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Desenvolvimento de hidrógeis 3D para regeneração axonal

Development of 3D hydrogels for axonal regeneration

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Engenharia Biomédica, realizada sob a orientação científica de Ramiro Daniel Carvalho de Almeida, Professor Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro, coorientação científica da Doutora Catarina Custódio, Investigadora Assistente e do Doutor João Mano, Professor Catedrático do Departamento de Química da Universidade de Aveiro.

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o júri

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palavras-chave

Lesão vertebro-medular, regeneração axonal, medicina regenerativa, hidrogéis, neurónios corticais, células da glia, células estaminais do tecido adiposo

resumo

Uma lesão na medula espinal é uma condição médica degenerativa e limitativa que não possui nenhum tratamento definitivo. A regeneração axonal é um passo fundamental para melhorar os resultados desta condição, mas os métodos tradicionais não oferecem soluções eficazes. Investigações mais recentes que incluem alternativas relacionadas com a medicina regenerativa têm emergido potencialmente como um novo tratamento desta lesão. De entre várias possibilidades, os hidrogéis são uma classe particular de biomateriais que se destacam devido à sua versatilidade de design, que pode corresponder a um conjunto específico de propriedades. Nesse sentido, estudamos a possibilidade de aplicar um hidrogel baseado em lisados de plaquetas no contexto de uma lesão vertebro-medular, como uma plataforma que pudesse promover a regeneração axonal. Os nossos resultados mostram que o hidrogel de metracriloílo e lisados de plaquetas (PLMA) não é ótimo em termos de adesão celular, mas que as suas propriedades podem ser melhoradas ao recorrer à utilização de um revestimento de poli-D-lisina (PDL). De seguida, investigamos o encapsulamento de neurónios corticais no interior da matriz do PLMA. Os resultados sugeriram que as células neuronais têm uma melhor adesão quando estão encapsuladas, o que se torna relevante numa perspetiva de aplicação do PLMA como um transportador de células. Por último, testamos a possibilidade da presença de outras células induzirem o desenvolvimento neuronal, nomeadamente a presença de células estaminais do tecido adiposo (ASCs) e células da glia (GCs). Para as ASCs, observamos que a presença destas células ofereceu suporte às células neuronais e que tiveram um efeito positivo no crescimento de neurites no interior dos géis de PLMA. No entanto, para as células da glia este resultado não se verificou, e futuras otimizações são necessárias para determinar se estas células poderão ser uma boa alternativa. No geral, os hidrogéis de PLMA poderiam ser usados como um *scaffold* para o crescimento axonal e como um transportador de células neuronais, e possivelmente oferecer melhores resultados quando combinado com células estaminais.

keywords Spinal cord injury, axonal regeneration, regenerative medicine, hydrogels, cortical neurons, glial cells, adipose stem cells

abstract Spinal cord injury (SCI) is a degenerative and limitative medical condition that has no definitive treatment. Axonal regeneration is a required event to improve the outcomes of this condition, but traditional methods do not offer effective solutions. Recent investigations involving regenerative medicine alternatives have emerged as a potential new treatment for SCI. Among various possibilities, hydrogels are a particular type of biomaterial that stand out due to their design versatility that can match a specific set of properties. Therefore, we studied the possibility to apply a platelet lysates-based hydrogel in the context of SCI, as a platform that could promote axonal regeneration. Our results show that the methacryloyl platelet lysates (PLMA) hydrogel is not optimal in terms of cellular adhesion, but its adhesive properties could be enhanced using a coating of poly-D-lysine (PDL). We next encapsulated cortical neurons inside the PLMA matrix. The results suggested that neurons would have a better adhesion when encapsulated, which is relevant in the perspective of using PLMA as a cell carrier. Lastly, we tested the possibility of the presence of other cells inducing neuronal development, namely the presence of adipose stem cells (ASCs) and glial cells (GCs). For ASCs, we observed that these cells offered support to neuronal cells and that had a positive effect in neurite outgrowth inside PLMA gels. However, for GCs this outcome was not verified and further optimizations are required to determine if they might be a good alternative. Overall, PLMA hydrogels could be used as a scaffold for axonal growth and a carrier for neural cells, and possibly offering a better outcome when combined with stem cells.

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Chapter 1

Introduction

1.1 Spinal Cord Injury

Spinal cord injury (SCI) is a devastating lesion in the spinal cord that has a high impact in the quality of life of those affected. The most common causes of SCI are vehicle accidents, falls, violence-related or sports-related injuries [1]. There are different outcomes associated with this lesion, depending on two different factors: the site where trauma occurs, leading to paraplegia (lower back injuries) or tetraplegia (cervical injury); and the extension of the damage, which results in a complete or incomplete loss of the nervous system functions [2]. The severity of this lesion is classified according to the American Spinal Injury Association (ASIA) impairment scale, represented on **table 1.1** [3, 4].

Table 1.1: ASIA impairment scale for SCI (adapted from McDonald et al., 2002.

ASIA grade	**Lesion description**
A	Complete – no sensory or motor function preserved in the sacral segments S4-S5
B	Incomplete – sensory but not motor function preserved below the neurological level, including S4-S5
C	Incomplete – motor function preserved below the neurological level and most key muscles have a grade lower than 3
D	Incomplete – motor function is preserved below the neurological level and most key muscles have a grade equal or higher than 3
E	Normal – regular motor and sensory functions

The treatment for this type of injury is very limited, and the innovation of the current solutions is deeply related to regenerative medicine. However, knowing the spinal cord system organization is of extreme relevance to understand the injury and potential targets for drug delivery or cell therapies.

1.1.1 General anatomical organization of the spinal cord

The spinal cord is one of the main components of the central nervous system (CNS), and it constitutes the connection between the brain and the peripheric nervous system (PNS) through the spinal nerves. Its main function is conducting information from the brain to the periphery and vice-versa, which makes it responsible for locomotion, organ functions and is the center of control for reflexes, for example [5, 6, 7, 8].

In terms of cellular organization, the nervous tissue that forms the spinal cord is composed of highly specialized cells: the neurons, which are the main functional units of the nervous system; and the neuroglial cells, that provide support and aid to the neuronal function [6, 9]. Functionally, neurons can be classified in sensory/afferent neurons, motor/efferent neurons, or interneurons. This classification is related to the role that neurons have within the nervous system, which may be of carrying signals from the CNS to the muscles (efferent), transmitting the information of stimuli towards the CNS (afferent), or decide and act upon the information that they have (interneurons) [6]. Furthermore, neurons can be classified according to their

structure, but it can be described using a general representation, as the one shown in **figure 1.1**. The main structure of a neuron is composed of a cell body or soma, a group of dendrites and an axon. Most cellular organelles, including the nucleus, are contained in the soma, which turns this structure into the major center of information of these cells. Dendrites are dense ramifications of the cell body and constitute the primary location for receiving information. On the other end, axons are responsible for the transmission of signals to other cells through the terminal arborization that will connect to other neurons' dendrites. Axons transmit electrical signals generated in the hillock, designated action potentials, and are covered in myelin sheaths that accelerate the transmission process [6, 9].

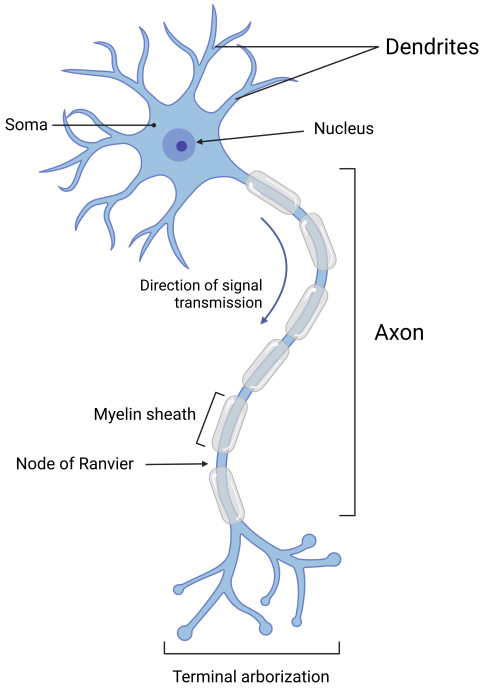
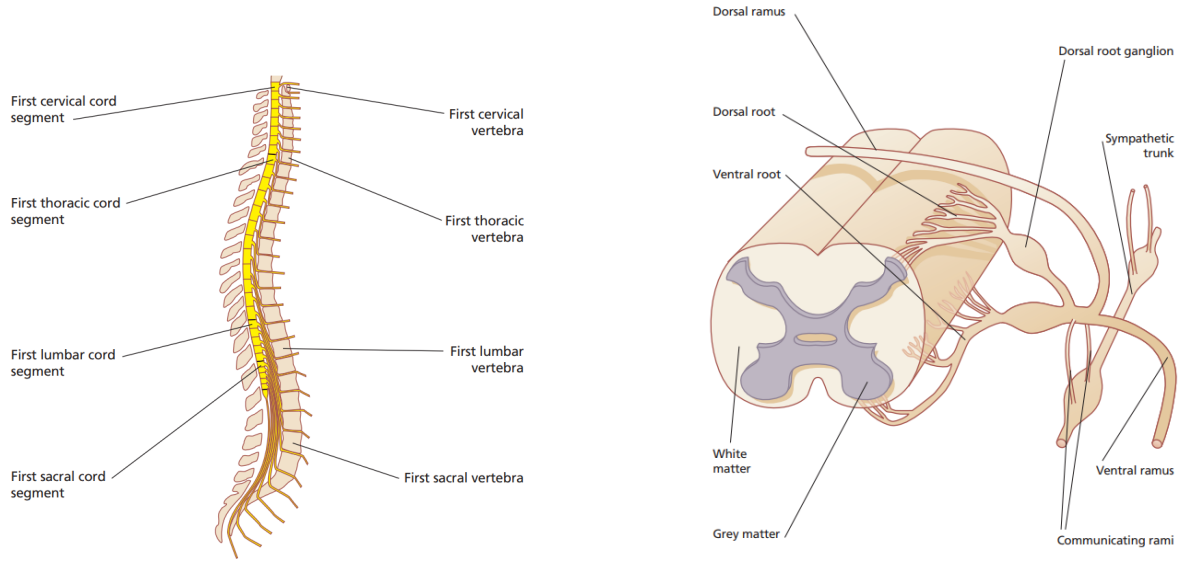


Figure 1.1: Representative structure of a neuron.

In addition to neurons, neuroglia or glial cells are also an important component of the nervous tissue. These cells have a supportive and protective role relative to neurons, and in the CNS there are four different types: astrocytes, microglia, oligodendrocytes and ependymal cells [6]. The most abundant glial cells are astrocytes, and they have a variety of functions inside the CNS, like the formation of the blood-brain barrier, the release of certain growth factors and protection from injuries by forming a scar around the lesion site, as it will be further described later [6, 9]. Oligodendrocytes are also vital to the nervous tissue, as they are responsible for synthesizing myelin sheaths around the axonal segments.

The spinal cord is inserted in the vertebral canal, which provides structural protection. There are 31 pairs of spinal nerves that arise from the spinal cord, denominated segments, and each of those nerves results from the junction of a ventral and a dorsal root. Each segment has a correspondent vertebra, so they are divided in five regions: cervical (8 segments), thoracic (12 segments), lumbar (5 segments), sacral (5 segments) and coccygeal (1 segment). The cervical pairs exit the canal through an intervertebral space above their corresponding vertebra, except for C8. The thoracic, lumbar and sacral pairs exit through the same space,

but below their corresponding vertebra. However, the spinal cord length does not fully extend to the length of the vertebral canal, which creates an enlargement in the last third of the canal. This space, represented in **figure 1.2a** is occupied by the lumbar, sacral and coccygeal nerves, that extend from the end of the spinal cord, the medullary cone, to their respective vertebra to exit the canal [6, 8]. Each spinal nerve is connected to the spinal cord through two roots: the anterior/ventral root, and the posterior/dorsal root. This connection is represented in **figure 1.2b**. These roots have different functions, carrying different types of information, as it will be further described. The dorsal roots have a ganglion, where the cell bodies of the neurons that constitute this pathway are located [6].



(a) (b)

Figure 1.2: General anatomy of the spinal cord. **(a)**: Insertion of the spinal cord in the vertebral canal. **(b)**: Organization of the ventral and dorsal roots (adapted from Sheerin, 2004).

