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BIOHYBRIDS FOR NEURAL TRACTS REGENERATION

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Resumen

Las lesiones del sistema nervioso que implican la interrupción de haces axonales son devastadoras para el individuo. La regeneración autónoma de los tractos axonales dañados o degenerados es poco frecuente, ya que intervienen una gran cantidad de factores que limitan esta recuperación. Hoy en día, la medicina convencional no cuenta con tratamientos efectivos y exitosos para estas lesiones, y el tratamiento de los síntomas suele ser la mejor solución. Para revertirlo y lograr la reconexión funcional de las neuronas, la ingeniería de tejidos actualmente opta por el uso de soportes tridimensionales biocompatibles, células y moléculas bioactivas. Específicamente, una de las estrategias propuestas han sido los conductos nerviosos guiados, no solo para lesiones de nervios periféricos sino también para tractos del sistema nervioso central.

En esta Tesis Doctoral, se propone la combinación de un conducto tubular hueco de ácido hialurónico (HA) relleno con fibras de ácido poli-L-lactida (PLA) en su lumen, y con células de Schwann (SC) pre-cultivadas como células de soporte de la extension axonal para superar los obstáculos que limitan la regeneración de axones *in vivo*. Se ha demostrado que el conducto de HA y las fibras de PLA mantienen la proliferación de las SC, las cuales forman una estructura cilíndica denominada 'vaina de SC' en la pared interna del lumen del conducto y a su vez crecen de forma direccional en las fibras de PLA. El conjunto unidireccional paralelo formado por las fibras PLA y las SC recapitula las características direccionales de los tractos axonales en el sistema nervioso. Al sembrar un explante de ganglio de la raíz dorsal (DRG) en uno de los extremos del conducto, se ha conseguido el crecimiento de los axones del DRG y se ha estudiado las características de las SC, los axones crecidos y su asociación, comprobando que el biohíbrido es capaz de soportar el crecimiento axonal.

Además, se propone un concepto multimodular para superar las limitaciones típicas de la regeneración axonal a larga distancia, con la combinación de haces de fibras de PLA en el lumen de varios conductos o módulos de HA individuales más cortos que se posicionan uno detrás del otro, diseñando conductos nerviosos guiados con la longitud deseada, junto con SC pre-cultivadas. El

conducto multimodular demostró ser eficaz para promover el crecimiento dirigido de axones. Además, se ha desarrollado un constructo compuesto por la estructura formada por las fibras de PLA y las SC, denominado 'cordón neural', tras eliminar el conducto de HA, lo que abre la puerta a la generación de una estructura neural *in vitro* para su trasplante.

Resum

Les lesions de el sistema nerviós que impliquen la interrupció de feixos axonals són devastadores per a l'individu. La regeneració autònoma dels tractes axonals danyats o degenerats és poc freqüent, ja que intervenen una gran quantitat de factors que limiten aquesta recuperació. Avui dia, la medicina convencional no compta amb tractaments efectius i reeixits per aquestes lesions, i el tractament dels símptomes sol ser la millor solució. Per revertir i aconseguir la reconnexió funcional de les neurones, l'enginyeria de teixits actualment opta per l'ús de suports tridimensionals biocompatibles, cèl·lules i molècules bioactives. Específicament, una de les estratègies proposades han estat els conductes nerviosos guiats, no només per lesions de nervis perifèrics sinó també per tractes de sistema nerviós central.

En aquesta tesi doctoral, es proposa la combinació d'un conducte tubular buit d'àcid hialurònic (HA) farcit amb fibres d'àcid poli-L-lactida (PLA) en el seu lumen, i amb cèl·lules de Schwann (SC) pre-cultivades com a cèl·lules de suport de l'extension axonal per superar els obstacles que limiten la regeneració d'axons *in vivo*. S'ha demostrat que el conducte d'HA i les fibres de PLA mantenen la proliferació de les SC, les quals formen una estructura cilíndica anomenada 'beina de SC' a la paret interna de l'lumen de l'conducte i al seu torn creixen de manera direccional en les fibres de PLA. El conjunt unidireccional paral·lel format per les fibres PLA i les SC recapitula les característiques direccionals dels tractes axonals en el sistema nerviós. A l'sembrar un explantament de gangli de l'arrel dorsal (DRG) en un dels extrems de l'conducte, s'ha seguit el creixement dels axons de l'DRG i s'ha estudiat les característiques de les SC, els axons crescuts i la seva associació, comprovant que el biohíbrido és capaç de suportar el creixement axonal.

A més, es proposa un concepte multimodular per superar les limitacions típiques de la regeneració axonal a llarga distància, amb la combinació de feixos de fibres de PLA en el lumen de diversos conductes o mòduls de HA individuals més curts que es posicionen un darrere l'l'altre, dissenyant conductes nerviosos guiats amb la longitud desitjada, juntament amb SC pre-cultivades. El conducte multimodular

va demostrar ser eficaç per promoure el creixement dirigit d'axons. A més, s'ha desenvolupat un constructe format per l'estructura formada per les fibres de PLA i les SC, denominat 'cordó neural', després d'eliminar el conducte d'HA, el que obre la porta a la generació d'una estructura neural *in vitro* per al seu trasplantament.

Summary

Injuries to the nervous system that involve the disruption of axonal bundles are devastating to the individual. Autonomous regeneration of damaged or degenerated axonal tracts is infrequent since a large number of factors are involved limiting this recovery. Nowadays, conventional medicine does not have effective and successful treatments for these injuries, and the treatment of symptoms is often the best solution. In order to reverse it and achieve the functional reconnection of neurons, tissue engineering currently opts for the use of biocompatible three-dimensional supports, cells, and bioactive molecules. Specifically, one of the proposed strategies has been nerve guidance conduits, not only for peripheral nerve injuries but also for tracts of the central nervous system.

In this Doctoral Thesis, we propose the combination of hyaluronic acid (HA) single-channel tubular conduit filled with poly-L-lactide acid (PLA) fibres in its lumen, with pre-cultured Schwann cells (SC) as cells supportive of axon extension to overcome the obstacles limiting axon regeneration *in vivo*. We have proved that HA conduit and PLA fibres sustain the proliferation of SC, which form a cylindrical structure named 'SC sheath' on the inner wall of the lumen of the conduit and in turn grow directionally in the PLA fibres. The parallel unidirectional ensemble formed by PLA fibres and SC recapitulates the directional features of axonal pathways in the nervous system. Planting a dorsal root ganglion (DRG) explant on one of the conduit's ends, we have followed axon outgrowth from the DRG and studied the features of SC, the grown axons and their association, checking that the biohybrid is capable of supporting axonal growth.

Furthermore, we propose a multimodular concept to overcome the typical limitations of long-distance axonal regeneration, with the combination of PLA fibres bundle in the lumen of several shorter individual HA conduits or modules which positioned themselves one behind the other, designing nerve guided conduits with the desired length, together with pre-cultured SC. The multimodular conduit proved effective in promoting directed axon growth. Moreover, we developed a construct consisting of the structure formed by the PLA fibres and

the SC, named ‘neural cord’, after eliminating the HA conduit, that opens the door to the generation of a neural structure *in vitro* for transplantation.

Glossary

BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
DAPI	4’ 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DRG	Dorsal root ganglion
DVS	Divinyl sulfone
ECM	Extracellular matrix
FBS	Foetal bovine serum
FDA	Food and Drug Administration
GA	Glutaraldehyde
GAP43	Growth associated protein 43
GDNF	Glial cell line-derived neurotrophic factor
H-E	Hematoxylin-eosin
HA	Hyaluronic acid
IGF1	Insulin-like growth factor type-1
IL-1	Interleukin-1
IL-1α	Interleukin-1-alpha
iPSCs	Induced pluripotent stem cells
LFBP	Luxol fast blue with picric
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein

N-CAM	Neural cell adhesion molecule
Neural cord	Defined for the first time on page 72
NF	Neurofilament heavy polypeptide
NGC	Nerve guidance conduit
NGF	Nerve growth factor
NSPCs	Neural stem/progenitor cells
NT-3	Neurotrophin-3
OMgp	Oligodendrocyte myelin glycoprotein
P/S	Penicillin/Streptomycin
p75	p75 NGF receptor
PB	Phosphate buffer
PCL	Poly-ε-caprolactone
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PLA	Poly-L-lactic acid
PLGA	Poly-glycolic acid
PNI	Peripheral nerve injury
PNS	Peripheral nervous system
S100β	S100 calcium-binding protein β
SC	Schwann cells
SC sheath	Defined for the first time on page 47
SCI	Spinal cord injury
SEM	Scanning electron microscopy
SNpc	Substantia nigra pars compacta
TBI	Traumatic brain injury
TEM	Transmission electron microscopy

TNF-α	Tumour necrosis factor-alpha
Tuj1	Neuron-specific class III β-tubulin
VEGF	Vascular endothelial growth factor

Introduction

1. Cell biology of the nervous system

1.1. Nervous system structure

The nervous system is one of the most complex systems in the human body, and understanding the structure, the organization, and the cellular components of the nervous system is the first and necessary step for treating the nervous injuries.

The nervous system is a complex assemblage of cells in charge of directing, supervising and controlling all the functions and activities of our internal organs and body in general. It receives input from a variety of sources and transmits the proper signals. The nervous system controls our physical movements, maintains homeostasis of many internal variables, and also initiates our higher thought processes and emotions [1].

The nervous system is subdivided anatomically into the central nervous system (CNS) and the peripheral nervous system (PNS), and functionally into the somatic nervous system and the autonomic nervous system (visceral). The CNS is formed of the brain and spinal cord. The skull encloses the brain, and the spinal cord is at the centre of the vertebral column. The PNS comprises the nerves emerging from the brain (named cranial nerves) and from the spinal cord (named spinal nerves).

The nervous systems integrate a lot of function like sensation and body movements, which can be divided into sensory or motor signals. The sensory signals are transmitted through the sensory or ascending pathway to processing

centres in the spinal cord and brain, resulting in an appropriate response for motor activity. This response is carried through the nervous system to the muscles and glands along motor or descending pathways [1].

1.2. Nervous system cells and organization

The primary type of cell in the nervous system is the neuron, which is the excitable cell type in charge of send and receive signals through action potentials. The neuron comprises four structurally defined regions: a cell body or soma (1) that extend a single nerve process named axon (2), which ends at presynaptic terminals (3) containing boutons, and a variable number of branching processes named dendrites (4) (Figure 1A). Neurons can be structurally classified in multipolar (one axon and multiple highly branched dendrites), bipolar (one axon and one dendrite), and pseudounipolar neurons (two axons: peripheral and central) [2] (Figure 1B); and functionally classified in sensory or afferent neurons (carry information toward the CNS), interneurons (relay messages within the CNS), and motor or efferent neurons (carry information away from the CNS to muscles and glands).

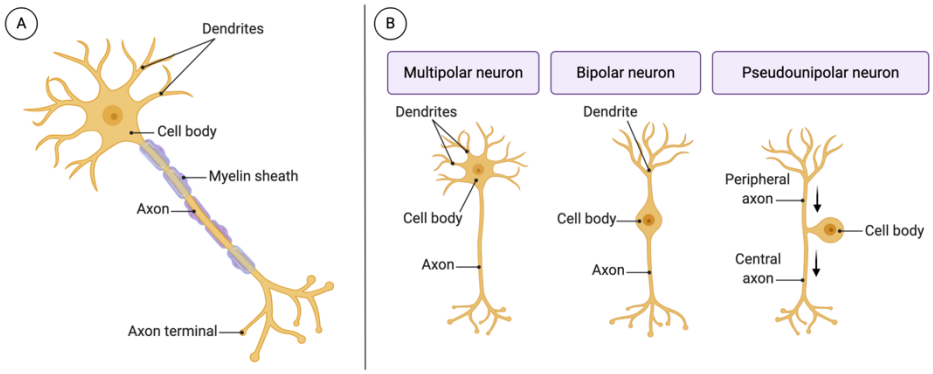

Figure 1. The neuron: the primary cell in the nervous system.
(A) Structural parts of a neuron. (B) Different structurally classes of neurons. Figure created in BioRender.com.

The other cell type in nervous tissue is the smaller and more prevalent neuroglia (glia), the supportive cells that surround the somas, axons, and dendrites of neurons in both the CNS and PNS [1].

The PNS glia consist of Schwann cells (SC) that surround neuron axon and perineuronal satellite cells surrounding the neuron cell body (Figure 2A) [3]. SC wrap themselves around a short segment of an axon many times as a sort of insulating blanket, creating a shiny white protective layer around the axon named myelin sheath and forming the myelinating axons. The unmyelinated axons are also encased in SC. Satellite cells are flat cells that enclose and support the cell bodies of neurons in the PNS. Both SC and satellite cells, envelop and separate unmyelinated axons from each other and are located in the interneuronal space between neurons [4].

The CNS glia includes astrocytes (astroglia), oligodendrocytes (oligodendroglia), microglia, and ependymal cells (Figure 2B). The astrocytes have several processes and are in charge of anchoring capillaries and neurons cells bodies and dendrites in place, make contact with ependymal cells of the ventricular system, regulating the extracellular environment of the brain, aiding in the formation of the blood-brain barrier, and repairing damaged brain tissue [5]. The oligodendrocytes are the equivalents of SC of the PNS fabricating and maintaining CNS myelin, with the difference that a single oligodendrocyte can extend its processes wrapping around multiple axons [6]. Microglial are cells with functions related to both immune response and maintaining homeostasis. The ependymal cells are cuboidal glial cells that line the central canal of the spinal cord and the ventricles of the brain, and they are involved in the production and circulation of cerebrospinal fluid [4].

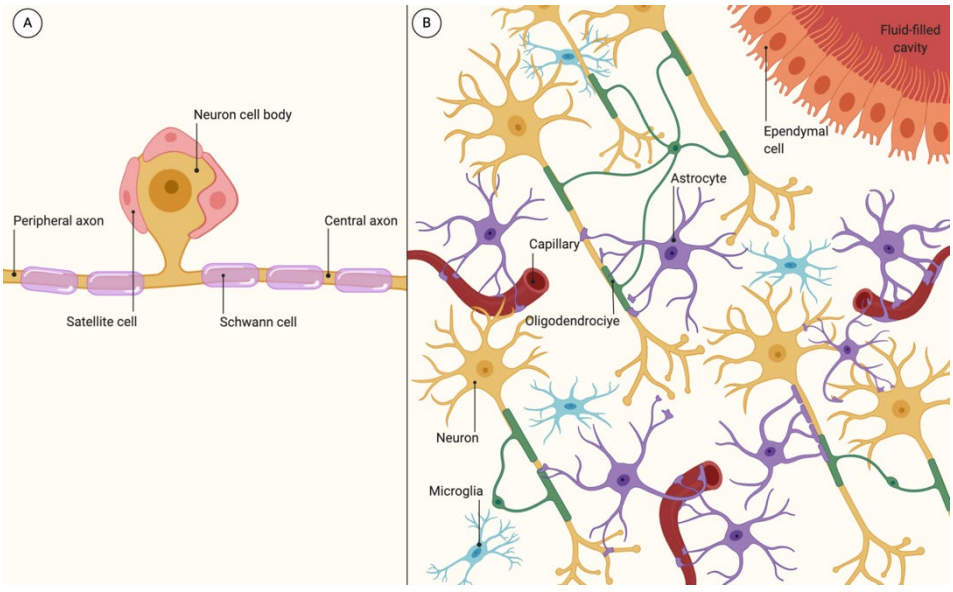

Figure 2. Nervous system cell organization.
(A) Cells and organization in the peripheral nervous system. (B) Cells and organization in the central nervous system. Figure created in BioRender.com.

In brief, there are many roles in which the glia is involved. Glial cells give the CNS structural support and protection for neurons networks and guide the developing migrating neurons from their sites of origin to their correct destination and the outgrowth of their axons. Also produce trophic and growth factors to the nervous system regeneration and plasticity, and myelin sheaths to insulate the axon and increase the velocity of action potential propagation (oligodendrocytes in CNS and SC in PNS) [5–8]. Microglia remove debris produced following injury or neuronal death and monitors the CNS, and astrocytes act as bridges that transport nutrients from the capillaries to the neurons and also proliferate to develop astrocytic scars to repair nervous tissue following an injury (reactive gliosis) [1,4].

1.3. Bundles of axons in the nervous system: peripheral nerves and central nervous system tracts

Bundles of axons receive such specific names as nerve (in the PNS) and tract or fasciculus, among others (in the CNS).

Nerves contain only the axon part of the neuron. The cell bodies of sensory neurons are located in adjacent structures to the spinal cord (dorsal root ganglion (DRG)) or in cranial ganglia, while the cell bodies of motor neurons are within the CNS (spinal cord or brainstem) [2]. Each nerve of the PNS consists of three essential tissue elements: axons, SC (and myelin sheaths), and connective tissue (endoneurium, perineurium, and epineurium) (Figure 3A). In the PNS, there are also ganglia, formed by cell bodies and satellite cells associated with the peripheral nerves. A peripheral nerve consists of numerous nerve fibres formed by axons and associated SC. Each axon–SC unit is surrounded by a basal lamina sheath composed by collagen, laminin and fibronectin and the endoneurium wraps all this structure. Groups of insulated nerve fibres are bound together into fascicles by the perineurium. In turn, groups of fascicles and blood vessels, which serve to nourish cells, are bound together by the epineurium. The myelin sheath, which surrounds an axon inside a nerve fibre, is a structure composed of many continuous spiral laminated layers of the plasma membrane and allow transmission of faster and more efficient electrochemical impulses. Nearly all nerve fibres over 3 μm in diameter are myelinated, and those under <3 μm are unmyelinated. Only one SC encapsulates a myelinated nerve fibre, but a group of unmyelinated fibres might share the same SC (Figure 3B) [9].

Bundle axons of the CNS are located in the white matter. Within the white matter of the spinal cord, the sensory fibres of the pathways form groups called ascending tracts or fasciculi, carrying information up to the brain. Fibres of the motor pathways form groups referred to as descending tracts, carrying information from the brain to the peripheral effectors (muscles and glands). So, the spinal cord consists of a large number of ascending and descending tracts, each located in particular parts of the dorsal, lateral, or ventral column of the white matter (Figure 3C) [1]. In the brain, there are many axonal pathways. One example is the axonal pathways between the substantia nigra pars compacta (SNpc) and the striatum, which is altered in Parkinson's disease. SNpc neurons send long-projecting axons to the striatum, and in Parkinson's disease there is a selective loss of the dopaminergic neurons, so this neurodegeneration deprives

the striatum of crucial dopaminergic inputs and cause ineffective feedback of motor pathway [10] (Figure 3D).

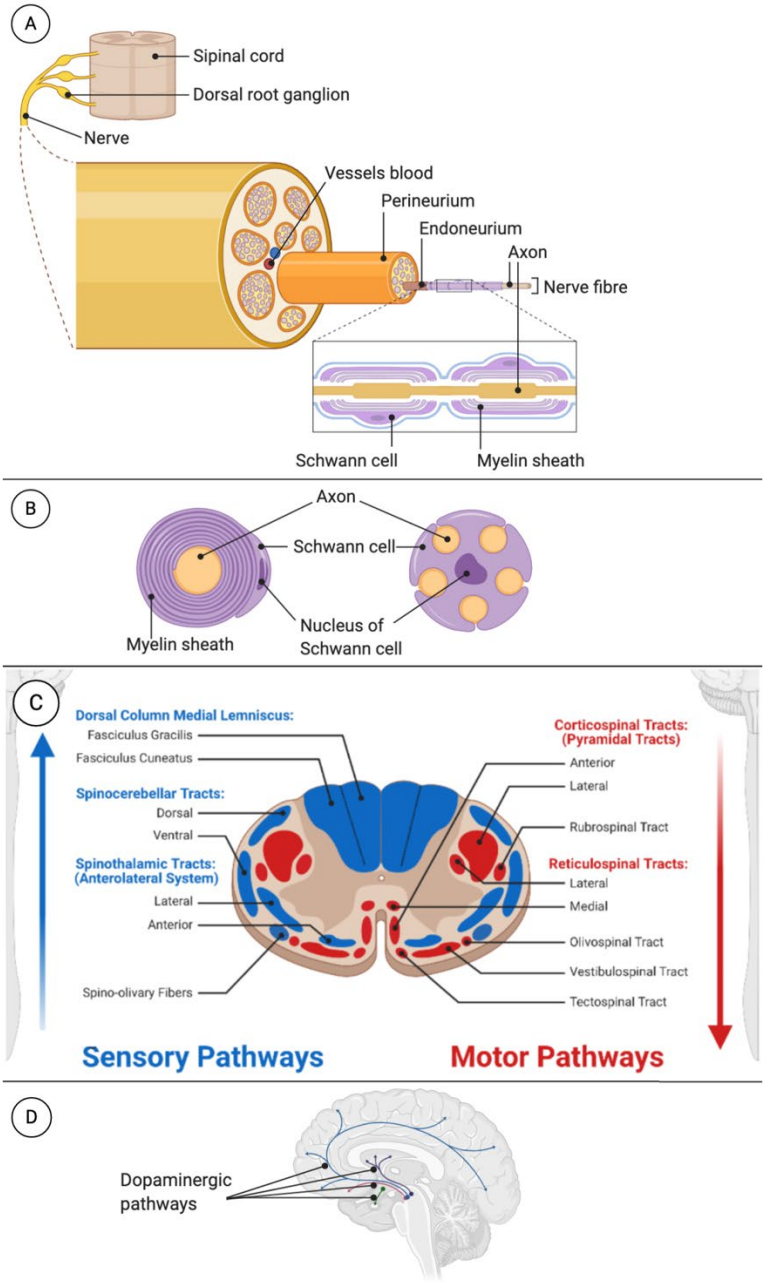

Figure 3. Bundles of axons in the nervous system.

(A) Peripheral nerve anatomy. (B) Myelinated (left) and unmyelinated (right) nerve fibres in the peripheral nervous system. (C) Spinal cord tracts divided in sensory or ascending tracts (blue) and motor or descending tract (red) to illustrate the complexity of the tracts organization in the spinal cord. (D) Dopaminergic pathways as an example of the large number of pathways inside the brain. Dopaminergic pathways are the bundles of neurons projections in the brain that synthesize and release the neurotransmitter dopamine. Alterations in these pathways may be involved in multiple diseases and disorders such as Parkinson's disease, addiction or attention deficit hyperactivity disorder. Figure created in BioRender.com.

In summary, axonal bundles represent an aligned and unidirectional architecture that makes axonal nervous tissue development possible. When peripheral nerve injury (PNI), spinal cord injury (SCI), traumatic brain injury (TBI), or neurodegenerative disease occur, the intricate architecture undergoes alterations leading to growth inhibition and loss of guidance [11].

2. Regeneration in the peripheral and central nervous system

Precisely because of all the marked differences between CNS and PNS discussed above, the mechanisms involved after a damage occurs and their potential for regeneration are very different [6]. Moreover, the evolution and outcome of the regenerative situation may depend on very different factors.

Traumatic injuries, interruption of blood supply or neurodegenerative diseases can damage axons, cell bodies or somas in the nervous system and produce a disconnection of axonal bundles [12–14].

