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Regulation of ECM mimetics during oligodedrocytes differentiation *in vitro*

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Doutor Mário Grãos (Biocant) e do Professor Doutor Carlos Jorge Alves Miranda Bandeira Duarte (Universidade de Coimbra)

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Abstract

Oligodendrocytes (OLs) are the myelin-producing cells of the central nervous system (CNS). Their differentiation undergoes different maturation stages: neural progenitors, oligodendrocyte precursor cells (OPCs), pre-OLs, immature OLs, non-myelinating mature OLs and myelinating mature OLs. The latter provide the insulation of axons and facilitate the conduction of action potentials. Loss of the myelin sheath occurring in demyelinating diseases leads to anomalous nerve transmission and neuronal cell death, as in multiple sclerosis (MS). The failure in remyelination and inhibition of *de novo* oligodendrocytes differentiation observed in neurodegenerative diseases may be partially caused by modifications in the extracellular matrix (ECM) composition and rigidity due to scar tissue formation following lesion of the affected areas. This may result in imbalance of the normal physiological ECM-induced signal transduction into cells present in those areas.

Extracellular matrix composition and its mechanical properties play a key role in cellular differentiation. OLs differentiation is modulated by ECM proteins like laminin, vitronectin and fibronectin. These ECM components bind to and activate integrins, triggering multiple intracellular signalling events, in which the actin cytoskeleton and myosin motor proteins seem to play a key role. For example, laminin-2 (merosin) is known to bind α6β1-integrins and promote OLs differentiation and myelination. Additionally, this differentiation process seems to be directly affected by the substrate elasticity, as showed recently.

Mammalian tissues present different rigidities, ranging from 0.1 to 30,000,000 kPa. Cells also sense the rigidity of their environment when cultured *in vitro* and seem to respond better when on substrates that mimic the stiffness of their native microenvironment. Depending on the substrate elasticity, neural stem cells either become neuronal when cultured on soft substrates (0.01 to 0.5 KPa); or glial, when cultured on slightly stiffer ones (1 to 10 KPa). This effect has been suggested to recapitulate the

development of the CNS, since neurons develop first (on a softer environment) and glial cells later (stiffer environment), hence each cell type is originated *in vitro* in response to its respective compliant substrate.

We have created a setup based on functionalised polyacrylamide hydrogels with different degrees of stiffness, that was used for cell culture. We obtained hydrogels with a range of stiffness between 0.1KPa and 6.5KPa (stiffness of brain ranges from 0.1KPa to 10KPa). Hydrogels were functionalised with poly-D-lysine (PDL), fibronectin (FN), PDL/FN or PDL/merosin, and CG-4 cells [oligodendrocyte-type 2-astrocyte (O-2A) cell line] were successfully seeded on these hydrogels. CG-4 cells cultured on the stiffest hydrogel (about 6.5KPa) in proliferation medium seemed to respond to the substrate stiffness and the ECM proteins tested, displaying a more progenitor-like bipolar morphology or a more branched and differentiated-like morphology more effectively when in presence of fibronectin or merosin, respectively. Additionally, CG-4 cells seemed to differentiate faster on functionalised hydrogels than on coated coverslips when in presence of differentiation medium.

Key words: Oligodendrocytes, extracellular matrix, mechanotransduction, integrins

Resumo

No sistema nervoso central os oligodendrócitos são as células responsáveis pela produção da bainha de mielina. Durante o processo de diferenciação estes passam por diferentes fases de maturação: progenitores neurais, células progenitoras de oligodendrócitos, pré-oligodendrócitos, oligodendrócitos imaturos, oligodendrócitos maduros não mielinizantes e oligodendrócitos maduros mielinizantes. A mielinização é um processo que promove o isolamento de axónios, facilitando a condução de potenciais de ação. A perda da bainha de mielina – desmielinização - leva a uma condução anómala da transmissão nervosa e à morte neuronal. A falha na remielinização e a inibição da diferenciação de oligodendrócitos *de novo* observada nas doenças neurodegenerativas, como por exemplo, na esclerose múltipla pode ser causada em parte por alterações na composição e rigidez da matriz extracelular, devido às lesões do tecido nas zonas afetadas. O que pode conduzir a um desequilíbrio da transdução de sinais entre a matriz extracelular e as células nas zonas afetadas.

A composição e as propriedades mecânicas da matiz extracelular são cruciais para a diferenciação celular. A diferenciação de oligodendrócitos é modulada por proteínas da matriz extracelular, como por exemplo: laminina, vitronectina e fibronectina. Estes componentes ligam e ativam integrinas que por sua vez ativam múltiplas vias de sinalização, nos quais o citoesqueleto de actina e as proteínas motoras, como a miosina, parecem ter um papel importante. Por exemplo, é sabido que a ligação da laminina-2 (merosina) a integrinas α6β1 promove a diferenciação de oligodendrócitos e mielinização. Foi demonstrado recentemente que este processo parece ser afetado pela rigidez do substrato.