Beyond the protection provided by the vertebral spine, the spinal cord is also surrounded by layers of connective tissue that separate the soft tissue of the CNS from their protective bones [6, 7]. These layers are designated meninges and there are three around the spinal cord. The most superficial and thick one is the dura mater, which separates the spinal cord from the vertebrae through the epidural space. This space is mainly composed of vascularized adipose tissue. Following this layer, the arachnoid is a very thin membrane of epithelium and forms a gap to the pia mater that contains cerebrospinal fluid. Lastly, the pia mater is another thin layer that is connected to the surface of the spinal cord. Along the cord, extensions of this layer fix the cord to the vertebral canal, and at the bottom of the spinal cord, the pia mater is a fibrous extension that attaches the spinal cord to the coccyx [6].

Internally, the spinal cord has a cylindrical shape and is constituted by two different types of nervous tissue: the gray matter and the white matter. The gray matter forms a H-shaped nuclei, with posterior/dorsal, anterior/ventral and lateral/intermediate horns in each half, as shown in **figure 1.3**. It is composed mostly of the neural cell bodies, dendrites and axonal terminations [6, 8]. The gray matter is the central site for the processing of information,

because is where the synaptic contacts between neurons occur. On the other hand, the white matter that surrounds the gray core is composed of bundles of myelinated axons, divided into tracts, that are responsible for transmitting signals between different parts of the body. These tracts can be classified in ascending or descending, according to their function.

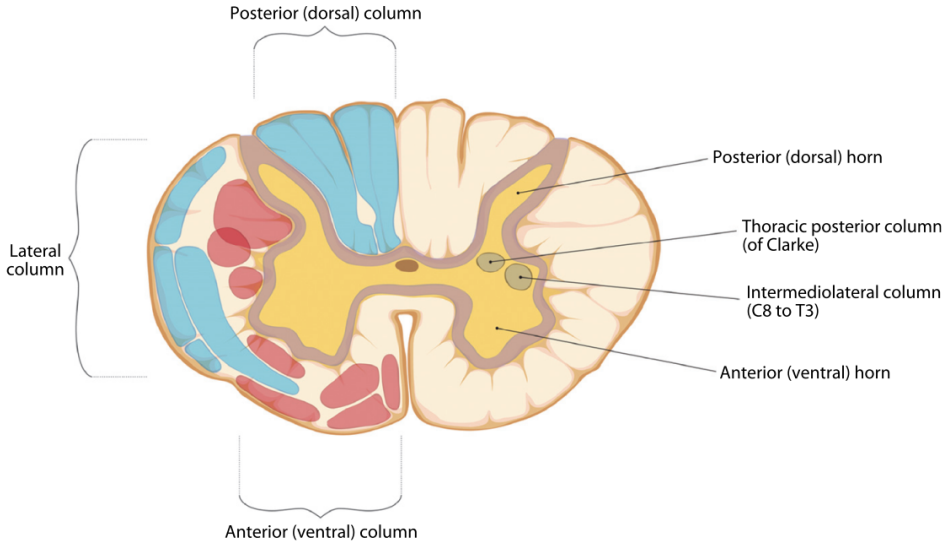


Figure 1.3: Cross-sectional division of the spinal cord, with the ascending (blue) and descending (red) pathways represented (adapted from Cho, 2015).

Ascending or sensory tracts are responsible for carrying sensorial information, like pain, temperature changes or pressure, from the receptors in the body to the cerebral cortex [6, 8]. The ascending pathway transports the nerve impulses across three distinct neurons, denominated first-order, second-order and third-order neurons. The first-order neuron, which has their cell body located in the dorsal root ganglion, transmits the information along the spinal cord, towards the brainstem, where connects to the second-order neuron. This neuron extends upon the thalamus to meet the third-order neuron, responsible for transmitting the nerve impulse to the cerebral cortex [5, 6].

The descending tracts transmit motor signals from the brain to muscles or organs, controlling voluntary and involuntary movements and regulating organ functions [5]. In this case, the impulses travel from the cord to the muscles and organs through the ventral root, formed by motor nerves. Two different neurons compose this pathway: the upper motor neuron, that originates in the brain cortex and prolongs to the spinal cord; and the lower motor neuron, that connects the cord and the target system [5, 6].

1.1.2 Pathophysiology

Spinal cord injury is a complex and progressive lesion, and it can be categorized in two main mechanisms: the primary and the secondary injuries [3, 10, 11, 12]. The primary injury consists in the initial mechanism that induces the lesion and causes the mechanical disintegration of the spinal cord [11]. Excessive forces of compression, laceration, shearing or stretch can cause an injury [10]. This event occurs within minutes and results in the disruption of blood vessels, axons and cell membranes [4, 11].

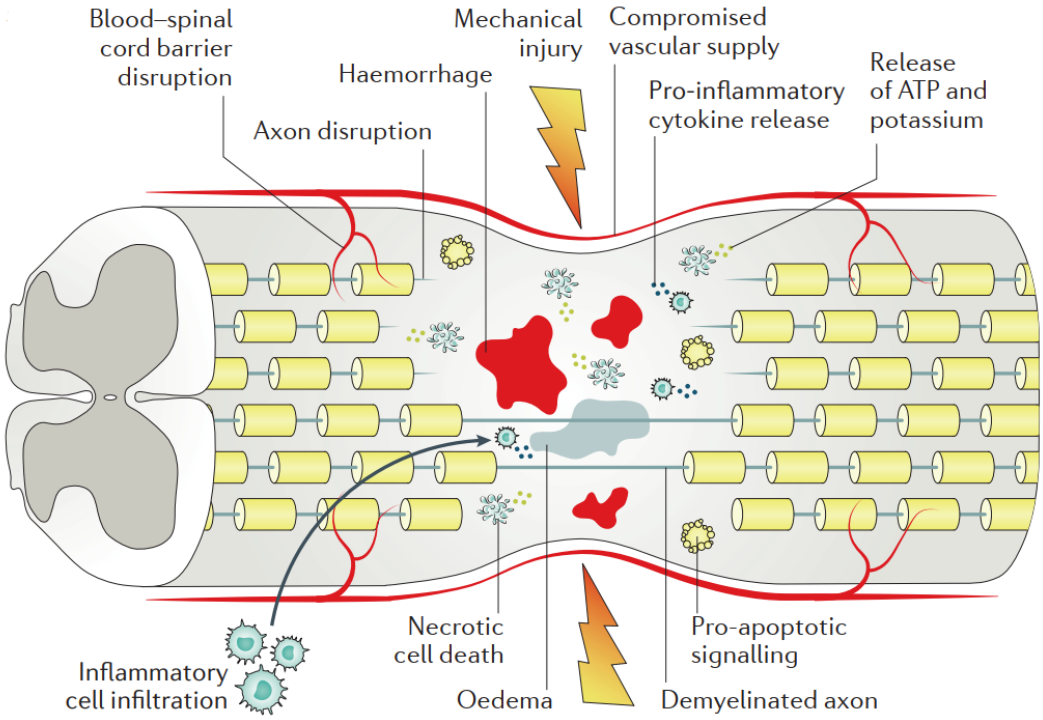


Figure 1.4: Pathophysiology of SCI: primary injury and acute phase (adapted from Ahuja et al., 2017).

On the other hand, the secondary phase is a progressive injury that determines the severity of SCI, characterized by successive events that occur over time, up to months after the initial trauma [10]. The series of events that constitute the secondary injury may be divided in four main stages: acute, subacute, intermediate and chronic. Immediately after the primary injury, the disruption of blood vessels causes hemorrhage and inflammation within the gray matter, and the blood-spinal cord barrier (BSCB) is damaged. As a result, an infiltration of macrophages, T-cells, microglia and astrocytes occurs and there is an upregulation of inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukins (IL-1α, IL-1β and IL-6) [4, 10].

Furthermore, a series of biochemical imbalances take place to cause more damage and cell death. Excitotoxicity is one of the main chemical consequences of tissue damage, and it consists in the overproduction of excitatory neurotransmitters, like glutamate, as a result of cell membranes disruption [4, 11]. This excess of glutamate creates an increase in the intracellular concentration of Ca2+, causing an overload that activates calcium-dependent proteases that results in mitochondrial dysfunction and aggravates cell apoptosis. In the white matter, these chemical disturbances also damage oligodendrocytes and, consequently, leads to the demyelination of surviving axons [10, 13]. In addiction to excitotoxicity, the production of free radicals and reactive oxygen species (ROS) affect cellular function, by damaging proteins, nucleic acids and lipids [4, 10].

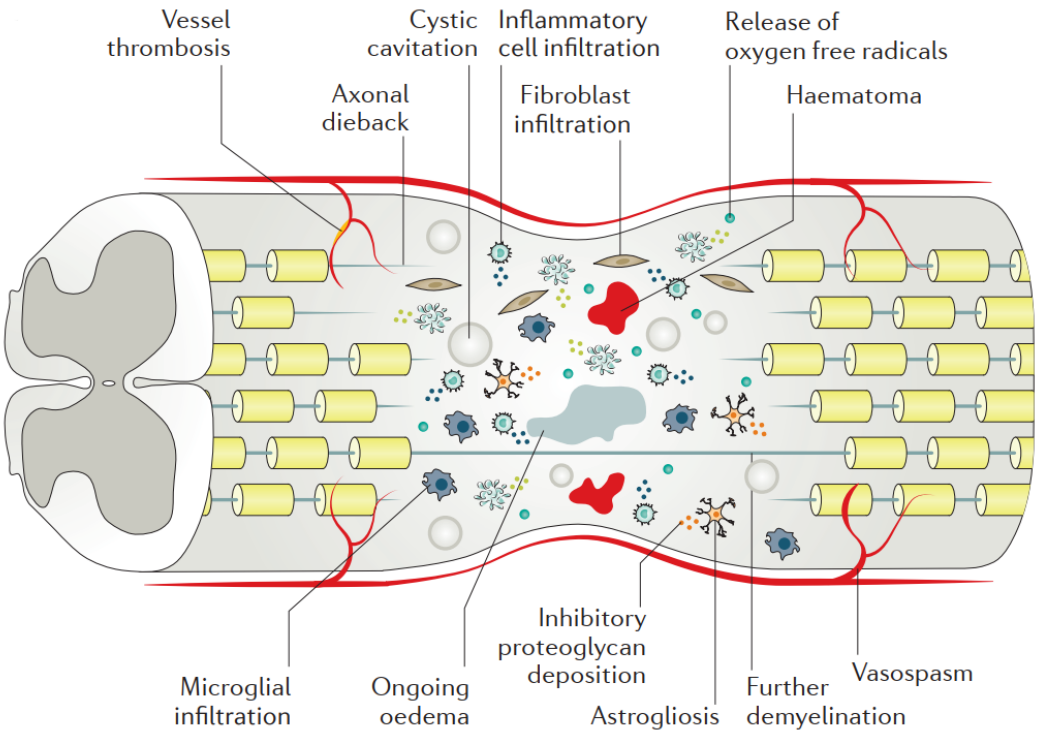


Figure 1.5: Pathophysiology of SCI: subacute phase (adapted from Ahuja et al., 2017).

The evolution of this injury through the intermediate and chronic phases (from 2 weeks to 6 months post-injury) conducts the spinal cord to a state of permanent damage. At these phases, a full-developed cystic cavity has emerged as one of the main barriers to neural tissue regeneration, and it is surrounded by a glial scar composed of reactive astrocytes, microglia, fibroblasts, $NG2^+$ oligodendrocytes precursor cells (OPCs) and pericytes [10, 13]. Moreover, an upregulation on the levels of chondroitin sulfate proteoglycans (CSPGs) during the acute phase of SCI also gives a significant contribute to inhibiting axonal regeneration upon scar tissue formation [4, 13]. Despite the negative impact of the glial scar and being one of the main barriers to allow healing and regeneration of the neural tissue after SCI, it also has a protective and stabilizing function. This scar tissue is also a main barrier preventing the uncontrolled spread of tissue damage [4, 10].

1.1.3 Current treatments and solutions

Nowadays, there is not a definitive cure for SCI, either it is through pharmacological treatments or other alternative therapies, such as cell transplantation therapies [4]. The current treatment methods only allow to improve the clinical outcome of the injury, but do not overcome the damage caused by the primary injury. Furthermore, these treatments are time-sensitive because the time window for their application is often very short due to the rapid evolution of the secondary injury.