In injuries of the PNS, functional restoration following significant nerve lesions in length (>10 mm) is generally poor due to insufficient axonal reinnervation of distal targets [15,16]. The situation is less desperate when only the reconnection of the distal and proximal ends of the injured axonal bundle in relatively short distances is needed [17,18]. In this situation the distal part of the axon, which is disconnected from the cell body, undergoes Wallerian degeneration, resulting in fragmentation and disintegration of the axon in the first 18–48 hours. SC surrounding the axon survive and produce pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-1-alpha (IL-1α), playing a critical role in macrophage recruitment to the injury site [19]. After one to two

weeks, reasonably fast as compared to the CNS, macrophages and SC remove axonal and myelin debris. Besides, macrophages secret cytokines such as interleukin-1 (IL-1) and platelet-derived growth factor (PDGF) that stimulate SC to divide, de-differentiate and proliferate distal to the injury. These stimulated SC are induced to secrete growth factors such as nerve growth factor (NGF) or insulin-like growth factor-1 (IGF-1), in order to improve neuron survival after axotomy and stimulate axons regeneration. It is in this moment when the Band Büngner is formed [20]. The Band Büngner consists in SC and their basal lamina conforming a cylinder that serves as a pathway to guide axon and their growth cones, from proximal end to distal end, across the gap. In optimal conditions, the growth cones can extend at a rate of 1–3 mm/day [12].

Moreover, SC and regenerating axons express cell surface adhesion molecules like neural cell adhesion molecule (N-CAM) and N-cadherin, which promote regeneration [21]. Also, SC secret neurotrophic factors that promote axonal elongation, the survival of injured neurons and vascularisation of the distal nerve. These factors include brain-derived neurotrophic factor (BDNF), NGF, neurotrophin-3 (NT-3), glial cell line-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF) [20,22,23]. Finally, SC myelinate the re-growing axon forming new myelin sheaths [24]. Meanwhile, at the site of axonal damage, a motile growth cone-like structure is developed. Changes in gene expression and protein production occur within the cell body, and new proteins are transported to the growing axon tip, such as tubulin alpha 1 and the growth-associated protein GAP43 (GAP43), which is upregulating in both sensory and motor neurons, until axons reconnect with their targets (Figure 4A).

The damage and regeneration produced in axonal tracts of the CNS are more complicated than in the PNS. David and Aguayo [25,26] confirmed that CNS mature neurons could regenerate in the presence of permissive peripheral nerve grafts, suggesting that the peripheral nerve glial environment was permissive to central axonal regrowth. After these findings, the pro-regenerative environment in PNS, and inhibitor factors in CNS for the axonal growth were identified. The inhibitor factors in the CNS include the limited intrinsic ability of CNS axons to regenerate [27,28], the long-distance required for regrowth, a local inhibitory environment [29], migrating cells to the injured area forming a glial scar, and the chemical composition of the extracellular matrix (ECM) at the formed scar [30,31]. After a CNS axon is injured, Wallerian degeneration in the damage tract occurs.

However, the degenerating myelin and axonal debris after the apoptosis of oligodendrocytes persist much longer due to the insufficient recruitment of macrophages. These molecules that inhibit neurite growth are contained in myelin, for example, Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp). Nogo-A is a potent inhibitor of neurite growth and blocks axonal regeneration in the CNS. Moreover, a few hours after injury, microglia are the first glial cells to react. Astrocytes become reactive and collaborate in forming the glial scar, with inhibitory molecules, like proteoglycans, remaining for several weeks to months and complicating the axonal regeneration [32] (Figure 4B).

Therefore, due to the limitations of the body to regenerate axonal bundles discussed above, it is necessary to develop a biomimetic approach that can establish a bridge across the lesion while providing optimal morphological, chemical and biological signals for the restoration of nervous tissue.

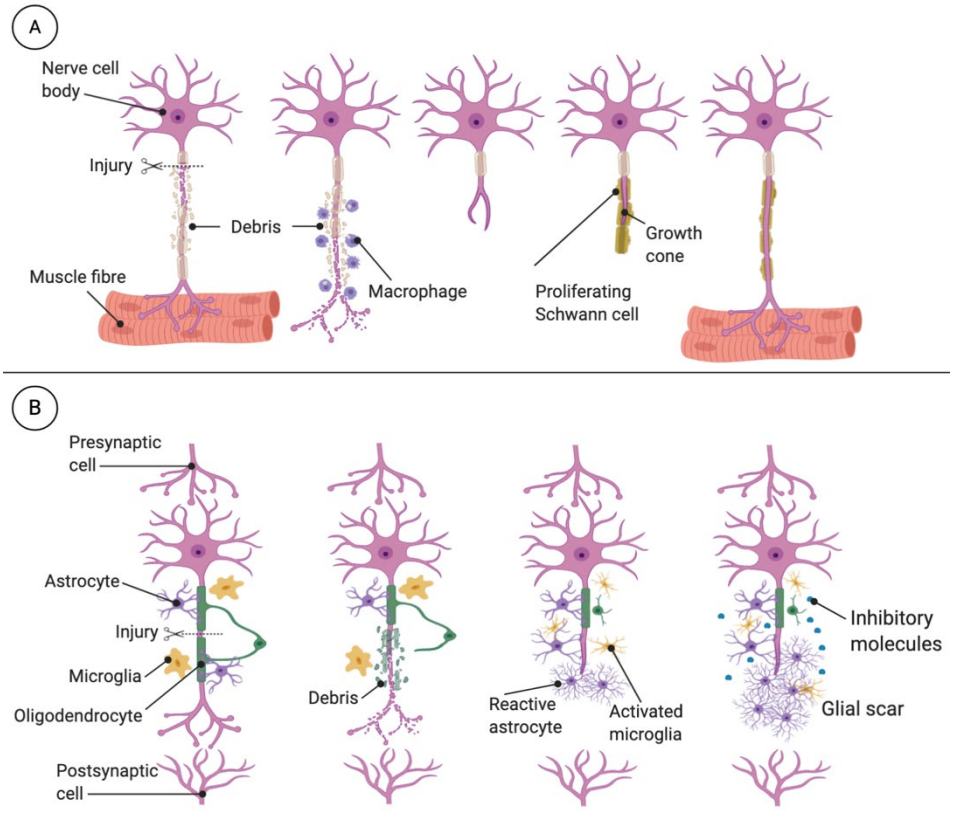

Figure 4. Regeneration mechanisms after injuries.
(A) Regenerative situation in PNS. Proliferating SC and macrophages work together to remove myelin debris, release neurotrophins, and lead axons toward their synaptic targets, resulting in restored neuronal function. (B) Regenerative situation in CNS. The few neurons that survive axotomy attempt regeneration and subsequently meet an impenetrable glial scar composed myelin and cellular debris, as well as astrocytes, oligodendrocytes, and microglia. Figure created in BioRender.com.

3. Tissue engineering

With the development of the most precise cell analysis tools and the discovery that the cells of an adult organism maintained a certain capacity to generate new tissue, even in the CNS, a new approach emerged to try to technically deal with any damage or pathology: the tissue engineering. According to Langer and Vacanti [33], tissue engineering consists of applying the fundamentals of biology

and engineering to develop functional substitutes for damaged tissue, with three general components: scaffolds for cell transplantation and support, cells that can create a functional matrix, and bioactive factors that support and regulate the activity of cells (Figure 5). A functional substitute refers to an ideal solution that fully restores affected function, integrates as well as possible with the host's surrounding tissue, and lasts for the rest of the affected person's life. So, for example, any orthopaedic prosthesis or even heart valves, do not belong to the field of tissue engineering because they have a useful life of around 15 years, need aggressive surgical intervention for their implantation, an immunosuppressive medication to avoid immune rejection, and aspire to replace the tissue instead of regenerate it [34].

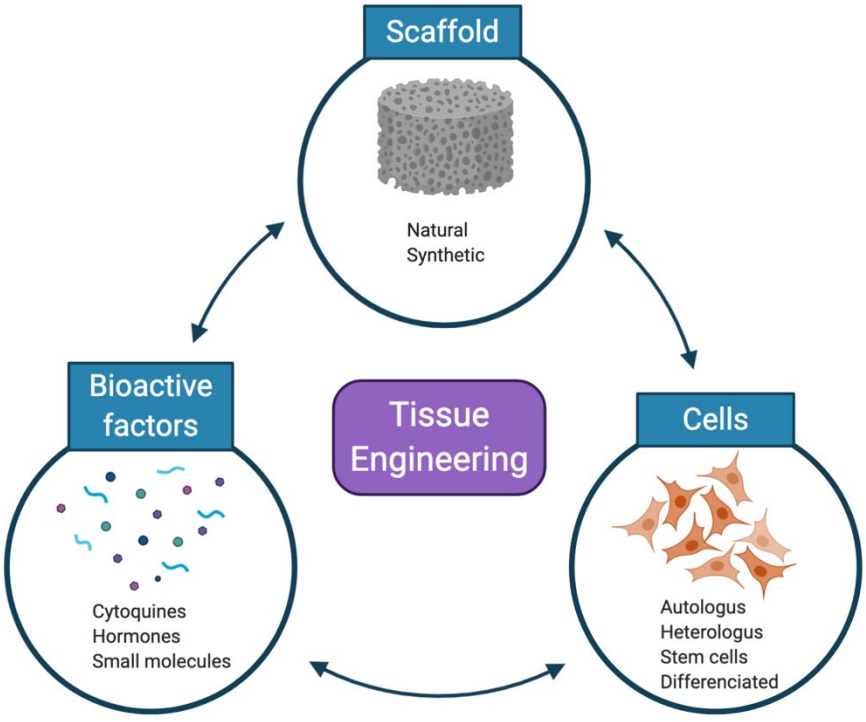

Figure 5. The tissue engineering triad.
Scaffolds, cells, and bioactive factors are used in isolation or in combination to recapitulate the desired tissue. Figure created in BioRender.com.

3.1. Therapies for tissue engineering

Tissue engineering therapies have obtained relevant advances in the reconstruction of useful and healthy tissue in many tissues and organs of the human body [35–37] during the last 20 or 30 years. However, recovering lost connections in the nervous system has traditionally been a very complex challenge in which researchers have had to be especially cautious and methodical.

Diseases and injuries of the human nervous system are devastating to the individual and have substantial societal implications and costs. Injuries and diseases of the human nervous system are highly prevalent, affecting more than 1 billion people around the world [38]. They are devasting for the patient and have a tremendous social and economic impact.

As mentioned above, after an injury in the PNS, the situation for the regeneration is more straightforward than in the CNS. When the gap resulting from the injury is very small, nerve endings can rejoin with sutures [39], but when this is not possible because of the high tension in the nerve endings the gold-standard traditional technique is the autograph, employing a nerve segment taken from a less important site from the same individual [40]. Autograft offers encouraging results, and it is not easy to find a device that gives an equally satisfactory result [41], being currently used as the standard reference or positive control of other experimental solutions [42,43]. However, autografts have limitations like the low availability of donor nerve [44], the dimensions mismatches [45], host immune response and the comorbidity associated with additional surgery [46]. Another traditional option is to use donor cadaveric nerve allografts or xenografts (from an animal), but, as with allograft, they have limitations like requirement of immunosuppression or decellularization to prevent immune rejection [47–49].

Others trials have been performed with other more abundant but similar tissues such as tendon collagen [50], muscle fibre epimysium [51] or even CNS ECM [52], to avoid healthy sectioning nerves. However, although the results are promising, the trend is to reach therapies that do not require the use of host tissues due to associated drawbacks such as limited donor supply and often immune complications.

Besides, the regeneration in the CNS is minimal, and there is no clinical treatment with a proved improvement of the CNS repair. In SCI, TBI, and degenerative

disease, initial damages result in fast acute primary injury events. Current treatments, instead of trying to recover damaged tissue, try to prevent the secondary injury with high doses of steroids in SCI [53], and maintaining adequate oxygen supply, blood flow and pressure in TBI. For more severe brain injuries, surgery is required to remove hematomas and repair contusions [54].

Due to the limited ability of the body to regenerate axonal bundles, it is necessary to develop a biomimetic strategy that can provide a bridge across the lesion while giving optimal morphological, chemical and biological signals for the recovery of nervous tissue. So, tissue engineering in the nervous system have provided new alternative therapeutic avenues. Neural tissue engineering defends the use of external biomaterial supports, with cells and bioactive molecules [33] to overcome the inhibitory environment and achieve a successful repair of the nervous system. The first idea to overcome the drawbacks of the previous techniques was to develop materials with dimensions, morphology and characteristics determined as an implantable devices or biomaterials. The objective is to obtain biocompatible structures that are integrated into the surrounding tissue, and that can be invaded or replaced by native cells and recover lost functionality.

The first biomaterials used, and still in use, had a natural origin and an identical or very similar nature to the tissue to be restored. Natural biomaterials are widely used in tissue engineering because of their bioactivity and microstructure that mimics the ECM, thus supporting adhesion, differentiation, or oxygen and nutrient transport. Matrices based on generic mixtures of ECM substances have been used [55], but also scaffolds of purified ECM molecules like collagen [56] or other proteins [57], hyaluronic acid [58] and some polysaccharides [59]. Scaffolds are defined as three-dimension porous stable biomaterials designed to have some or all of these characteristics: promote cell-biomaterial interactions, cell adhesion and ECM deposition, allow the exchange of oxygen, nutrients and factors, be biodegradable and non-cytotoxic. However, although biomaterials of natural origin can be manufactured in a multitude formats such as *in situ* gellable solutions, scaffolds, fibres or tubular conduits, the truth is that usually their mechanical properties are poor and many times they biodegrade too quickly to allow adequate support [60].

Another common approach is the use of biomaterials of synthetic origin. For example, poly-ε-caprolactone (PCL) [61,62], poly-L-lactic acid (PLA) [63,64], poly (lactic-co-glycolic acid) (PLGA) [65,66], among others [67,68]. These materials

seemed to make up for the shortcomings of natural biomaterials since they can be manufactured with a multitude of techniques and architectures adapted to the type of tissue to be regenerated [69]. Furthermore, by choosing the proper polymer or its composition, it is possible to obtain greater control over biodegradability [70]. A wide variety of formats can be obtained, such as aligned scaffolds [71,72], fibres or filaments [63,73] and tubular structures [74–76]. However, they also have certain limitations, such as reduced bioactivity that increases the risk of rejection after implantation. Therefore, the choice of the origin of the biomaterials to be used is especially important, being widespread systems composed of combination of various types of biomaterials [77].

Apart from afore mentioned, and as a part of the search for therapies with regenerative potential, cell transplantation appeared. In the first place, the transplantation of mature cells from the patient, obtained through biopsies was tested [78]. However, the number of cells obtained by biopsies was not always sufficient, and the cell proliferation rate was relatively low. Adult stem cell transplantation, which offered great potential thanks to its multipotentiality, was also tested [79,80]. Nevertheless, their availability is limited, their survival rate is low after transplantation. In 2006, Takahashi and Yamanaka artificially converted differentiated cells into a pluripotent state *in vitro*, naming them induced pluripotent stem cells (iPSCs) [81]. This discovery represented a promising alternative approach for regenerative medicine thanks to the possibility of a cell autotransplantation that can be better long term integrated by being compatible with the host's immune system. However, there is a reluctance to use these cells due to the lack of regulation and the tumorigenic and immunogenic properties of iPSCs [82]. Besides, there is a growing trend in the use of iPSCs in clinical trials, but it would be necessary more time to consider it a viable alternative.

Unfortunately, to date, cell transplantation alone in the nervous system is not an option for tissue recovery, due to the associated problems such as limited availability of some cell sources or low survival rates after transplantation. Therefore, tissue engineering advocates combining a cell source with the use of the biomaterials to contain cells and create an environment conducive to growth and prevent undesirable external factors. Biomaterials would provide, on the one hand, a three-dimensional environment in which accommodate the cells, and on the other hand, they could be devices for controlled release of bioactive molecules in a localized way in the desired tissue [83,84]. The cells would play a

vital role by producing growth factors and ECM molecules, promoting tissue recovery.

3.2. Hyaluronic acid

Among all natural polymers, hyaluronic acid (HA) has been used in this Doctoral Thesis. HA is an unbranched glycosaminoglycan made up of pairs of glucuronic acid and N-acetylglucosamine units. HA is present naturally in all vertebrates and is a significant constituent of the ECM in many parts of the human body. It can be found in the form of small oligosaccharides or macromolecule around more than 10^6 Da molecular weight.

It was described for the first time in 1934 when Karl Meyer and John Palm isolated a novel glycosaminoglycan from the vitreous humour of bovine eyes [85]. They discovered that this substance contained a uronic acid and an aminosugar, so they named it “hyaluronic acid”.

This material has gained significant interest in the area of tissue engineering thanks to its properties:

1. HA is a major component of connective tissue, being a key factor in cell growth and differentiation [86].
2. HA is biodegradable, bioresorbable, and biocompatible. HA is approved by the Food and Drug Administration (FDA) for clinical use.
3. HA structure contains functional groups like carboxylic or hydroxyl that can be used to modify it, introducing functional domains or crosslinking to produce a hydrogel [87].
4. HA is involved in the wound healing process, so in the field of tissue engineering, it can be used to accelerate this process [88].
5. HA has low non-specific adsorption of proteins. Moreover, HA could be modified with cells receptors (CD44, RHAMM, ICAM-1) to enhance cell growth and tissue repair [89].
6. High molecular weight HA (usually > 60 kDa) has anti-adhesion properties for cells [90]. Therefore, in the case of the nervous system, it could inhibit glial scar formation and maintain astrocytes in a quiescent and non-reactive state [91,92]. On the other hand, the smallest fragments, which are formed

when damage occurs, are recognized by microglia and astrocytes, among others, activating the immune response, the localized angiogenesis and the proliferation and differentiation of astrocytes which become reactive [93,94].

Purified HA of animal or bacterial origin is widely used in cosmetic and plastic surgery as injectable subcutaneous filler, due to its function in tissue regeneration and its hydrophilic nature. Furthermore, they have been used to prevent adhesions after surgical interventions [95] or even in regenerative approaches in the nervous system [58,96].

Despite their attractive properties, the use of HA scaffolds is problematic due to their short residence time and low mechanical integrity in aqueous solution. However, this limitation has been addressed through chemical modification and crosslinking, resulting in biomaterials with physicochemical properties that may significantly change from the native HA, but generally retain its biocompatibility and biodegradability.

Many modifications can be made [97], although they could be summarized as chemical crosslinks, physical crosslinks, and the union of specific molecules or drugs for their controlled release.

Specifically, for this work, crosslinking with divinyl sulfone (DVS) is of great interest [98,99] since it is very convenient to obtain cross-linked in a short time and with very mild cross-linking conditions. The chemical crosslinking occurs between the hydroxyl groups of the HA chains and the DVS forming an infinite network of sulfonyl bis-ethyl crosslinks. This method is easy since DVS crosslink the HA in aqueous alkaline solutions at room temperature in a few minutes. Different DVS crosslinked HA scaffolds haven been used successfully [100,101]. There are commercial products based on HA crosslinked with DVS approved by the FDA for clinical use [102].

3.3. Polylactic acid

Another material used in this Doctoral Thesis is polylactic acid. Polylactic acid is obtained from lactic acid. Lactic acid is a natural organic acid that can be produced by chemical synthesis or fermentation. By fermentation, an excellent specificity of the product is obtained, producing pure D (-) or L (+) isomers, in

addition to having a low cost of substrates, low production temperature and low energy consumption [103,104].

Polylactic acid was discovered for the first time in 1932 by Carothers (DuPont) who produced a low molecular weight product by heating lactic acid under vacuum [105]. Later, in 1954, DuPont patented a higher molecular weight product [106]. In the same way that there are two isomers in lactic acid, by polymerization, it can be obtained poly-L-lactic acid, poly-D-lactic acid, and poly-DL-lactic acid [107,108]. Poly-L-lactic acid (PLA) has obtained significant attention because of its outstanding biocompatibility and mechanical properties [109], so it has been used in biomedical devices [110].

The FDA and European regulatory authorities have approved the polylactic acid for all food type applications and some surgical applications as suture materials [111], resorbable plates and screws [112], or drug releasing systems [113].

In the field of nervous tissue engineering polylactic acid has been employed in the development of single or multilayer guidance conduits [114], nanofibrous conduit scaffolds with single or multiple microchannels [115,116] or as fibres allocated in the lumen of tubular conduits [72,117] providing a support for cell adhesion, migration and elongation in a guided way.

4. Tissue engineering approaches in the nervous system

Despite its complexity and the limitations that have already been described to achieve regeneration of the nervous system, various hopeful strategies have been developed for the regeneration of this tissue, such as directly injected gels [58], porous three-dimensional scaffolds similar to a sponge [118], meshes with regular pore geometry [119], elongated and oriented microfilaments [63] and other configurations that are useful for other tissues. However, the best approximations are the so-called nerve guidance conduits (NGCs) due to their tubular structure that serves as a physical guide to direct axons sprouting, contain the growth factors secreted by the injured tissue, and reduce the infiltration of scar tissue [120]. These guided nerve conduits have been widely used in nerve regeneration with promising results [121], but also to regenerate spinal cord [61] or even brain [62,122,123].

4.1. Tissue engineering approaches in the peripheral nervous system: nerve guidance conduits

NGC is understood to be any device with a stable and elongated structure whose purpose is to support the growth of axons that have lost the ability to establish synaptic connections due to injury or degeneration.

Initially, simple hollow silicone conduits were used for PNI regeneration [16] [124]. However, these were quickly replaced and optimized because they were not degradable, they formed an excessive fibrous capsule and due to problems with compression of the nerves. With the silicone conduit approach, the different phases of regeneration into a hollow NGC were described in a 10 mm nerve gap from the rat sciatic nerve, corresponding to the sequenced phases of Wallerian degeneration and resulting regeneration mechanism. First, the fluid phase, where the conduit is filled with plasma exudate containing neurotrophic factors and ECM molecules (around 12 h after the injury). Second, the matrix phase, where fibrin cables are formed along the gap (around 1 week after injury). Third, the cellular phase, where SC invade the gap, migrate and proliferate, aligned along the fibrin cable, forming the Bands of Büngner. Fourth, the axonal phase, where the first axons are visible migrating to their distal targets (around 2 weeks after injury). Fifth, the myelin phase, where SC produce myelin and wrapped around each axon (around 4 weeks after injury) [16].

Hence, several FDA approved devices are consisting of hollow NGC that can be classified in these categories: non-resorbable devices, natural resorbable devices, and synthetic resorbable devices [125].

However, in longer gaps, the formation of the fibrin cable is compromised. SC, cannot align through the injury site, reducing the formation of the Bands of Büngner, necessary structures for the guidance of re-growing axons [126]. Moreover, it is known that hollow NGC can derive in inadequate reinnervation. So, to control the axon growth, many strategies focus on filling the lumen with a diversity of configurations to provide a supporting structure, either mechanical or biological, that promotes cells growth, guidance, and correct targeting [127,128].

Different studies have clarified the parameters that would be a significant advantage in achieving glial cell survival and axon extension along the different NGCs. Therefore, there are many design variables that an ideal NGC should consider and incorporate (Figure 6):

1. The biomaterial employed must be biodegradable and biocompatible and produce no inflammatory response [129].
2. The NGC must be flexible and soft to prevent compression of regenerating axons and limit tissue inflammation, respectively.
3. The NGC should present a guidance cue for the extending growth cone to reduce misdirection. The use of films or fibres as lumen filler has been proposed as an excellent way to obtain a structure as similar as possible to nervous tissue [121,130] so that the filler serves as a guide structure and intermediate support. Moreover, surface topography has been shown to influence cellular behaviour, including cell morphology, guidance, proliferation, and differentiation [131].
4. The NGC could incorporate intraluminal channels. Neural cells tend to grow and develop best on spherical or cylindrical structures, presumably by analogy with similar structures such as nerves, spinal cord, or brain tracts.
5. The biomaterial must be porous to allow an exchange of oxygen, nutrients, and factors between the lumen of the NGC and the interstitial fluid [132–134].
6. The NGC should prevent fibrous tissue ingrowth into the injury site. For example, HA inhibits the glial scar formation after brain damage [91].
7. The NGC should present angiogenic cue to provide nutrients to the new cells that invade it. This can be achieved, both by the presence of structures that allow the growth of endothelial cells and development of new blood vessels, and by the presence of angiogenic factors and certain micro-topographies [135,136].
8. The NGC must satisfy technical requirements for further production with the desired dimensions, sterilization, long-term storage, surgical handling, or suturing.
9. The NGC could incorporate supporting cells. Almost all the studies published in this field show a perceptible improvement in the result obtained with the NGC *in vivo* when they contain pre-cultured neuroglia in the lumen in comparison with the empty NGC. The benefits of cell therapies for tissue engineering have already been discussed previously, but thanks to the fact that the cells are confined in the scaffolds, the cells multiply their effect by not dying or migrating to other areas, which was one of the main drawbacks of

cells transplantation technique. For example, SC are of great interest for neural tissue engineering and help in PNI after introduced in a NGC [76,127,137]. SC can be isolated from nerve tissue, cultured and expanded *in vitro*, and incorporated in the NGC [138]. It should be noted that although SC are neuroglia originating in the PNS [67], there are precedents for the reconnection of tracts in the CNS [25,66,139].