Os tecidos dos mamíferos apresentam uma gama de rigidez entre 0,1 e 30.000.000kPa. As células têm mecanismos que as tornam capazes de 'sentir' a rigidez do substrato quando cultivadas *in vitro* e parecem responder melhor em substratos que mimetizem a rigidez do seu microambiente nativo. Em resposta à alteração da rigidez do substrato as células estaminais neurais podem-se diferenciar em linhagens neuronais em

substratos mais moles (0,01 a 0,5 KPa); ou em linhagens de glia quando cultivadas em substratos mais duros (entre 1 e 10 KPa). Este processo tem sido sugerido como uma recapitulação do desenvolvimento do sistema nervoso central, uma vez que os neurónios se desenvolvem primeiro num microambiente mais mole e, seguidamente, ocorre o desenvolvimento das células da glia num ambiente mais rígido. Assim, cada tipo celular pode ser originado *in vitro* em resposta à rigidez do substrato.

Foram criados hidrogéis de poliacrilamida com diferentes níveis de rigidez, os quais foram funcionalizados com diferentes proteínas e, posteriormente, utilizados em cultura de células. Os hidrogéis obtidos apresentam uma gama de rigidez entre 0,1KPa e 6,5KPa (a gama de rigidez do cérebro varia entre 0,1 e 10 KPa). Os géis foram funcionalizados com poli-D-lisina (PDL), fibronectina (FN), PDL/FN ou PDL/merosina e, seguidamente foram cultiva células CG- 4 (linha celular de oligodendrócito-tipo 2-astrócito) nos diferentes géis com sucesso. As células CG-4 cultivadas no gel mais rígido (cerca de 6,5KPa) em meio de proliferação aparentaram responder à rigidez do substrato e às proteínas da matriz extracelular testadas, apresentado uma morfologia mais bipolar, assemelhando-se a células progenitoras de oligodendrócitos ou uma morfologia mais ramificada, assemelhando-se a células diferenciadas, quando cultivadas na presença de fibronectina ou merosina, respetivamente. Além disso, na presença de meio de diferenciação a diferenciação das células CG-4 pareceu ser mais rápida nos hidrogéis funcionalizados do que nas lamelas com ‘coating’.

Palavras-chave: oligodendrócitos, matriz extracelular, mecanotransdução e integrinas

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List of abbreviations

AEP	Anterior entopeduncular area
AFM	Atomic force microscopy
ANOVA	Analysis of variance
bFGF	Basic fibroblast growth factor
bHLH	Basic helix–loop–helix
BMPs	Bone morphogenetic proteins
Cbp	Csk-binding protein
CG-4	Central glial 4
CGE	Caudal ganglionic eminence
CNS	Central nervous system
Csk	COOH-terminal Src kinase
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
E	Elastic modulus
ECM	Extracellular matrix
FAK	Focal adhesion kinase
Fas	Focal adhesions
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FN	Fibronectin
G	Shear modulus

GAP	GTPase-Activating Proteins
GDP	Guanosine diphosphate
GPCR	G-protein-coupled receptor
GTP	Guanosine-5'-triphosphate
H	Hour
hnRNP A2	Heterogeneous nuclear ribonucleoprotein A2
HOG	Human oligodendroglioma
Hz	Hertz
ICAMs	Inter-Cellular Adhesion Molecules
KPa	Kilo Pascal
KO	Knock out
LGE	Lateral ganglionic eminence
MBP	Myelin basic protein
MGE	Medial ganglionic eminence
MF	Microfilaments
MN	Merosin
mQ	MilliQ
MS	Multiple sclerosis
MSCs	Mesenchymal stem cells
MT	Microtubules
NCAM-L1	Neural cell adhesion molecule L1
NEPs	Neuroephithelial precursors cells
NHS	*N*-Acryloxysuccinimide
NPCs	Neural precursors cells

NSCs	Neural stem cells
O-2A	Oligodendrocyte-type 2-astrocyte
OLs	Oligodendrocytes
ON	Overnight
OPCs	Oligodendrocytes precusor cells
PDGFAA	Platelet derived growth factor alpha A
PDGFRα	Platelet derived growth factor receptorα
PDL	Poly-D-lysine
PKC	Protein kinase c
Ptc	Patched
pMN	Motor neuron progenitor
RT	Room temperature
SD	Standard deviation
Shh	Sonic hedgehog
Smo	Smoothened
SVZ	Subventricular zone
T3	Triiodo-L-thyronine
T4	Thyroxine
TEMED	Tetramethylethylenediamine
TH	Thyroid hormones
V	Poisson ratio
VZ	Ventricular zone
Y	Tyrosine