The initial care provided post-trauma is a key piece to determine the severity of a spinal cord injury, and it is a time-sensitive matter. In all cases of confirmed or suspected injuries, the spinal cord immobilization is crucial to avoid further damage and narrow the secondary

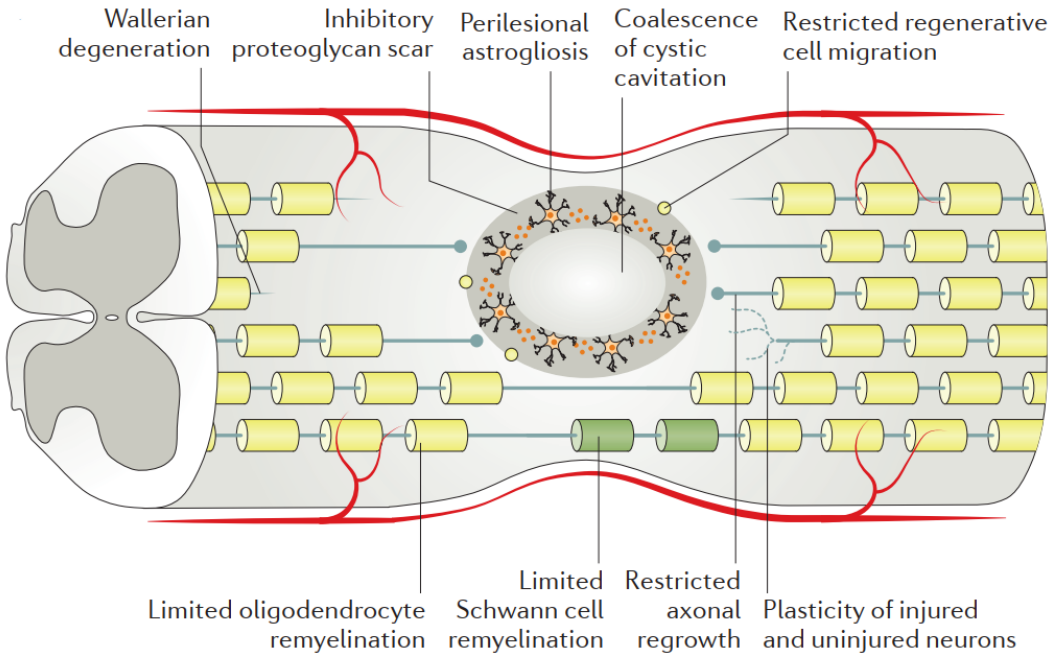


Figure 1.6: Pathophysiology of SCI: intermediate and chronic phases (adapted from Ahuja et al., 2017).

injury effects [3, 10].

Among the pharmacological treatments studied over the years, the administration of methylprednisolone (MPSS) was the first to be applied clinically [3]. MPSS is a glucocorticoid that is thought to be associated with increasing anti-inflammatory cytokine levels and reducing oxidative stress [10]. Around 1990, a high-dose 48 hours administration of MPSS within 8 hours post-injury was shown to reduce the negative outcomes of SCI. However, further studies on this treatment showed it was related with a number of complications that outweighed the benefits, since it did not reverse any of the damage that was already caused previously to its administration. In the same studies, an alternative 24-hour protocol presented less severe side effects while maintaining the improve on the neurological condition of patients [3, 10].

1.2 Hydrogels

Biomaterials have evolved in the past decades from being almost inert and having minimal interaction with biological systems, to being one of the most promising solutions for tissue engineering (TE) and regenerative medicine (RM), which implicates a positive biological response. On this note, hydrogels have emerged as one of primary solutions for scaffolding cells in the TE and RM fields. Their versatility in terms of mechanical properties make them suitable materials to be applied in both soft and hard tissues, and also for scaffold applications in a broad variety of cell types.

1.2.1 Characterization

Hydrogels are a hydrophilic type of biomaterial that started emerging as biomedical applications in the 1960s [14]. By definition, hydrogels are three-dimensional (3D) networks, formed by crosslinked chains of polymers, and their main characteristic is the ability to absorb a large amount of water [15, 16, 17]. The first report of hydrogels as we know them nowadays appeared in 1960 [18], when there were many issues regarding polymers being used as biomaterials due to their poorly suited physical properties. Since then, many research has been made around the properties of hydrogels and their application as biological systems for drug delivery or as scaffolds for tissue engineering, for example.

In comparison with other types of biomaterials, hydrogels present some advantages for clinical applications since they are biocompatible, may be engineered to fit a specific set of mechanical or physical properties [16], and the high-water content makes them very similar to the extra cellular matrix (ECM) [19]. Furthermore, hydrogels can be designed to be applied in different ways, such as a structural scaffold to provide support for cell growth, or as an injectable material, which is less invasive and more localized [20]. Biodegradability is also an important feature for some kinds of applications, like regenerative medicine where the hydrogel may be important for initial cell development and then degrades when is not needed.

There are many ways in which hydrogels can be classified, from their preparation to the type of cross linking, ionic charge, or structure [15]. However, the main classification present in the literature refers to the source of the polymer or polymers that constitute the hydrogels, and according to it, hydrogels can be considered natural, synthetic or hybrid. Natural polymers can be based on two main organic compounds: proteins and polysaccharides. This type of polymers presents the advantage of enhanced biological properties, since they can be found in the human body and are inherent biocompatible materials [21], thus being associated with minimized toxicity and inflammatory reactions. However, they exhibit mechanical properties that can be hard to manipulate, and are more limited in terms of supply, compared to synthetic ones. Regarding natural-based hydrogels, both protein and polysaccharides have been used for various applications, from which can be highlighted collagen and fibrin in the protein category, and alginate and chitosan in the polysaccharide category [19, 21, 22, 23].

On the other hand, synthetic polymers can also be suitable for biological applications, since it has been shown that some properties that can influence the biocompatibility of the material can be easily controlled and manipulated, such as degradation rate and shape. Nevertheless, the bioactivity of this type of polymers is not as strong as natural-based polymers' due to more hydrophobic surfaces and the lack of cell-recognition signals. There are many different synthetic polymers that can be used to form hydrogels, such as PLA and PGA, and methacrylate-based polymers such as pHEMA [19, 23].

From the need to enhance physical and/or biological properties of natural or synthetic polymers that are not always ideal, hybrid hydrogels started to emerge. Although there are diverse definitions for this term [24], in general it refers to multicomponent hydrogels that, in addition to the base polymer that forms the biomaterial, can contain a smaller fraction of another polymer, biological factors, proteins, peptides or nano- and microstructures in their physical or chemical composition [25]. The main objective of the incorporation of these components in the main matrix of a hydrogel is usually associated with improvement of biological properties like cell adhesion, proliferation and differentiation or bioactivity when growth factors or proteins are involved, or the reinforcement of mechanical and physical

properties, when other polymers or nano/microparticles are added.

1.2.2 Applications

In biomedical research, hydrogels present themselves as a solution for many applications due to their biocompatibility, their flexibility and their unique mechanical properties that are very close to the properties of some living tissues. Hydrogels can have applications both *in vitro* or *in vivo*, and both in injectable and non-injectable form. The most common application of hydrogels *in vitro* is as a scaffold for cell culture [16, 26, 27]. Since the hydrogel properties are easy to manipulate to best correspond to the needs for a certain type of cell or tissue, they are a good way to grow and proliferate cells in 3D scaffolds and produce larger amounts when they are needed.

One of the most important and related applications of hydrogels *in vivo* is for drug delivery systems. The porosity and degradability of the hydrogels are controllable features of a gel matrix that favor the use of hydrogels to deliver drugs in better conditions and with less limitations associated with the traditional drug intake systems [15, 16, 26]. A porous structure can be loaded with different substances and provides protection for the drug from the hostile biological environment in the human body, and the degradability and diffusion rate of the drug through the scaffold control its release period. Hydrogels have been used as delivery systems for cells, specific molecules for certain treatments or biological factors, like growth and anti-inflammatory agents [17].

Another important field where hydrogels are applied is tissue engineering. There are reports of the application of hydrogel scaffolds for the regeneration of numerous types of tissues, like skin, bone, tendons, ligaments and neural, for example [26, 28]. The fundamental approach to create scaffolds for regenerative medicine is the combination of a hydrogel with cells, that will allow the growth of a new tissue to replace a damaged one or will provide the medium to regeneration in vivo. Once again, depending on the type of tissue being engineered, very specific physical, mechanical and biological properties are required, so the material and design selection for the scaffold, as well as the type of cells that will incorporate it, play an important role [15]. Both natural and synthetic derived materials have been used to form hydrogels for tissue engineering, since both types present different advantages for this type of application. Natural hydrogels are inherently similar to the ECM, which works favorably for *in vivo* applications, and synthetic hydrogels are controllable in terms of mechanical properties and chemical composition, thus facilitating the construction of a suitable scaffold with the necessary properties.

Other applications of hydrogels include biosensors and contact lenses. The latter was actually the first time a hydrogel was applied in the biomedical field, in 1960 by Otto Wichterle [18], using PHEMA, and marked the beginning of research for soft lenses. In the biosensors field, the fundamental role of hydrogels is to preserve parts of a biosensor from undesired interference, when they are responsible for biological signals recognition, or to allow biological elements to stay active for enough time so that recognition occurs. One example is the use of hydrogels as a matrix to enzymes that react with the substrate and allow the determination of analyte concentrations, or the formation of composites of hydrogels and microorganisms, such as cells or bacteria, to detect chemical variations in a biological system [15].

1.2.3 Regenerative medicine

Regenerative medicine refers to the field of biomedical applications to repair damaged tissues [29]. Hydrogels have significant impact in this research area for many sorts of tissues, especially for soft tissues due to their "smoothness", although some more rigid tissues like bone and cartilage are also included. Either as a scaffold to provide specific conditions for cell growth, migration and differentiation, or as an injectable material to be applied *in vivo* and promote regeneration, hydrogels are able to create a favorable environment for tissues regeneration due to their adaptable properties, biochemical resemblance to the ECM or capacity to cultivate/deliver biological factors in a localized way.

Bone tissue is vascularized and composed of mostly collagen and hydroxyapatite, hence has some self-healing potential and therefore the application of biomaterials to help the regeneration of this kind of tissue is more related with providing a medium to help that healing process [26]. Associated with hydrogels, special cues may include the addition of cells, growth factors with osteogenic effects or the creation of composites with ceramic materials like hydroxyapatite, calcium phosphate-based compounds, or bioactive materials like Bioglass or carbon nanotubes. These latter materials have been successfully used alone for bone injury's treatment but present the disadvantage of needing a highly invasive procedure to be applied [28]. The combination of ceramic-based materials with a hydrogel matrix allows the increase of porosity, which may be an important feature for bone tissue regeneration, and the reduction of the need for invasive procedures.

On the other hand, tendon injuries, for example, show increased difficulty in terms of regeneration and healing due to their acellularized character and short vascularization [26]. In this case, hydrogels have an important role to promote the reconstruction of tendon tissue. Fiber-reinforced hydrogels present a considerable solution for tendon regeneration since they can help provide alignment and guidance for tenocyte growth and proliferation, besides the inherent conditions that the application of a hydrogel scaffold supplies to the biological environment in the lesion site, like controlled drug delivery of growth factors or scar formation inhibitors [28].

Other applications of hydrogels for regenerative medicine include cardiovascular and skin tissue [26]. Myocardial regeneration after a heart attack or an infarction is difficulted by the lack of electrical conductivity after the injury, thus combining the potential of hydrogels with other micro or nanoparticles that can promote electrical activity may be a relevant matter [28]. Another important issue regarding cardiovascular tissue engineering is the load and stretch that the myocardium is subjected to during heart contractions, for which hydrogels are advantageous materials due to their viscoelastic behavior. Concerning skin tissue regeneration, hydrogels introduce a viable alternative to autologous transplants of skin that are usually very limited in terms of supply [26].

1.2.4 Hydrogels applied to SCI

One of the most challenging fields around regenerative medicine is the neural tissue regeneration. When an injury occurs in the nervous system, a very hostile environment gets settled at the lesion site and damaged tissue hardly possesses any capability to regenerate. This is aggravated when the injury happens in the central nervous system, since the peripheral nervous system usually presents a greater ability to self-regenerate than the central system [22, 30]. One of the main targets to improve the harm done to the system is axon regeneration, since

axons are the main channel of electrical communication in a neuron, and their disruption leads to the failure of that function, which is the primary of the nervous system. Hydrogels have been applied to both peripheral and central nervous systems regeneration, and in the CNS it includes the brain and the spinal cord. In both cases, injectable systems stand out and hydrogels, as water-soluble and potentially biodegradable polymers, become a primary choice for neural tissue engineering applications. Additionally, hydrogels can work as drug or cell delivery systems, which may help to overcome the difficulty of neural tissue to regenerate [31].

Concerning the CNS, hydrogels emerged as an effective approach for SCI regeneration therapy mainly due to their physical and mechanical properties. The soft character of hydrogels that inherently mimics the CNS structure [22, 23] and the versatility to best fit certain properties, for example by easily modifying parameters like the cross-linking density or varying the polymer components that compose the matrix, turn them into excellent materials for this kind of application. Furthermore, one of the main advantages in comparison with other materials is the capability to be applied in an injectable form, allowing a more localized and less invasive treatment [32, 33]. The high water content of hydrogels presents as an advantage for their application, since it better mimics the ECM environment of neural tissue [23], and their versatility that may allow the incorporation of biological factors, cells or other substances can help with the inflammatory and inhibitory environment that develops during the SCI.