10. The NGC could release bioactive factors incorporated into the biomaterial, to modulate the behaviour of cells and improve their survival and growth. The biomaterial could also be functionalized with microparticles of a different material loaded with the bioactive molecule of interest [140]. Neurotrophic factors such as NGF are of particular interest [141].
11. The NGC could present conductive biomaterials. Studies on conductive polymers and electrical stimulation appear as a relatively novel approach to increase neurite extension and axonal regeneration [142].

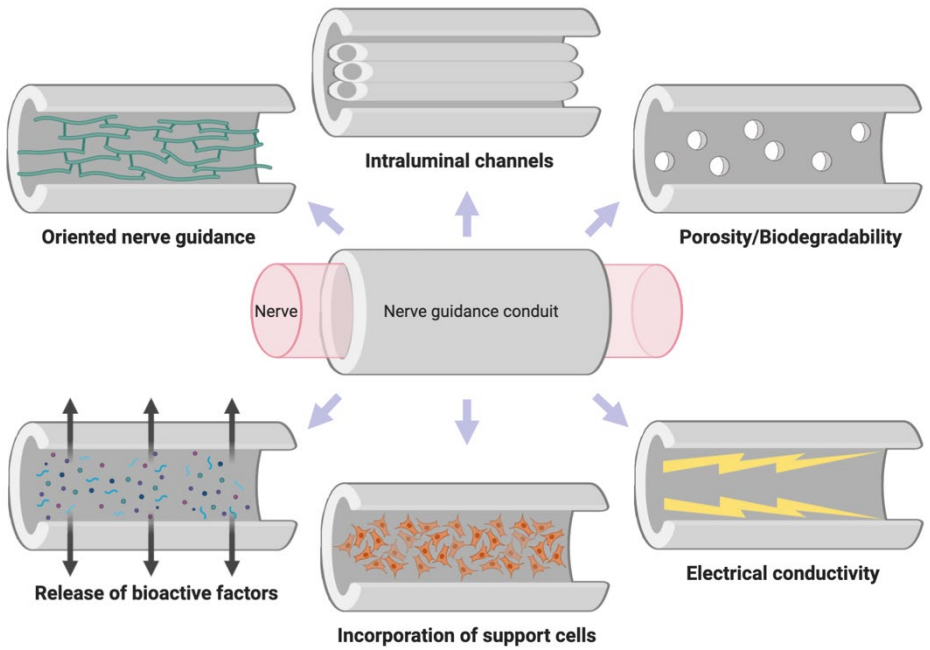

Figure 6. Properties of the ideal nerve guidance conduit.
Figure created in BioRender.com.

A multitude of strategies has been developed for the regeneration of PNI taking all these factors into account [74]. A vast diversity of NGCs formats has been employed, including porous cylindrical structures of collagen [143], structures based on PLGA and PCL microfilaments [65,144], tubular conduits with one or more channels [145], HA with fibres inside the lumen [100] and conduits with PLGA aligned conductive fibres [146].

4.2. Tissue engineering approaches in the central nervous system: spinal cord injury, traumatic brain injury, and neurodegenerative disease

As mentioned above, axonal bundles represent a unidirectional and aligned architecture allowing systematic axonal development within the nerve tissue. However, when an injury or disease in the PNS or CNS occurs, this complex architecture suffers disruption leading to the inhibition of growth and loss of guidance, whose recovery is especially tricky in the CNS [147].

Commonly, human SCI occurs after a contusion injury that compresses the spinal cord. However, many of SCI consists of complete transection of the spinal cord, with a complete disruption of the entire spinal cord. Moreover, there are other circumstances like large lesions or chronic situations where an injury has occurred some time previously, where it may be necessary to use some form of an oriented bridging substrate that bypasses the injury site and restores continuity across a traumatized area [148].

The spinal cord consists of a considerable amount of ascending and descending tracts, each located in particular parts of the dorsal, lateral, or ventral column. When SCI occurs, ideally, this spatial organization should be maintained by implanted structures, allowing that regenerating axons can find their proper targets in the grey matter behind the injury site. For this reason, biomaterials with channels or guides that are oriented parallel to the spinal cord tracts, like NGCs, among others, seem to constitute the optimal configuration. Under this premise, many strategies have been developed for SCI such as HA conduits with PLA fibres in the lumen [149], scaffolds with highly aligned conduits made of fibrin

[150], conduits made of aligned PLA nanofiber [63], multichannel [151] or individual cannel agarose scaffold [152], poly(lactide-co-glycolide) multiple channel bridges [153], among others [154–156].

Another group of disorders of the CNS affect the brain, such as TBI or neurodegenerative diseases such as Parkinson's disease. In the same way that occurs with SCI or PNI, they all have in common the loss of long-distance axonal connections.

The brain is a tremendously complex organ, and a multitude of tissue engineering approaches have been carried out to address the brain injuries. Hydrogels have been attractive for application to the brain due to their consistency similar to soft brain tissues and high permeability that allows the exchange of oxygen and nutrients [157,158]. Even micro and nanoparticles also offer advantages in brain tissue engineering. As systems are small and can be customized according to the polymer or molecules used, they are capable of crossing the blood-brain barrier, being carriers of molecules or drugs of interest [159,160].

However, taking into account the idea of regenerative bridges to facilitate long-distance axonal regeneration, several recent studies of great interest have been developed. For example, micro-columns containing tubular agarose hydrogels with collagenous matrix inside have been produced, making use of the confining capacity of astroglia cells to create a neural guide pathway [161,162]. A more complex system of micro-tissue engineered neural networks, containing mature primary cortical neurons and long axonal tracts in the lumen of hydrogel micro-columns, have also been developed to mimic grey and white matter [123], proposed as a possible treatment for Parkinson's disease [163].

Conclusively, from a neurological perspective, the challenge will be to design scaffolds that sustain multiple functions, a structure that can modulate a variety of cellular and signalling functions, and that can effectively support and guide the regenerative growth of axonal bundles or axonal descending and ascending tracts from diverse sources.

Objectives

Axonal bundles represent an aligned and unidirectional architecture that makes axonal nervous tissue development possible. When peripheral nerve injury (PNI), spinal cord injury (SCI), traumatic brain injury (TBI), or neurodegenerative disease occur, the intricate architecture undergoes alterations leading to growth inhibition and loss of guidance through large distance. Axon regeneration in the central nervous system (CNS) is severely restricted. In contrast, peripheral nervous system (PNS) axons can regenerate in some cases allowing functional recovery when the damage involves a relatively short distance, but in most cases, lesions are much longer, and the regenerative process becomes complicated. Therefore, approaches that facilitate axonal guidance have gained considerable interest in the regeneration of axonal bundles, both in the PNS and the CNS.

The present doctoral thesis hypothesises that the combination of hyaluronic acid (HA) single-channel tubular conduit filled with poly-L-lactide acid (PLA) fibres in its lumen, with pre-cultured Schwann cells (SC) as cells supportive of axon extension, could overcome the obstacles limiting axon tracts regeneration *in vivo*.

In the same way, a multimodular concept, combining the PLA fibres in the lumen of several shorter individual HA conduits or modules which positioned themselves one behind the other, and the pre-cultured SC could overcome the typical limitations of long-distance axonal tracts regeneration in the nervous system.

In order to demonstrate our hypotheses, in this Doctoral Thesis, we have addressed the following objectives:

1. To design of a material system to achieve axonal extension at different lengths.
2. To check the performance of the different components of this system in the process of axonal growth.
3. To explore the multimodular hypothesis to achieve long-distance axonal extensions.
4. To study the viability and behaviour of the biological structures formed in vitro within the material system.

Chapter I

Engineered axon tracts within tubular biohybrid scaffolds

1. Introduction

Axon regeneration in the CNS is severely restricted after TBI, stroke, SCI and related conditions that involve axonal disruption. In contrast, PNS axons can regenerate in some cases allowing functional recovery when the damage involves a relatively short distance [17,18]. After damage, CNS axons have an intrinsically limited capability to regenerate and they are surrounded by a local inhibitory environment [29]. Moreover, the loss of neuronal populations and synaptic connections is most irreversible due to the limited outgrowth capacity of mature neurons [27]. The perineuronal network, including reactive and migrating cells to the injured area, and the chemical composition of the ECM at the formed scar, constitute part of the inhibitory factors [30].

In axonal pathways, the axons of neurons form parallel bundles (nerves in the PNS, tracts in the CNS). Therefore, approaches that facilitate axonal guidance have gained considerable interest in the regeneration of axonal pathways, both in the PNS and the CNS. Strategies for promoting axon guided growth *in vivo* after injury include reversing the inhibitory stimuli for axon growth [164], increasing intrinsic neural regenerative programs [165], providing a substrate to guide axon growth [25], adding neural elements facilitating axon growth [166], and cell transplantations. Cells transplants, in most cases, are limited to local effects on the host's circuits without rebuilding the damage. For instance, numerous attempts have been made to integrate various types of cells after SCI, like neural stem/progenitor cells (NSPCs) [167] or SC [168]. Transplantation approach has mostly shown poor or null ability to rebuild damaged circuits and reinstating the cytoarchitecture of damaged or lost CNS tracts [169]. However, Kumamaru and collaborators recently showed that grafted foetal NPCs into the injured spinal cord adopt certain sensory and motor spinal interneuron fate, which innervate to growing host damaged corticospinal tract recapitulating motor domains without the need of additional exogenous guidance [170].

Glial cells have gained considerable interest due to their fundamental role in the regeneration process after injury [171]. SC play an important role in neuroregeneration and protection in the PNS [172] and are responsible to myelinate axons. Moreover, after injury, SC activate, divide, de-differentiate and proliferate distally contributing to nerve repair [173]. SC are a source of neurotrophic, angiogenic factors, and surface proteins that are involved in the maintenance of normal nervous system function and the activation of an innate

immune response after injury [20], Their regenerative potential when transplanted in the CNS has been also assessed [168]. For these reasons, use of SC has been proposed for regenerative purposes both in the PNS [174,175] and the CNS [176,177].

In order to overcome the limitations of purely cell-based therapies, the tissue engineering philosophy has emerged. This approach proposes the combination of natural or artificial nerve guidance conduits with cell transplantation and/or growth factor delivery to facilitate guiding axonal regrowth [178]. A nerve conduit should provide a suitable environment for neuron survival and axonal extension, guide axonal projections, and mimic the biomechanics with adequate mechanical properties [179]. Functionalized hydrogels are capable of emulating the biochemical and physical properties of the ECM, aiding in cell delivery and retention within the injured area [77]. Use of biocompatible scaffolds represents a benefit when used as a vehicle with minimal tissue invasiveness in comparison with intra-parenchymal transplantation approaches, conferring protection of the transplanted cells against the hostile environment generated at the injury site [180]. Different fabrication techniques are available to produce biomaterials that guide axonal outgrowth such as aligned scaffolds [71], fibres or filaments [77] and tubular structures [65,74,181].

In the present study, we wanted to combine a synthetic nerve conduit with supportive cells into a construct able to overcome the common limitations of axon regeneration and to establish an experimental model to check axon growth *in vitro* within the developed construct. Two different types of biomaterials were employed in our conduit scaffolds: a HA hollow conduit and PLA microfibers in their lumen [100,182,183]. HA is a natural glycosaminoglycan, an essential component of the ECM of many tissues [86]. HA has already shown good biocompatibility, biodegradability, and therapeutic benefits on neuronal regeneration processes [184], and exhibits mechanical properties similar to soft nervous tissues [185]. Previously [100,182,183], we found that SC seeded into HA conducts with suitable diameters and wall pore structure were able to proliferate and self-organize into a continuous cylindrical structure named ‘SC sheath’ spanning the whole distance of the conduit. PLA is a synthetic polyester with a long history as a biomedical material due to its outstanding mechanical properties, biodegradability, and biocompatibility [109]. PLA fibres gain interest as part of the tubular conduit concept when allocated in the lumen [117,186]

providing a support for cell adhesion, migration and elongation in a guided way. In our previous study [149] we found that HA conduits filled with PLA fibres induced preferential neuronal differentiation of progenitor cells *in vitro* and showed total biocompatibility and beneficial effects *in vivo* in a SCI model.

In the present work, we cultured SC into our HA-PLA tubular scaffolds until they had developed the above-mentioned cell 'sheath'. After this time, our experimental model for axon growth consists of planting at one end of the construct a DRG explant, as a source of projecting neurons. The axon extension stemming from this source into the SC-seeded conduit lumen was then followed in time and characterized. We investigated the effects on the axon growth of having or not the SC and the PLA microfibers in the HA tubular scaffolds. This biohybrid construct proved effective in promoting directed axon growth, and the results may thus be of interest for the goal of reconstructing axonal bundles in the nervous system.

2. Materials and methods

2.1. Cell source

Primary rat SC (P10301, Innoprot) expanded with SC Medium (P60123, Innoprot) were employed at 4-5 cell passage for cell cultures in the materials. Sprague–Dawley rats from Charles River and SD-Tg(GFP)2BalRrrc from Rat Resource & Research Center (University of Missouri Columbia, Columbia, MO, USA) were used for DRG explants dissection and were maintained following the National Guide to the Care and Use of Experimental Animals (Real Decreto 1201/2005). After the sacrifice by decapitation, whole DRG or DRG-GFP explants were dissected from the spinal column of neonatal rats (P3-P4) and transferred into ice-cooled Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific) containing 10% foetal bovine serum (FBS; Thermo Fisher Scientific), using a dissecting microscope to remove the remaining nerves and connective tissue.

2.2. Preparation of hyaluronic acid conduits and hyaluronic acid conduit with poly-L-lactic acid fibres

The synthesis of HA from *Streptococcus equi* (1.5–1.8 MDa, Sigma-Aldrich) conduits was carried out as previously described [100,182]. Briefly, PCL

(PolySciences) fibres of 400 μm were extruded in Hater Minilab and a polytetrafluoroethylene thin block with 1.5 mm-wide grooves with a single PCL fibre of 400 μm diameter was used as a mould for the conduits. 5% (w/v) of HA was dissolved for 24 h in sodium hydroxide 0.2 M (Scharlab). Then, the HA was crosslinked with DVS (Sigma-Aldrich) in a 9:10 DVS:HA monomeric units molar ratio, and this solution was mixed and injected in a mould. Later, the solution in the mould was lyophilised for 24 h (Lyoquest-85, Telstar, Spain). Finally, the conduits were hydrated in distilled water, the PCL fibres were extracted, and the conduits were cut to 6 mm length (HA hereafter). In several conduits, 20 aligned PLA (AITEX Textile Research Institute, Spain) fibres of 30 μm diameter were placed inside the channel of the 6 mm length conduits. The group constituted of conduit and PLA fibres inside was named HAf hereafter. Before the seeding of the cells, the conduits were sanitised for 2h with 70º ethanol (Scharlab), then conduits were immersed in 50º, 30º ethanol and distilled water for 10 min and were conditioned with culture medium overnight.

2.3. Cell culture and cell seeding within conduits

SC (4-5 cell passage) were grown in flasks until confluence at 37ºC, 5% CO_2, in SC medium. SC were seeded at a density of 100,000 cells/6-mm conduit suspended in 3 μl of SC medium with Hamilton syringe (SGE Analytical Science), inserting the syringe at one end on the conduit, both in HA and HAf groups. SC were maintained in the incubator for 30 min before adding the SC medium. After 10 days of SC culture, DRG explants were placed in direct contact with one end of the conduit with the help of tweezers, and the conduits were then transferred into 48-well plates, which was maintained with a specific DRG medium (Neurobasal medium, D-glucose 2mg/mL, L-glutamine 100X, 1% FBS, 1% penicillin/streptomycin (P/S), 2% B27 supplement, 0,1% NGF (Thermo Fisher Scientific)) refreshed every 2 days until 21 additional days counted from the moment the DRG was seeded. When indicated, in some experiments, DRG explants expressing GFP (DRG-GFP explants) were employed. In order to assess the different effect of the different components of the construct, four different experimental groups were established: (1) HA conduit with the DRG explant (named as HA+DRG), (2) the HA conduit with the 20 PLA fibres inside (HAf) with the DRG explant (HAf+DRG), (3) the HA conduit seeded with SC (HA+SC) with the DRG explant (HA+SC+DRG), and (4) the HA conduit with the

20 PLA fibres inside seeded with SC (HAf+SC) with the DRG explant (HAf+SC+DRG). A schematic representation of the working groups is shown below (Figure 7).

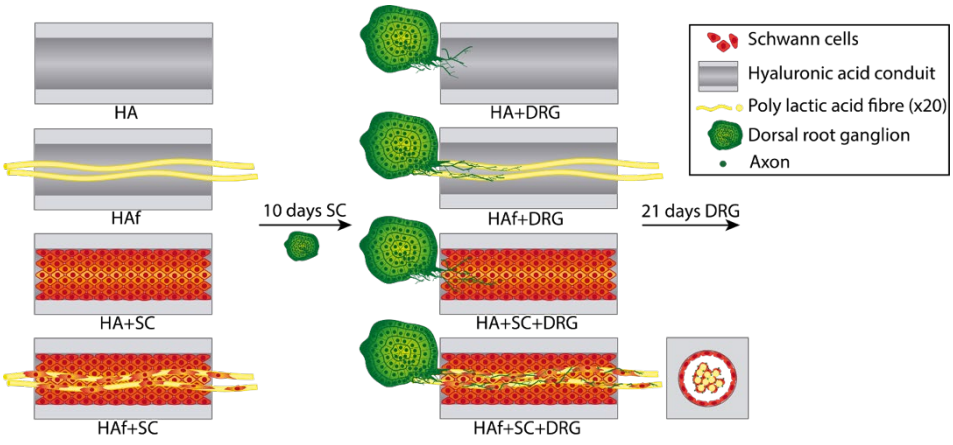

Figure 7. Experimental setup.

Schematic representation of the different experimental groups employed in work. In the groups with SC, SC were seeded and maintained in the conduits for 10 days before the planting of the DRG, which was then cultured for another 21 days. In the left, groups before the seeding of DRG explant (HA, HAf, HA+SC, HAf+SC). In the right, groups after the seeding of the DRG explant (HA+DRG, HAf+DRG, HA+SC+DRG, HAf+SC+DRG). All groups were analysed at the experimental day 21 after DRG seeding.

2.4. Scanning (SEM) and Transmission electron microscopy (TEM)

After culture, samples for SEM were washed in phosphate buffer (PB) 0.1M and fixed in 3.5% glutaraldehyde (GA; Electron Microscopy Sciences) solution for 1h at 37ºC, post-fixed with 2% OsO_4 (Electron Microscopy Sciences) and dehydrated. Later, conduits were processed in a critical point dryer (critical point values: 328C, 1100 psi). The conduits were cut longitudinally to expose their internal lumina and coated with an ultrathin layer of gold and observed at an acceleration tension of 10 kV in a scanning electron microscope (Hitachi S-4800, EE.UU.). Regarding TEM, samples were washed in PB 0.1M and fixed in 2% paraformaldehyde (PFA; Sigma-Aldrich)-2.5% GA solution for 5h at 37ºC, post-

fixed with 2% OsO_4 and dehydrated in a series of ethanol solutions of increasing concentration. Later, conduits were stained with 2% uranyl acetate (Electron Microscopy Sciences), embedded in araldite resin (Sigma-Aldrich) and allowed to solidify at 70ºC for 72h. The conduits embedded in araldite resin were cut crosswise in semithin sections (1.5 µm), stained with 1% toluidine blue (Sigma-Aldrich) and sections of interest were detached from the glass-slides by repeated freezing and thawing in liquid N_2. Selected sections were further sectioned with and ultramicrotome (Leica EM UC6, Leica, Spain) to obtain ultrathin sections (50-60 nm). The proximal, medium, and distal part of each conduit were visualised and studied with a transmission electron microscope (FEI Tecnai Spirit G2, EE.UU.). Quantification of the number of axons at the proximal and distal part of HAf+SC+DRG was performed employing representative TEM images with a common area.

2.5. Staining and immunocytochemistry

SC seeded for 10 days were characterised with by Coomassie staining. The samples were rinsed with PB 0.1M and fixed in 4% PFA for 20 min. The cells were then stained in a 0.2% solution of Coomassie brilliant blue R250 (Sigma-Aldrich) in methanol:acetic acid:distilled water, 46.5:7:46.5 (v/v/v) (Sigma-Aldrich) for 1h and rinsed several times in distilled water. The structure formed by the SC inside the lumen were extracted cutting the conduits and images were acquired with a Nikon Eclipse i80 microscope (EE.UU.). Glial and neural population and neurite outgrowth were identified analysing the expression of different markers by immunofluorescence with confocal laser scanning microscopy: neuron-specific class III β-tubulin (Tuj1; neurons), myelin basic protein antibody (MBP; glial cells and myelin membrane), S100 calcium-binding protein β antibody (S100β; glial cells), growth associated protein 43 (GAP43; growth cones), p75 NGF receptor antibody (p75; glial marker), and neurofilament heavy polypeptide antibody (NF; neurons). For confocal microscopy, the conduits were rinsed thoroughly with PB 0.1M and fixed in 4% PFA for 20 min. Cells were permeabilised and blocked with 0.1% Triton X-100 (Sigma-Aldrich), 10% FBS in PB 0.1M for 2h. Conduits were then incubated in at 4°C overnight with primary antibodies: mouse monoclonal Tuj1 (1/300; Neuromics) and MBP (1/400; Abcam); rabbit monoclonal S100β (1/200; Abcam) and GAP43 (1/400; Abcam);

rabbit polyclonal p75 (1/200; Abcam) and NF (1/800; Abcam). Secondary antibodies, goat anti-mouse IgG Alexa Fluor® 488 and goat anti-rabbit IgG Alexa Fluor® 633 (1/200; Thermo Fisher Scientific), were used for a further 2 hours of incubation at room temperature in the darkness. Afterwards, samples were incubated with 4' 6-diamidino-2-phenylindole (DAPI, 1/5000; Sigma-Aldrich) during 10 min to stain nuclei. It was necessary to make a longitudinal cut of the conduits before performing the immunocytochemistry assay to obtain a complete view of the lumen before using a confocal microscope (LEICA TCS SP5, Leica microsystems, Spain). The confocal images were processed with an overlay to make a reconstruction of the total length of the conduits. Neurite length on conduit and fibres were measured in the reconstruction of the confocal fluorescent images using Image J software.

2.6. Statistical analyses

Each experiment was performed at least four times unless otherwise noted. For all the experiments, three independent replicates (n=3) of each studied group were employed. Data were expressed as mean ± standard deviation. The Shapiro-Wilk test was used to confirm the data normality on GraphPad Prism 8. Results were analysed by t-student test on normal data and Mann-Whitney test in the opposite case. A 95% confidence level was considered significant. An asterisk * indicates statistically significant differences, indicating a p-value below 0.05.

