channels favour the re-growth of neurites along the surface of the channel [186].

The most common materials employed for brain regeneration purposes are the mentioned polyacrylates and aliphatic polyesters, but other families of synthetic polyesters have also been tested, such as poly (trimethylene carbonate) (PTMC). PTMC can be obtained with different biodegradabilities, the PTMC with fast degradation and slow resorption showing a similar degree of brain regeneration than PLGA. Oppositely, PTMC with a fast rate of degradation and resorption presents a faster and more efficient tissue recovery than common PLGA [204]. Silicon substrates are components also employed in neural interfaces such as electrodes and their functionalization with the protein of neuron adhesion, L1, has shown reduction in

astrocyte reaction, fibroblast adhesion, and microglia and macrophages reaction. Moreover, L1-functionalized silicon increases neurons density at the implant-tissue interface [205].

CNTs have also a great interest in neural tissue engineering due to their electrical properties, and their problems of biocompatibility can be elicited by chemical modifications. Indeed, both hydrophobic and hydrophilic modified CNTs have been implanted in the damaged brain of rats resulting in functional recovery. Their benefits in brain regeneration can be enhanced by combining NPCs and CNTs; these systems reduce the gliogenesis of NPCs promoting the differentiation into functional neurons, which restore electrical activity and lead to synaptic formation [206].

7. Biomaterials for spinal cord regeneration

SCI is usually caused by traumatic injuries, including traffic accidents, acts of violence, falls and sports injuries. Damages in spinal cord are due to a mechanical insult following a fractured vertebra or disk intruding into the spinal canal, and can lead to a partial or complete spinal cord transection. The majority of patients die for respiratory complications, because the injury at any level of the spinal cord implies difficulties in respiratory function due to the destruction of the descending motor tracts, which regulate muscles, such as diaphragmatic, thoracic, and abdominal, implied in the breathing process. Consequently, the patients with SCI require assisted breathing, and the clinical treatments are limited to reduce the pain and prevent secondary injuries, including the administration of anti-inflammatory drugs. However, the classical clinical treatments do not achieve tissue regeneration nor recovery of the lost functions.

In early stages, the damaged tissue presents necrosis due to physical trauma, hemorrhage and disruption of the BSCB leading to ischemic processes. The disruption of the BSCB produces the infiltration of foreign components, including fibroblasts, macrophages and cytokines, which are involved in the activation of astrocytes leading to the formation of the gliotic scar and inflammatory processes. After a few days, the apoptotic pathways are activated by the presence of a toxic microenvironment, producing glial and neurons loss without the possibility of replacement by the absence of sufficient endogenous progenitors. The primary axonal loss is followed by a continuous axonal degeneration due to demyelination by oligodendrocytes loss, the block of signalling and the lack of a permissive microenvironment for axon regrowth. Moreover, the axon degeneration progresses over months and years, even it can affect the distal ends of several axons (Wallerian degeneration) leading to the loss of entire spinal tracts.

The axons in the spinal cord form longitudinal bundles, tracts, and they are similar to the nerve fascicles that compose the peripheral nerves. Thus, several researchers have proposed the use of nerve conduits for this pathology due to their success for peripheral nerve repair. However, the spinal cord presents some physiological differences and the regeneration is more complex due to the limitation of regeneration in the CNS. Owing to the complex geometries, partial transection founded at spinal cord lesions and the need of invasive surgeries for the implan-

tation of guidance conduits, many researchers have considered more convenient the use of injectable hydrogels.