Chapter I

Introduction

I - Introduction

I.1 - Oligodendrocytes

The central nervous system (CNS) is constituted by neurons (the nerve-impulse transmitting cells) and glial cells: macroglia (originated from the neural tube) and microglia (originated from hematopoietic cells). Microglia is the resident population of macrophages of the CNS and has an important function in both immune surveillance and defence. There are two major classes of macroglia: oligodendrocytes (OLs) and astrocytes and these constitute the neuronal-supporting cells of the CNS.[1]

Astrocytes ("star like") have many functions: (i) structural and trophic support for neurons (ii) interaction with blood vessels and induction of the formation of the blood-brain barrier and (iii) regulation of CNS synaptogenesis and synaptic transmission. Oligodendrocytes ("sparsely branching") play a key role in the production of myelin sheath around axons. Additionally, oligodendrocyte precursor cells (OPCs) express voltage- and ligand-gated ion channels and have synaptic input from neurons.[1]

Myelin is a lipid-rich membrane composed by approximately 70% lipid and 30% protein, in contrast with other common membranes, that have only 30-50% lipid composition. The low capacitance, high resistance membrane wraps and electrically insulates axons, allowing for the rapid and efficient propagation of nervous impulses[2]. In demyelinating disorders, the loss of the myelin sheath causes anomalous nerve impulse transmission and neuronal death, leading to incapacity and sometimes decease. Multiple sclerosis (MS) is the most common non-traumatic demyelinating disorder and OLs are lost in the course of the disease[3]. Transplantation and differentiation of OPCs may help to restore nerve fibres re-myelination and function in future clinical applications.

I.1.1 - Oligodendrocytes differentiation

I.1.1.1 - Oligodendrocytes differentiation during development

The molecular events underlying oligodendrocyte proliferation, survival, and maturation are poorly understood, yet knowledge of these processes is of great value for the development of therapeutics for demyelinating diseases like MS. OLs differentiation goes through several identifiable maturation stages characterized by distinct morphological and antigenic changes (which can be used as lineage and maturation markers), before finally associating with axons and elaborating myelin sheaths. Such stages include precursor cells, oligodendrocyte-type 2-astrocyte (O-2A) progenitors, pre-OLs, immature OLs, non-myelinating mature OLs, and myelinating mature OLs [4] (Figure 1).

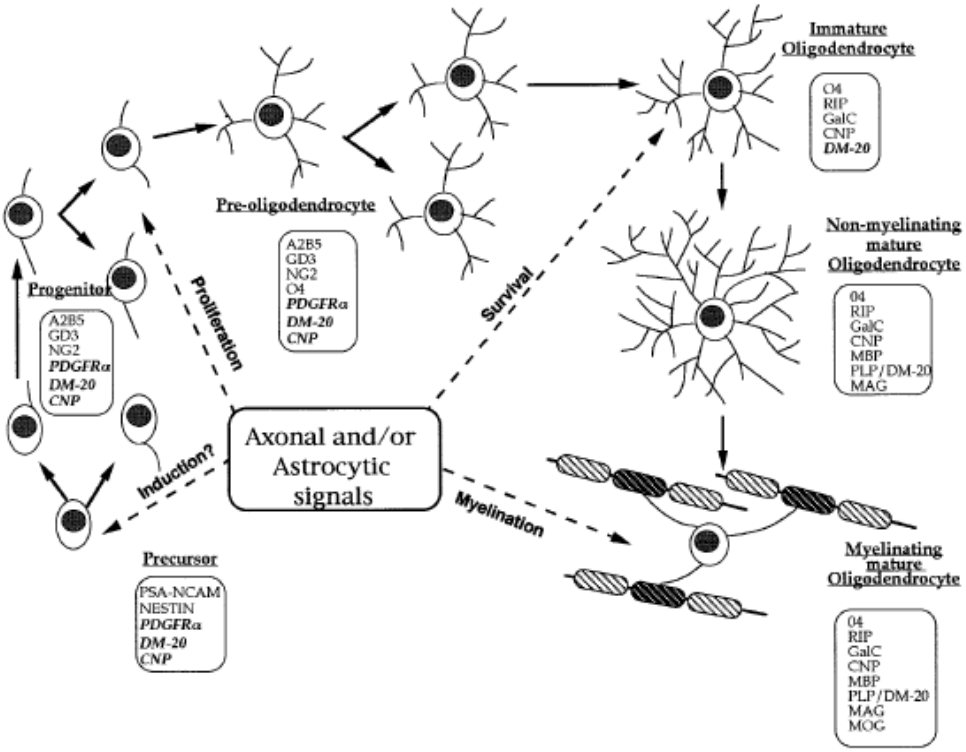


Figure 1| The oligodendrocyte (OL) lineage. Morphological and markers expression from precursor cells to myelinating mature oligodendrocytes, through progenitors, pre-oligodendrocytes, and immature non-myelinating oligodendrocytes. Timing of neuronal and astrocytic signaling is indicated. Stage-specific markers and RNA (italic) are boxed (Adapted from Baumann et al. 2001).[5]