3. Results

3.1. Schwann cells seeded inside the lumen of hyaluronic acid conduits generate an internal 'Schwann cell sheath'

As in previous studies [100,182] we seeded rat SC in the lumen of the conduit and cultured them for 10 days (Figure 8). The SC form a cylindrical sheath-like tapestry continuously spanning to the whole length of the internal lumen (Figure 8A, blue), composed by several layers of cells that contact tightly, the 'SC sheath'. These cells presented elongated nuclei, lax chromatin, and homogeneously distributed organelles in their cytoplasm (Figure 8B). These cells also presented junctional complexes between cells (Figure 8C). The SC formed a tight cell

mantle with a consistency that kept them together so that it could easily be detached from the channel's surface and taken outside, as seen in the Figure 8D, stained in blue with Coomassie. The consistency of this 'SC sheath' structure allows its folding without breaking, as can be seen on Figure 8D, where the more intensely coloured regions correspond to overlapped crimped parts of the length of the cell cylinder.

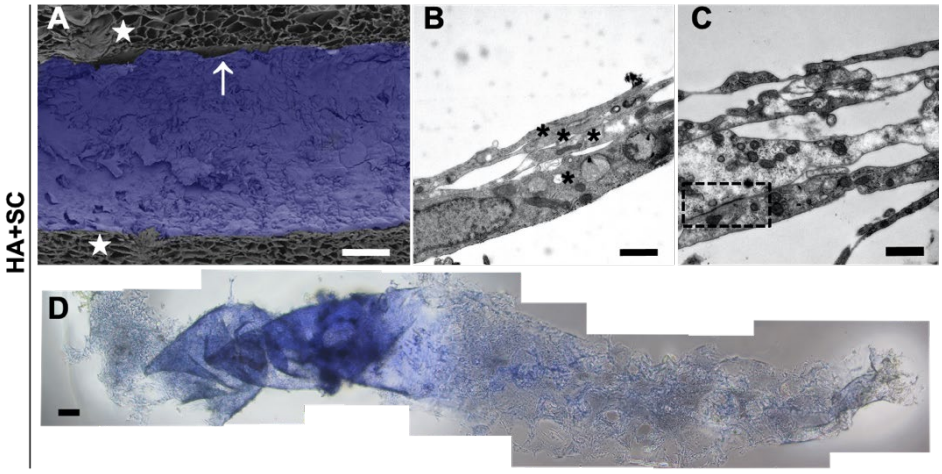

Figure 8. Schwann cells grown inside the hyaluronic acid conduits.
(A) Scanning electron microscope image from a longitudinal cut, dividing the HA+SC conduit into two parts, at day 10 of culture. The SC form a continuous 'sheath' coating the inside surface of the HA conduit's lumen, which presents a smooth surface (arrow), here blue-coloured for the sake of contrast with the material of the conduit's wall, whose porous structure can be clearly seen (stars). (B) Transmission electron microscope image from a transversal section of the 'SC sheath' in the HA conduit for the same culture time. Asterisks indicate the different cell layers that form the 'SC sheath'. (C) Transmission electron microscope image from a transversal section of the 'SC sheath' in the HA conduit. The dashed square indicates the zone where a junctional complex can be observed as a darker line between the membranes of two cells. (D) Bright field photograph of an entire and wrinkled 'SC sheath' in a culture dish after having been extracted from the HA conduit. The cell cylinder is stained with Coomassie brilliant blue R250. Scale bar: 100 μm (A, D), 1 μm (B, C). Figure 8D is enlarged in the Appendix.

3.2. Pre-seeding of hyaluronic acid conduits with Schwann cells improves axonal extension after 21 days culture of dorsal root ganglion explants

The postnatal P3-4 rat DRG explants were cultured for 21 days at one end of the HA conduit without pre-seeded SC (Figure 9A-9D) and with pre-seeded SC cultured for 10 days (Figure 9D-9H) in order to study the influence of the pre-seeded SC on the axonal extension from the DRG explant.

Groups HA+DRG and HA+SC+DRG showed significant differences in what refers to axon outgrowth. Figures 9A and 9B are macroscopic images of both kinds of samples, post-fixed with OsO_4 to have the cellular content stained in black for better visual identification. Both samples show the DRG at the ends (indicated with a white arrow), but only the channel of sample HA+SC+DRG appears black-stained. Cells coming from the DRG had not appreciably penetrated the HA conduit in group HA+DRG. This was further verified in longitudinal cuts (Figure 9C) and with confocal fluorescent images showing the axonal outgrowth immunostained with Tuj1 in green (Figure 9D, 9E). In the group HA+DRG without SC the DRG explant maintained its original rounded shape without axonal growth out of the DRG explant, without showing signs of cytotoxicity, so the axonal extension could not be quantified. In contrast, the group HA+SC+DRG with pre-seeded SC showed an axonal extension (0.66 ± 0.01 mm) in a significant way, though without any preferred directionality (Figure 9E).

A cross-section normal to the lumen's axis of the HA+SC+DRG conduit was analysed by bright field microscopy after toluidine staining and by TEM (Figures 9F-9G). Figures 9F and 9F' show the compact and continuous SC layer resting at the internal surface of the lumen without adhesive contacts to the conduit substrate ('SC sheath' indicated with an arrow and HA conduit indicated with a star). As previously, the TEM images showed the tight multilayer 'SC sheath' now also in the HA+SC+DGR conduit (Figure 9G). Figure 9H shows that the SC in the HA+SC+DRG sample possess a well-organized cytoskeleton distribution without picnotic nuclear bodies, indicating a good tolerance of the SC to the DRG growth medium after 21 days of culture. In fact, the 'SC sheath' that appeared here is similar to the 'SC sheath' after 10 days of SC culture (Figure 8A) being continuous and coating the entire lumen, and the transversal section (Figure 9G) is also like that in a 10-day SC culture with SC growth medium (Figure 8B).

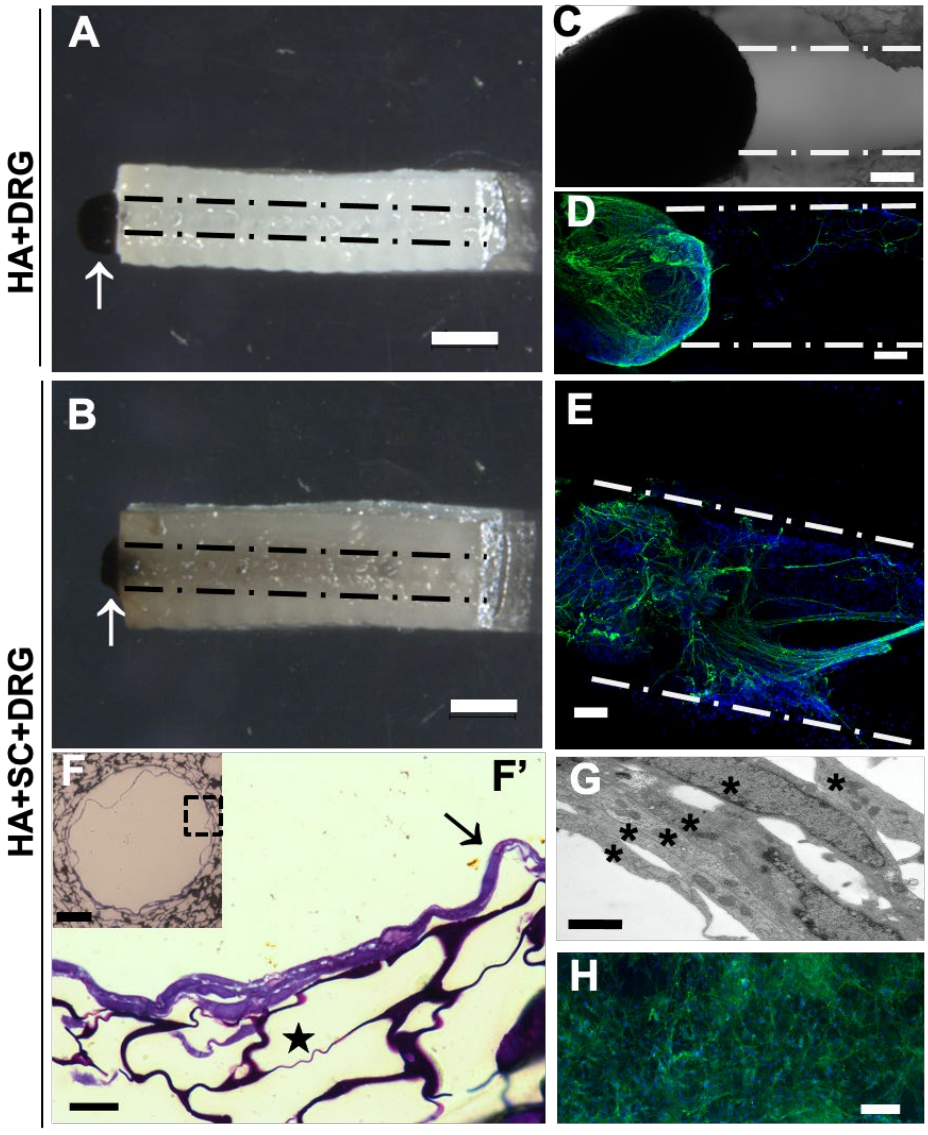

Figure 9. Influence of pre-seeding Schwann cells on cell invasion of the conduit by dorsal root ganglion (DRG) cells after 21-day DRG culture.

Macroscopical view of hydrated (A) HA+DRG and (B) HA+SC+DRG conduits and (C) bright-field photograph of a longitudinal section of HA+DRG conduit post-fixed with OsO4 (cells stained in black) showing the DRG explant in one end of the conduit indicated with a white arrow. Confocal fluorescent image showing the longitudinal section of (D) HA+DRG and (E) HA+SC+DRG conduits after nuclear staining with DAPI (blue) and

neuronal staining with Tuj1 (green). (F) Bright field photograph of the transversal section of HA+SC+DRG conduit showing Toluidine blue staining of SC in the internal lumen of the sample and a detail (F') of the 'SC sheath' (indicated with an arrow). The porous structure of HA is indicated with a star. (G) Transmission electron microscopic image from a transverse section of the 'SC sheath' in the HA+SC+DGR conduit for the same culture time. Asterisks indicate the different layers that form the 'SC sheath'. (H) Confocal fluorescent image of a longitudinal section of HA+SC+DRG after nuclear staining with DAPI (blue) and f-actin with Phalloidin (green). Dash lines delimit the conduit's lumen: black for samples before the longitudinally cut and white for longitudinal sections (A, B, C, D, E). Scale bar: 1 mm (A, B), 100 μm (C, D, E, F, H), 1 μm (F'), 1 μm (G).

3.3. Schwann cells coat poly-L-lactic acid fibres while still making the sheath-like structure at the hyaluronic acid lumen's inner surface

HAf samples, of HA conduits filled with PLA fibres, were seeded with SC and cultured for 10 days as previously shown. Longitudinal and transversal cuts of these HAf+SC samples were analysed with confocal fluorescent, SEM and TEM microscopies (Figure 10). Images of a longitudinal section of HAf+SC after nuclear staining with DAPI and glial staining with S100β in the lumen show that the presence of PLA fibres in the lumen did not hinder the formation of the 'SC sheath' at the conduit's lumen surface (Figures 10A), being similar to the 'SC sheath' formed in conduits without fibres (Figure 8A). SC attached to and grew on the PLA fibres too, and completely covered them (Figures 10B, 10C). SC on PLA fibres acquired elongated shapes oriented in the direction of the PLA fibre axis (Figure 10C), which contrasts with the rounded and flattened SC shape that have the cells in the 'SC sheath' (Figure 10A). The cells coat so densely the fibres, that individual cells connect one PLA fibre to another, making the cell-fibre bundle into an aggregate unit (Figure 10C). Bright field photograph of the cross-section of HAf+SC conduit (Figure 10D) shows toluidine blue staining of SC surrounding the PLA fibres and, in detail (Figure 10E), we could see how SC embrace two PLA fibres (indicated with f). The TEM image of the HAf+SC conduit shows the tight packing of the SC on the fibres' surface (Figure 10F).

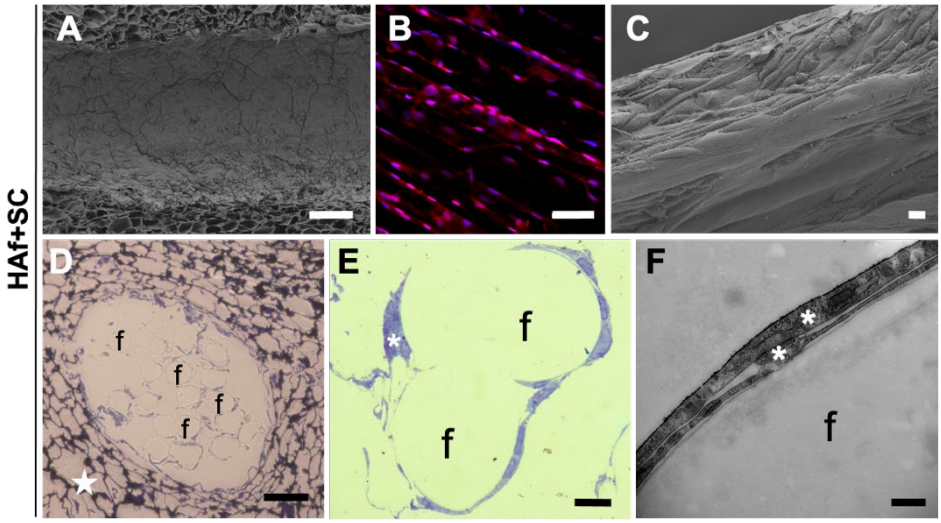

Figure 10. Schwann cells distribution inside the hyaluronic acid conduits with poly-L-lactic acid fibres.

Scanning electron microscopic image from a longitudinally cut of the SC tubular 'sheath' in the lumen (A) and PLA fibres (C) of HAf+SC after 10 days of SC culture. (B) Confocal fluorescent images of a longitudinal section of HAf+SC after nuclear staining with DAPI (blue) and glial staining with S100β (red) in PLA fibres (B) for the same culture time. (D) Bright field photograph of the transversal section of HAf+SC conduit showing toluidine blue staining of SC surrounding the PLA fibres and a detail (E) with two fibres. (F) Transmission electron microscopic image from a transverse section of HAf+SC showing SC encircling a PLA fibre. The star indicates the HA, the f indicates a PLA fibre and asterisks indicate the different layers of SC. Scale bar: 100 μm (A, D), 50 μm (B), 10 μm (C, E), 500 nm (F).

3.4. Schwann cells together with poly-L-lactic acid fibres induce directed axon outgrowth from one end to the other of the conduit

DRG explants were cultured for 21 days on one extreme of conduits without pre-seeded SC (HAf+DRG) and with pre-seeded SC (HAf+SC+DRG) for 10 days, as explained above, to study the influence of aligned PLA fibres on the axonal growth. The DRG explants attached faster and remained at the end of the conduit more consistently than in the HA group, where it was not infrequent for the explants to detach in contact with the culture medium. Macroscopical observation of the entire conduits post-fixed with OsO_4 (cells stained in black) shows higher

cell density throughout the lumen of HAf+SC+DRG (Figure 11A) than in HAf+DRG (Figure 11C) conduits. Confocal fluorescent reconstructions in Figures 11B, 11D, completely spanning the longitudinally cut conduit's whole length, show a nuclear staining with DAPI (blue) and neuronal staining with Tuj 1 (green). The DRG projecting axons fully invade the lumen of the HAf+SC+DRG conduit (Figure 11D). In the absence of SC (HAf+DRG group), fewer axons were identified (Figure 11B). A closer look at the fibres of the HAf+SC+DRG constructs shows that the projecting axons stained with Tuj1 (green; Figure 11E) and the SC stained with p75 (red; Figure 11F) closely co-exist (Figure 11G).

In the HAf+DRG group, axons elongated through the lumen reaching a length of 1.97 ± 0.82 mm (Figure 11B), a longer extension than in the HA+DRG (Figure 9C) and HA+SC+DRG groups (Figure 9H) groups. The outgrowth found in HAf-DRG group was also more ordered. In the case of the HAf+SC+DRG group, after the 21 days of DRG explant culture, all the analysed specimens showed axons reaching the opposite end of the HA conduit (6 mm) and even exceeding this length growing on the fibres that showed out of the conduit (Figure 11D). The elongation of axons that could be thus measured reached 7.52 ± 0.71 mm. As fibres protrude at both ends of the HA conduit, axons extended all through the fibres' length, even without the HA enclosure. In the presence of SC, Figure 11D, axonal growth is ordered parallelly on the SC-coated fibres, in the direction of the fibre axis (Figure 11D, 11E-11G). The SC coating the fibres served as guiding support of the axons all along the whole length.

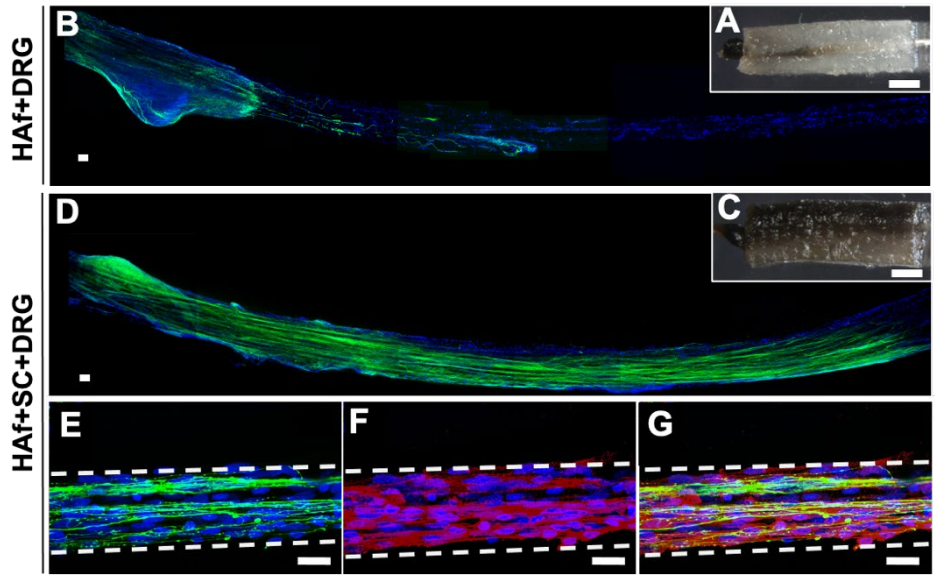

Figure 11. Cells distribution and axon growth in hyaluronic acid conduits with poly-L-lactic acid fibres after 21 days.

Macroscopical view of hydrated (A) HAf+DRG and (C) HAf+SC+DRG conduits post-fixed with OsO_4 (cells stained in black) showing the DRG explant at one end of the conduit. Representative confocal reconstruction of the conduit's complete length of (B) HAf+DRG and (D) HAf+SC+DRG conduits, after nuclear staining with DAPI (blue) and neuronal staining with Tuj1 (green). Confocal fluorescent image of a longitudinal section of HA+SC+DRG after (E) nuclear staining with DAPI and Tuj 1 in green, (F) DAPI and glial marker p75 in red and (G) merge. Dash lines delimit one PLA fibre. Scale bar: 1 mm (A, C), 100 μm (B, D), 20 μm (F, G, H). Figures 11B and 11D are enlarged in the Appendix.

GAP43 was employed to trace the active axon outgrowth along the HAf+SC+DRG conduits (Figure 12A-12B; green). The axon growth cones ultrastructure was analysed by TEM. Figure 12A shows a confocal reconstruction of the conduit's complete length, made from a series of confocal fluorescent images of longitudinal sections of a HAf+SC+DRG conduit and also a detail (Figure 12B) for GAP43 immunostaining showing a continuous and abundant staining along the entire axonal projections. We could confirm the presence of growth cones along the entire length thanks to the TEM images, showing growth cones at the proximal (Figure 12C) and distal (Figure 12D) transversal sections

of samples. Growth cones (indicated with an asterisk) appeared located between other axons (*Ax*) and glial processes (*SC*) (Figure 12B, 12C).

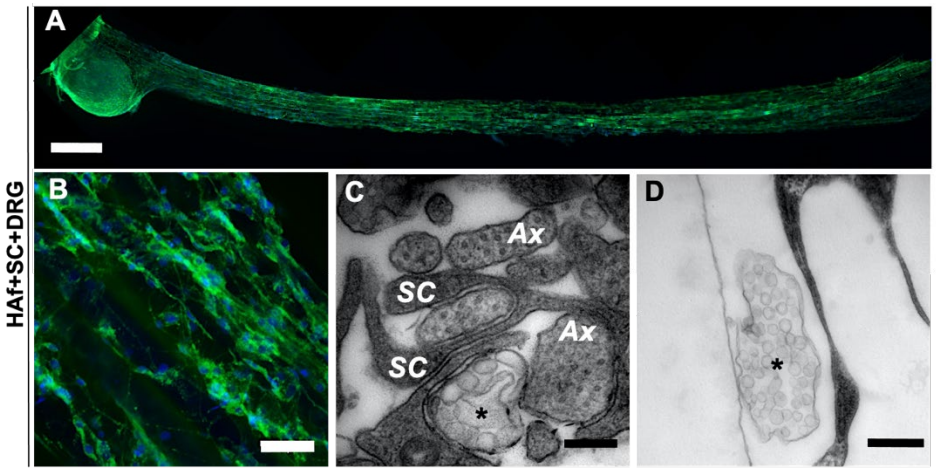

Figure 12. Axonal outgrowth trace in HAf+SC+DRG group.
(A) Representative confocal reconstruction of the conduit's complete length of longitudinal sections of HAf+SC+DRG conduit and (B) a detail, after nuclear staining with DAPI (blue) and growth cones staining with GAP43 (green). (C, D) Transmission electron microscopic images from a transverse section of the HAf+SC+DRG conduit showing SC (indicated with *SC*), axons (indicated with *Ax*) and growth cones (indicated with an asterisk). Scale bar: 100 μm (A), 20 μm (B), 500 nm (D), 200 nm (C). Figure 12A is enlarged in the Appendix.

3.5. Different cells from the dorsal root ganglion explant migrate and coexist with the pre-seeded Schwann cells within HAf+SC+DRG

Besides the axons outgrowth from the DRG explants, different cells can migrate from DRG explants such as SC and fibroblast. To identify these, a DRG-GFP explant was used in the experiment. Figure 13 shows that GFP+ cells are present within the HAf+SC+DRG samples after 21 days of DRG explants culture. Red-stained S100β+ SC coexist with green-stained GFP+ cells stemming from the explant all along the entire length of the samples, on the lumen's surface of the conduit and on the PLA fibres (Figure 13A). TEM images of transversal cuts revealed a large number of cells that appeared to be fibroblasts (marked with an

asterisk on Figure 13B). Fibroblasts appear as thin cells, elongated nuclei and condensed chromatin, with a developed rough endoplasmic reticulum and caveolae. In the direct neighbourhood, many collagen fibres, typically found in these cells, were observed (indicated with arrows on Figure 13B). Besides fibroblasts, SC migration from the DRG explant into the conduit could also be ascertained by the co-localization of both markers (S100β red and GFP green, giving rise to the yellow colour in the merged image shown in Figure 13A).