As stated, the regeneration of the spinal cord requires guidance structures for a correct axon regrowth, since the tracts in the spinal cord are formed by aligned bundles of axons. Particularly, tracts in the spinal cord own diameters about 100-1000 μm , so the implanted scaffolds in the spinal cord must be permissive to the formation of axon bundles around these diameters. Moreover, the myelinated axons in the spinal cord environment show diameters around 20 μm , thus the SCI scaffolds should be able to guide individual neurite outgrowth as well. The degree of swelling of a material under physiological conditions is important to maintain an appropriate alignment and avoid the compression of the regenerating tracts. Other properties, similarly to what happens with brain regeneration, are important to design scaffolds for spinal cord regeneration; they include biocompatibility, controlled biodegradability, high surface/volume to permit cell attachment and the correct nutrient transport, similar mechanical properties to the host tissue, etc. Guidance structures can be fabricated by different techniques; the most common ones include rolled sheets of aligned electrospinning, scaffolds obtained by freeze-drying of polymeric solutions or moulding combined with particle leaching to generate porosity, or printing the polymers previously designed by CAD software.

7.1. Natural biomaterials

The natural polymers employed in spinal cord regeneration are similar to the ones proposed for brain regeneration, and include fibrillar proteins, polysaccharides, glycosaminoglycans and carbohydrates. The most common are the ECM components collagen and hyaluronic acid, polymers derived from algae such as agarose and alginate, and proteins derived from blood plasma such as fibrin and fibronectin. They are potential materials for the purpose of spinal cord regeneration for their possibility of forming hydrogels *in situ*, avoiding invasive surgeries. However, they are commonly fabricated as porous scaffolds and guidance conduits to be implanted in the spinal cord lesions and to permit axon regeneration in an aligned manner.

Collagen has been successfully employed in peripheral nerve guidance channels, resulting in conduits approved by the FDA as Neuromatrix™ and Neuroflex™. Collagen shows mechanical properties similar to spinal cord tissue, and possesses peptide-motifs for cell adhesion, migration, proliferation, etc. It has a good biocompatibility and integration with the host tissue of the spinal cord, and can be obtained in different shapes, including the aforementioned guidance conduits [207,208]. However, this material lacks ability to support and promote axon growth, and consequently its injection to obtain a gel *in situ* has resulted in dense inclusions within the gel and cavities at the interface biomaterial/host tissue which impede axon growth [207]. In most cases, axon attachment and guidance can be promoted by topographical cues. For this reason, aligned and random collagen electrospun fibres have been developed forming conduits for SCI models, which have shown good results concerning the integration with the spinal cord tissue: reduction of the acute inflammatory response, non-formation of glial scar at the interface between the lesion site and the scaffold, cellular infiltration into the scaffold and axonal sprouting [208]. However, this system has resulted in low axonal sprouting and the aligned conduit does not promote neurite alignment. That is, collagen alone does not

promote axon regeneration or alignment, which is a fundamental task in spinal cord repair, and its use requires functionalization to incorporate molecular cues, or to combine it with other permissive substrates of axon guidance and growth. Indeed, collagen has been employed as filling of other polymers' conduits, resulting in the absence of axonal ingrowth and regrowth, but when collagen includes growth factors (FGF or NT3), the axons grow into the channels [209]. The immobilization of peptides such as laminin improves axonal regeneration in the SCI; thus, laminin gradients in a collagen gel filling have shown to increase axonal length and provide directional guidance to the collagen fibres [210].

HA, like collagen, shows a good compatibility with spinal cord tissue but does not promote axon attachment and growth, impeding regeneration. However, the implantation of HA gels in spinal cord lesions is able to reduce the lesion cavity, reduces inflammation and gliotic scar in the surrounded tissue. The reduction of inflammation and gliosis is due to the own nature of HA: the negative charge of the HA chain inhibits cell attachment and their binding site interactions with the cell membrane receptor CD44 of macrophages, limiting the inflammatory effects [211]. HA can be functionalized or combined with other polymers to improve axon attachment and regeneration. For example, the incorporation of poly-L-lysine into HA gels can promote neurite attachment, while the incorporation of the nogo66 receptor antibody, which has an agonist interaction with the inhibitory molecule nogo66, promotes axon regrowth. This system has yielded to the reduction of the glial scar at injury sites promoting wound healing and has facilitated angiogenesis; it has even shown improvements in axon regeneration, leading to both myelinated and unmyelinated axons [212]. The combination of HA with methylcellulose (HAMC) produces gels *in situ*, which are employed in brain damages and SCI, too. This gel has the advantage of covering entirely the lesion cavity, and the possibility of incorporating factors or other neuroprotective compounds enhancing SCI regeneration. For instance, the incorporation of EGF and EPO in the HAMC gels has improved neuroprotection at the implant site and increased neuron density [213,214].