The neuronal and glial cell types present in the mature CNS are generated from an initially homogeneous pool of neural precursor cells (NPCs) in the embryonic neural tube, the so-called neuroepithelial precursor cells (NEPs). The neurons are generated first, followed by glial cells[1]. In the spinal cord, the first OPCs are generated from NEPs from a specific domain of the ventricular zone (VZ) called motor neuron progenitor (pMN) that also generates motor neurons[1]. OLs in the spinal cord are derived from both ventral and dorsal sources[6]. Generation of the ventral precursors starts approximately two days earlier than that of the dorsal precursors[6] (Figure 2). In the forebrain three waves of oligodendrocyte production have been described. In mice, oligodendrocyte precursors are first generated from the medial ganglionic eminence (MGE) and anterior entopeduncular area (AEP) at embryonic day 11.5; from the lateral and caudal ganglionic eminence (LGE-CGE) around embryonic day 15 and from the cortex after birth. Both in spinal cord and forebrain, oligodendrocyte precursors seem to compete for populating the available area. In the postnatal forebrain, the most ventral oligodendrocyte precursors die and the more dorsally-derived oligodendrocyte precursors predominate[7]. When OPCs first appear, they have a distinctive radial morphology. Radial glia may differentiate in both oligodendrocytes and astrocytes[1] and before OLs migrate, they must be specified[8].

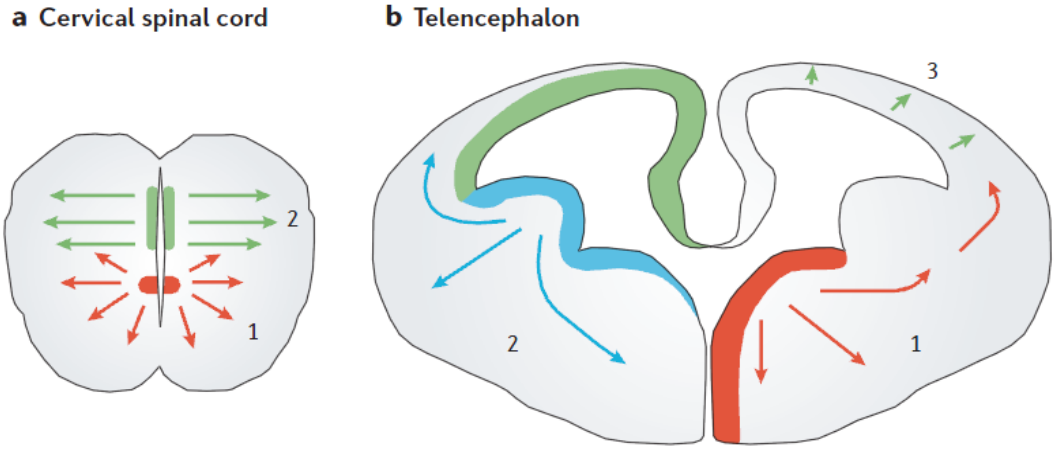


Figure 2| Origins and migration of oligodendrocyte precursors in the rodent cervical spinal cord and telencephalon. A) In the mouse spinal cord, ~85% of OPCs from pMN in the ventral ventricular zones (1), starting at about embryonic day 12.5. About embryonic day 15 a second wave of precursors starts in more dorsal regions by trans-differentiation of radial glia[9]. B) In the telencephalon, the ventral-most precursors in the MGE are produced from about embryonic day 12.5 (1), production of the LGE derived precursors starts a few days later[10](2) and production of the cortex-derived precursors occurs mainly after birth (3)[6]. (Adapted from Richardson *et al.* 2006).[6]