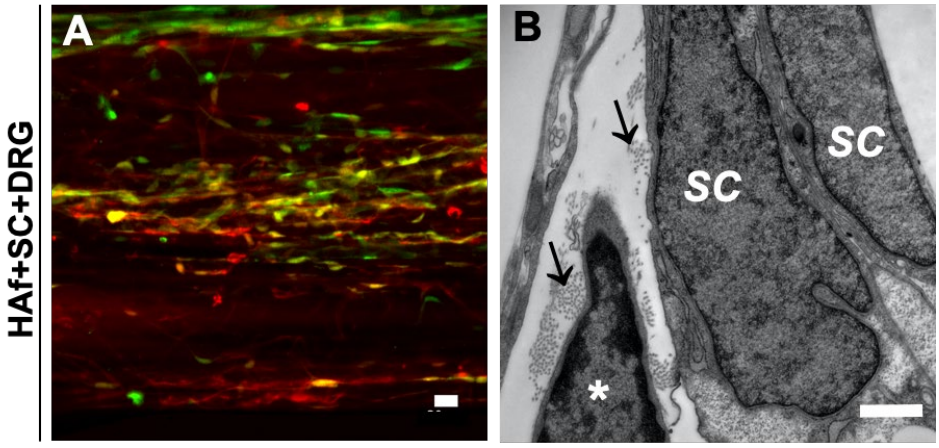

Figure 13. Coexistence of pre-seeded Schwann cells and dorsal root ganglion-GFP explant cells in HAf+SC+DRG group after 21 days.
(A) Confocal fluorescent image of a longitudinal section of HAf+SC+DRG conduit, after immunostaining showing S100β glial marker (red) and DRG-GFP explants cells (green). (B) Transmission electron microscopic image from a transverse section of the HAf+SC+DRG conduit showing fibroblast. An asterisk indicates fibroblast and arrows indicate collagen fibres. Scale bar: 20μm (A), 1μm (B).

3.6. Ultrastructural analysis of HAf+SC+DRG shows Schwann cells surrounding unmyelinated bundles of well-preserved axons

We performed an ultrastructural analysis to gain a general sight of the distribution and interaction of the cells inside the HAf+SC+DRG samples after 21 days of DRG explants culture. Figure 14A presents a sketch of the sections analysed in TEM. In all proximal, medium, and distal sections SC could be found surrounding a number of unmyelinated bundles of well-preserved axons. The cross-sections of the axons had the expected cytoplasmic contents of microtubules and neurofilaments, as can be seen in Figure 14E. Also, SC had the normal morphology and structure seen in other *in vitro* studies [187]. Some SC enclosed scattered single axons (Figure 14E). In some larger bundles, a cytoplasm-rich large SC revealed a complex interaction with the axons by extending several cytoplasmic processes that seemingly subdivided the axon bundle into smaller groups (Figure 14B, 14C). All these events were found over the entire length of the conduits. We found that the diameter of the axons was approximately the same along the entire length of the conduit, being <3 μm. Immunostaining for neurites with NF marker (Figure 14G) and for myelinating SC with MBP marker (Figure 14H) revealed the association of both cell types again (Figure 14I). Although the MBP marker is associated with myelinating SC, in the TEM images (Figure 14B-14E) the characteristic structures belonging to the myelin sheath, an electron-dense multi-layered covering around the axons, were not observed, maybe because the myelinating process has a longer time requirement than the short time of the experiments.

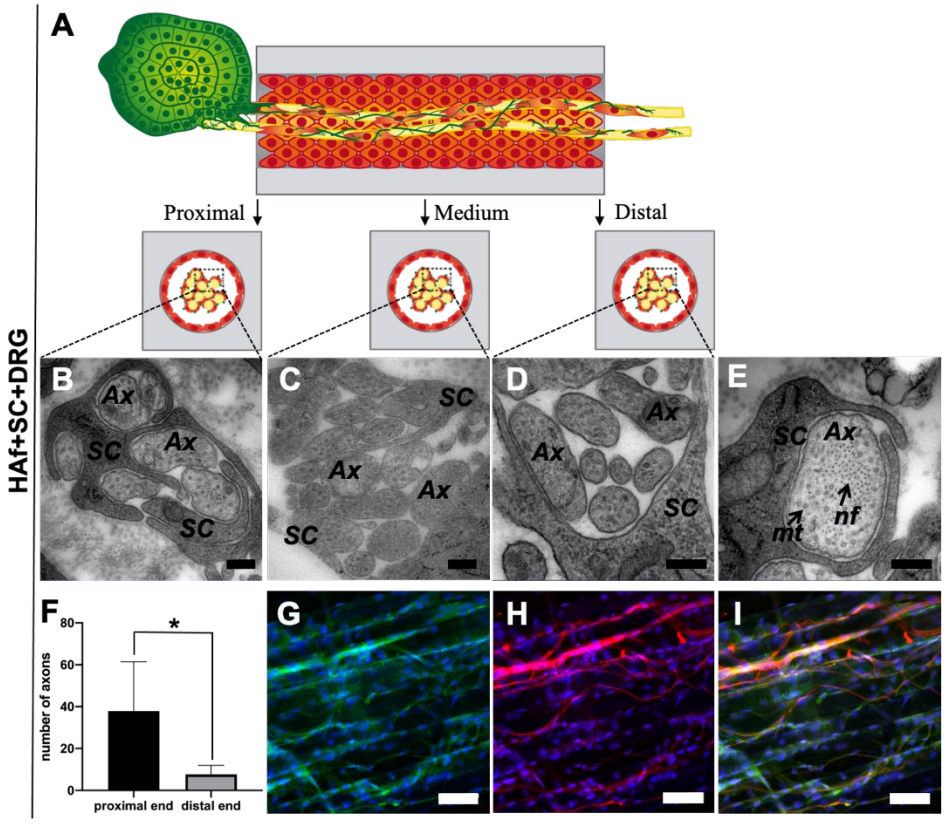

Figure 14. Cell ultrastructure in the HAf+SC+DRG group.

(A) Sketch of the locations of the proximal (B), medium (C) and distal (D) cross-sections (normal to conduit axis) taken for transmission electron microscopy (TEM). TEM images of the HAf+SC+DRG conduit at (B) proximal, (C) medium and (D) distal sections. (E) Representative TEM image from a cross-section showing a SC (indicated with *SC*) and an axon (indicated with *Ax*) with its cytoplasmic content of microtubules (indicated with *mt*) and neurofilaments (indicated with *nf*). (F) Quantification of the number of axons at the proximal and distal sections of the conduit. Confocal fluorescent image of a longitudinal section of HAf+SC+DRG after (G) nuclear staining with DAPI in blue and neurites marker NF in green, (H) DAPI and myelinating SC marker MBP in red and (I) merge. Scale bar: 200 nm (B, C, D, E), 50 μm (G, H, I).

4. Discussion

In this work, a biohybrid construct has been designed to mimic the *in vivo* physiological situation produced during neural regeneration, trying to act as an *in vitro* predictive model. The tubular scaffold made by crosslinked HA acts as a template for the development of a 'SC sheath' and could act as a physical shelter in case of cell transplantation. Additionally, PLA fibres support the directed growth of SC, and SC constitute the necessary auxiliary cell support for axon outgrowth. The conjoint association of SC and PLA fibres recapitulate the directional features of axonal pathways. Our *in vitro* experimental model includes the seeding of a DRG explant at solely one end of the HA construct. In this way, the experimental *in vitro* model allows both for a study of the kinetics of unidirectional axon growth and recapitulates some features of physiological situations in which axons outgrowth starts from a neuronal population (proximal neurons in PNS or substantia nigra in the case of the nigrostriatal tract in CNS).

The biohybrid construct (HAf+SC+DRG group) shows a total axonal extension of 7.52 ± 0.71 mm by the end time of the experiment (21-days end-time point) in all specimens (Figure 11D). Moreover, other cells from the DRG explant migrate through the lumen of the biohybrid construct (Figure 13A).The supporting cells in the DRG explant that could migrate out from the ganglia are SC and fibroblasts [187]. We could observe (Figure 13B) SC and fibroblasts all along the entire conduit of HAf+SC+DRG.

The pre-seeded SC cultured for 10 days within the HA conduits developed the sheath-like continuous cell structure that we had previously described in [100] (Figures 8A and 10A, 10C), and also grew on PLA fibres. (Figure 10B, 10C). This 'SC sheath' has integrity and resists manipulation and could be easily detached from the conduit (Figure 8D). The formation of this type of cellular structure is explained by the highly hydrophilic nature of HA, which allows only for a weak cell adhesion to its surface [99]. In this way, the HA conduit acts as a template of the 'SC sheath' but not as an attachment substrate. The HA-SC bond is weaker than the stronger SC-SC bond, and thus proliferation of the SC leads to an integral one-piece 'SC sheath'. In contrast, the more strongly bonded SC attached to the hydrophobic PLA fibres remain on them. PLA fibres, being more hydrophobic, adsorb more ECM proteins and thus are more efficient for cell adhesion [188]. In this way, after co-culture the HA scaffold can be easily removed, and we are left with the grown biohybrid structure formed by the 'SC

sheath' and the SC-coated PLA fibres. This biohybrid structure is the object of a forthcoming study. The SC adhered on the PLA fibres and surrounded the whole fibre bundle. The more elongated shape of the SC on the fibres (Figure 10C) as compared with their more rounded shape (Figure 10A) in contact with the HA surface indicates the sensitivity of these cells to the radius of curvature (much greater in the case of the HA lumen surface than in the case of the PLA fibres). Hence, SC morphology is influenced both by the different HA or PLA chemistries but also by the curvature radius.

In order to have an *in vitro* model that simulated the *in vivo* situation of directed axonal growth in tracts originating from one neuronal population, we located the DRG explant at one of the ends of the conduits. Chemotactic stimuli secreted by the SC within the conduit together with the presence of the PLA fibres made axons in HAf+SC+DRG group sprout from the explant, extend along the fibres, and reach the end of the tube by the end of experimental time (Figure 11D). So, the differences between HAf+DRG group, where the axonal growth was discrete, and HAf+SC+DRG group must most probably lie in that the growing axons of the HAf+SC+DRG group do not have to wait for migrating SC and find larger amounts of neurotrophic factors in the conduit. This difference stresses the importance of a rich supply of auxiliary cells (here SC) in aiding and potentiating the regeneration possibilities innate naturally in neurons. In contrast to related works that employ tubular constructs with a gel filler in the lumen [181], our concept critically makes use of both the PLA fibres and the SC. They proved to be effective both in promoting and guiding the axon growth through topographical cues (supplied by the fibres) and biochemical signals contributed by the SC. SC without PLA fibres exerted a positive but discrete effect on axonal growth (experimental group HA+SC+DRG, Figure 9E). Fibrillar structures have been seen to improve neural regeneration [117,186], and in our work, they lent support to the proliferative capacity of cells and guided their migration and growth, partly due to their cell-adhesive properties. These adhesion properties were advantageous not only for the axonal extension but also for keeping the DRG stably positioned at one end of our constructs at the beginning of the experiments (Figures 9A and 9B). The introduction of the PLA fibres (Figure 11) meant an improvement in the handling of the DRG since the bundle of PLA fibres was a very adherent substrate and protruded through both ends of the conduits.

In humans, axon growth rate during peripheral nerve regeneration can reach 1 mm/day [189], and this rate is significantly reduced in the CNS [190]. DRG axons grow with rates between 130 and 300 μm/day *in vitro* [191]. In the present work, we can estimate the axonal extension rate achieved *in vitro* to lie between 350 and 400 μm/day, a value obtained by dividing the maximum length reached at day 21 by axons through that time. It must be born in mind that this figure represents a lower bound for the actually occurring growth rate since we cannot know whether axons had covered the maximum length of our conduits before day 21. Considering that the most effective implantable medical devices for functional regeneration currently do not exceed rates of 0.5-1 mm/day [192], our biohybrid construct compares favourably with those types of devices.

Axons growing in our HAf+SC+DRG samples had growing neurites, as evidenced by the appearance of growth cones stained with GAP43 (Figure 12). During development, axons extend from the neuronal cell body and grow, frequently over long distances, in order to connect with a target. It is the growth cone that recognises the molecular cues present in the environment and integrates the information to determine the pathway the growing axon will take. This response process requires both cell adhesion and subsequent triggering of an intracellular signalling cascades. The cell adhesion molecules that the growth cones recognised are mostly expressed by glial cells [193]. Moreover, growth cones use integrins to select a pathway of ECM molecules on which to extend processes [194]. *In vitro*, DRG adhesion is a prerequisite for neurite extension, and the PLA has demonstrated to serve as a substrate for cells and also ECM [195]. GAP43 is a neuron-specific, membrane-associated phosphoprotein and the most abundant proteins in neuronal growth cones [196]. However, this protein is not confined to neurons but is also expressed by glial cells like SC precursors and in mature non-myelin-forming SC [197]. So, fluorescence images presented here could show these different cell types (Figure 12A, 12B). However, it could be confirmed that there are growth cones along the entire length thanks to the TEM images (Figure 12C, 12D), similar to other authors results [190].

As previously mentioned, SC surround unmyelinated bundles of well-preserved axons (Figure 14B-14E), extending cytoplasmic processes that embrace and subdivide the axon bundle. These axons presented a typical cytoplasmatic content of microtubules (*mt*) and neurofilaments (*nf*). Figure 14F shows an estimation of the number of axons at the proximal and distal section using TEM

images. This quantification is not representative of what happens throughout the section, it should be understood as an approximation, since we have not monitored the intermediate zone of the conduits. Approximately 21% of the axons we count at the beginning of the conduit reach the end of the 6 mm conduit after 21 days of DRG explant culture.

SC with myelinic potential were observed thanks to the MBP marker (Figure 14H, 14I), although in the TEM images (Figure 14B-14E) the characteristic structures of the myelin sheath were not observed. Probably the absence of myelin was due to the insufficient time in which the experiments are carried out for the myelination process to develop [198]. It could be observed that the neurites were in contact with the SC (Figure 11E-11G) and the way they appeared to extend throughout the cells resembles the neuronal behaviour *in vivo* during regeneration when SC align longitudinally to form the bands of Büngner that regrowing axons use to extend [199]. The ultrastructure of neurites and all the neural tissue developed within the HAf+SC+DRG constructs after 21 days is similar to an unmyelinated nerve *in vivo*, where SC interact with multiple small axons (<3 μm), resulting in non-myelinated nerve fibres formed by axonal bundles surrounded by SC processes [200]. It remains hypothetical whether these structures would evolve towards myelinated structures with more time of culture or with a contribution of additional factors.

In summary, the biohybrid proposed here recapitulates directional hallmarks of axonal tracts and may represent a step towards the regeneration of injured axonal pathways like nerves in PNS and neural tracts in CNS.

5. Conclusions

Taking into account the results shown in this chapter, it can be concluded that:

1. SC proliferate within the HA tubular conduits forming a ‘SC sheath’ in the lumen, made up of a thin and dense layer of cells, thanks to the three-dimensional tubular structure that confines them and the hydrophilic nature of HA.
2. In HA conduits with the PLA fibre bundle in its lumen, the ‘SC sheath’ maintains its structure. Furthermore, the SCs adhere and grow on the PLA fibres, acquiring a shape oriented in the direction of the axis of the PLA fibres.

3. The conjoint association of SC and PLA induces a more efficient process of axon extension from a DRG explant than either SC or PLA fibres per separate after 21 days of culture.
4. The configuration of the biohybrid formed by HA conduits with PLA fibres and pre-seeded SC allowed the neuron somas placed on one end project their neurites and reach the other end of the tube after 21 days of culture.
5. The SC showed a good tolerance to the DRG growth medium after 21 days of culture.
6. The biohybrid construct proved effective in promoting directed axon growth, guided by the PLA fibre bundle covered by SC.

Chapter II

Multimodular bio-inspired organized structures guiding long-distance axonal regeneration

1. Introduction

Axonal bundles or axonal tracts represent an aligned and unidirectional architecture that makes axonal nervous tissue development possible. Loss of communication between the CNS and the PNS is the cause of countless disorders that compromise the quality of life of affected people. This loss of communication can be due to PNI, SCI, TBI, or neurodegenerative disease like Parkinson's disease. When one of them occurs the intricate architecture of the nervous tissue undergoes alterations leading to growth inhibition and loss of guidance [11].

Injuries in peripheral nerves can result in a gap between two nerve stumps. When nerve endings are unable to rejoin without tension, a bridging section of nerve is used, and two end-to-end sutures are performed [39]. In this case, a nerve graft is used, usually from the patient (autograft), or tissue from a cadaveric human donor (allograft). However, the use of autografts have limitations [44–46]. Allografts overcome several of the drawbacks of autografts but require immunosuppression or decellularization to prevent immune rejection [49]. These problems drive the search for a tissue engineering alternative to this treatment.

The FDA has approved several conduits based on natural and synthetic degradable biomaterials to repair nerve defects arising from PNI [125], all limited to use in relatively short distance peripheral nerve reconnection but with advantages in comparison with autografts [46]. However, in most cases, these nerve conduits are not good enough repairing the injury like the autograft. In general, failure of nerve regeneration across long gaps seems to be the result of a lack of the formation of an initial fibrin cable, which is necessary for SC migration into the constructs and the formation of the bands of Büngner which are aligned columns of SC and laminin [16]. There is a limitation in nerve regeneration if the gap is >10 mm in rats [16]. Synthetic conduits have only been used successfully clinically to bridge the injured nerve stumps when the gaps are <10–12 mm [201] but recently researchers have provided promising results in the rat sciatic nerves [202]. Most research focuses on filling the lumen of nerve guides with scaffolds, fibres, cells, and/or drug delivering therapies in order to improve the regeneration capacities in long gap PNI [147], such as porous cylindrical collagen structures whose ellipsoidal cavities have a preferential direction [143], or aligned polymer fibre-based constructs [73]. Although the most common is to use nerve guidance

conduits with one or more parallel through channels [76] that serve as a preferential path or guide to the axons, or combined systems of the above [72,75].

In the case of CNS regeneration, there is no clinical treatment with a proved recovery. In SCI, TBI, and degenerative disease, initial damages result in fast acute primary injury events. Current treatments, instead of trying to recover damaged tissue, try to prevent the secondary injury [53,54]. So, tissue engineering is working in developing strategies to provide a bridge across the lesion to recover of nervous tissue. In the case of a complete transection, large lesions, or chronic situations in the spinal cord where biomaterials with channels or guides that are oriented parallel to the spinal cord tracts, have been produced [63,151,153]. With the same guiding cues idea for the treatment of brain lesions, researchers propose tubular micro-columns containing hydrogel with the capacity of astroglia cells to create a neural guide pathway [161], or more complex mico-column system containing mature primary cortical neurons and long axonal tracts proposed as a possible treatment for Parkinson's disease [163].

SC are key player in the neural repair process in the PNS and constitute the necessary auxiliary cell support for axon outgrowth. SC can secrete neurotrophic factors, which is important for nerve regeneration, as well as cell adhesion molecules [20]. The SC supporting regeneration effect *in vitro* [203] and *in vivo* [168] has been reported, and different approaches have been employed in combination with materials in order to induce an alignment to orient *in vitro* neuronal growth [204]. Moreover, nerve guides pre-filled with SC have been proved to support regeneration in rat PNS [67] and CNS [139].

In our previous studies [100,182,183], we found that SC seeded into HA conduits with PLA microfibers in their lumina (forming a biohybrid construct) were able to proliferate and self-organize into a continuous cylindrical cell ‘sheath’ in the lumen of the conduits. Also, SC grew on the PLA fibres, spanning the whole distance of a 6 mm length biohybrid construct. We demonstrated that the biohybrid construct proved effective in promoting directed axon growth along the entire 6 mm length. HA is a natural glycosaminoglycan, an essential component of the ECM of many tissues [86], and biocompatible and biodegradable. HA has already shown therapeutic benefits on neuronal regeneration processes [184] and exhibits mechanical properties similar to soft nervous tissues [185]. PLA is a synthetic polyester with mechanical properties, biodegradability, and biocompatibility [109]. PLA fibres raise interesting as part of the tubular conduit concept when allocated

in the lumen [117] providing support for cell adhesion, migration and elongation in a guided way.

Here we propose a multimodular concept to overcome the typical limitations of long-distance axonal regeneration, introducing PLA fibres bundle in the lumen of several shorter individual HA conduits or modules which positioned consecutively, designing nerve guidance conduits with the desired length. We seeded and cultured SC into the 18 mm multimodular conduit for 5 days (Figure 15A). Then, we put at one end of the construct a DRG explant, as a source of projecting neurons, and the axon extension stemming from this source into the SC-seeded multimodular conduit lumen was observed (Figure 15B). We compare the multimodular conduit with a unimodular conduit with the same length in order to study the cells viability and distribution, and the axonal extension of neurons coming from the DRG explant.

Moreover, after these 5 days of SC culture, we developed a construct consisting of the PLA fibres together with the SC that we have named ‘neural cord’ spanning the interior of the multimodular conduit, eliminating the HA cover protection and culture for 5 additional days. With the development of this stable ‘neural cord’, we can obtain a living nerve *in vitro* for transplantation without the HA cover if desired, alone [205,206] or within a different protection structure [207].

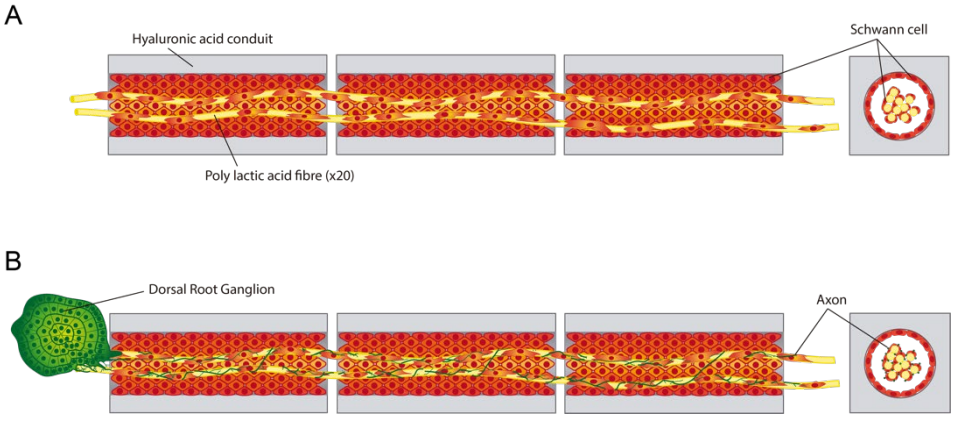

Figure 15. Schematic view of the long-distance axonal regeneration strategy proposed.

(A) Longitudinal and transversal view of a multimodular conduit formed by three 6 mm length individual modules of HA and PLA fibres passing through its lumens. We seed SC inside for 5 days and SC form a tapestry in the inner lumen and also grow on the PLA fibres. (B) After 5 days of SC culture, we seed a DRG explant at one end of the multimodular conduit and culture for 21 days expecting the axons of projecting neurons extent through the multimodular conduit. We observer the axon growth to prove the effectiveness of promoting directed axon growth in long distances.

2. Materials and methods

2.1. Cells source

Primary rat Schwann cells (P10301, Innoprot) expanded with SC Medium (P60123, Innoprot) were employed at 4-5 cell passage for cell cultures in the materials. Sprague–Dawley rats from Charles River were used for DRG explants dissection and were maintained following the National Guide to the Care and Use of Experimental Animals (Real Decreto 1201/2005). After the sacrifice by decapitation, whole DRG explants were dissected from the spinal column of neonatal rats (P3-P4) and transferred into ice-cooled DMEM medium with a high glucose level (4.5 g/L) (Thermo Fisher Scientific) containing 10% FBS (Thermo Fisher Scientific), using a dissecting microscope to remove the remaining nerves and connective tissue.