Agarose hydrogels, like collagen and hyaluronic acid, is not adequate for axon infiltration, but is a good substrate when it is functionalized with protein gradients, of laminin for example. Axons grow, preferably, up the laminin gradient *in vitro*, which means promotion of axon extension and guidance [215]. Agarose permits to obtain conduits with an internal structure of aligned linear pores, by means of a freeze-drying process with cooling gradients [216]. These materials show a good biocompatibility and integration within the host tissue, because they do not cause fibrous encapsulation, nor foreign response, the scaffold-host tissue interface is continuous and the cystic cavity is rarely observed. The immobilization of BDNF into the channels promotes blood vessels formation and alignment along the longitudinal orientation of the channels and enhances axonal infiltration into agarose scaffolds [115]. Agarose can also be employed as an *in situ* forming gel, because it is a thermoresponsive hydrogel at temperatures over 17°C, but gelation upon cooling is a drawback of injectable hydrogels. However, Kaoka et al. employed a customized device for quickly cooling; they implanted agarose gelling *in situ* with steroids, for SCI models, resulting in a reduction of macrophage infiltration and lesion cavity [217].

Alginate is another polymer derived from algae employed for spinal cord tissue engineering. This material has the ability of forming gels by the interaction of multivalent ions, especially Ca^{+2} ions. It has been employed to obtain conduits for spinal cord repair by an ionic diffusion method, where Ca^{+2} ions in an alginate solution diffuse by capillarity forming parallel and aligned conduits with adjustable pore diameters [218]. Alginate gelation *in situ* under physiological conditions can be performed by Ca^{+2} ions forming hydrogels, thus this material can be employed to form gels in the spinal cord cavities. These gels permit axonal support and extension and inhibit the gliotic scar formation in the injured spinal cord [219]. The use of alginate for spinal cord tissue engineering instead of agarose is due to its biodegradability.

Fibrin and fibronectin are two glycoproteins derived from plasma: fibrin is a fibrillary acidic protein glycoprotein polymer and fibronectin is a plasma glycoprotein dimer. Fibronectin gels can be obtained with linearly aligned fibres, which orient axonal growth in spinal cord implants [220] and are more permissive for axon ingrowth than collagen or fibrinogen [210]. However, fibronectin gels implanted alone in the spinal cord generate cavities due to their slow gelation ability. Fibrin has been employed to fill synthetic conduits, made of poly(2-hydroxyethyl methacrylate-co-methyl methacrylate) P(HEMA-MMA) for example, resulting in a greater axonal regeneration specially from vestibular neurons, even obtaining improvements in locomotor function after complete spinal cord transaction at T8 in rats [209]. Fibrin can be modified with heparin to incorporate neurotrophic factors, such as NT-3 [221]. The incorporation of NT-3 into the fibrin matrix reduces the activation of astrocytes inhibiting the glial scar formation. The reduction of the inhibitory components for axon regeneration can also be performed by incorporating the enzyme chondroitinase ABC (ChABC), which cleaves the CSPGs chains generating a more permissive environment for axon regeneration [222]. Fibrin gels are an adequate vehicle to encapsulate embryonic stem cells; its implantation along with pluripotent cells and growth factors, including NT-3 and PDGF in subacute rat model of SCI has been found to increase the survival of transplanted cells and resulted in more differentiated neurons [223]. However, the use of fibrin gels for these applications is falling into decline for its fast degradation. Because of this drawback, fibrinogen has been combined with fibronectin to obtain more durable and efficient gels [207]. The fibronectin/fibrinogen gels have demonstrated a good integration with the host tissue, axonal ingrowth associated with SCs infiltration and deposition of laminin, abundant vascularization and non-formation of cavities. That is, fibronectin/fibrinogen gels are more suitable for SCI regeneration than any of them separately, even than collagen gels.