Despite the suitability of hydrogels for being applied in SCI, they are often combined with cell therapies or biological factors to improve the outcome. Cell transplantation is one of the therapies that emerged as a possible regenerative solution for repairing the damage associated with SCI [23]. Among different types of cells investigated, stem cells stand out due to their potential for differentiation and, as it has been more recently investigated, their ability to release multiple types of growth factors and biological factors [34, 35]. Stem cells (SCs) of different origins have been investigated as a potential cell therapy for SCI, from which can be highlighted neural stem cells (NSCs), induced pluripotent stem cells (iPSCs), embryonic stem cells (ESCs) and mesenchymal stem cells (MSCs) [23, 34, 36].

On this topic, mesenchymal stem cells have gained extreme relevance, as this group of SCs is not associated with major ethical or safety concerns. Moreover, MSCs are relatively abundant and easy to obtain from multiple sources: bone marrow (BM-MSCs), adipose tissue (ASCs), and the umbilical cord (UC-MSCs) [37, 38]. MSC implantation following SCI has been shown to improve the harsh outcomes of the lesion in animal models, but the mechanisms associated with this improvement are yet to be clarified. In contrast to other groups of stem cells, MSCs do not have a high ratio of neural differentiation, so their potential for promoting neuronal regeneration is being associated with their paracrine effect rather than their multipotency [38]. Consequently, one alternative for a RM therapy using MSCs is to explore the benefits of their secretome that may have an immunomodulatory and anti-inflammatory action [37].

Whereas cell therapies present some improvement regarding neural regeneration in SCI context, the reports of clinical trials often present very limited and insufficient outcomes [37]. Furthermore, as previously stated, the lesion site is adverse and does not promote survival of the transplanted cells, which is also one of the main reasons for the unsuccessful application of regenerative cell therapies [36]. The combination of hydrogels with these therapies has emerged in order to overcome their inadequacy when applied alone. **Table 1.2** resumes research made regarding the application of hydrogels in SCI animal models.

Table 1.2: Hydrogels applied in animal models of SCI, in combination with cells therapies and/or biological factors.

Ref.	Materials	Biological factors	Encapsulated cells	Results
[39]	Polyacrymalide (PA)	IKVAV peptide and polylysine (PL)	-	Accelerated neuronal differentiation of embryonic and adult neural progenitor cells; promoted the formation of focal adhesions containing $\beta 1$ integrin; promoted neurogenesis, maturation and neurite outgrowth of NSCs
[40]	Hyaluronan and methylcellulose (HAMC)	KAFAK peptide and brain-derived neurotrophic factor (BDNF)	-	Cell proliferation of PC12 cells and release of KAFAK and BDNF; positive behavioral results regarding the recovery of locomotor functions in rats; inflammatory cytokines regulation and inhibition
[41]	Heparin-poloxamer (HP)	Nerve growth factor (NGF)	-	Good cell survival and neurite outgrowth in PC12 cells; locomotor function recovery observed in SCI rat models; HP-NGF showed inhibition of the glial scar formation and chronic ER stress-induced apoptosis
[42]	Laponite (Lap) and heparin (Hep)	Fibroblast growth factor 4 (FGF4)	-	Lap/Hep@FGF4 group showed motor functional recovery and axonal regeneration after SCI in rat models; suppression of inflammatory reactions, increase of remyelination and reduction of glial scaring

[43]	Hydroxyphenyl derivative of hyaluronic acid (HA-PH) + fibrinogen (F)	Integrin-binding peptide arginine-glycine-aspartic acid (RGD)	Human Wharton's jelly derived mesenchymal stem cells (hWJ-MSCs)	Proliferation of hWJ-MSCs in HA-PH-RGD/F hydrogels; both HA-PH-RGD and HA-PH-RGD/F promoted axonal ingrowth in a SCI model, but the hydrogel seeded with hWJ-MSCs showed an enhanced result; no significant effects were observed in terms of functional recovery, blood vessel regeneration or glial scar density
[44]	Collagen (C) and laminin (L)	5-Nonyloxytry-ptamine (5-NOT)	-	Progress observed *in vitro* with neurite outgrowth, migration and fasciculation of cerebellar neuronal cells; 5-NOT promoted survival and neurite lenght of cortical neurons when co-cultured with glutamate-challenged astrocytes; C/L+5-NOT hydrogels applied in SCI mice presented motor recovery
[45]	Peptide amphiphile (PA) functionalized with IKVAV	Brain-derived neurotrophic factor (BDNF)	-	BDNF was bioactive under physiological conditions and stimulated neurite outgrowth; the implantation of BDNF-loaded PA/IKVAV hydrogel promoted the preservation of axonas, reduction of astrogliosis, but did not present any improvement in BBB scores
[46]	Gelatin and methacrylate (GelMA)	-	Induced pluripotent stem cell (iPSC) derived neural stem cells (iNSCs)	iNSCs survived and differentiated when encapsulated in the GelMA hydrogel and presented robust neurite outgrowth; GelMA/iNSCs implanted in SCI rat models showed improvement in motor function recovery, reduction of cystic cavity and the inflammation, and promoted axonal regeneration

[47]	Macroporous functional hydrogel (MFH) composed of pHEMA and HepMA	-	Neural stem cells (NSCs)	MFH-2, containing 0.50 wt% of HepMA, was selected for *in vitro* and *in vivo* experiments; the combination of MFH-2 with bFGF and collagen promoted the proliferation of NSCs; implanted MFH-2+bFGF+collagen and MFH-2+bFGF+collagen+NSCs showed the best improvement in BBB scores and the presence of neurons along the conduit; the NSCs implanted in the MFH-2+bFGF+collagen hydrogel contributed to a better density of regenerated neurons
[48]	Gelatin with shape-memory polymer fibers	-	Embryonic stem cells (ESCs)	The composite hydrogel promotes viability and differentiation towards motor neurons of ESCs; ESCs loaded hydrogel transplanted to SCI animals improved neuronal tissue regeneration and motor function recovery
[49]	Gellam gum (GG)	GRGDS	Adipose tissue-derived stem cells (ASCs) and olfactory ensheating cells (OECs)	ASCs and OECs can be cocultured in 2D and 3D GG-GRGDS hydrogel; *in vitro* axonal outgrowth evaluation showed that the GG-GRGDS+ASCs presents the biggest increase in neurite area; the combination of the hydrogel with ASCs and OECs resulted in the greater improvements in the locomotor function of SCI rat models

[50]	Collagen (Col), laminin (Lam) and hyaluronic acid (HA)	-	Neural progenitor cells (NPCs)	NPCs from E14-E15 rat embryos differentiate into oligodendrocytes with a 66.8 % efficiency in Col-HA-Lam hydrogels; the Col-HA-Lam hydrogel combined with NPCs presented the best results in terms of functional recovery of SCI mice; the hydrogel contributed to reduction of the lesion area and scar tissue formation, and the hydrogel with NPCs contributed to a massive presence of oligodendrocytes
[51]	Chondrotin sulfate methacrylate (CSMA)	-	Neural stem cells (NSCs)	NSCs survived and proliferated in the CSMA hydrogel; *in vitro* assays showed a significant decrease in NSCs differentiation into astrocytes and a higher percentage of differentiation into neurons for the 3D CSMA hydrogel; SCI rat models presented better functional recovery in the CSMA/NSCs group and also a decreased lesion cavity; the differentiation into astrocytes was also reduced *in vivo*, which facilitated neural regeneration after SCI

[52]	Alginate and fibrinogen	Glial-derived neurotrophic factor (GDNF)	-	The release profiles for the two formulations of GDNF encapsulation were different: GDNF release was slower for the GDNF microspheres-loaded hydrogel; PC12 cells differentiated into a neuronal phenotype when cultured in the GDNF-loaded hydrogels; *in vivo* assays showed that the injection with GDNF-loaded hydrogels promote the development of neurofilaments at 6 weeks and 3 months post-injection; the hydrogel-treated mice presented less GFAP (astrocytes) staining and more endothelial and nerve fiber staining within the lesion site

1.3 Methacryloyl platelet lysates (PLMA) hydrogel

In order to overcome recent issues concerning cell culture methods, hydrogels have emerged as a possible solution to create 3D platforms for this application. However, despite the emerging of hydrogels based on extracellular matrix components as biocompatible and bioactive materials, their mechanical properties and stability are often poor. The work developed by Santos et. al. proposed a formulation for a hydrogel based on platelet lysates (PL) conjugated with methacrylic anhydride (MA), originating the PLMA hydrogel [53].

Blood is a source of different therapeutic products, divided into cellular and plasma components. These products are mostly indicated for transfusional applications, but in the last years, the use of platelet components has been investigated to form new biomaterials to be applied in regenerative medicine and tissue engineering [54]. The healing and tissue repair processes that happen in vivo are the key to understanding and developing blood-based biomaterials [55]. Platelets have a key role in blood coagulation, which is the primary biological defense against trauma involving vascular injury. They contain different types of granules that are responsible for releasing bioactive molecules and growth factors upon platelet activation [55, 56]. Substances like platelet-derived growth factors (PDGF) and vascular endothelial growth factor (VEGF), which are associated with the CNS development and neuroprotection, are examples of two growth factors contained in α-granules [55]. The release of these growth factors, combined with other substances, is associated with the formation of a fibrin clot that will be responsible for wound healing and vascularization and tissue repair [54]. The physiological action of platelets makes them appealing for regenerative medicine applications, leading to an increase in interest for platelet-based materials. Platelet-rich plasma (PRP) is a plasma solution with a high concentration of platelets, obtained from centrifugation of whole blood [54, 53]. Following activation, PRP is reported to be able to form gels that can release

biological factors [55, 56].

For the development of PLMA hydrogels, the principle of conjugating MA with protein molecules was applied to platelet lysates, as it will be further described. As a result, Santos et al. were able to develop a new platform suitable for tissue engineering applications, rich in biological factors and that can be obtained from an allogenic source [53]. These hydrogels will be further described and characterized in this chapter.

1.3.1 Synthesis

The chemical modification of protein-based materials by reaction with methacrylic anhydride has been largely investigated, particularly regarding methacrylamide-modified gelatin (GelMA). Gelatin can form hydrogels by physical crosslinking at room temperature, but it presented some issues related to the fact that the crosslinking can be thermally reversed [57, 58]. GelMA is the result of the reaction of gelatin with methacrylic anhydride, and hydrogel formation occurs in the presence of a photoinitiator by UV irradiation. This new method of hydrogel formation with natural materials represented a significant improve in the physical and mechanical properties of these gels [58, 59]. Several synthesis conditions, like the photoinitiator concentration, the photopolymerization method or the degree of methacryloyl substitution in the protein molecule, can be used to modify the mechanical properties of MA-modified materials [59].

The process to obtain the platelet lysates is composed of two steps: firstly, platelet-rich plasma (PRP), a type of platelet concentrates, is obtained from a differential centrifugation applied to whole blood; and then, the PRP undergoes freeze/thaw cycles to obtain the final PLs product [53]. In this study, the previous chemical modification with MA was applied to platelet lysates. PLs contain proteins with hydroxyl and amino groups that will react with the methacrylic anhydride to form methacrylate and methacrylamide groups, as shown in **figure 1.7**, to originate the final composition of PLMA. The degree of methacrylation (DM), i.e. the number of proteins that are conjugated with MA, is an important factor and can vary by changing the molar ratio between PL and MA [53]. In this case, the formulation used was the one with lower degree of methacrylation, PLMA100.

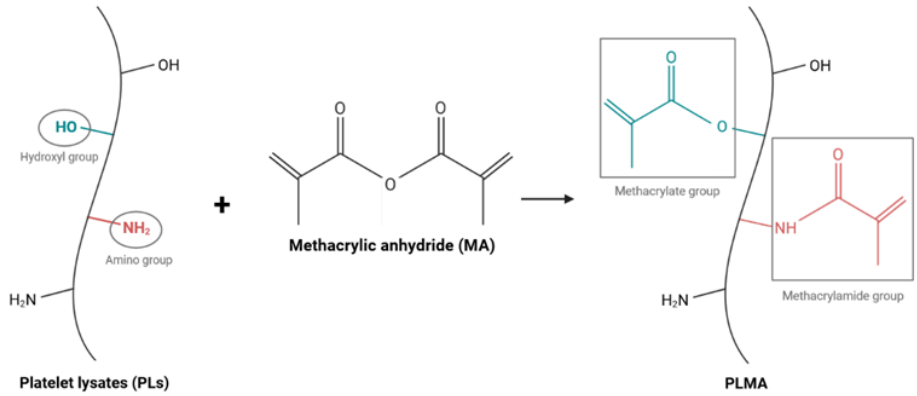


Figure 1.7: Chemical conjugation of platelet lysates with methacrylic anhydride.