2.2. Preparation of hyaluronic acid unimodular and multimodular conduits with poly-L-lactic acid fibres

The synthesis of HA sodium salt from *Streptococcus equi* (HA; 1.5–1.8 MDa, Sigma-Aldrich) conduits was carried out as previously described [100,182]. Briefly, PCL (PolySciences, Mw = 40 kDa) fibres of 400 μm were extruded in Hater Minilab, and a polytetrafluoroethylene thin block with 1.5 mm-wide grooves with a single PCL fibre of 400 μm diameter was used as a mould for the conduits. 5% (w/v) of HA was dissolved for 24 h in sodium hydroxide 0.2 M (Scharlab). Then, the HA was crosslinked with DVS (Sigma-Aldrich) in a 9:10 DVS:HA monomeric units molar ratio and this solution was mixed and injected in a mould. Later, the solution in the mould was lyophilised for 24 h (Lyoquest-85, Telstar, Spain) to generate HA microporous matrices. Finally, the conduits were hydrated in distilled water for 2 h, the PCL fibres were extracted, and the conduits were cut to the desired length. In several conduits, 120 aligned PLA (AITEX Textile Research Institute, Spain) fibres of 10 μm diameter were placed inside the channel of the conduits. In order to obtain the unimodular conduit, PLA fibres bundle was introduced in a long unimodular conduit (12 mm or 18 mm length). To form the multimodular conduit PLA fibres bundle was introduced in the lumen of three shorter individual conduits or modules (4 mm or 6 mm length) which positioned themselves one behind the other. Fixing structures were added at both ends of the PLA fibres bundle that protrudes from the conduits to hold the unimodular conduit and individual modules in the multimodular conduit together. In this work, we have opted to use three individual modules for the multimodular conduit and thus have two inter-module zones. We have not chosen to work with more individual modules due to the long culture time that this would entail.

Before the seeding of the cells, the unimodular and multimodular conduits were sanitised for 2h with 70º ethanol (Scharlab) and 10 min with 50º, 30º ethanol and distilled water. Then, conduits were conditioned with culture medium overnight.

2.3. Cell culture and cell seeding within conduits

We used 18 mm length unimodular conduits and multimodular conduits of three 6 mm length modules for all the experiments with cell culture. SC 4-5 cell passage were grown in flasks until confluence at 37ºC, 5% CO_2, in SC medium. SC were seeded at a density of 600,000 cells in a total length of 18 mm (unimodular or

multimodular conduit) and 9 µl of SC medium. SC were seeded with a 1-10 micropipette, inserting the tip at one end on each individual modules that form a multimodular conduit (3 µl in each module) or in the two ends in the case of the unimodular conduit (4,5 µl in each end), to ensure the homogeneous distribution of cells in the lumen, and were maintaining in the incubator for 30 min before adding the SC medium. SC were cultured for 5 days in SC medium. After that, DRG explants were placed in direct contact with one end of the unimodular or multimodular conduits and were then transferred into 6-well plates, which was maintained in a tissue culture incubator at 37ºC, providing a humidified atmosphere containing 5% CO_2 with a specific DRG medium (Neurobasal medium (Thermo Fisher Scientific), D-glucose 2mg/mL (Sigma-Aldrich)L-glutamine 100X (Thermo Fisher Scientific), 1% FBS, 1% P/S (Thermo Fisher Scientific), 2% B27 supplement (Thermo Fisher Scientific), 0,1% NGF (Thermo Fisher Scientific)) refreshed every 2 days until 21 additional days counted from the moment the DRG was seeded.

2.4. Scanning electron microscopy

The unimodular and multimodular without cells (three 4 mm length modules) conduits with a total length of 12 mm were dried for SEM visualisation. The conduits were cut longitudinally to expose their internal lumina and coated with an ultrathin layer of gold and observed at an acceleration tension of 10 kV in a scanning electron microscope (Hitachi S-4800, EE.UU.).

To observe the samples after 5 or 10 days of SC cultures, samples for SEM were washed in PB 0.1M and fixed in 3.5% GA (Electron Microscopy Sciences) solution for 1h at 37ºC, post-fixed with 2% OsO_4 (Electron Microscopy Sciences) and dehydrated. Later, samples were processed in a critical point dryer (critical point values: 328C, 1100 psi). Samples with conduits were cut longitudinally to expose their internal lumina and observed in a scanning electron microscope. Quantification of the transversal dimension of the structure formed by the PLA fibres and the SC (‘neural cord’) was performed employing SEM images and measured using Image J software.

2.5. MTS assay

To evaluate the cell proliferation inside conduits, MTS assays (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) were carried out on the unimodular and multimodular conduits (n=5 each). When incorporated to the cells, the MTS was reduced by metabolically active cells at 5 days of SC cell culture in a rate proportional to the number of live cells. After 1 h of incubation with the MTS reagent, the medium was removed, and its absorbance was measured with a Victor Multilabel Counter 1420 spectrophotometer (Perkin-Elmer) at 490 nm.

2.6. Immunocytochemistry

After the SC culture or co-culture with DRG explant, SC and neural population were identified analysing the expression of different markers by immunofluorescence with confocal laser scanning microscopy: F-actin (ActinRed™ 555 ReadyProbes™ Reagent; for SC) and neuron-specific class III β-tubulin (Tuj1; for neurons). For confocal microscopy, the conduits were rinsed thoroughly with PB 0.1M and fixed in 4% PFA for 20 min. Cells were permeabilised and blocked with 0.1% Triton X-100 (Sigma-Aldrich), 10% FBS in PB 0.1M for 2h. Conduits were then incubated in at 4°C overnight with primary antibody mouse monoclonal Tuj1 (1/300; Neuromics) Secondary antibody goat anti-mouse IgG Alexa Fluor® 488 (1/200; Thermo Fisher Scientific) and ActinRed™ 555 ReadyProbes™ Reagent (2 drops/ml; Thermo Fisher) were used for a further 2 hours of incubation at room temperature in the darkness. Afterwards, samples were incubated with DAPI (1/5000; Sigma-Aldrich) during 10 min to stain nuclei. It was necessary to make a longitudinal cut of the conduits before performing the immunocytochemistry assay to obtain a complete view of the lumen and the PLA fibres before using a confocal microscope (LEICA TCS SP5, Leica microsystems, Spain). The confocal images were processed with an overlay to make a reconstruction of the total length of the conduits. Neurite length on conduit and fibres were measured in the reconstruction of the confocal fluorescent images using Image J software.

2.7. Development of 'neural cord'

After the 5 days SC culture inside the multimodular conduits, using a stereoscope for visual guidance, we made a longitudinal cut with a scalpel of the different modules to eliminate the HA conduits protection to obtain a complete 'neural cord' formed by the PLA fibres and the associated SC. The 'neural cord' was transferred with sterile surgical forceps into a 6-well plate and cultured for 5 days more in SC medium.

Moreover, we performed a proof of concept seeding DRG explants to confirm that it is possible to grow and guide the axons through the 'neural cord'. After 5 days of SC culture, a DRG explant was placed in one end of the multimodular conduit and cultivated for 10 days with DRG medium. Then, we eliminate the HA conduits and cultivate the 'neural cord' with the DRG explant for 4 additional days.

2.8. Statistical analyses

Each experiment was performed at least four times unless otherwise noted. For all the experiments, three (SEM and immunocytochemistry assays) or five (MTS assay) independent replicates (n=3 or n=5) of each studied group were employed. Data were expressed as mean ± standard deviation. The Shapiro-Wilk test was used to confirm the data normality on GraphPad Prism 8. Results were analysed by t-student test on normal data and Mann-Whitney test in the opposite case. A 95% confidence level was considered significant. An asterisk * indicates statistically significant differences, indicating a p-value below 0.05.

3. Results

3.1. The composition of the multimodular conduit with short individual modules minimizes failures in the manufacturing process

Unimodular and multimodular HA conduits were obtained with the same procedure as previously described [100,182] with 1-mm width, an internal cylindrical channel of 400 μm and variable in length. In Figure 16 we show an unimodular conduit of 12 mm length and a multimodular conduit formed by three individual modules of 4 mm length each, in the fully hydrated stage. A

macroscopic view of both conduits in aqueous solution is shown in Figure 16A and 16B. SEM images of longitudinal cuts of the unimodular (Figure 16C) and multimodular (Figure 16D) conduits revealed the lumen of the conduits containing the PLA fibres, laid out parallel to the conduit's axis. In the multimodular concept, the PLA fibres are the common element to all the individual modules forming the multimodular conduit. For this reason, the multimodular conduit is more adaptable to different lengths, covering this length with the required number of individual modules traversed by a bundle of PLA fibres (Figure 16B, 16D, 16E). Modular manufacturing to cover a long length, versus building a single conduit covering that long length, minimizes failures in the manufacturing process. In the same way, the multimodular conduit is more versatile to adapt to different situations, for example to a curvature in which the unimodular conduit could break in some point if the length is long (Figure 16E).

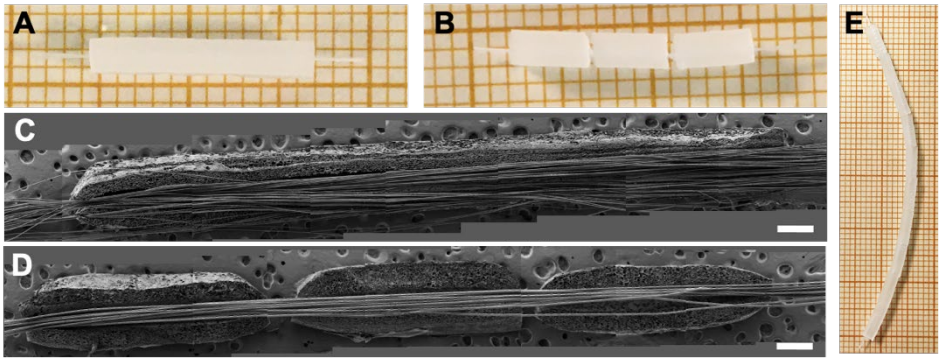

Figure 16. Unimodular and multimodular conduits concept.
Macroscopical view of hydrated (A) unimodular and (B) multimodular conduits. Scanning electron microscope images from a longitudinal cut of (C) unimodular and (D) multimodular conduits, dividing the conduits into two parts (only the part with de PLA fibres is shown). (E) Macroscopical view of hydrated multimodular conduit formed by 10 modules in order to cover a long and curved distance. The distances between the modules in multimodular conduits is exaggerated for better visualization. Scale bar: 500 μm.

3.2. The density of Schwann cells is lower and unevenly distributed in long-unimodular conduit

We seeded rat SC in the lumen of the 18 mm length unimodular conduit and multimodular conduits of three 6 mm length modules and cultured them for 5 days (Figure 17). MTS assay performed after 5 days of SC culture (Figure 17A) shows that the number of cells in unimodular conduits was significantly lower than in the multimodular conduits. A macroscopic view of both conduits after the MTS assay is shown in Figure 17B. The brown colour is due to the reduction of MTS reagent, and we could observe areas with a discontinuity in SC density, indicated with a black dash lines box, explaining the higher cell density in the multimodular conduits.

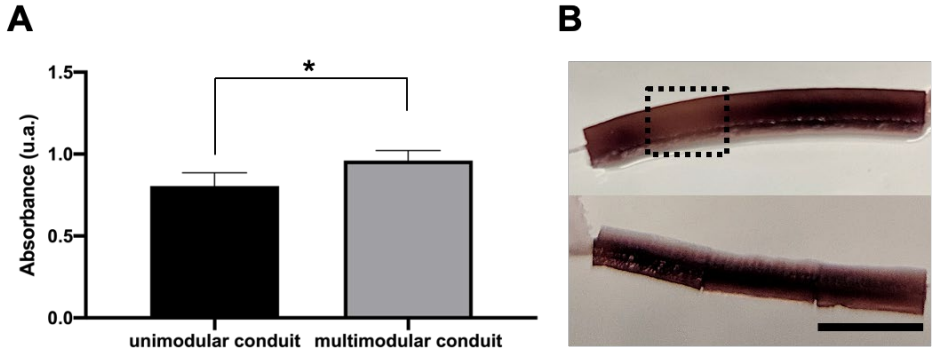

Figure 17. Differences of Schwann cells density between unimodular and multimodular conduits after 5 days.

(A) MTS assay on SC cultured for 5 days inside unimodular and multimodular conduits. In multimodular conduit cell proliferation was increased. An asterisk * indicates statistically significant differences, indicating a p-value below 0.05. (B) Macroscopical view of hydrated (top) unimodular and (bottom) multimodular conduits after MTS assay (revelled with colour brown). Zone without cellular continuity in unimodular conduit is indicated with a black dash lines box. Scale bar: 6 mm.

3.3. Schwann cells grow without discontinuity within the multimodular conduit

Unimodular and multimodular HA conduits were seeded with SC and cultured for 5 days as previously shown. Longitudinal cuts of these samples were analysed with confocal microscopy (Figure 18). Fluorescent reconstructions in Figure 18A, 18F, completely spanning the whole length longitudinally cut of unimodular and multimodular conduits, show nuclear staining and F-actin of SC. As previously described in an HA conduit of 6 mm length [100,182], the SC form a cylindrical sheath-like tapestry continuously spanning to the whole length of the internal lumen and SC attached to and grew on the PLA fibres too, and completely covered them. We could observe that SC growing in the lumen of the unimodular and multimodular conduits with a total length of 18 mm. SC grew on the PLA fibres at the beginning, at 6 mm, at 12 mm and 18 mm in the unimodular sample (Figure 18B, 18C, 18D, 18E). Moreover, SC grew at the beginning and the end of multimodular samples, but also at the inter-module zone between modules 1 and 2 and the inter-module zone between modules 2 and 3 (Figure 18G, 18H, 18I, 18J).

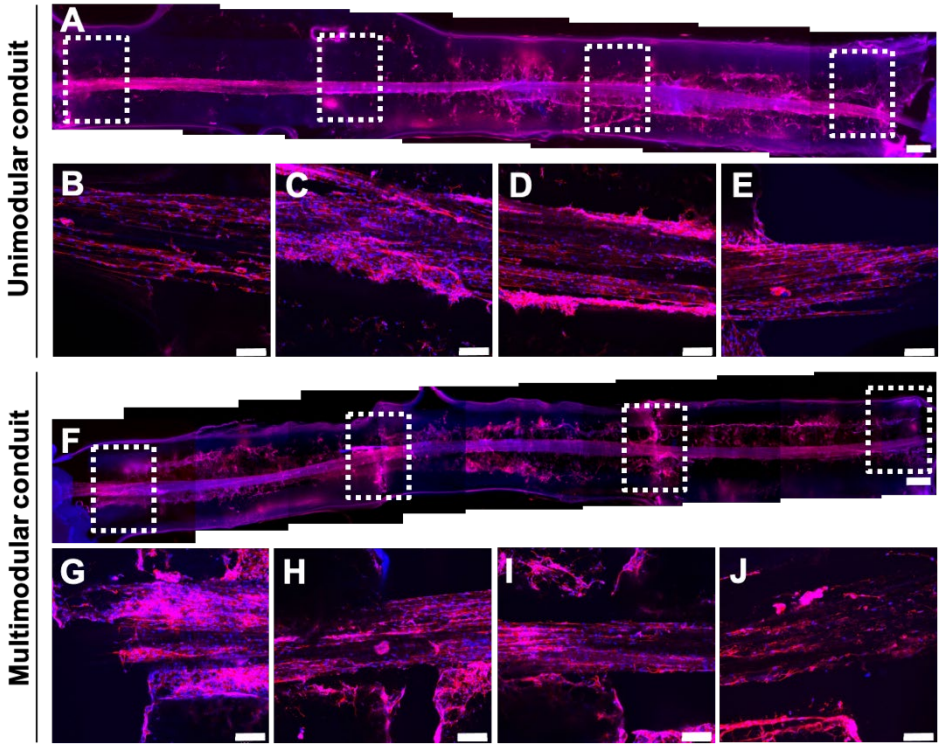

Figure 18. Schwann cells distribution in unimodular and multimodular conduits after 5 days.

Representative fluorescence reconstruction of the conduit's complete length of (A) unimodular and (F) multimodular conduits, after nuclear staining with DAPI (blue) and F-actin of SC staining with ActinRed™ 555 ReadyProbes™ Reagent (red). Confocal fluorescent image of a longitudinal section of unimodular conduit at (B) 0 mm, (C) 6 mm, (D) 12 mm, (E) 18 mm, and multimodular conduit at (G) 0 mm, (H) inter-module zone between modules 1 and 2, (I) inter-module zone between modules 2 and 3, (J) 18 mm, showing continuity in SC growth even in the absence of the HA conduit. Details in A and F are indicated with a white dash lines boxes. Scale bar: 500 μm (A, F), 100 μm (B, C, D, E, G, H, I, J). Figure 18A and 18F are enlarged in the Appendix.

3.4. Axons extend through the multimodular conduit crossing the inter-module zones

The postnatal P3-4 rat DRG explants were cultured for 21 days at one end of the unimodular and multimodular HA conduit with pre-seeded SC cultured for 5 days (Figure 19) in order to study the axonal extension from the DRG explant, by neuronal staining with Tuj 1 (green). The DRG projecting axons fully invade the lumen of the multimodular conduit (Figure 19B), reaching the opposite end of the multimodular 18 mm length conduit, while in the unimodular conduit the maximum length was around 11 mm (Figure 19A). In the multimodular conduit fibres pass through the individual modules, axons extended all through the fibres' length, even without the HA conduit in the inter-module zone between modules 1 and 2 (indicated with m1, m2 in Figure 19C) and the inter-module zone between modules 2 and 3 (indicated m2, m3 in Figure 19D). The number of axons that reached the end of the multimodular conduit was less than at the beginning (Figure 19E).

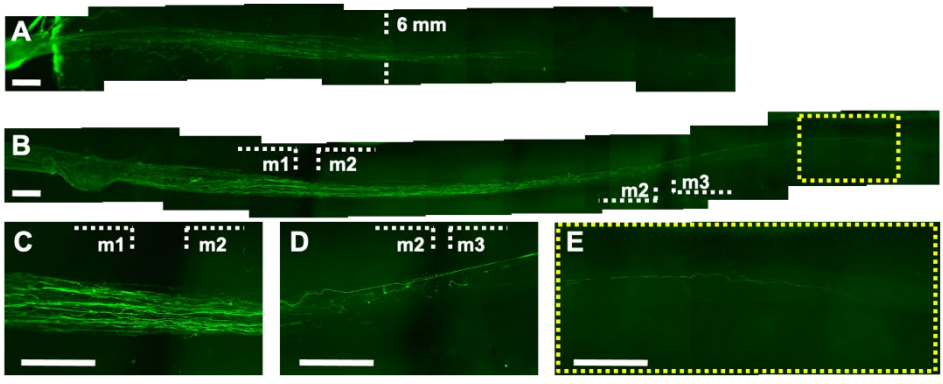

Figure 19. Axon growth in unimodular and multimodular conduits after 21-days dorsal root ganglion explant culture.

Representative fluorescence reconstruction of the (A) unimodular and (B) multimodular conduit's complete length after neuronal staining with Tuj1 (green), showing greater axonal extension of the multimodular conduit. Fluorescent image of a longitudinal section of (C) inter-module zone between modules 1 and 2 (m1, m2), (D) inter-module zone between modules 2 and 3 (m2, m3), (E) final zone in module 3. White dash lines delimit the modules of the multimodular conduit, and yellow dash lines box delimit the final zone of the module 3 (16-17 mm long from the DRG explant). Scale bar: 500 μm. Figure 19A and 19B are enlarged in the Appendix.

3.5. 'Neural cord' extracted from the hyaluronic acid conduit can survive after 5 additional days in culture

Multimodular HA conduits were seeded with SC and cultured for 5 days. Then we eliminate the HA conduit making a longitudinal cut and keep in culture this structure 5 more days. We obtained a 'neural cord' formed by the PLA fibres and the associated SC (Figure 20). Figure 20A shows a scheme of longitudinal and transversal view of the 'neural cord' obtained. Figure 20B and 20B' show the 'neural cord' in culture before fixing. Figure 20C shows a fluorescence reconstruction of the 'neural cord''s complete length, made from a series of fluorescent images of longitudinal sections of a 'neural cord' showing SC F-actin staining, and its detail in Figure 20C'. After the 5 additional days, we could observe that the 'neural cord' was intact after manipulation 5 days before to eliminate the HA conduit and also before fixing it. We could prove the stability against manipulation of the 'neural cord' without altering it with the SEM reconstruction in Figure 20D and it detail in Figure 20D', where we could see a compact 'neural cord'.

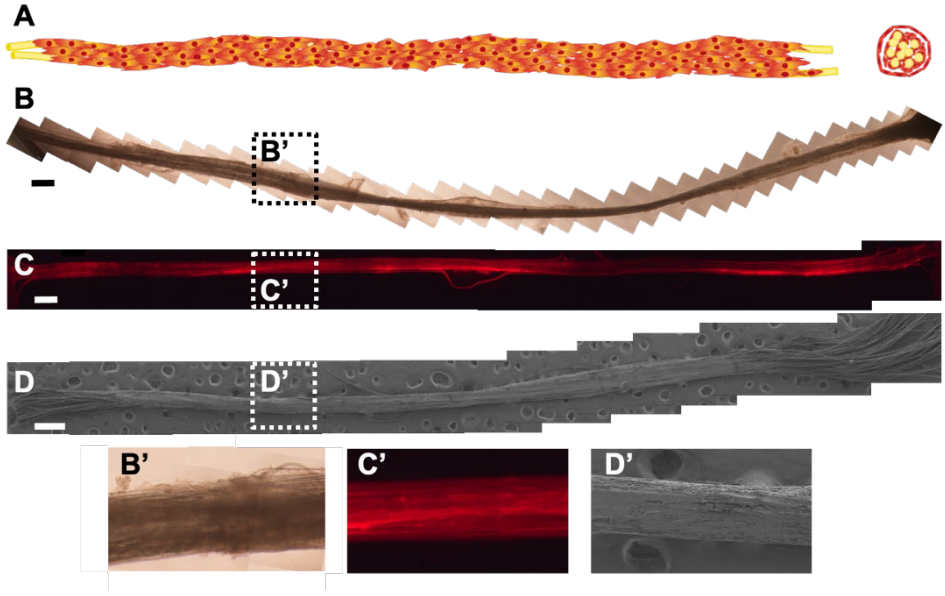

Figure 20. Schwann cells distribution in the 'neural cord' after 5 additional culture days.

(A) Longitudinal and transversal schematic view of 'neural cord'. (B) Representative phase-contrast reconstruction of the 'neural cord''s complete length in culture before fixing. (C) Representative fluorescence reconstruction of the 'neural cord''s complete length after F-actin of SC staining with ActinRed™ 555 ReadyProbes™ Reagent (red). (D) Representative SEM reconstruction of the 'neural cord''s complete length, showing a compact structure after the manipulation of the sample. Details in reconstructions are indicated with dash lines boxes in B', C' and D'. Scale bar: 500 μm. Figure 20A, 20B and 20C are enlarged in the Appendix.

3.6. The 'Schwann cell sheath' is incorporated into the 'neural cord' in the 5 additional days of culture

Figure 21A and 21B show SC associated with PLA fibres in multimodular conduit after culture for 5 and 10 days, respectively. The multimodular conduits were cut longitudinally to obtain the internal view (Figure 21A and 21B). Figure 21C and 21D show the 'neural cord' after 5 additional days in culture after eliminating the HA conduit. In Figure 21A and 21B, we could observe a double structure formed by SC, SC growing on PLA fibres and covering them, and the 'SC sheath' detached from the internal lumen and starting to attach to the SC-PLA fibres structure. In Figure 21C and 21D we could not distinguish these two different cellular structures. On the contrary, this 'neural cord' shows a more abundant and compact SC structure in comparison with multimodular conduits after 5 and 10 days of culture. The transversal dimension of the 'neural cord' (Figure 21C, 21D) rage between 237 μm in the thinnest areas and 285 μm in the thickest areas in comparison with 202 μm and 250 μm for the structure after 5 days of culture (Figure 21A) and 178 μm and 273 μm after 10 days of culture (Figure 21B).