SAPNs are an interesting strategy to address SCI, due to their ability to be injected and form nanofibrous gels *in situ*, acting as contact guidance substrates for axon outgrowth without employing any invasive surgery. As an example, a negatively charged peptide amphiphile incorporating the neuroactive pentapeptide epitope from laminin, IKVAV, has been injected after laminectomy of spinal cord of mouse at the T10 vertebral segment, resulting in a reduction of astrogliosis and oligodendrocyte apoptosis. Moreover, functional recovery was assessed by the Basso, Beattie and Bresnahan (BBB) locomotor scale modified for mouse at 9 weeks after SAPN injection [224]. However, the stability of many SAPNs is poor and need structural modifications. In this sense, the RAD16-I (Puramatrix™) ionic peptide (multiple of the RADA

amino acid sequence) has been modified with bone marrow homing motifs to enhance its stability and bioactivity in the injured spinal cord environment after its gelation [225]. The injection of this modified RAD16-I has shown to promote the upregulation of trophic factors and ECM remodelling proteins, an increment in cellular infiltration, together with laminin and collagen, axon regeneration after 7 days and some improvements in the motor and coordination function 8 weeks after implantation. RAD16-I has been implanted in the transected dorsal column of spinal cord of rats, carrying SCs and embryonic NPCs to replace dead cells and promote neuroprotection [226]. The implanted cells have survived after 6 weeks, SCs mature with a tubular morphology and NPCs differentiate into neurons, astrocytes and oligodendrocytes. This system has shown a good integration with the host tissue without gaps between the implants and the injured tissue at 6 weeks. In addition, host cells have been found to migrate into the gel and extensive blood vessels have been formed.

7.2. Synthetic biomaterials

The synthetic polymers permit to obtain a wide range of structural designs for spinal cord repair, including conduits to form bridges between the rostral and caudal ends of the injured spinal cord, and their properties are more controllable than those of natural polymers. The synthetic polymers most employed for spinal cord tissue engineering are either biodegradable materials, mainly the aliphatic polyesters (PLA, PGA, PLGA, PCL), or bioestable ones, such as acrylate-based hydrogels (PHEMA and PHPMA).

PLA is a good choice for spinal cord regeneration because the FDA has approved its use for several clinical applications, including absorbable sutures and fabrication of peripheral nerve grafts. PLA scaffolds have been designed as conduits for axon guidance in the environment of the spinal cord by a freeze-drying technique, which allows obtaining aligned pores to guide axon growth. However, the implantation of these conduits with entrapped BDNF in the transected adult rat thoracic spinal cord does not improve axon regeneration [227]. Further studies have incorporated engineered SCs expressing BDNF and NT-3 into porous PLA scaffolds, resulting in modest axon regeneration after 6 weeks, but only a few implanted cells survived after one week [228]. Regeneration employing PLA can be improved incorporating topographical cues for axon growth and guidance. For example, PLA conduits have been developed by rolling random and aligned PLA electrospun fibres [229]. These conduits, implanted into a 3 mm gap created after complete transaction of thoracic rat spinal cord, increased cell proliferation and allowed host cells and tissue infiltrate into the lumen of the conduit, filling the scaffold 4 weeks after implantation, closing the gap and forming a continuous tissue. Moreover, the PLA fibres promote the regeneration of supraspinal and propriospinal neurons and glial cells, and aligned fibres promote longer bridge regeneration without adding other cues for axon regeneration, pointing out the importance of topographical cues for axon growth and guidance. However, the electrospun PLA conduits are structurally unstable, easily fragmenting and collapsing.