Initial specification of spinal cord oligodendrocytes takes place during embryonic development. This process requires precise interplay between cell-intrinsic and regionally-restricted extrinsic factors[8]. A program of NEP cell fate specification is initiated by diffusion of Sonic hedgehog (Shh) from the notochord that generates a morphogenetic gradient and complementary signals from the roof plate[1]. Ventral oligodendrogenesis is dependent on Shh signaling, involving activation or repression of a set of homeobox, paired-box (Pax) and basichelix–loop–helix (bHLH) transcription factor genes. Expression of bHLH transcription factors Olig1 and Olig2 occurs during embryonic development in the ventral spinal cord, in the specialized domain that also gives rise to motor neurons (pMN), and in the ventral forebrain. Olig expression is essential for OL-lineage specification and seems to persist postnatally in the spinal cord and brain[7]. The mechanistic details of Shh signaling in vertebrates is still unclear. Shh binds to its transmembrane receptor Patched (Ptc), causing disinhibition of its co-receptor Smoothened (Smo), a seven-pass transmembrane G-protein-coupled receptor (GPCR), eventually promoting nuclear translocation of the full-length form of the transcription factor family Gli (Figure 3).

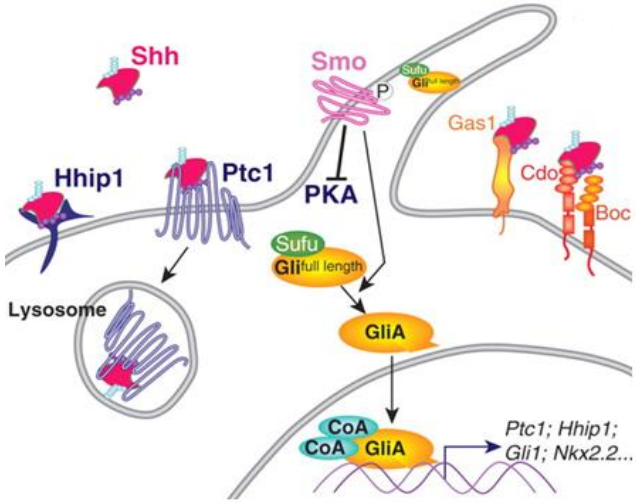


Figure 3| The Shh signalling pathway involves two transmembrane proteins: Patched (Ptc) and Smoothened (Smo). Smo acts as a signal transducer, but in the absence of the Shh, Ptc interacts with and inhibits Smo. In the presence of the ligand, the interaction of Ptc and Smo is loss and Smo is no longer inhibited. Gli proteins may then enter the nucleus and function as transcriptional modulators. Mouse Gli1 is not proteolytically cleaved and acts as a transcriptional activator with redundant functions during development, while Gli2 possesses both activator and repressor activities. Gli3, on the other hand, is thought to act as an inhibitor of Shh signalling[1]. (Adapted from reference Miller 2002).[11]

Downstream signalling that involves Gli transcription factors leads to high expression of Olig1 and -2, and results in oligodendroglial differentiation of OPCs and eventually in myelination[9]. Olig 1 and 2 are important effectors in oligodendroglial fate decision[9]**.**

Another factor that stimulates oligodendrogenesis is platelet-derived growth factor (PDGF), a survival factor and a mitogen for various cell types including neurons and oligodendrocytes. PDGF stimulates the proliferation of OPCs, and in the absence of PDGF, OPCs exit the cell cycle and differentiate prematurely. Additionally, PDGF inhibits neuronal and astrocytic differentiation of neural stem cells (NSCs) by inducing oligodendrocyte differentiation *in vitro*. PDGF also plays a direct role during myelination.[9]

Development of OLs is promoted by thyroid hormones (TH), which induce proliferation of OPCs, promote their differentiation, and finally enhance morphological and functional maturation of post mitotic oligodendrocytes. TH-enhanced myelination is delayed in hypothyroid animals.[9]

Oligodendrogenesis may be activated by cell–intrinsic determinants, like Sox genes. For example Sox-9 is necessary for glial fate decision of NSCs in the developing spinal cord. Sox9,-10, and -17 are required for developmental oligodendrogenesis, hence the Sox family plays a key role in OLs development.[9]

Oligodendrocytes differentiation is inhibited by Notch downstream targets Hes1 and Hes5, which inhibit oligodendroglial differentiation and promote astroglial fate. Bone morphogenetic proteins (BMPs) also promote astrocytes differentiation and reduce neuronal and oligodendroglial differentiation of cultured NSCs and *in vivo*, during development. Wnt signalling also inhibits generation and maturation of OLs. Other inhibitors of oligodendrocytes differentiation are protein kinase C (PKC), RhoA, and ROCK[9] (the mechanism of action of Rho proteins is addressed below in greater detail).

The final step in the generation of oligodendrocytes is the development of a mature myelinating phenotype, and this is largely regulated by axonal signals[11] and extracellular matrix (ECM) components. For example, knock out (KO) mice for the laminin-α2 subunit of the extracellular protein laminin exhibit a severe myelin deficit in the

forebrain[7]. ECM components play a key role regulating proliferation, migration, and differentiation of OPCs,[9] which will be explored in more detail below.