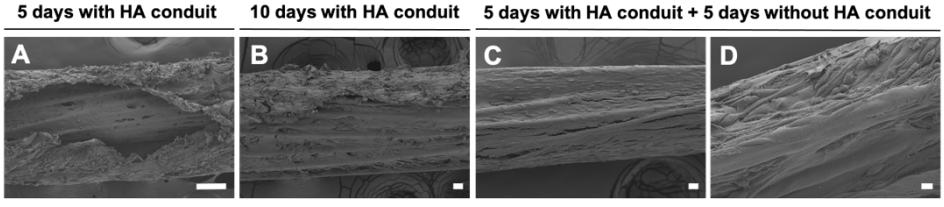

Figure 21. Schwann cells distribution in multimodular conduits and 'neural cord'. Scanning electron microscopic image from a longitudinally cut of the SC on PLA fibres after (A) 5-days culture and (B) 10-days culture in multimodular conduits. (C, D) Scanning

electron microscopic image from a longitudinally cut of the 'neural cord' formed after 5-days culture without the HA conduit. Scale bar: 100 µm (A), 20 µm (B, C), 10 µm (D).

3.7. Axons extend through the 'neural cord' after a short-term co-culture with dorsal root ganglion explants

Preliminary results of the 'neural cord' with a DRG explant positioned at one end seem to confirm that it is indeed possible to grow and guide the axons through it (Figure 22). We have been able to observe the axonal extension with DRG explants for a total 14 days (10 days with the HA conduits and 4 days without the HA conduits). We maintained the HA conduits for 10 days to ensure the adhesion and growth inside the lumen of the conduits of DRG explants. We performed a short-term culture as a proof of concept to study the axonal extension. The distance covered by the axons was around 4 mm long.

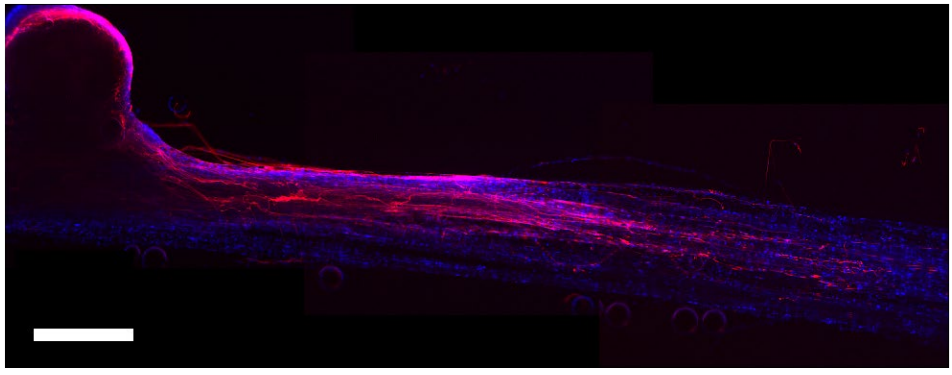

Figure 22. Axon growth in 'neural cord' after short-term co-culture with dorsal root ganglion explant culture.
Representative confocal reconstruction of the first 4-5 mm of longitudinal sections of the 'neural cord' showing nuclear staining with DAPI (blue) and neuronal staining with Tuj 1 (red) after 10 days with Ha conduits and 4 additional days without the HA conduits. Scale bar: 500 µm.

4. Discussion

Biohybrid approaches seem to have better results in terms of functional recovery in comparison with purely material-based constructs [208]. Besides SC have demonstrated to enhance axonal growth *in vitro* [209], support the recovery of spinal cord function [210] and regenerate nerves in combination with implants [211,212].

In this work, we propose a multimodular concept to create guided nerve conduits of great length to address the large lesions of the axonal tracts of the nervous system. In previous studies [100,182] we have verified that the porous HA conduits are capable of containing SC inside them, which form a 'SC sheath' covering the entire lumen. Furthermore, these SC grow on the PLA fibres along them, in a guided manner. The combined use of PLA fibres and SC recapitulate the directional characteristics of the *in vivo* physiological situation produced during neural regeneration. We have also verified that this biohybrid construct is capable of supporting the axonal growth of axons from a DRG positioned at one end of the conduit. These axons grow unidirectionally and reach the end of the 6 mm conduits. However, in a real clinical situation when a neural lesion occurs, the lesions are usually larger and the gap to be regenerated is often critical. Frequently, it is not possible to regenerate lesions <10-12 mm [201], although it is increasingly common to find promising results in longer lesions, even at critical distances of 14-15 mm [76,202]. Moreover, lesions produced in CNS such as SCI or TBI lead to the loss of guidance through large distance [11]. Due to the limitations and our previous experience, we have developed a guided nerve conduit composed of several individual modules arranged one after the other with a bundle of PLA fibres as a common element to all of them (Figure 16). In this way the construct can be adapted to different lengths (Figure 16E) in a versatile and simple way.

The multimodular conduit presents the main advantage compared to a single conduit of the same length: failures in the manufacturing process of the conduits are avoided or reduced, which are more significant when the conduit becomes longer and longer. The multimodular concept also has additional advantages when cells are seeded inside. We have verified that there is a higher cell density in the multimodular conduit than in the unimodular conduit (Figure 17A) and the SC growth is not continuous throughout the lumen of the unimodular conduit (Figure 17B). This fact can be explained by the way SC are seeded in the interior.

In the case of the unimodular conduit, the pipette can only be inserted through two points of the conduit, the two ends, and during the 5 days of culture, the SC cannot self-organize to completely cover the entire interior of the conduit, since the supply of nutrients will also be decimated in the central area of the lumen, farthest from the open ends of the unimodular conduit. However, this situation is different in the multimodular conduit. In this case, by seeding each individual module, the SC can cover the entire lumen of each 6 mm individual modules. Besides, the modules are kept as close as possible thanks to fixing structures at the two ends of the multimodular conduit, with the advantage of a non-hermetic inter-modular zone, allowing a better exchange of nutrients and resulting in a higher accumulation of SC in these areas (Figure 18H-18I). It has been reported that increased permeability improves axonal regeneration [213], even being an option to eliminate the conduit cover and obtain promising results in axonal regeneration [205,206].

The multimodular conduit, in addition to being able to contain a well-organized SC structure inside, has been shown to support axonal growth practically until the end of the 18 mm multimodular conduit (Figure 19B). Human axons growth during peripheral nerve regeneration have a rate of approximately 1 mm/day [189], and this rate is significantly lower in the CNS [190,214]. DRG axons grow *in vitro* with rates between 130 and 300 μm/day [191]. In our present study, we can estimate the axonal grow rate *in vitro* for the multimodular conduit of approximately 600-700 μm/day, a value obtained by dividing the maximum length reached at day 21 by axons through that time. Moreover, the most effective implantable medical devices for functional regeneration currently do not exceed rates of 0.5-1 mm/day [192]. The axons from the DRG seeded at one end of the multimodular conduit, after 21 days, extended along the 3 individual modules, even in the two inter-module zones without the protection of the HA. Moreover, axons reached the last individual module, although in fewer numbers than in the initial area of the construct. Furthermore, this absence of the HA conduit in the inter-module zone does not prevent SC from growing on the PLA fibres in a continuous and unidirectional manner (Figure 18F-18J). In this way, the axons, which grow accompanied by the SC attached to the PLA fibres, can grow through the entire length and inter-module areas, positioning the modular concept as a perfectly valid approximation for axonal growth. This fact, added to the previously mentioned advantages of the modular concept, makes HA modular conduits with

PLA fibres and pre-seeded with SC an option for the regeneration of long lesions of the nervous system.

Besides, the structure that is formed within the multimodular conduit, composed of the PLA fibres and the pre-seeded SC, can lack these HA conduits. The significant compaction of the cells in these PLA fibres (Figures 20 and 21) makes the 'neural cord' have consistency and integrity on its own and can be manipulated without compromising the stability of the structure. SC survive after removing HA conduits with a scalpel and can remain alone in culture (Figure 20B). Moreover, preliminary results in a short-term co-culture with DRG explants showed that the distance covered by the axons was around 4 mm long. For this reason, it is not unreasonable to think that in a long-term co-culture of SC with DRG explants experiment, the 'neural cord' will be able to support a more significant axonal extension (Figure 22). The short axonal growth, in this case, is probably because of the process of eliminating the HA conduits was too aggressive because it was not optimized as it was a proof of concept. For this reason, axons could be damaged in the process. Therefore, these results are not conclusive regarding the support of axonal growth.

The generation of this 'neural cord' makes it possible to develop directly transplantable structures without conduit, being more accessible the SC and the neurotrophic factors they release [20] and thus guide the axonal regrowth across the regenerating site [143,215]. In the case of peripheral nerve regeneration, the 'neural cord' could be transplanted employing the microsurgical epineurial sheath tube technique [143]. In nerves and spinal cord, it could be sutured directly to the stumps [205] or introduced the 'neural cord' in other nerve guidance conduit. In brain injuries, the 'neural cord' could be directly injected into the brain using a thin-walled needle, or inside a different nerve guidance conduit or encasement [123]. In the case of employing a new encasement for the 'neural cord', the encasement could be functionalized with bioactive molecules in order to support nerve regeneration over longer gaps and/or deal with a possible inflammatory response [140].

Concluding, in this work, it has been possible to construct neural communication structures of long length (18 mm) to reconnect the CNS and/or PNS by modular assembly from elementary modules of shorter length in which neuronal precursors and support cells have been cultured, making possible a new perspective for the approach of long tissue defects in the nervous system.

5. Conclusions

From the results obtained in this second chapter it can be concluded that:

1. The idea of the biohybrid formed by an HA conduit with a bundle of PLA fibres inside and the pre-seeded SC can be adapted to both a longer unimodular concept and a multimodular concept.
2. A multimodular biohybrid can be developed due to the PLA fibres that are the common element to all the elemental modules that make up the modular biohybrid.
3. SC are viable and proliferate within the lumen of unimodular and multimodular conduits. Moreover, SC adhere and grow on PLA fibres even in inter-module areas of the multimodular conduit.
4. SC proliferate and are distributed more effectively in the multimodular conduit than in the long unimodular conduit.
5. The multimodular biohybrid could promote directed axonal extension all through the fibres' length, even in the inter-module zone between modules of the multimodular conduit.
6. SC together with PLA fibres generate a ‘neural cord’ which is a viable and easy to handle structure spanning the interior of the multimodular biohybrid, after removing the HA conduits.

Discussion

The regeneration of the nervous system raises a multitude of challenges that, as mentioned previously, have been tackled in very different ways.

The situation of a PNI is much less desperate, considering the more favourable regenerative capacity of the PNS [17]. Besides, FDA-approved devices for the treatment of PNI exist in clinical practice and are often successful [125]. However, they are devices indicated for the treatment of short injuries, and many times the injuries found in the clinic exceed these limits, complicating the regenerative process and the quality of life of patients. To solve this problems, many researchers have been developing alternative tissue engineering strategies capable of achieving tissue recovery over long distances, leading to different biomimetic designs with the tissue to be regenerated [74,148].

For the development of these tissue engineering approaches, the choice of materials to use is essential. They must be soft and flexible to prevent compression of the growing axons and limit the inflammatory response. They must also be biodegradable and biocompatible and prevent the development of fibrous tissue at the injury site [129]. To this end, in this Doctoral Thesis HA has been chosen, which meet all the qualities to fulfil the regenerative process in the nervous system [91,129]. In order to improve its mechanical properties and rapid

biodegradability, HA has been crosslinked with DVS. In this way, using the appropriate moulds and technique, we have been able to obtain porous tubular conduits with a hollow interior. Furthermore, there are already devices on the market made of HA crosslinked with DVS approved by the FDA [102], therefore, it would not be necessary further clinical trials to guarantee our material safety [102].

We have opted for developing NGCs taking into account that this architecture is the most appropriate for PNI, but also considering that it could also be applied in injuries or diseases of the CNS such as SCI or the disruption of specific axonal pathways in the brain. NGCs have a multitude of advantages because, thanks to their tubular structure, they serve as a physical guide to direct the growing axons, contain the growth factors secreted at the injury site, and reduce scar tissue infiltration [120].

As shown in detail in *Chapter I*, the chosen material, HA, is perfectly compatible with the growth and proliferation of the supporting cells selected for this work, the SC. As seen in previous studies of the group where this Doctoral Thesis has been developed [100][183], the SC formed a 'SC sheath' that supposes a continuous structure that covers the lumen of the conduit but with weak cell adhesion to its surface. This situation is due to the high hydrophilicity nature of HA. In a two-dimension situation it might have been detrimental to the adhesion and retention of the SC but, in this case, the tubular three-dimensional conformation confines the cells in a relatively small and curved space, resulting in a very interesting architecture for the generation of this 'SC sheath'. In *Chapter II* there is a change regarding obtaining the 'SC sheath'. While in *Chapter I* 100,000 SC are seeded in a 6-mm HA conduit and kept in culture for 10 days, in *Chapter II*, we opted to double the seeding cell density (200,000 cells in a 6-mm HA conduit) for 5 days of culture. In a previous study, we verified that the resulting 'SC sheath' was comparable to that produced with the conditions of *Chapter I* and we decided to carry out this change in order to reduce the SC culture times.

Another material used in this Doctoral Thesis is PLA in fibre format. PLA has many advantages, such as its mechanical properties and biodegradability [110], and it is a material that is also approved for clinical use by the FDA. In this work, PLA fibres have been chosen as a guide element. It is known that the introduction of elements that favour guidance has advantages to guide and direct axons growth inside the NGC towards the tissue behind it [121]. In this case, the PLA

shows excellent compatibility with the adhesion and growth of the SC, which also grow elongated and guided in the same direction as the fibres. Moreover, the introduction of the PLA fibres in the HA conduits does not alter the formation of the 'SC sheath'. So, the PLA fibres are not only a physical guide for the axons but also for the pre-seeded SC, which accompany and guide the axons during their extension (*Chapters I and II*).

The topography, hardness and other physical parameters of the environment in which cells grow are of great importance [216], as well as bioactive molecules that act as chemotactic stimuli [217]. All of this can influence different cell behaviour, such as growth and directionality. PLA fibres have been shown to promote SC adhesion. This property of PLA fibres allows the adsorption of ECM proteins, and this causes a better adhesion of SC via integrins, generating focal adhesions that promote cell survival. Integrins directly activate survival pathways via the phosphoinositide 3-kinase and mitogen-activated protein kinase pathways [218]. Moreover, the fact that PLA is adherent favours cell migration compared to other substrates [219].

Furthermore, PLA fibres are a physical support for SC, colonizing the construct more efficiently. Furthermore, this association of SC attached in an elongated fashion onto the PLA fibres recapitulates microarchitectures such as Bügner's bands formed by SC strands that selectively guide growing axons containing growth-promoting molecules like glycoproteins, laminin and fibronectin [20]. The strategy of creating tubular implants with artificial Bügner bands is something that has been explored previously [199].

In *Chapter I*, the PLA fibres used are 30 μm in diameter. This same construct as that described in *Chapter I* (HA conduit with 30 μm diameter PLA fibres) was tested in an approach for ependymal cell transplantation in the spinal cord during this Doctoral Thesis development [149]. It was in these in vivo experiments that Dr Victoria Moreno Manzano from the Neuronal and Tissue Regeneration Laboratory in Centro de Investigación Príncipe Felipe warned that perhaps the fibres were too stiff for the spinal cord tissue and could damage it. That was the reason why we switched to the use of 10 μm diameter PLA fibres (*Chapter II*). Associated with *Chapter II*, an in vivo experiment for PNI has been carried out, which will be discussed later in *Work in progress and future*. We used PLA fibres of 10 μm, reducing the stiffness of the fibres without altering their guidance

function, as can be seen in multimodular experiments and the 'neural cord' (*Chapter II*). Furthermore, in vitro studies of guidance cues on curved surfaces have shown that in small diameters such as 10 μm and 30 μm, guided axonal growth occurs in the same way, which does not occur with larger diameter fibres (100-300 μm) where the preferential growth along the long axis decreases and the axons grow in all directions [220,221].

Another key element of the biohybrid proposed in this Doctoral Thesis, together with PLA fibres, are SC. SC play an essential role in neuroregeneration and protection of the PNS [172]. They are also a pump for neurotrophic and angiogenic factors, in addition to providing the surface proteins that intervene in the maintenance of the normal function of the nervous system and the activation of the immune response after damage [20]. Besides, they have not only been used in regeneration strategies of the PNS [174,175] but also in the CNS [176,177]. Also, they are being used as an autologous cell transplant to treat chronic spinal cord injuries within the Miami Project [168]. In this work, SC are useful not only as a source of factors but also, as they are pre-seeded and aligned with the PLA fibres, as a guide for the axons of the projecting neurons of the DRG explants. The 'SC sheath' will secrete ECM and bioactive factors (like BDNF, NGF, NT-3, GDNF and VEGF) that will concentrate in this space, thanks to the HA conduit architecture, and promote axonal extension.

Moreover, SC aligned in the PLA fibres could intervene in the extension of the growth cone of the axon pathfinding, which is responsible both for the neuronal circuitry development in the brain [222], and for the regrowth of damage axonal tracts after an injury or disease [223]. Growth cone behaviour such as advancing, retracting, turning, and branching is regulated by the structural reorganization of the F-actin and microtubules. This structural reorganization modifies different intracellular pathways that results in the formation of focal adhesions in the growth cone during axonal extension [224] due to the recognition of cell external adhesion molecules expressed by glial cells such as SC [193] (Figure 23).

Figure 23 can serve as a speculative model of the biohybrid proposed to assist neural regeneration in axonal tracts, taking into account everything discussed above. In brief, the external conduit hinders invasion of surrounding microglia, reactive astrocytes and other cells. Simultaneously, the PLA fibres and SC create a bridge between the proximal and distal damaged areas. SC contribute with an

ECM and trophic factors and appear like bands of Büngner. Finally, axons from the proximal area, through their growth cones, regenerate and extend along this pro-regenerative bridge to the distal area. At last, myelinated nerve fibres will be developed to complete nerve regeneration and functional restoration. This last phenomenon has not been deeply investigated to date (in vivo experiment for PNI has been carried out, which will be discussed later in *Work in progress and future*, but the results are preliminary).

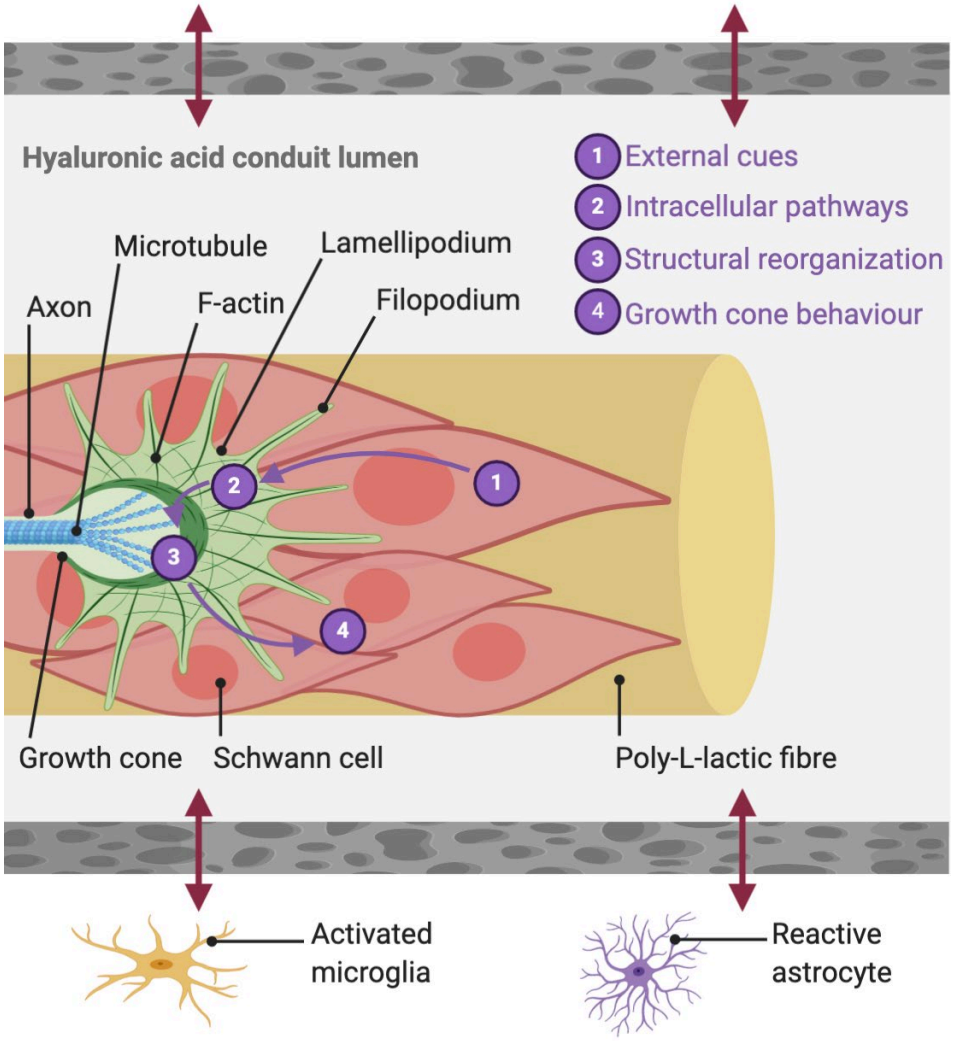

Figure 23. Speculative scheme of the biohybrid here proposed in the enhancement of neural regeneration in axonal tracts.

The external HA conduit is a barrier for microglia, reactive astrocytes and other cells but permits the exchange of nutrients and oxygen. Moreover, HA conduit provides adequate support for the SC and the bundle of PLA fibres in its lumen. Only one representative PLA fibre is shown in the scheme. SC proliferate inside the lumen of the HA conduit and also on PLA fibres acquiring elongated shapes oriented in the direction of the PLA fibre axis. Chemotactic stimuli secreted by the SC within the HA conduit together with the presence of the PLA fibres made axons sprout from the DRG explant, extend along the fibres. So, (1) external cues result in (2) activation of intracellular signalling pathways, which in turn (3) cause alterations in cytoskeletal structures (actin filaments in green, microtubules in blue) and membrane dynamics. Finally, these effects (4) cause changes in growth cone behaviours. Figure created in BioRender.com.

In practice, SC can be isolated from patient nervous tissue, cultured and expanded *in vitro*, and then incorporated into NGCs [138]. In fact, transplant autologous human SC is used in the clinic to treat chronic spinal cord injuries within the Miami Project [168]. It is true that on certain occasions, especially for long-gap lesions, the use of differentiated SC is unsatisfactory [225]. Renewable and lineage-restricted SC precursors are considered highly desirable and promising cell sources for the production of SC to obtain large amounts of SC but are extremely limited. Several cell types are capable of differentiating to SC, such as human pluripotent stem cells [226,227] and multipotent stem cells [228–230]. To date, human pluripotent stem cells with high expansion and differentiation capacity are considered the ideal renewable sources to generate high numbers of SC. However, their broader uses are still limited by long differentiation time and low functionality [226,231].

Therefore, we think that the proposed biohybrid is an option not only for PNI but also for axonal tracts of the CNS. However, in clinical reality, lesions of the nervous system often derive in long distances damages. The initial approach was to extend the HA conduit as much as possible to cover bigger lengths, but in practice, this supposes several problems such as failures in the manufacturing process. The more we tried to lengthen the conduits, the more problems we encountered related to keeping the PCL fibre centred inside the grooves of the polytetrafluoroethylene mould or the cracks at some point in the conduit after lyophilisation. Regarding cells, it could be challenging to get a homogeneous cellular distribution inside the conduits, and nutrient supply to the cells in the central zones is complicated. So, a modular solution seemed a better approach.