Lactic acid is normally copolymerized with another aliphatic polyester, glycolic acid (GA), to obtain the copolymer PLGA, which enhances the properties of implantable conduits and allows a better control over the degradation rate. Such as PLA, PLGA can be employed to

fabricate conduits for spinal cord regeneration. Thus, aligned porous conduits of PLGA with interconnected walls have been obtained by a gas foaming/particulate leaching process for spinal cord regeneration of rats [230]. These conduits allow the infiltration and longitudinally alignment of different cells including fibroblasts, macrophages, glia, endothelial cells and neurofilaments, which grow up to 2.5 mm from the proximal end of the injured spinal cord. Macrophages are activated in the acute inflammation step but their activity decreases over time, while astrocytes are localized around the structure and the CSPGs levels surrounding the bridge decrease. These conduits can be fabricated with different porous structures, either with small pores (less than 38 μm) or larger ones (63-108 μm), showing differences in the distribution of cell ingrowth; small pores lead to a less uniform cell ingrowth but an increment of endothelial cell infiltration and formation of vessel-like structures. However, conduits of both sizes provided some functional recovery in rats after 14 days, indicating the implant stability and the plastic organization.

The PLGA copolymer has been employed in combination with neurotrophic factors to promote neuroprotection and enhance regeneration in mice models of SCI [231]. As an example, PLGA was shaped as microspheres containing sonic hedgehog (Shh), which is a multifactorial growth factor related to the spinal cord development, and then it was injected in spinal cord lesions. This system reduces the glial scar at the injury site, not affecting the inflammatory response, and provokes an increment in the number of proliferative cells, including NPCs, which provide a permissive environment for regeneration. These benefits result in improvements in the locomotor function recovery, that is, some kind of regeneration. PLGA conduits have also been loaded with neurotrophins such as NT-3 and BDNF [232]. These PLGA conduits were fabricated by a gas foaming/particulate leaching method, injecting a mixture of PLGA microspheres and salt particles into an aluminium mould with oriented wires and subjecting the mixture under pression to obtain a continuous and porous PLGA multichannel. Then the microchannels were filled with hydroxyapatite complexed with a lentivirus encoding luciferase. Once implanted into a rat model of hemisectioned spinal cord, these conduits allowed fibroblasts and macrophages infiltration, but astrocytes were only observed in the surrounding tissue. The axon density per channel is increased by releasing neurotrophins in the rostral position, suggesting the beneficial effect of NT-3 and BDNF in neuroprotection and regeneration. Moreover, the myelination was enhanced in the channels with lentivirus encoding both neurotrophins. The neuroprotective effect can also be achieved by incorporating cells secreting neurotrophic and growth factors into the PLGA conduits. The incorporation of SCs into PLGA multichannel conduits has demonstrated robust axonal regeneration, but not functional improvements though [233]. The effect of different channels diameters in these structures has been studied [234], and those with diameters of 450 μm and 660 μm demonstrated axon regeneration after 3 months post-implantation in the transected rat spinal cord, but the longest axon fibres were found in the smallest channels. Other cells have been seeded in PLGA conduits and implanted in rat model of transected spinal cord, including NSCs [235]. In this work, PLGA conduits fabricated by a moulding-solvent evaporation process seeded with SCs or NSCs were implanted after an SCI, resulting in similar axonal regeneration in both seeded conduits, but relatively poorer than in the control healthy group, and none functional recovery. However, the system with NSCs seeded in the conduits led to an interesting result,

since NSCs preferentially differentiated into neurons with some cells with astrocyte phenotype.

PCL has also been proposed for spinal cord regeneration, due to its good mechanical properties and its slower degradation rate compared with the other aliphatic polyesters. Porous PCL conduits have been fabricated by a salt-leaching and an injection-moulding process, obtaining different morphologies for spinal cord regeneration purposes, including cylinders, tubes, multichannels, open-path architectures with a central core and open-path architectures without such central core [236]. The different morphologies influence the regenerative process of the spinal cord and provide different results relative to cell infiltration and tissue response. In the cylindrical, tubular and multichannel architectures, cell growth has been found to occur along the outside of the implants, the interior walls being filled with cellular debris, macrophages, and fibroblast-like cells after 1 month. However, the result most discouraging of these three structures was the secondary cell death within and around the implants after 3 months. Besides, both open-path structures produced growth of tissue into and along the entire length of the conduits and less cysts and fibrotic tissue around the implants and the stumps. In addition, the open-path structures promoted astrocyte infiltration and sufficient contact guidance to allow the extension of axons across the entire defect length, even many fibres accomplished to be myelinated.