Following the PLs reaction with MA, the final PLMA solution undergoes a process of lyophilization. then, the lyophilized PLMA is dissolved in a photoinitiator solution of 0.5% w/v 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone in PBS. The solubility of PLMA

allows it to be dissolved up to a concentration of 20% w/v while maintaining a low viscosity character [53]. For the hydrogel formation, this PLMA solution is subject to a photopolymerization process under ultraviolet (UV) radiation at a relatively low intensity of 0,95 W/cm^2, for 20 to 60 seconds.

1.3.2 PLMA characterization

Several parameters were studied in order to characterize the PLMA hydrogels, which are relevant to understand the potential of these gels for biological and tissue engineering applications.

Firstly, mass spectrometry (MS) was performed in PLs and PLMA100 to evaluate and compare their protein content and modifications. Platelets have a great protein content, with over 1100 different proteins that can be converted to more than 1500 bioactive factors, including growth factors, enzymes and their respective inhibitors, and other factors involved in tissue repair [60]. The MS analysis identified the main protein components in PLs and PLMA100, which are referred in **table 1.1**, and also the total and modified peptide sequences of the PLMA100 formulation. The results show that methacrylation did not significantly affect the protein content in PLMA100, as most of the main components of PLs are present in that formulation. Furthermore, the degree of peptide modification in PLMA100 was 14% and most of the modification sites are in the serum albumin peptides, which is the most abundant protein in PLMA [53].

Table 1.3: Main protein components found in PL and PLMA100 (adapted from Santos et al., 2018).

#	**PL**	**PLMA100**
1	Serum albumin	Serum albumin
2	Apolipoprotein B-100	Serotransferrin
3	Complement C3	Complement C3
4	α2-Macroglobulin	α2-Macroglobulin
5	Serotransferrin	Keratin type II cytoskeletal 1
6	Complement C4-B	Apolipoprotein B-100
7	Keratin type I cytoskeletal 10	Keratin type I cytoskeletal 9
8	Immunoglobulin γ1 heavy chain	Apolipoprotein A-I
9	Keratin type II cytoskeletal 1	Keratin type I cytoskeletal 10
10	Apolipoprotein A-I	Haptoglobin
11	α1-Antitrypsin	Immunoglobulin γ1 heavy chain
12	Haptoglobin	α1-Antitrypsin
13	Keratin type II cytoskeletal 2 epidermal	Keratin type II cytoskeletal 2 epidermal
14	Ceruloplasmin	Immunoglobulin heavy constant mu
15	Keratin type I cytoskeletal 9	Immunoglobulin heavy constant α1

16	Apolipoprotein A-IV	Ceruloplasmin
17	Hemopexin	Hemopexin
18	Immunoglobulin mu heavy chain	Inter-α-trypsin inhibitor heavy chain H4
19	Complement factor B	Keratin type II cytoskeletal 5
20	Complement factor H	Antithrombin-III
21	Immunoglobulin heavy constant alpha 1	Keratin type I cytoskeletal 14
22	Vitamin D-binding protein	α1-B-glycoprotein
23	Complement C5	Acting cytoplasmic 2
24	Acting cytoplasmic 2	Immunoglobulin kappa light chain
25	α1-B-glycoprotein	Immunoglobulin heavy constant γ2
26	Fibrinogen γ chain	Complement factor B
27	Fibrinogen α chain	α1-Acid glycoprotein 1
28	Inter-α-trypsin inhibitor heavy chain H4	Vitronectin
29	Inter-α-trypsin inhibitor heavy chain H2	Inter-α-trypsin inhibitor heavy chain H1
30	Immunoglobulin kappa light chain	Kininogen-1
31	α1-Antichymotrypsin	Fibrinogen α chain
32	Antithrombin-III	α1-Antichymotrypsin
33	Prothrombin	Complement C4-B
34	Apolipoprotein E	Apolipoprotein A-IV
35	Fibrinogen β chain	Angiotensinogen

In terms of mechanical and physical properties, structural characteristics like porosity, stiffness, and water content were evaluated. Cross-section images of PLMA100 hydrogels were obtained by scanning electron microscopy (SEM) to analyze and measure pore size at different degrees of methacrylation and PLMA concentrations. For PLMA100, the hydrogels present a heterogenic matrix and the decrease in pore size with the increase of concentration is minor [53]. Another parameter that does not change significantly with concentration is the water content, that is above 90% for all samples. As mentioned before, these are important features for hydrogels applied in cell culture and tissue engineering, as they may affect cellular survival. Moreover, mechanical properties like the Young's modulus (YM), ultimate strain and ultimate stress were evaluated. The Young's modulus and ultimate stress results show that for all concentrations of PLMA100, these values are low (< 5 kPa for YM and < 15 kPa for ultimate stress) and, therefore, this formulation presented mechanical properties that are more suitable for soft tissues applications.

For the *in vitro* studies, L292 mouse fibroblasts and human-derived adipose stem cells (hASCs) were encapsulated in PLMA hydrogels. The first assay using mouse fibroblasts was performed using PLMA100 at a concentration of 10% w/v for 7 days, and cell viability and proliferation results, obtained using fluorescence microscopy, showed that these cells adhered and survived in the hydrogel. However, the assay using hASCs on PLMA100 at 10% w/v concentration did not present the same successful results. The experiment was repeated using

PLMA100 at a concentration of 15% w/v, which has a stiffer character, and the results showed that this formulation provided a stronger support for cell viability [53]. For further analysis, DNA quantification and MTS assays were performed for these cultures, which confirmed the previously described results.

Beyond the successful cell cultures performed in PLMA hydrogels, another important feature of these gels is the release of bioactive molecules. Protein and growth factors release is associated with hydrogels, as it facilitates cellular processes like adhesion, growth and proliferation. A protein quantification was performed in PLMA100 for the concentrations of 10% and 15% w/v. The results showed that there was a fast release in the first 12 hours, and from that timepoint to the last one, performed at approximately 240 hours, the release of protein content was consistent and sustained [53].

1.4 Objectives

Spinal cord injury, along with several other neurodegenerative conditions, is associated with an extremely inhibitory scaring that does not allow or promote any type of regeneration post-lesion. The biochemical environment established is nearly impossible to come around through traditional methods, which lead to the introduction of regenerative medicine techniques that have been largely investigated over the past years. In this context, biomaterials offer a remarkable wide set of possible solutions as carriers of cells or biological substances that may help overcome some chemical conditions, while also working as scaffolds for the local cells to grow and proliferate.

In this sense, we aimed to work with a natural-based hydrogel, that presents the possibility to be of an autologous source, and study its ability to promote axonal regeneration. For this purpose, we started by evaluating the cellular adhesion of cortical neurons to these gels, and the influence of adding a biological factor – BDNF – or an adhesive coating – poly-D-lysine – to them.

Later, we hypothesized if these gels could act as scaffolds for a three-dimensional growth of axons, so we tested the encapsulation of neurons within PLMA hydrogels. We assessed the viability of neuronal cells when cultured in these gels, and also investigated the possibility of improving that viability by co-culturing neurons with adipose-derived mesenchymal stem cells and glial cells.

Chapter 2

Methods

2.1 Cell preparation

2.1.1 Primary cortical neurons

Primary cortical neurons were obtained from E18 pregnant rats. Following brain dissection, the cortices were treated with trypsin (1.5 mg/mL) and deoxyribonuclease in Hank's balanced salt solution (HBSS) for 15 minutes at 37 ^{o}C. Then, the HBSS solution containing trypsin was removed and the tissue was washed with 1 mL of plating medium containing 10 % fetal bovine serum (FBS), 0,7 % glucose and 1 mM sodium pyruvate. The cortices were centrifuged for 1 minute at 1000 rpm and the plating medium was removed, to remove the last traces of trypsin. A suitable volume of plating medium was added, and the cells were suspended mechanically using a P1000-pipette.

2.1.2 Human-derived adipose stem cells

Human-derived adipose stem cells (ASCs) were cultured in Minimum Essential Alpha Medium (α-MEM), supplemented with 10 % FBS and 1 % antibiotic/antimyotic. After 7 days in culture, the cell suspension was obtained by washing the culture flasks with a 0.05 % trypsin-EDTA solution. After centrifugation at 300 g for 5 minutes, the cells were resuspended in the culture medium described above.

2.1.3 Glial cells

One week before cell culture was performed, glial cells were thawed in glial cells medium, containing 10 % horse serum, 0.7 % glucose and and 1 % penicillin/streptomycin, cultured in 60 mm Petri dishes, and the medium was changed twice during the week. To prepare the cell suspension, two of the Petri dishes were used. The culture medium was removed, and 1-2 mL of 0.05 % trypsin-EDTA solution was added to each dish. Following incubation with this solution at 37 ^{o}C for 1 minute, or until cells started to detach, 1 mL of culture medium was added, and the resulting solution was centrifuged for 5 minutes at 1000 rpm. After centrifugation, the trypsin was removed, and cells were resuspended in the medium described above.

2.2 Cell cultures

2.2.1 Neurons plated on PLMA's surface

10 μL of PLMA100 20 % w/v was added to individual wells of μ-slide angiogenesis plates (ibidi) and polymerized by exposure to a UV light for 40 seconds, at an intensity of 0.95 W/cm^2. Following polymerization, 50 μL of cells suspension in complete Neurobasal medium (NMB supplemented with 2 % B27, 0.5 mM glutamine and penincillin/streptomycin), were added on top of the PLMA hydrogel. Each cell suspension was prepared according to the desired density for each well. As a control, two wells of a μ-slide were coated with 50 μL of poly-D-lysine (concentration), and then washed three times with sterile miliQ water. The cells were plated as previously described for the PLMA hydrogels.

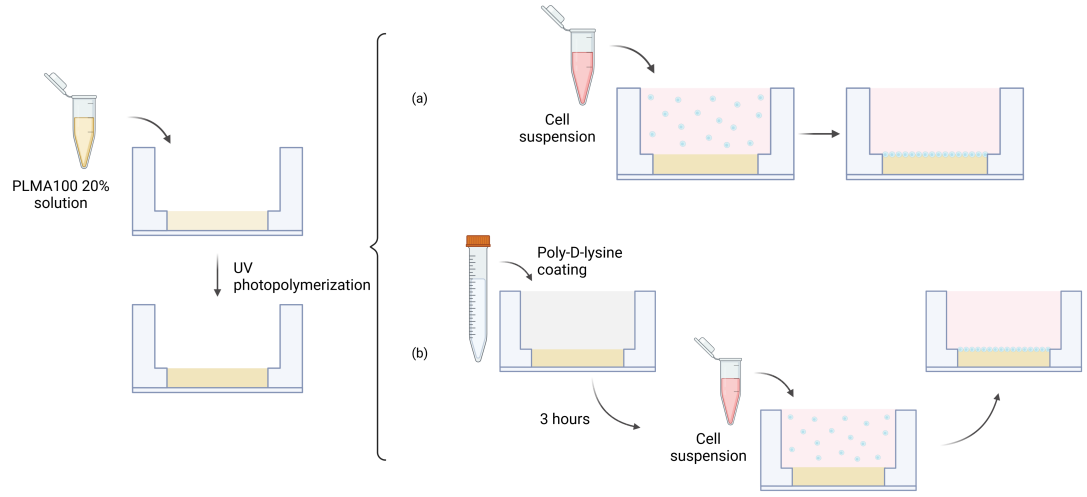


Figure 2.1: Graphic representation of the cell culture method where cells are plated on the top of PLMA100 hydrogels, using a μ-slide angiogenesis plate.

2.2.2 Encapsulated neurons in PLMA

The cell suspension with the desired cellular density was added to the PLMA solution before polymerization. For each hydrogel, 50 μL of the PLMA solution containing the cells were pipetted to a polydimethylsiloxane (PDMS) cylindrical mold with 6 mm diameter. The polymerization process occurred by irradiating the solution in the mold for 20 to 40 seconds with a UV light, with an intensity of 0.95 W/cm^2. Then, the hydrogels were placed in a 48-well plate and 200 μL of culture media were added to each well.