I.1.1.2 -Adult oligodendrocytes differentiation

In the adult CNS, new oligodendrocytes are derived from adult OPCs located throughout the white and grey matter. Another source of new OLs is from progenitor cells in the sub ventricular zone (SVZ)[12,13]. Adult OPCs are derived from the same progenitors as embryonic OPCs, and the two cell types show many similarities. However, the adult OPCs have a longer basal cell cycle time and a slower rate of migration[13]. PDGF and fibroblast growth factor (FGF) can induce proliferation and migration of OPCs *in vitro*, and are both up regulated during myelination[13].

There are four points of evidence suggesting that OPCs are the major source of remyelinating oligodendrocytes: (i) presence of dividing cells in normal adult white matter (which are likely but not proven to be adult OPCs) give rise to remyelinating oligodendrocytes; (ii) transplanted OPCs remyelinate areas of demyelination with great efficacy; (iii) demyelination areas in which both oligodendrocytes and OPCs die are repopulated by OPCs before new oligodendrocytes appear and (iv) cells with sequential expression of OPC and oligodendrocyte markers can be identified at the onset of remyelination.[13]

When myelin is lost, local OPCs undergo an activation step and switch from an essentially quiescent state to a regenerative phenotype. Activation is the first step in the remyelination process and involves both morphological changes and up regulation of several genes, which are associated with the development of oligodendrocytes (Olig2, NKX2.2, MYT1 and Sox2)[13]. Microglia and astrocytes are the major source of factors that induce OLs proliferation in response to injury. Following the activation step, OPCs are recruited, involving migration of OPCs in addition to the ongoing proliferation. OPCs must then be differentiated into remyelinating oligodendrocytes. The differentiation phase comprise three stages: establishing contact with the axon, expressing myelin genes and

generating a myelin membrane, and finally wrapping and compacting the membrane to form the sheath[13](Figure 4).

There are several factors that affect adult oligodendrogenesis, which include direct cellular interactions with astrocytes and/or microglia/macrophages, growth factors, cytokines, extracellular matrix molecules, and local neurotransmitters. Adult oligodendrogenesis may be affected by gender, age, pregnancy (the level of cell death and demyelination is lower in pregnant rats than in non-pregnant ones) and genetic background[12,13]. Nevertheless, remyelination may be incomplete or fail in case of specific diseases, such as in MS[7,13,14].

During early stages of MS, remyelination occurs, but this repair mechanism fails for