PCL can be chemically modified to obtain a biodegradable crosslinkable polymer by the addition of fumaryl chloride and PCL diol under ultraviolet light. This crosslinkable polymer, poly(ϵ -caprolactone fumarate) (PCLF), has been employed to fabricate multichannel conduits for spinal cord regeneration [237]. The benefits in spinal cord injuries of PCLF conduits have been compared with those of PLGA ones, resulting in a major number of axons in the first ones. PCL in combination with PLGA has been employed to obtain electrospun fibres conduits containing a filling of the RAD16-I gel to promote axon attachment and regeneration, and growth factors and neurotrophic factors (BDNF, CNTF, chABC and VEGF) to be used in a chronic SCI rat model [238]. The incorporation of RAD16-I into the lumen of the conduits led to a reduction of cavities, the gliotic scar occasionally appeared surrounding the implants but less frequently inside the lumen, promoted tissue infiltration, neurite regeneration and blood vessel formation and alignment. The incorporation of cytokines enhanced neuron maturation and the formation of axon bundles in all the microchannels, and the regeneration even continued after 6 months, measured by electrophysiological activity and locomotor improvements.

Poly- β -hydroxybutyrate (PHB) is a polymer produced by a wide variety of bacteria, it degrades slowly at physiological temperatures and the metabolites are secreted in urine. In contrast to aliphatic polyesters, the slow degradation rate and continuous elimination of the PHB avoid the formation of acidic debris, which can cause inflammation. PHB has been employed to fabricate conduits composed of two perpendicular layers containing parallel fibres for spinal cord regeneration [239]. These conduits have been coated with alginate, seeded with SCs and implanted into the gap generated after cervical SCI in rats, resulting in a reduction of the spinal cord cavity and the retrograde degeneration of the injured spinal tract. Further studies with PHB fibres conduits coated with alginate and seeded with SCs resulted in improvements in

cell attachment, proliferation and survival of SCs and promotion of axonal regeneration within the conduit [240]. However, only the descending raphespinal and sensorial neurons grew within and along the conduit, but not the rubrospinal ones.

PHEMA-and PHMA-based hydrogels are bioestable materials and due to their swelling rate, they show similar properties to those of the spinal cord tissue. The regenerative ability of both types of hydrogels has been compared in studies of adult and developing rat spinal cords [241]. Both hydrogels showed a good integration with the host tissue, presenting a minimal glial scar and cystic formation and the infiltration of connective tissue. They promoted the infiltration of blood vessels and axons into the pores, but axons grew into the central part of the hydrogels after eight weeks. Moreover, SCs infiltrated into the hydrogels and along the axons, suggesting the possibility of obtaining myelinated axons. These hydrogels can be modified with peptides and neurotrophic factors to improve axon regeneration and generate neuroprotection. Thus, the incorporation of the cell adhesive site of fibronectin RGD (NeuroGel™) into PHMA hydrogels with a porous structure obtained by freeze-drying has been found to promote axon regeneration in injured spinal cord [241]. These systems provoked an early inflammation a few days following implantation, which decreased gradually. One week after implantation, the proliferation of capillary sprouts was observed, along with a glial scar surrounding the implant and a few number of fibroblasts, fibrocytes and macrophages. Over the next weeks, the implants showed ingrowth of capillaries, infiltration of connective tissue, glial processes and axons. Moreover, the implants presented neither cystic formation nor secondary axon degeneration, and the axonal pathways were altered into the rostral stump, showing signs of morphological changes and regeneration indicating growth cone formation. Similar studies on PHPMA hydrogels with immobilized RGD and neurotrophic factors (BDNF and CNTF) resulted in a major number of axons growing into the hydrogels compared with unmodified ones [202].