In this sense, manufacturing problems do not exist since we continue to maintain the length of the individual conduit or modules at a relatively short distance. Moreover, as there is space between the inter-module areas, the exchange of nutrients and oxygen is guaranteed, as well as the contribution of factors between the lumen and the interstitial medium (Figure 18 and 19).

Furthermore, the modular concept has proven to be successful *in vitro*. The presence of PLA fibres as a common element to all the individual modules allows that, as we have seen in *Chapter I*, SC can adhere and grow in the same direction as the fibres. In this way, the growth of SC is continuous despite the presence of inter-module zones, and it is thanks to this that axons can extend to the end of the multimodular conduit accompanied by SC and in the same direction as the PLA fibres. Therefore, the same speculative model that we previously proposed for the 6-mm biohybrid (Figure 22) could also be applied to the multimodular conduit.

For these reasons, the modular concept could be thought of as a tailor-made biohybrid, as long as required by adding more or fewer individual modules. However, and although an *in vivo* test has been carried out with this concept, it is only possible to obtain preliminary conclusions about the success of this approach in PNI.

Furthermore, two additional interesting approaches are derived from the proposed biohybrid.

Since the HA conduit with the PLA fibres and the pre-seeded SC recapitulate the directional features of axonal bundles in the nervous system, it could be established as an experimental model to check axonal growth *in vitro* within the biohybrid, simulating the *in vivo* situation of directed axonal growth in axonal tracts originating from one neuronal population. In this work, a DRG explant has been used as a localized source of neurons. However, other different neuronal sources could be tested [232] or even using brain organoids [233].

The other approach is the named ‘neural cord’, as a transplantable structure without HA conduit, being more accessible the SC and the neurotrophic factors they release, and thus guide the axonal regrowth across the regenerating site (Figure 20 and 21). The ‘neural cord’ could be implanted directly [143,205] or even introduced into another different tubular conduit [123] which in turn could be functionalized with molecules or drugs of interest for neural tissue recovery.

In a proof of concept with a DRG explant positioned at one end, we confirmed the axonal growth (Figure 22). However, due to technical problems in the handling of the samples and the lack of time, we still do not have conclusive results in a long-term co-culture. Nevertheless, since the elements that make up the 'neural cord' are common to those present in the multimodular biohybrid, it is not unreasonable to think that it would result in a promising solution for neural regeneration.

In summary, we obtain a tubular conduit of HA with PLA fibres in its interior that promotes proliferation of SC inside the entire conduit, allowing a better axonal growth, and constitutes a structure that favours the axonal regeneration. Moreover, we have developed neural communication structures of long length by modular concept, and a structure named 'neural cord' as a directly transplantable construct.

Concluding, during the development of this Doctoral Thesis, we made the first step forward developing an *ex vivo* neural tract. In our approach, we manage to demonstrate a proof of concept about the possibility of developing an in vitro neural tract-like structure combining the use of tubular scaffold together with fibres as a guide and support cells. Even though the functionality of the tubular scaffold is essential in the first step of the development of the neural tract-like conduct since it promotes the arrangement of supportive cells as a 'SC sheath', we also hold the possibility to remove it obtaining just a 'neural cord' giving us the chance to replace it by another cover or even using it directly for regenerative therapies. It is noteworthy that, although our results are promising, these are preliminary steps toward the final goal, which is the creation of an artificial neural tract outside the body to use it as a transplantable structure. This goal also comprises the multicellular composition and the proper arrangement of these cells to better fit to the particular features of each particular neural tract.

Conclusions

After the experimental evidence shown previously concerning the development of a biohybrid for the regeneration of axon bundles or axons tracts, we can highlight the following general conclusions:

1. The biohybrid system formed by the HA conduit and the PLA fibres inside it together with the pre-seeded SC constitutes a structure that favours axonal regeneration at different lengths showing tight interactions between axons and SC.
2. The presence of SC together with the PLA fibres is imperative to achieve efficient axonal regeneration. The effect of the combination of these two factors is multiplicative since, in the presence of PLA fibres inside the lumen of the HA conduit with pre-seeded SC, the axons elongate longer than in either of the two systems alone.

3. The modular concept is a viable strategy to address the regeneration of long-distance axonal tracts since it combined the beneficial features of each individual module with the continuity and directionality of the fibres as a common element throughout the entire multimodule conduit.
4. After removing the HA conduit and performing an additional culture, a neural cord constituted by the PLA fibres and the SC growing in them is obtained. This structure is compact and manipulable and survives in the absence of the HA conduit constituting an alternative approach to the whole structure for regenerative therapies.

Work in progress and future work

The biomaterials developed in this Doctoral Thesis were designed initially based on the dimensions of the rat nigrostriatal tract. However, it is possible to introduce some modifications in the synthesis procedure for varying the shape and dimensions of the conduits. In this way, the conduits can be adapted to different uses, without causing them to lose their properties.

To verify the efficacy of the modular concept in the regeneration of tracts of the nervous system, a *in vivo* experiment has been performed using a model of sciatic nerve injury in rats. This work was done in collaboration with the group of Dr Victoria Moreno Manzano from the Neuronal and Tissue Regeneration Laboratory in Centro de Investigación Príncipe Felipe. The biohybrid had to be adapted to the dimensions of the rat's sciatic nerve (Figure 24D). Moulds with polytetrafluoroethylene channels (Figure 24A) have been used as in *Chapters I and II*, but in this case, their section is cylindrical, and the internal diameter of the lumen is 1 mm instead of 400 μm as in *Chapters I and II*. Furthermore, the bundle of fibres has been adapted to a cylindrical shape. In this way, the fibres of 10 μm of PLA are kept parallel in a kind of hollow cylinder thanks to two adhesive rings

that hold it together at the ends (Figure 24B). This cylinder of PLA fibres is inserted into the HA conduit and is attached to the ends of the conduit by perfectly matching the dimensions of the internal diameter of the conduit. Thus, the adhesive rings protrude at the ends to facilitate suturing in surgery (Figure 24C).

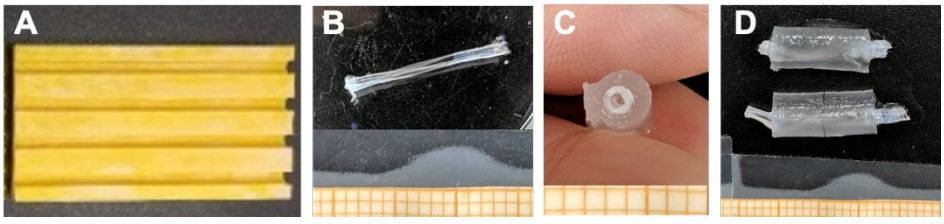

Figure 24. Adaptation of hyaluronic acid channels with poly-l-lactic acid fibres to a rat sciatic nerve injury model.
Macroscopic view of (A) the mould used for the fabrications of HA conduits, (B) the hollow cylinder of parallel 10 μm PLA fibres, (C) the cylinder of PLA fibres inside the HA conduit, and (D) the two kinds of constructs used for the *in vivo* experiment: 12 mm long conduits and 12 mm long modular conduits (two elementary modules of 6 mm long each).

In this experiment, conduits 12 mm long were used as a control group. In addition, two groups were tested: 12 mm long conduits with pre-seeded SC (biohybrid group) and 12 mm long modular conduits (two elementary modules of 6 mm long each) with pre-seeded SC (modular biohybrid group). In all groups, 450.000 SC were pre-seeded for 10 days in SC medium. Then the surgeries were carried out. The sciatic nerve of Sprague Dawley rats was sectioned, a portion of it was removed, and the different conduits were grafted. The distal and proximal stump were respectively sutured to the ends of the constructs in the ring that holds the PLA fibres together and protrudes from the HA conduits (Figure 25).

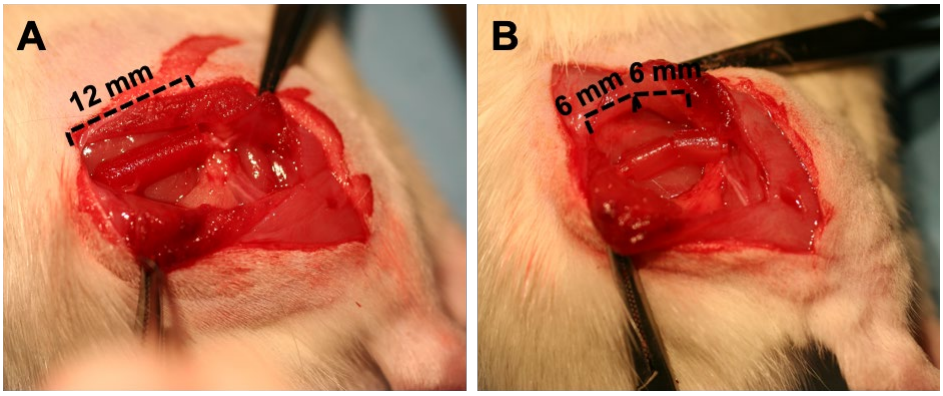

Figure 25. Implantation of biohybrids in a rat sciatic nerve injury model.
(A) Biohybrid conduit and (B) modular biohybrid conduit after suturing the distal and proximal ends of the nerve to the construct.

The rats were sacrificed at 8 weeks. To date, preliminary histochemical assays of the samples have been carried out. The samples were embedded in paraffin, and 5 um thick sections were cut. The sections were stained with hematoxylin-eosin (HE) and with a modification of the luxol fast blue stain that incorporates picric acid (LFBP) [234] and allows to differentiate myelinated nerve fibres (in blue), unmyelinated (greyish), and collagen (in red). In the three groups, a well-structured newly formed tissue has been observed thanks to the HE staining (Figure 26A, 26B, 26C). LFBP staining in the control group showed that most of the newly formed fibres correspond to collagen fibres (Figure 26D). On the other hand, both in the biohybrid and modular biohybrid group, myelinic nerve fibres are predominant (Figure 26E, 26F).

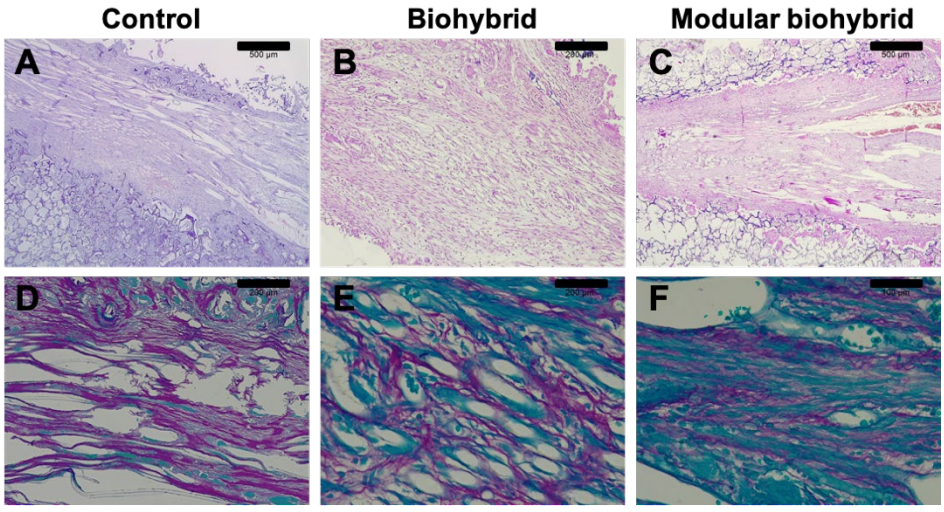

Figure 26. Preliminary histochemical assays after 8 weeks of implantation.
Bright field photograph of the (A) control, (B) biohybrid, and (C) modular biohybrid groups after H-E staining. Bright field photograph of the (D) control, (E) biohybrid, and (F) modular biohybrid groups after LFBP staining. Scale bar: 500 μm (A, C), 200 μm (B, D, E), 100 μm (F).

However, since the results are still preliminary, and there are still experiments to be concluded, no definitive conclusions can be drawn in favour of the modular concept.

On a separate issue, experiments are being carried out to draw definitive conclusions about the support of axonal extension in the 'neural cord'. Due to lack of time, it has not been possible to perform a long-term co-culture with DRG explants (Figure 22) that allow us to affirm that the axons coming from the projecting neurons of the DRG explant reach the end of the 'neural cord'.

Contributions

The work realised along this Doctoral Thesis has resulted in a large number of results. These results were diffused through several scientific and technical publications listed following.

Publications in scientific journals:

1. LAURA RODRIGUEZ DOBLADO; Cristina Martínez Ramos; José Manuel García Verdugo; Victoria Moreno-Manzano; Manuel Monleón Pradas. Engineered axon tracts within tubular biohybrid scaffolds. Acta Biomaterialia. 2020. ISSN 1742-7061. Under review.
2. Cristina Martínez Ramos; LAURA RODRIGUEZ DOBLADO; Eric Mocholi; Ana Alastrue-Agudo; Marina Petidier; Esther Giraldo Reboloso; Manuel Monleón Pradas; Victoria Moreno Manzano. Biohybrids for spinal cord injury repair. Journal of Tissue Engineering and Regenerative Medicine. 13, pp. 509 - 521. 2019. ISSN 1932-6254. DOI: 10.1002/term.2816

National and international conferences:

1. Title of the work: Guided axonal regeneration into hyaluronic acid conduits

   Name of the conference: 5th International Spinal Cord Repair Meeting (ISCORE 2019)

   City of event: Barcelona, España

   Date of event: 14/12/2019

   LAURA RODRIGUEZ DOBLADO; Cristina Martínez Ramos; Victoria Moreno-Manzano; José Manuel García Verdugo; Jorge Más Estellés; Manuel Monleón Pradas
2. Title of the work: Biohybrids for cell transplantation in spinal cord injury

Name of the conference: XLI Congreso de la Sociedad Ibérica de Biomecánica y Biomateriales (SIBB 2018)

City of event: Madrid, España

Date of event: 19/10/2018

LAURA RODRIGUEZ DOBLADO; Cristina Martínez Ramos; Eric López Mocholi; Ana Alastrue; Esther Giraldo Reboloso; Victoria Moreno-Manzano; Manuel Monleón Pradas

3. Title of the work: Electrical stimulation increases Schwann cells proliferation inside hyaluronic acid conduits

   Name of the conference: 1st EMF-Med World Conference on Biomedical Applications of Electromagnetic Fields (EMF-Med 2018)

   City of event: Split, Croatia

   Date of event: 13/09/2018

   Cristina Martínez Ramos; María José Morillo Bargues; Fernando Gisbert Roca; LAURA RODRIGUEZ DOBLADO; Tomás García Sánchez; Luis Mir; Esther Giraldo Reboloso; Victoria Moreno Manzano; Manuel Monleón Pradas

4. Title of the work: Hyaluronic acid conduits filled with poly-lactic fibres and ependymal progenitor cells as a new biohybrid concept for cell transplantation in spinal cord injury

   Name of the conference: 11th FENS Forum of Neuroscience

   City of event: Berlin, Germany

   Date of event: 11/07/2018

   Victoria Moreno-Manzano; Cristina Martínez Ramos; Ana Alastrue; LAURA RODRIGUEZ DOBLADO; Eric López Mocholi; Maravillas Mellado; P. Izquierdo; Esther Giraldo Reboloso; Manuel Monleón Pradas

5. Title of the work: Regeneration of long nerve tracts using biofunctionalized biomaterials. Qualitative studies

   Name of the conference: 11th FENS Forum of Neuroscience

City of event: Berlin, Germany

Date of event: 11/07/2018

M.A. Guedan Duran; N. Jemni Damer; J. Sanz Ortega; Y. Owen; M.D. Gimeno Garcia-Andrade; C. Largo Aramburu; I. Orueta Zenarruzabeitia; M. Minguet Rojas; R. Pelissier; A. Murciano; C. Sanchez Ramos; L. RODRIGUEZ DOBLADO; M. J. Morillo Bargues; C. Martínez Ramos; M. Monleón Pradas; F. Panetsos

6. Title of the work: A hyaluronic acid-based nerve guide conduit and electrical stimulation as combined strategies for neuroregeneration

   Name of the conference: II Congreso Nacional de Jóvenes Investigadores en Biomedicina. IV Congreso de Biomedicina Predocs Valencia

   City of event: Valencia, España

   Date of event: 24/11/2017

   María José Morillo Bargues; LAURA RODRIGUEZ DOBLADO; Tomás García Sánchez; Jorge Más Estellés; Cristina Martínez Ramos; Manuel Monleón Pradas

7. Title of the work: Hyaluronic acid conduits for neural tract regeneration

   Name of the conference: II Congreso Nacional de Jóvenes Investigadores en Biomedicina. IV Congreso de Biomedicina Predocs Valencia

   City of event: Valencia, España

   Date of event: 24/11/2017

   LAURA RODRIGUEZ DOBLADO; María José Morillo Bargues; Cristina Martínez Ramos; Manuel Monleón Pradas

8. Title of the work: Hyaluronic acid conduits filled with poly-lactic fibres and ependymal progenitor cells as a new biohybrid concept for cell transplantation in spinal cord injury

   Name of the conference: 4th International Spinal Cord Repair Meeting (ISCORE 2017)

   City of event: Barcelona, Spain

Date of event: 04/11/2017

Cristina Martínez Ramos; Ana Alastrue; LAURA RODRIGUEZ DOBLADO; Eric López Mocholi; Maravillas Mellado; Manuel Monleón Pradas; Victoria Moreno-Manzano

9. Title of the work: Biohybrid for neural tracts

   Name of the conference: 28th Annual Conference of the European Society for Biomaterials (ESB 2017)

   City of event: Athens, Greece

   Date of event: 08/09/2017

   LAURA RODRIGUEZ DOBLADO; Cristina Martínez Ramos; Manuel Monleón Pradas

10. Title of the work: Tubular-based hyaluronic acid biomaterial as a new biohybrid concept for cell transplantation in spinal cord injury

    Name of the conference: 28th Annual Conference of the European Society for Biomaterials (ESB 2017)

    City of event: Athens, Greece

    Date of event: 08/09/2017

    Cristina Martínez Ramos; LAURA RODRIGUEZ DOBLADO; Victoria Moreno Manzano; Manuel Monleón Pradas

Patents:

1. Monleón Pradas M, Martínez Ramos C, RODRÍGUEZ DOBLADO L, Gisbert Roca F. Dispositivo modular para regeneración nerviosa. OEPM ref. ES1598.113. In progress.

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Appendix

Figure 8. (D) Bright field photograph of an entire and wrinkled ‘SC sheath’ in a culture dish after having been extracted from the HA conduit. The cell cylinder is stained with Coomassie brilliant blue R250. Scale bar: 100 μm.

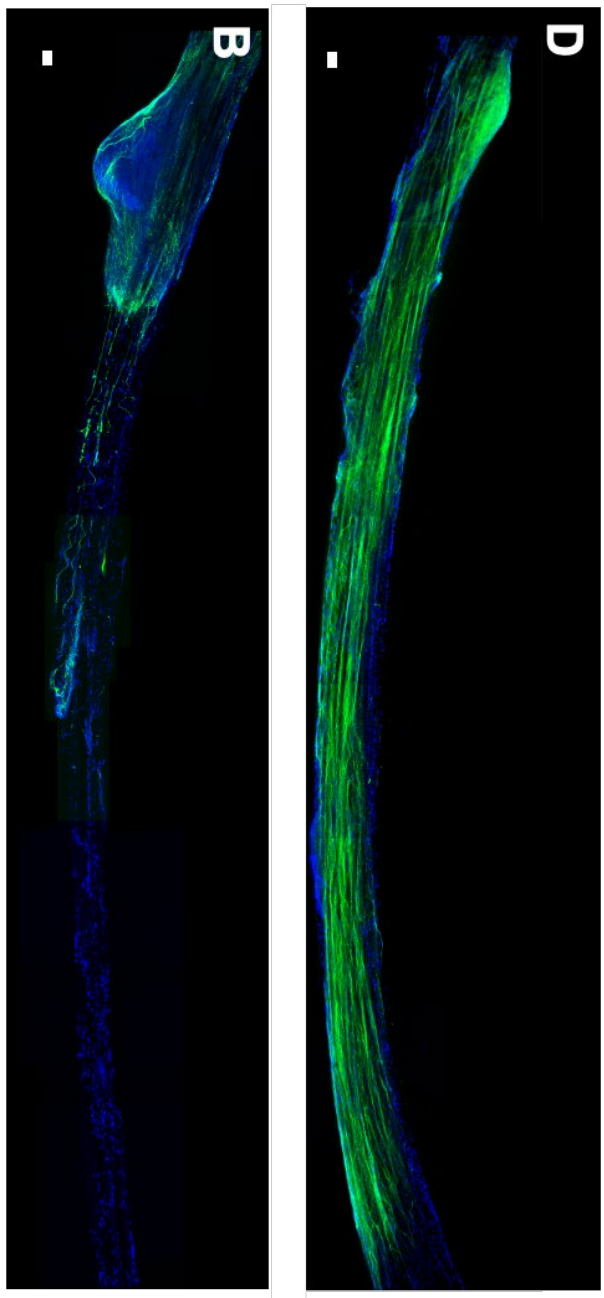

Figure 11. Representative confocal reconstruction of the conduit's complete length of (B) HAf+DRG and (D) HAf+SC+DRG conduits, after nuclear staining with DAPI (blue) and neuronal staining with Tuj1 (green). Scale bar: 100 μm.

Figure 12. (A) Representative confocal reconstruction of the conduit's complete length of longitudinal sections of HAf+SC+DRG conduit after nuclear staining with DAPI (blue) and growth cones staining with GAP43 (green). Scale bar: 100 μm.

Figure 18. Representative fluorescence reconstruction of the conduit's complete length of (A) unimodular and (F) multimodular conduits, after nuclear staining with DAPI (blue) and F-actin of SC staining with ActinRed™ 555 ReadyProbes™ Reagent (red). Scale bar: 500 μm.

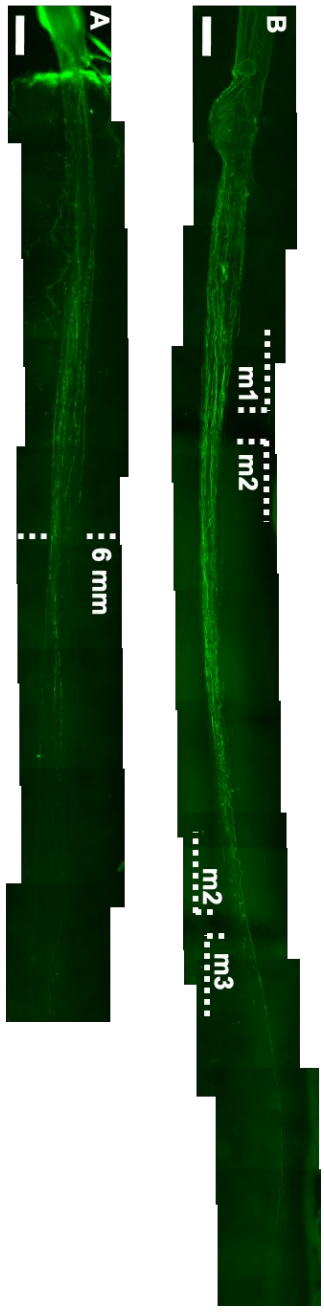

Figure 19. Representative fluorescence reconstruction of the (A) unimodular and (B) multimodular conduit's complete length after neuronal staining with Tuj1 (green), showing greater axonal extension of the multimodular conduit. Scale bar: 500 μm.

Figure 20. (B) Representative phase-contrast reconstruction of the ‘neural cord’’s complete length in culture before fixing. (C) Representative fluorescence reconstruction of the ‘neural cord’’s complete length after F-actin of SC staining with ActinRed™ 555 ReadyProbes™ Reagent (red) after. (D) Representative SEM reconstruction of the ‘neural cord’’s complete length, showing a compact structure after the manipulation of the sample. Scale bar: 500 μm.