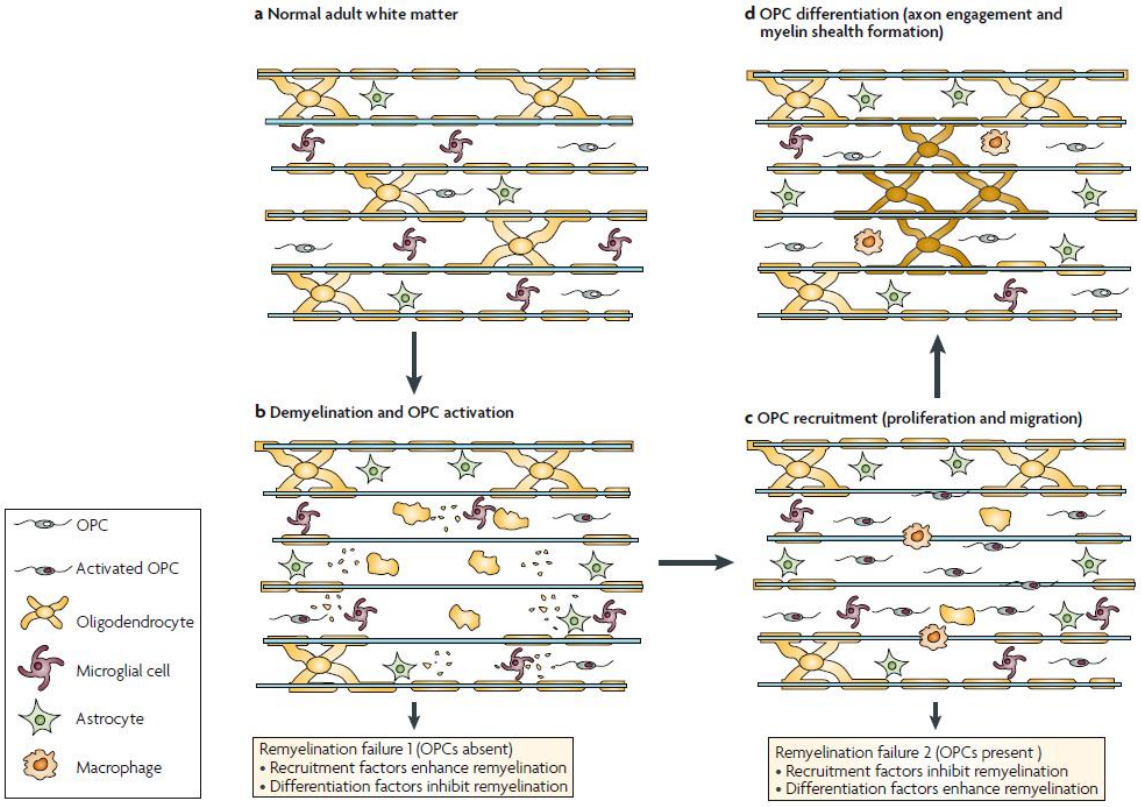


Figure 4| Phases of remyelination. A) Normal adult white matter contains myelinating oligodendrocytes, astrocytes, microglia and OPCs. B) OPCs activation. Following demyelination, microglia and astrocytes become activated, leading to the activation of nearby OPCs. C) Recruitment phase. Activated OPCs respond to mitogens and pro-migratory factors generated mostly by reactive astrocytes and inflammatory cells, resulting in proliferation and migration of OPCs in the demyelinated area. Macrophages eliminate the myelin debris. D) Differentiation phase. In the final phase of remyelination, the recruited OPCs differentiate into remyelinating oligodendrocytes.(Adapted from Franklin *et al.* 2008) [13]

as-yet unclear reasons. One typical reason put forward is that remyelination failure is due to depletion of OPCs after successive episodes of demyelination at the same site, finally exhausting the capacity of the system to adequately remyelinate[7]. Another reason is the presence of inhibitory factors or absence of stimulatory factors at the lesioned areas[5]. For successful myelination and remyelination to occur, it is also required that a particular balance exists between extracellular adhesion, matrix rigidity and intracellular contractile forces mediated by the oligodendrocytes actomyosin cytoskeleton[14](see below).

I.1.2 - Oligodendrocytes differentiation, ECM and integrins

Interactions between cells and their surrounding microenvironment (cellular niche) are crucial for the establishment and maintenance of cellular properties. The extracellular matrix (ECM) is a complex mixture of matrix molecules which are typically glycoproteins, including fibronectins, collagens, laminins and proteoglycans that assemble into fibrils or other complex macromolecular arrays. ECM components engage intracellular signal transduction through activation of transmembrane proteins called integrins.[15-17]

Integrins are heterodimeric transmembrane protein receptors that bind to ECM proteins and mediate cellular adhesion and signalling between the external environment, and the intracellular milieu (namely the cytoskeleton and signal transduction proteins). Ligand binding to the extracellular domain of integrins leads to integrin clustering and recruitment of actin filaments and signalling proteins to the cytosolic domain of integrins. ECM attachment and signalling centers are called focal complexes when they are still nascent and forming, or focal adhesions (FAs) when they have matured into larger and more stable complexes[18].

Integrins are composed by one of the 18 types of α subunits and one of the 8 types of β subunits identified so far, forming one of the 24 known combinations of integrin heterodimeric receptors. The α chains have a central role in determining ligand specificity, β subunits have a cytoplasmic tail that can bind to several intracellular anchor proteins, including talin (which plays an important role to link integrin clusters to the actin cytoskeleton), α-actinin [connects actin fibrils to the cytoplasmic tail of transmembrane receptors such as integrins, cadherins and inter-cellular adhesion molecules (ICAMs)] and

filamin (links actin filaments in orthogonal networks or parallel bundles). These anchor proteins can bind to actin or to other anchor proteins, thereby linking the integrin to actin filaments in the cellular cortex (Figure 5). This linkage leads to a clustering of the integrins and the formation of focal adhesions between cells and the ECM. Hence, integrins behave as signal transducers that trigger several signalling pathways when activated by the extracellular matrix upon binding[19].