Conduits of PHEMA-co-methyl methacrylate with different internal matrices –fillings- (collagen, fibrin, matrigel and methylcellulose) and growth factors (FGF and NT-3) have been compared with multichannel conduits and have demonstrated improvements in the regeneration of selective tracts after complete spinal cord transection at T8 in rats [209]. This study shows that all the internal matrices increase axon density within the channels except the collagen matrix without growth factors and the multichannel conduits without fillings. The regeneration of sensory axons was not influenced by the internal matrix and axon growth factors even hinder it. The different matrices and growth factors affect selectively to different tracts: fibrin promotes the greatest axonal regeneration of reticular neurons, whereas methylcellulose does of vestibular and red nucleus neurons, FGF increases the axonal regeneration of vestibular neurons, and NT-3 decreases the total amount of axons regenerating from brainstem neurons. Besides, fibrin filling and multichannels accomplish improvements in the locomotor function. With the aim of improving axon regeneration in the injured spinal cord, HPMA-and HEMA-based hydrogels have been combined with MSCs so as to deliver neurotrophic factors. In a preliminary study, the survival of MSCs within HPMA and PHEMA implants has been compared, being it higher in the HPMA hydrogels [242]. Besides, this work also demonstrated that most of the cells locate in the pores but a few cells migrate to the border

zones of the spinal cord, pointing out that the use of these hydrogels can be helpful as carriers of stem cells and their delivery in the lesion site of the spinal cord.

8. Biomaterials in neurodegenerative diseases affecting the striatum

Neurodegenerative disorders are characterized by a progressive loss of neuronal subtypes, and many of these diseases ultimately lead to a loss of the patient autonomy and even death. In this chapter, two neurodegenerative disorders that affect the striatum in the brain are dealt with PD and HD. Both show similar pathology, with many differences, affecting localized zones of the striatum in the earlier stages and causing the loss of neural subtypes: dopaminergic neurons in PD and GABAergic ones in HD. The experimental therapies are based on cell therapy and drug delivery, and the role of biomaterials is to incorporate cells or drugs to immobilize them into the site of interest.

PD is a neurodegenerative disease affecting around 1% of the population older than 60 years. It is caused by several factors, but researchers have identified six gene mutations associated with this incidence, including α -synuclein, parkin, UCH-L1, PINK1, DJ-1, and LRRK2/dardarin. This pathology is characterized by the loss of dopaminergic neurons in the substantia nigra of the nigrostriatal tract. For this reason, the striatum reduces dopamine levels and neuronal projections. Other aspect of PD is the generation of abnormal intraneuronal aggregates of protein, especially α -synuclein, called Lewy bodies. Further stages of this disease involve degeneration in the cerebral cortex and autonomic nervous system. The manifestations of PD include tremor, bradykinesia, rigidity, cognitive decline and finally death. The animal model commonly employed to study the PD is obtained by administration of 6-hydroxydopamine, which causes the lesion of dopaminergic pathways. In the initial stages of disease, L-DOPA administration is the most potent therapy to improve motor functions. However, this treatment becomes less effective in further stages, making necessary to find new therapies.

HD is a dominantly inherited neurodegenerative disease that results from the polyglutamine expansion in the gene encoding the huntingtin protein. This disease is characterized by the loss of brain striatal GABAergic neurons, and the degenerative process progressively extends to other brain regions including cortex. The pathogenesis of HD and the function of the huntingtin protein are not well understood, but this pathology is characterized by the loss of medium spiny neurons in the striatum and loss of neurons in the cortex leading to movement disorders (chorea), dementia, and eventually death. The early stages of HD show a localized damage in the striatum, which leads this disease to be a potential target for tissue engineering employing biomaterials. The animal models of HD are obtained by exposing neurons from striatum to drugs or poisons and by using transgenic animal models overexpressing polyglutamine expansions.