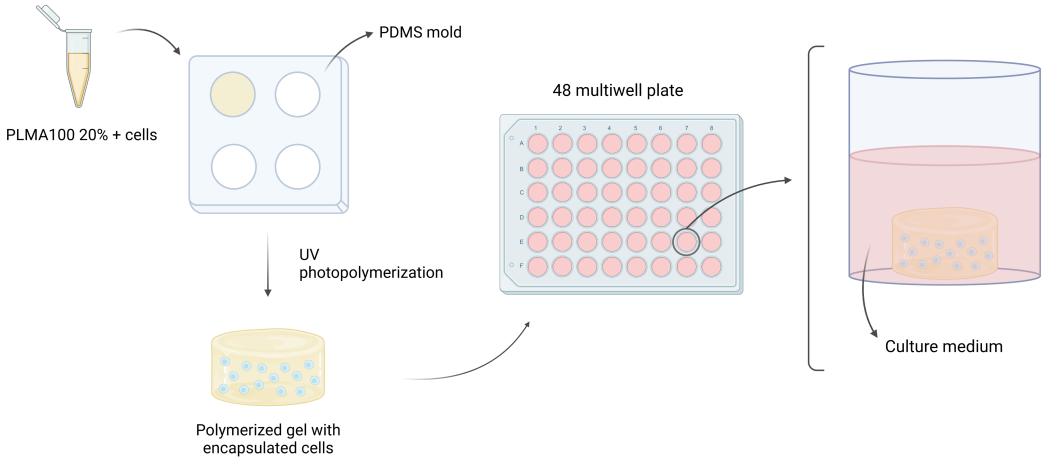


Figure 2.2: Graphic representation of the encapsulation of cells in the PLMA100 hydrogels.

2.3 Immunocytochemistry

2.3.1 Neurons plated in PLMA's surface

The culture medium was removed from each well and cells were fixed for 1 hour with 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Then, the samples were washed 3 times with PBS for 5 minutes each, permeabilized for 20 minutes with PBS-T (PBS + 0.25 % Triton) and blocked for 1 hour and 30 minutes with blocking buffer (3 % Bovine serum albumin (BSA) in PBS). The hydrogels were incubated with primary antibodies overnight at 4 ^{o}C, and after three washes with PBS for 5 minutes each, secondary antibodies for 2 hours at room temperature. The cells were also stained with a Hoechst solution (2 μg/mL) for 30 minutes.

2.3.2 Encapsulated neurons in PLMA

For these cultures, the previous protocol was applied but with some modifications. The culture medium was removed from each well and cells were fixed for 30 minutes with 4 % PFA in PBS. Then, the samples were washed 3 times with PBS for 5 minutes each, permeabilized for 15 minutes with PBS-T and blocked for 2 hours with blocking buffer. The hydrogels were incubated with primary antibodies overnight at 4 ^{o}C, and after three washes with PBS for 5 minutes each, secondary antibodies for 2 hours at room temperature. The cells were also stained with a Hoechst solution (2 μg/mL) for 30 minutes. For the single cultures, samples were incubated with anti-β-III tubulin (mouse) and a goat anti-mouse Alexa-conjugated 568 secondary antibody. For ASCs and neurons co-cultures, samples were incubated with anti-β-III tubulin (mouse) and a goat anti-mouse Alexa-conjugated 488 secondary antibody, and incubated with a phalloidin 594 solution (concentração), diluted in PBS, for 45 minutes at room temperature. For the glial cells and neurons co-culture, samples were incubated with anti-Neurofilament M (chicken), anti-GFAP (mouse) and goat anti-chicken Alexa-conjugated 488 and goat anti-mouse Alexa-conjugated 568 secondary antibodies.

2.4 Imaging and analysis

Images were acquired using a Zeiss LSM 880 with Airyscan microscope. The images for the plated neurons in the PLMA were obtained using widefield microscopy (Zen Blue software), with a 10x or a 20x objective. For the encapsulation samples, the images were obtained using confocal microscopy (Zen Black software), with a 20x or a 40x glycerin objective. Cell death and adhesion, in the plated samples, and neurite length, in the encapsulation samples, were quantified using the ImageJ software.

Chapter 3

Results

From previous results regarding the PLMA100 hydrogel (data not shown), we decided to proceed these studies using the PLMA100 20% w/v formulation since it presented the best results in terms of cellular adhesion and encapsulation compared to other formulations, namely the 10% and 15% w/v. Furthermore, different coatings have also been tested and the poly-D-lysine (PDL) coating was the most promising to improve cell adhesion to the PLMA hydrogels (data not shown).

Cell adhesion is one of the most important features that hydrogels must have in order to be successfully applied *in vivo*. Considering the previous optimizations, we started by investigating if the cell adhesion to the PLMA would vary with different cell densities. Cortical neurons were plated on top of the hydrogels, as described in chapter 2, with three different cell densities: 7500, 10000 and 12000 cells/well. At DIV-3 (data not shown) and DIV-7 (**figure 3.1**), cells were fixed and stained with Hoechst. It was observed that cells would adhere more to the coated hydrogels.

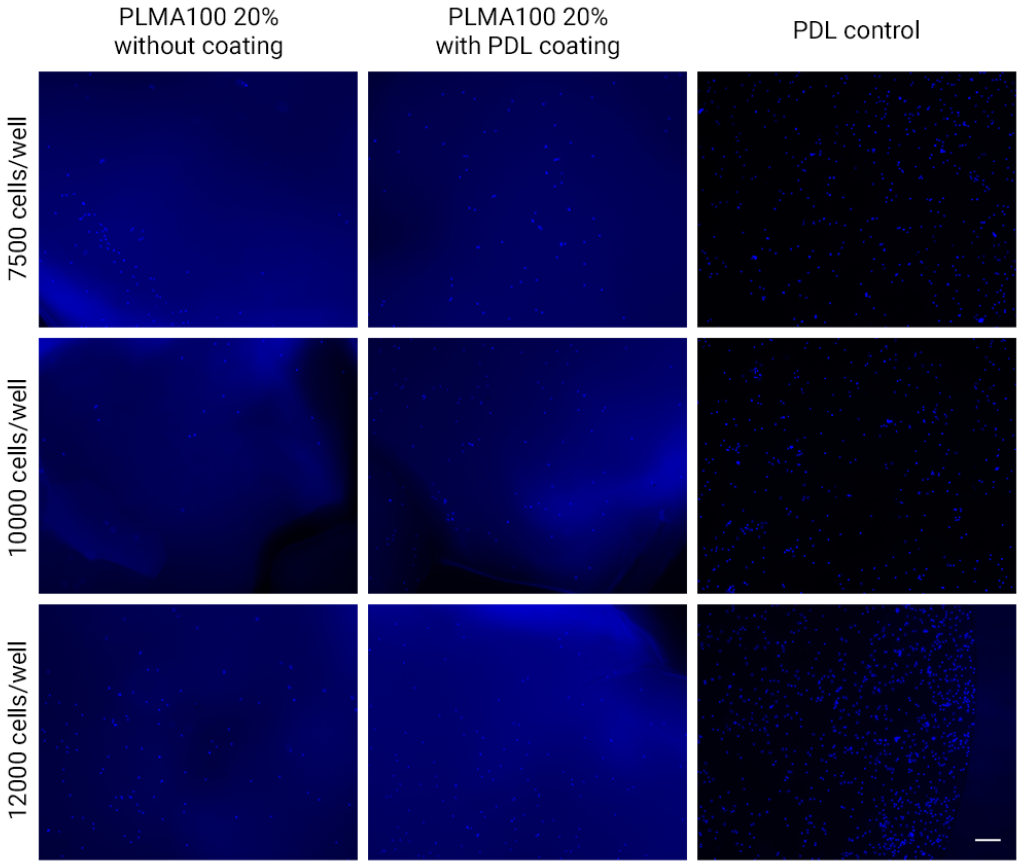


Figure 3.1: **Cortical neurons plated on PLMA100 20%**. Cells were plated at three different cell densities: 7500, 10000 and 12000 cells/well. At DIV-7 cells were fixed and stained with Hoechst, and imaging was performed using widefield microscopy. Scale bar: 100 μm.

To further analyze the previous results, we quantified the number of cells per area of the hydrogel samples at DIV-3 and DIV-7 (**figure 3.2**). Quantification results show that

from DIV-3 to DIV-7 there is a slight increase in the number of adhered cells for the coated hydrogels, while the opposite behavior is observed for the gels with no coating. Furthermore, the adhesion rate increases with higher number of plated cells for the PDL-coated hydrogels. As for the gels without coating, there is a minor decrease in the cellular adhesion from DIV-3 to DIV-7, which might suggest that cells were not surviving between the two time points and, therefore, detached from the substrate.

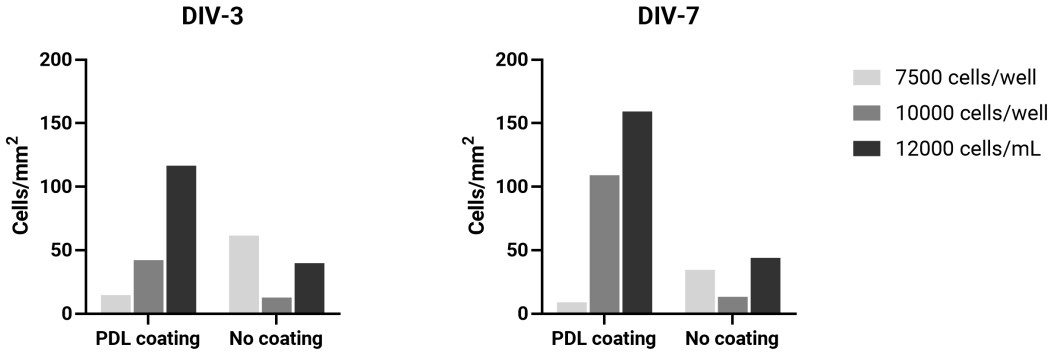


Figure 3.2: **Quantification of the number of adhered cells to the PLMA100 hydrogels per area**. Results show that the adhesion is bigger in the coated gels, and that there is an increase of adhered cells with the increase in the total number of cells plated.

Upon the result observed in the quantification of cellular adhesion, we hypothesized if a further increase in the cell density would improve the cell adhesion rate. Thus, we repeated the previous experiment but with higher cell densities: 15000, 17500 and 20000 cells/well. At DIV-3, cells were fixed and stained with Hoechst (**figure 3.3**). The results are similar to the previous experience, where the coated gels have better adhesion properties. These results suggest that the PLMA hydrogels can be a good scaffold for neuronal cells but further optimization is required.

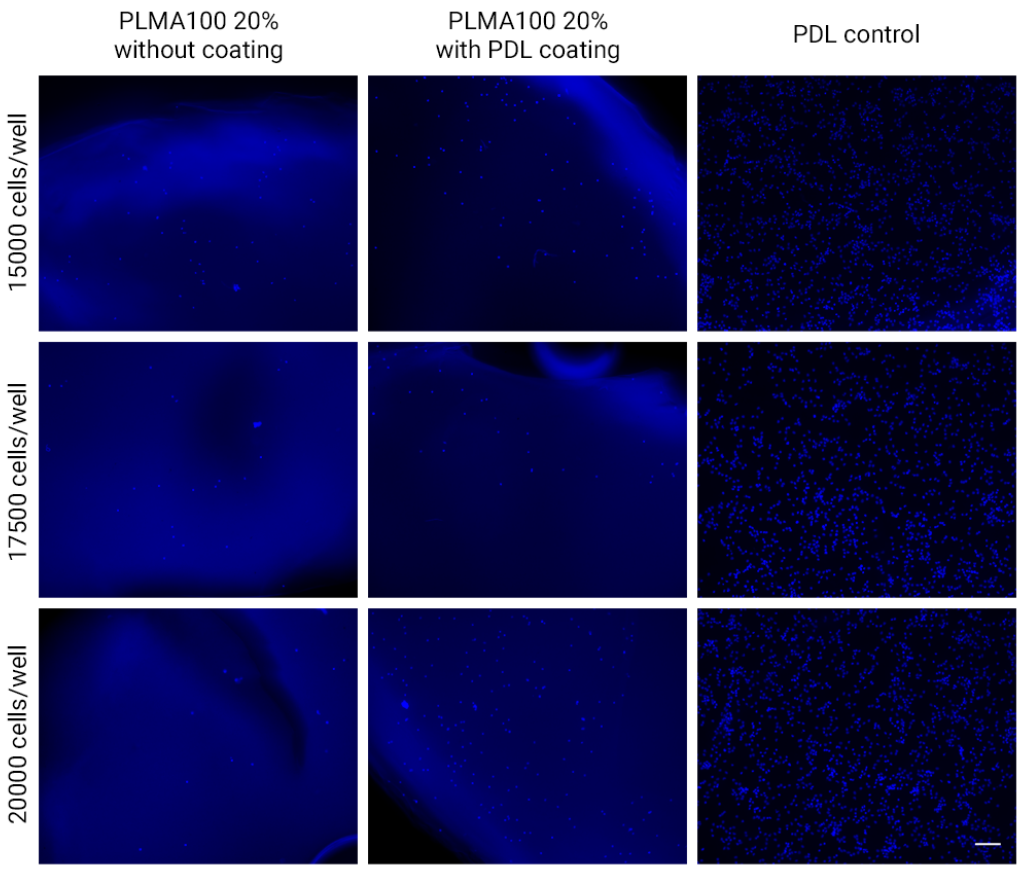


Figure 3.3: **Cortical neurons plated on PLMA100 20%**. Cells were plated at three different cell densities: 15000, 17500 and 20000 cells/well. At DIV-7 cells were fixed and stained with Hoechst, and imaging was performed using widefield microscopy. Scale bar: 100 μm.

We next asked if PLMA hydrogels would be suitable as a 3D scaffold for cell culture and, possibly, as a cell carrier for *in vivo* applications. To assess this possibility, cells were encapsulated in the PLMA matrix and cultured for 7 days with two different cell densities: 0.5×10^6 and 1×10^6 cells/mL. At DIV-7, cells were fixed and stained with Hoechst and against β-III tubulin (**figure 3.4**). Imaging was performed using confocal microscopy of the gels, that were placed on top of a coverslip with mounting media. The Z-stack images were obtained with the 20x and 40x objectives and projected on the XY plan. The cells appear to be well adhered to the PLMA matrix, but the outgrowth of neurites is not clear due to the fragmentation observed in the β-III tubulin staining images.

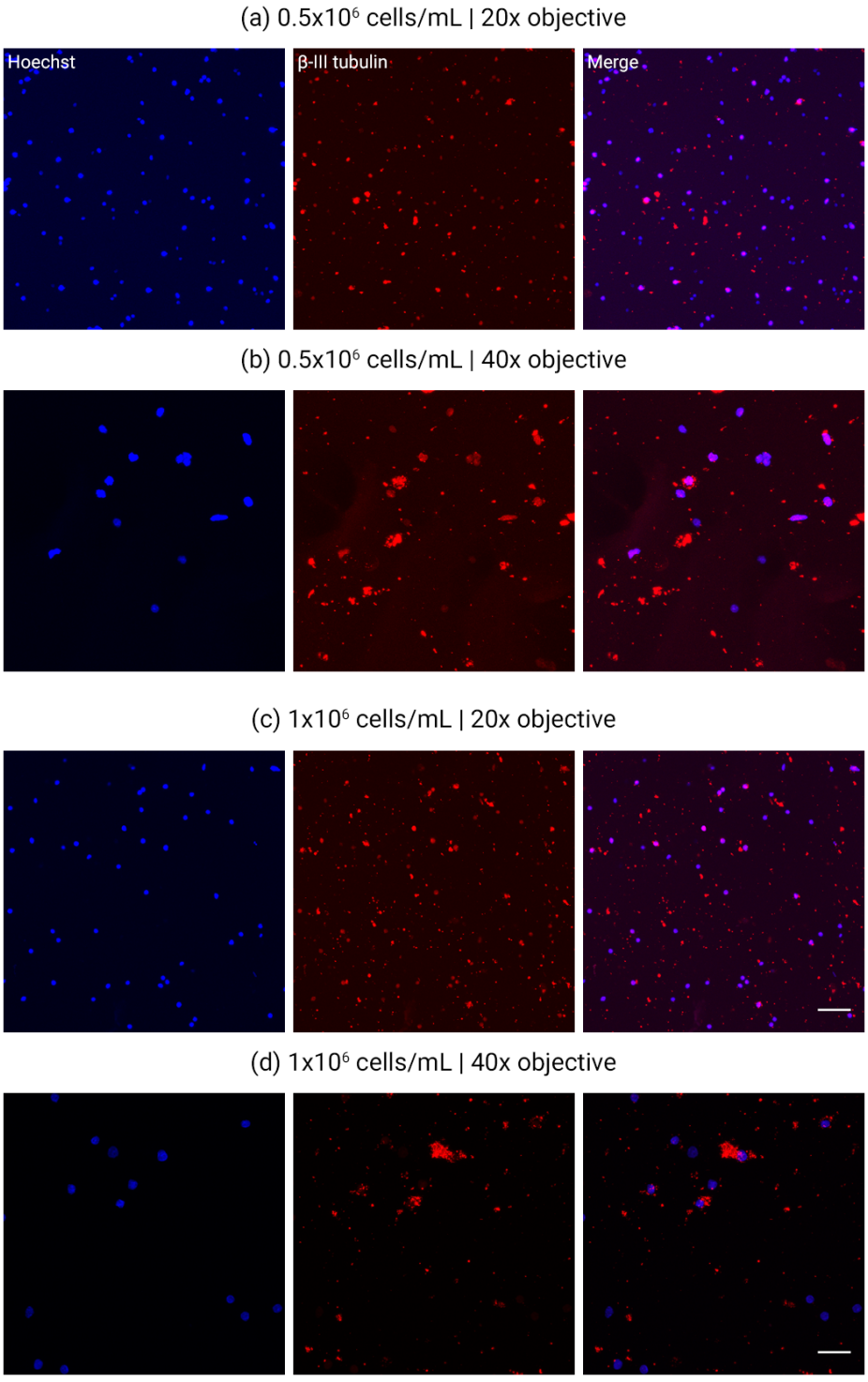


Figure 3.4: **Cortical neurons encapsulated in the PLMA100 20 % hydrogels.** Cells were introduced in the PLMA matrix at densities of 0.5×10^6 (a,b) and 1×10^6 (c,d) cells/mL, and cultured for 7 days. At DIV-7, cells were fixed and stained with Hoechst (blue) and against β-III tubulin (red). Images were obtained using confocal microscopy, by applying the Z-stack function and compressing images in the XY plan. Scale bar: (a,c) 50 μm and (b,d) 25 μm.

Since neuronal development was not clear, we then asked if the increase in the cell density would improve the contact between neurons and, consequently, the intercellular support for cells to grow. So, we increased the cellular density to 2×10^6, 4×10^6 and 8×10^6 cells/mL. At DIV-7 cells were fixed and stained with Hoechst and against β-III tubulin (**figure 3.5**). The results obtained were similar. Once again, the β-III tubulin staining appears to be fragmented within the hydrogels. This could be due to the lack of trophic support leading to degeneration of the neuronal cells or due to a manipulation-induced mechanical stress.

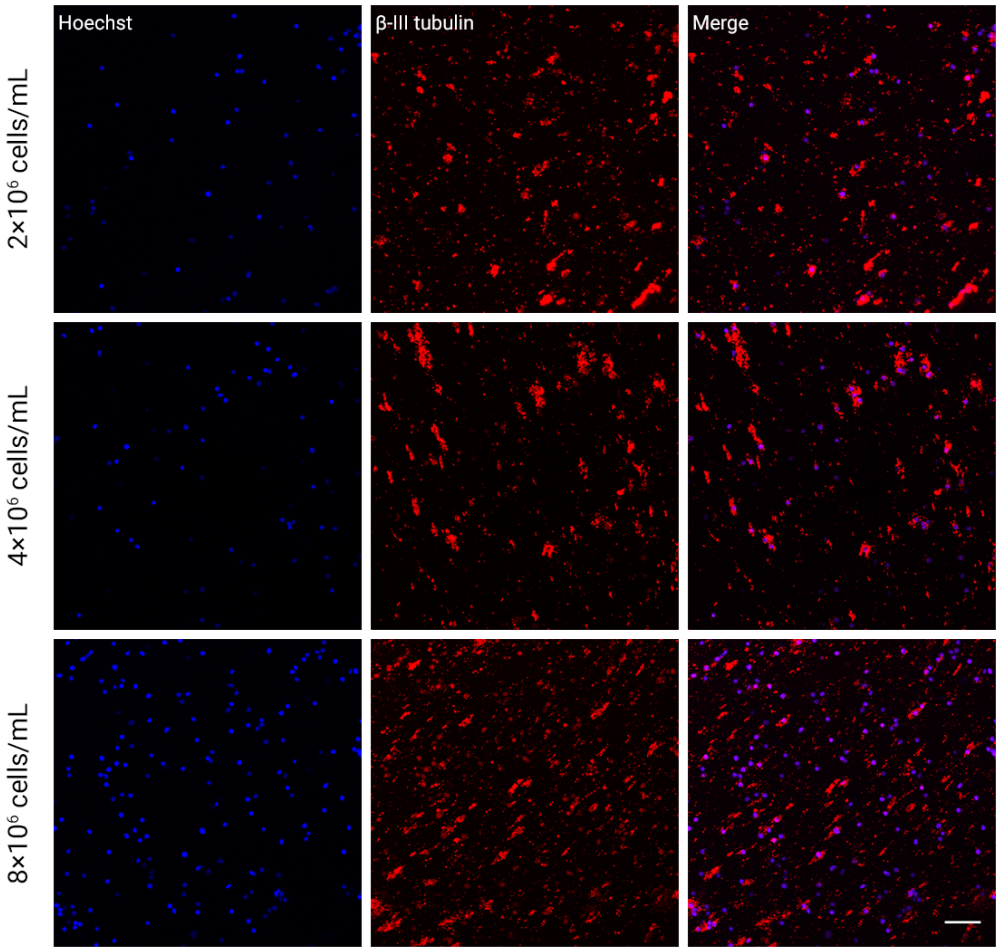


Figure 3.5: **Cortical neurons encapsulated in the PLMA100 20 % hydrogels.** Cells were introduced in the PLMA matrix at densities of 2×10^6, 4×10^6 and 8×10^6 cells/mL, and cultured for 7 days. At DIV-7, cells were fixed and stained with Hoechst (blue) and against β-III tubulin (red). Images were obtained using confocal microscopy, by applying the Z-stack function and compressing images in the XY plan. Scale bar: 50 μm.

In the context of another study, we observed that adipose tissue-derived stem cells (ASCs) promoted neuronal development and neurite outgrowth of cortical neurons. As previously stated, hydrogels can be conjugated with other cell types or biological factors that may possibly enhance the development and outgrowth of neurites. Mesenchymal stem cells, such as ASCs, have been shown to support axonal growth and have a positive effect on neuronal de-

velopment. Furthermore, the culture of ASCs on the PLMA100 hydrogels has been performed successfully, with immunostaining showing good development and proliferation of these cells within the PLMA100 15 % gels. Thereby, we next hypothesized if the co-culture of ASCs with cortical neurons would enhance neuronal survival upon hydrogel encapsulation. The culture of ASCs has been performed successfully at a cell density of 3×10^6 cells/mL, so from there we designed an experiment with two different total cell densities: 1.5×10^6 and 3×10^6 cells/mL, where the ratio of ASCs and cortical neurons was of 1:1. To ensure that ASCs would develop in the PLMA100 20 % hydrogels, we used a control with only ASCs in culture at a density of 3×10^6 cells/mL. After 7 days in culture, cells were fixed and stained with Hoechst and phalloidin, for all samples, and labeled against β-III tubulin, for the co-culture samples (**figure 3.6**). By contrast with the previous experiments, these results showed evident neurite outgrowth, combined with the development of ASCs. However, the fragmentation of the β-III tubulin staining is still present, and by analysis of the nuclear staining, it is possible to conclude that a fraction of the cells did not survive.

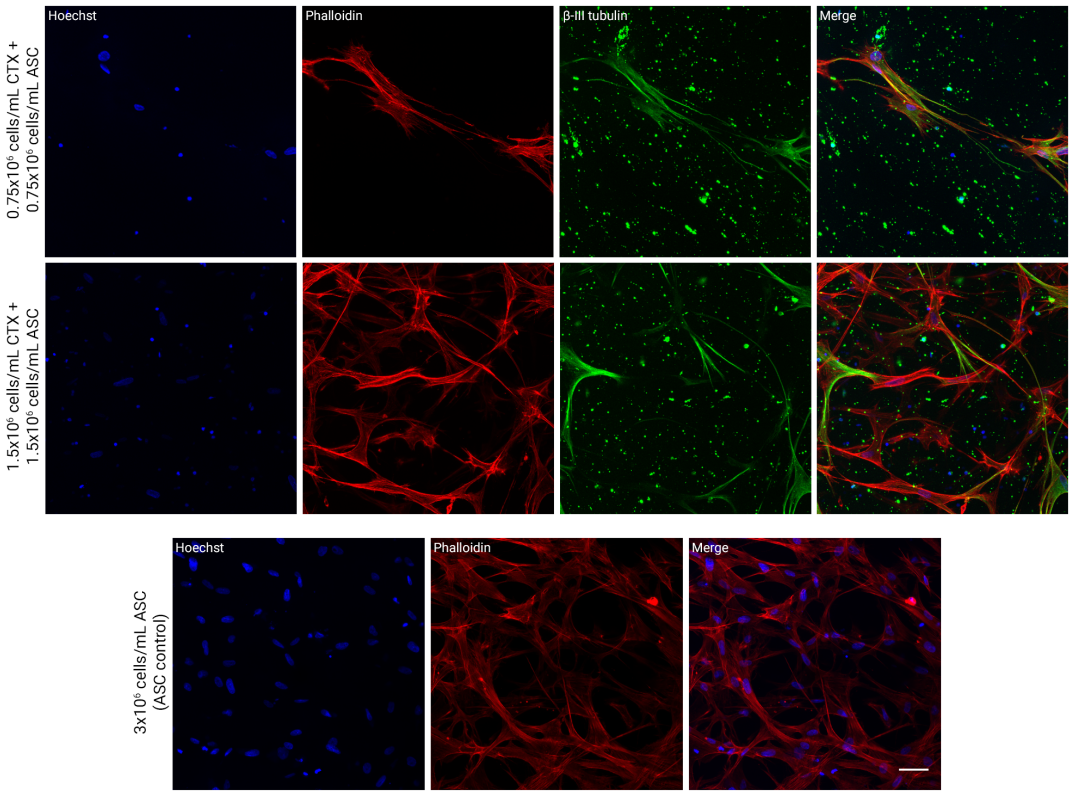


Figure 3.6: **Co-culture of human adipose stem cells (ASCs) and cortical neurons (CTX), encapsulated in PLMA100 20 % hydrogels.** Both cell types were incorporated in the PLMA gel matrix and cultured for 7 days. At DIV-7, cells were fixed and stained with Hoechst (blue) and phalloidin (red), and for the co-cultured samples, against β-III tubulin (green) to highlight neurite outgrowth. Fluorescence images were obtained using confocal microscopy, by applying a Z-stack function and compressing the images in the XY plan. Scale bar: 50 μm.