Biomaterials for drug delivery are promising since they can be easily modified to adjust the release kinetics to the target organ, tissues or cells, they can selectively cross biological membranes including the impermeable BBB, and the release can be controlled by modulating the biodegradable properties of the biomaterial carriers. The most common structures

employed for drug delivery in these neurodegenerative diseases are nanoparticles, liposomes and hydrogels. The principal problem of the oral administration of L-DOPA is that its prolonged use entails tolerance, and its efficacy is limited by the permeability of the BBB. The implantation of dopamine-loaded biomaterials into the striatum would be a more efficient method of controlled release to address PD. The incorporation of dopamine in hydrogels has demonstrated good results, employing non-cytotoxic biomaterials such as dextran crosslinked with gelatine for models of PD [243]. The delivery of dopamine can be accomplished during weeks and produces motor functional recovery in models of hemiparkinsonian rats. However, the release of dopamine occurs during short times and the behavioural bias reappears. GDNF is another molecule employed in PD studies, since it has a trophic effect in the dopaminergic system increasing the production of dopamine, stimulating the regeneration of dopaminergic neurites [244]. Therefore, the incorporation of GDNF in PLGA microparticles and their posterior implantation into the striatum of parkinsonian rats has demonstrated improvements in motor function and increments of fiber density in the striatum [245]. The delivery of GDNF in the striatum in PD models has also been achieved by transfected fibroblasts expressing this trophic factor immobilized into alginate-poly-L-lysine microparticles [246]; this implantable device has shown to increase cell survival and release GDNF during around six months in parkinsonized rats, resulting in improvements of behaviour.

The immobilization of cells within biomaterials is usually carried out by encapsulating cells into polymeric microparticles or by attaching them on the biomaterials' surfaces. The use of biomaterials pursues to increase cell survival of transplanted cells, control cell fate, maintain cell phenotype and promote cell engraftment into the interest site. The deficit of dopamine and dopaminergic neurons loss typical of PD disease could be improved by the incorporation of cells expressing this neurotransmitter or by stromal cells with the ability to differentiate into dopaminergic neurons. In both cases, cell survival would be improved by combining cells with biomaterials. For example, collagen-coated dextran microparticles (Cytodex 3®) together with chromaffin cells have been implanted into the striatum of rats with 6-hydroxydopamine-induced injuries of the substantia nigra [247]. The implantable biohybrids did not cause any inflammation response, increased cell survival and produced functional benefits after 8 months. Similar results have been obtained by incorporating foetal ventral mesencephalon (FVM) cells into the same Cytodex3® microcarriers. Another system to incorporate chromaffin cells into the striatum of PD animal models has been developed by employing glass bead microparticles, which has led to similar results, even long-term functional recovery after implantation in hemiparkinsonian rats [248]. Alginate is another natural polymer employed to encapsulate cells for the treatment of neurodegenerative diseases affecting the striatum. Its microparticles have been combined with choroid plexus cells, which secrete several neurotrophic factors, to study their effect in HD rat models [249]. This device had a neuroprotective effect by the neurotrophic factor release, a reduction of the lesion cavity and improvements in the motor function. Synthetic polymers have also been employed in the treatment of neurodegenerative diseases affecting the striatum. As an example, NT-3 immobilized into PLGA microparticles with LN in their surface have been employed to attach and expand MSCs, and implanted in hemiparkinsonian rats [250]. LN improves the attachment of MSCs to the surface, while the neurotrophic factor NT-3 promotes the differentiation of MSCs into neurons. This

device led to improvements of neuron differentiation and the regeneration of the nigrostriatal pathway, and subsequently to modest improvements in the behaviour.

9. Concluding remarks

There is a high interest in the use of biomaterials to repair damage in the brain in many of the most prevalent and burden causing neurologic diseases. Biomaterials can provide a better environment for the homing and survival of neurons, glia and brain blood vessels, act as guidance cues for promoting axonal growth, and provide a proper stimulus for the formation of new synapses and to promote neural plasticity.

Although there is still no clinical application of this research, the wealth of data obtained may foresee a successful solution in the near future, which still will have to go through all the regulatory and clinical essays process.

If we are able to provide a way to regenerate and reconstruct the damaged brain, we will turn the available concept of clinical neurosciences, from procedures that are directed to protect the damaged brain from further injuries into truly reconstructive neurosciences.

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