As previously descried, glial cells are an important component of nervous tissue and have protective and supportive roles towards neuronal cells. Additionally, a study performed by Raimondi et al. [61] shows that the co-culture of astrocytes and neurons in collagen-based hydrogels induces extensive neuronal differentiation. Therefore, we hypothesized if the presence of glial cells within PLMA100 hydrogels would induce neurite outgrowth. For this purpose, , we performed a co-culture at three different time points – 7, 14 and 21 days – and with two cell ratios – 1:5 and 1:10. The total number of cells per gel was fixed at 100.000, and the number of neurons (CTX) and glial cells (GCs) was adapted for each ratio: 90.000 CTX + 10.000 GCs for the 1:10 ratio, and 80.000 CTX + 20.000 GCs for the 1:5 ratio. At the three time points, cells were fixed and stained with Hoechst, and against Neurofilament M and GFAP. The result presented in **figure 3.7** is representative of all time points, despite the images being from the DIV-14 samples. In contrast to what was expected, neither neuronal nor glial development was observed for this experiment. However, it must be taken into consideration that the protocol regarding the extraction of glial cells after their proliferation was not optimized, which could result in their death prior to culture within the PLMA gels.

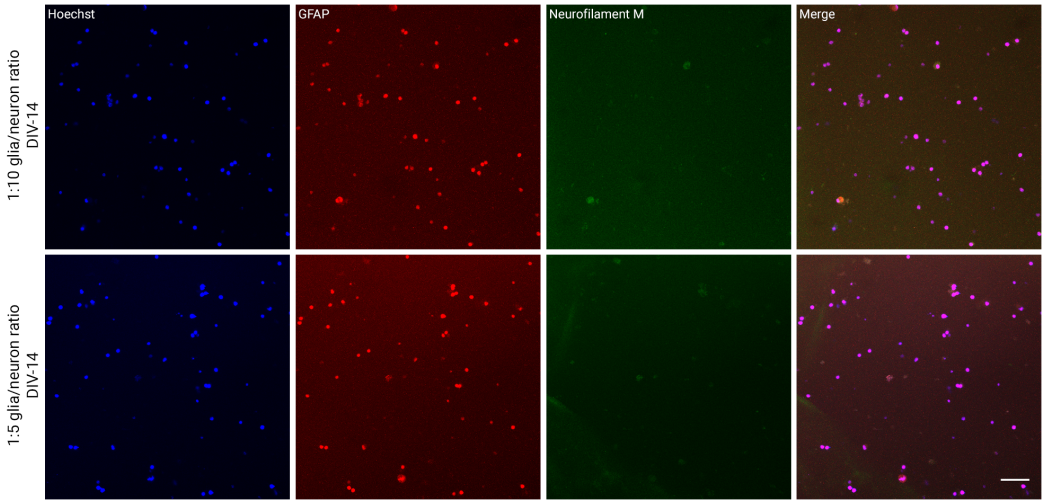


Figure 3.7: **Co-culture of glial cells (GCs) and cortical neurons (CTX), encapsulated in PLMA100 20 % hydrogels.** Both cell types were incorporated in the PLMA gel matrix and cultured for up to 21 days. At DIV-7, DIV-14 and DIV21 cells were fixed and stained with Hoechst (blue), and against Neurofilament (green) and GFAP (red). Fluorescence images were obtained using confocal microscopy, by applying a Z-stack function and compressing the images in the XY plan. Scale bar: 50 μm.

Chapter 4

Discussion

Spinal cord injury is a medical condition that carries a highly negative impact in the quality of life for patients, being associated with both health, economic and social consequences. In addition, the current treatments are extremely limited and do not allow functional recovery of the damaged motor or sensory systems. This is associated with the biochemical environment that is generated at the lesion site, which is highly inhibitory and toxic and translates into a biological barrier for regeneration and, consequently, rehabilitation. Recently, biomaterials have introduced the prospect of new and more complex treatments for a variety of pathologies, including degenerative conditions like SCI. In this context, hydrogels are presented as enhanced biomaterials for regenerative medicine applications due to their versatility in terms of physical properties. Furthermore, the possibility of being designed to fit a specific set of traits also makes them desirable for both *in vitro* and *in vivo* applications. In our study, we worked with a PLMA hydrogel, developed from the chemical modification of platelet lysates, to determine if it could potentially be applied to induce axonal regeneration. Being a natural-based material, PLMA is rich in bioactive substances, and its chemical conjugation provides the ability to manipulate certain mechanical properties. On this note, we proceeded to study if this biomaterial would be suitable to be applied as a 3D scaffold for axonal regeneration, or as a carrier of neuronal cells to the injury site.

The first step was to analyze if cells would be able to adhere to the PLMA hydrogel. Cellular adhesion is an important feature regarding biomaterials when they are sought to be applied *in vivo*, and it is often a point of failure. Following prior optimizations, this studies proceeded using the PLMA100 20 %, conjugated with the addition of BDNF (50 ng/mL) to the culture media. We firstly compared the cellular adhesion to these gels with and without a coating of poly-D-lysine, which is an adhesive molecule, and with different cell densities – 7500, 10000 and 12000 cells per well (**figures 3.1 and 3.2**). Our results clearly show that the presence of an adhesive molecule in the surface of these hydrogels improves considerably the adhesion of cortical neurons. However, this process was not optimal a high number of cells did not adhere. Thus, we hypothesized if an increase in the cell density would strengthen the contact between cells and, accordingly, the cellular support could help the adhesion to the PLMA. For this purpose, we increased the cell densities up to 15000, 17500 and 20000 cells per well (**figure 3.3**) and repeated the previous experiment with the exception that only the DIV-3 time point was performed, since no obvious difference between DIV-3 and DIV-7 was observed. Nonetheless, the increase in the number of cells plated did not induce the expected outcome. The results for this experiment exhibit an identical behavior to the lower densities, where the coated hydrogels have a higher rate of adhesion but still far from the PDL-coated coverslips.

We next investigated if encapsulation of cortical neurons within the PLMA matrix would be a promising strategy. This would allow to investigate the capability of PLMA hydrogels to act as a cell carrier or as a three-dimensional scaffold for neural cells to grow, two conditions that are crucial for an application in SCI. Interestingly, cortical neurons appeared to stay attached to the hydrogel matrix upon encapsulation (**figure 3.4**), contrary to what was observed for the cultures on the PLMA surface. Moreover, the immunostaining against β-III tubulin suggests that neurons might be able to develop and outgrow neurite extensions, but it is likely that degeneration occurred, resulting in the fragmented staining observed in the images. The source of this degeneration can be related to several experimental conditions, and it was firstly thought to be associated with the time of cell fixation. This time was increased relative to the standard cell culture protocol, based on the assumption that cells were not in direct contact with PFA when encapsulated within the PLMA gels. However,

some optimization procedures, we established that the fixation step was set to 30 minutes. Since no change was observed, we next hypothesized if the cell densities were not high enough to have cell-cell interaction inside the hydrogels. After further research, higher cell densities were tested (**figure 3.5**), but the outcomes were similar to prior experiments. There were still some possibilities regarding the degenerated staining observed for β-III tubulin, and one hypothesis was that the antibody was targeting certain components of the PLMA itself. To test this theory, hydrogels with no neural cells encapsulated were incubated with the same antibodies and the previous observations did not occur, confirming that the β-III tubulin was effectively staining a cellular component. Lastly, we thought that, since the encapsulation culture method allowed a slight movement of the hydrogel inside each well, it could be causing the shattering of axons and dendrites. This would require adapting the current protocol for encapsulation and further optimizations that were not performed.

To proceed with this study, we then decided to progress to the co-encapsulation of cortical neurons with other cells. The first co-culture performed was with adipose stem cells, that have a positive effect on neuronal development and were already known to grow and proliferate when cultured in PLMA hydrogels. The ASC control experiment showed an extensive and healthy development of these cells when cultured within PLMA100 20 % hydrogels, which had never been tested. Furthermore, the co-culture results exhibit a clear neurite outgrowth, combined with the ASC development, which represents an interesting outcome (**figure 3.6**). These results are in line with previous observations from our laboratory and others where, which indicate that mesenchymal stem cells induce neuronal survival and development. More importantly, when using PLMA hydrogels, stem cells can provide structural support for neurons to grow. Nevertheless, this experiment has a drawback associated with the culture medium that was used. Even though neuronal development occurred, the α-MEM medium is not the ideal culture medium for cortical neurons, and further experiments using complete NMB should be conducted. We further explore this line of investigation by co-culturing cortical neurons with glial cells. As opposed to what would be expected, this experiment setting did not lead to neuronal development as neither axonal nor glial cells extensions were observed (**figure 3.7**). In theory, the presence of glial cells would induce higher neurite growth since they are inherently associated with supporting the development of nervous tissue. Nonetheless, this has not been proved for the encapsulation in PLMA hydrogels and there are a few considerations that could justify this outcome. The first consideration is the cell dissociation protocol for the glia, which was optimized for a different purpose and could be causing the death of these cells prior to encapsulation. In addition, the substrate of PLMA gels could not be ideal for this type of cells to grow and proliferate, thus they would not be able to provide support to neurons when co-cultured. Equally, the polymerization method of PLMA, that involves the irradiation of cells with UV light, could be harmful for glial cells and be inducing their death. Although the expectations of this assay were not fulfilled, further studies must be conducted to further explore this experimental hypothesis.

Chapter 5

Conclusions

Spinal cord injury is an example of CNS degenerative conditions that are extremely difficult to overcome due to an adverse biochemical environment generated at the lesion site. In this context, axonal regeneration is a key part to induce recovery of nervous functions following SCI.

The main objective of this work was to develop a hydrogel that would be able to induce axonal regeneration. For this purpose, we performed our studies with a platelet-based hydrogel, that was known to be rich in bioactive molecules that may favor CNS neuron regeneration. Thus, we proceeded with the study following two different directions: the first one being related to the cell adhesion properties of the PLMA hydrogel; and secondly the possibility to use these gels as a scaffold for axonal growth and regeneration.

Summarily, the PLMA hydrogel is not an optimal substrate for the adhesion of neuronal cells at its elementary composition. The presence of an adhesive molecule – PDL – can improve the outcome, but it is still far from being optimal. Likewise, the increase between cell interactions by plating a higher number of cells on the PLMA surface did not strengthen its adhesion ability. However, when cells are introduced to the PLMA matrix, the paradigm is different. After encapsulation, cortical neurons appeared to adhere to the hydrogel matrix and, possibly, be able to develop and outgrowth neurites. Our results were not completely conclusive regarding the single cultures of neurons, due to the need of an alternative protocol that would eliminate some factors that could be causing their degeneration. Despite that, the co-culture of CNS neurons with ASCs clearly showed that the presence of these stem cells supported and enhanced neuronal development inside the PLMA hydrogels. This effect, however, may be associated with a structural support or with a paracrine regulation caused by the ASCs secretome. Lastly, we aimed to understand if the presence of glial cells, which are a vital support for neurons in a physiological context, would produce a similar result. Unfortunately, these results were not so successful and additional optimization for the preparation and encapsulation of glial cells is required to determine if GCs are a good supporting cellular population.

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