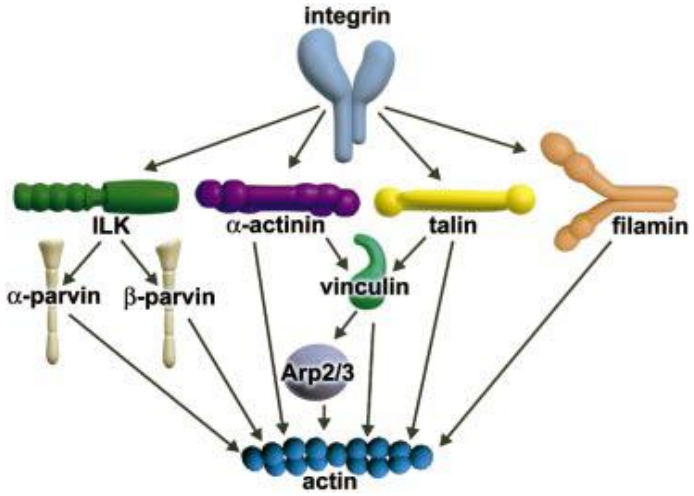


Figure 5| Overview of different partners through which integrins can link to the actin cytoskeleton[18]. Adapted from (Brakebusch *et al.* 2003).[18]

During development, the expression of integrins is regulated, and different cell types express distinct sets of integrins, depending on their lineage and differentiation stage. Oligodendrocytes express different types of α and β subunits depending on the differentiation stage and each has distinct roles during development: αvβ1 promotes migration, αvβ3 proliferation, αvβ5 differentiation and α6β1 both differentiation and survival of newly formed OLs[20] (Figure 6). The expression of integrins during oligodendroglial maturation is not only regulated by the genetic developmental program, but is also affected by ECM components, influencing the integrin expression repertoire of OLs[21].

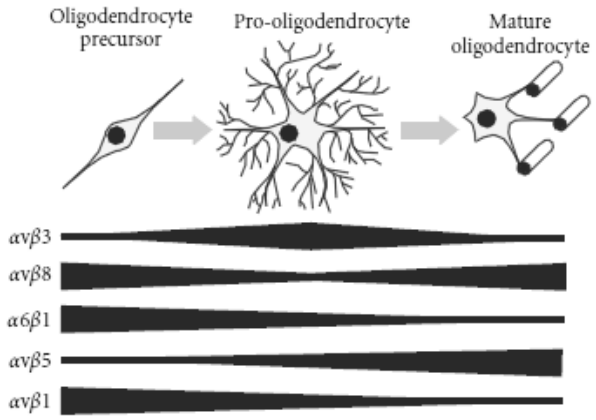


Figure 6| Representation of integrin receptor expression over the course of oligodendrocyte development. Oligodendrocytes originate as precursor cells with simple morphology and subsequently differentiate into pro-oligodendrocytes, characterized by extension of intricate process meshwork. The integrin expression profile changes along the maturation stage of the cell, as indicated. Adapted from (O'Meara et al. 2011).[21]

Activated integrins recruit and activate Src family kinase (SFK) proteins. SFKs are non-receptor tyrosine kinases that integrate external signals received through both integrins and growth factor receptors[22]. One of these SFKs, Lyn, is expressed in the oligodendroglial lineage. Another one, Fyn, is expressed in the brain (neurons and glial cells), but the peak of its activity during development can be correlated with myelination and is involved in the oligodendrocyte differentiation process[20].

Lyn is associated with the PDGFR–αVβ3 integrin complex and upon activation, contributes to proliferation of OPCs. Integrin αVβ3 activation promotes phosphorylation of Lyn on tyrosine residue Y397 and its subsequent activation [22]. On the other hand, C-terminal Src kinase (Csk) and Csk - binding protein (Cbp) are up regulated in the absence of laminin and phosphorylate Fyn on inhibitory tyrosine residue (Y531)[22,23] (Figure 7-1). Hence, activation of αVβ3 integrin and Lyn (for example by fibronectin) mediates OPC proliferation and, in the absence of laminin signalling through α6β1 integrin and Fyn, final differentiation of OPCs into OLs is restrained (Figure 7-1).

OPCs differentiation into oligodendrocytes requires activation of Fyn, and its kinase activity and expression level are up regulated during this final differentiation stage. Fyn is activated by integration of signals triggered by laminin (present in the extracellular matrix) through integrins (e.g.: α6β1) and axonal neural cell adhesion molecule L1 (NCAM-

L1), which binds to the contactin–integrin complex. Full activation of Fyn is thus achieved by differential phosphorylation of the two regulatory tyrosines of Fyn[24] (Figure 8.IA). Laminin binding to integrins promotes dephosphorylation of the inhibitory C-terminal tyrosine (Y531), allowing the kinase to become fully activated by single phosphorylation of the activating tyrosine (Y420)[22] mediated by the L1-contactin axis (Figure 8.I-A and 8.I-B). Laminin signalling suppresses Csk and Cbp, restraining phosphorylation of the inhibitory tyrosine residue (Y531) and thus promoting Fyn activation and oligodendrocyte differentiation (Figures 7-2 and 8.II)[23].The individual effects of laminin or L1 lead to similar levels of activation of Fyn, but by different mechanisms, while the combined effect of both synergistically results in maximal activation of the kinase (Figure 8).

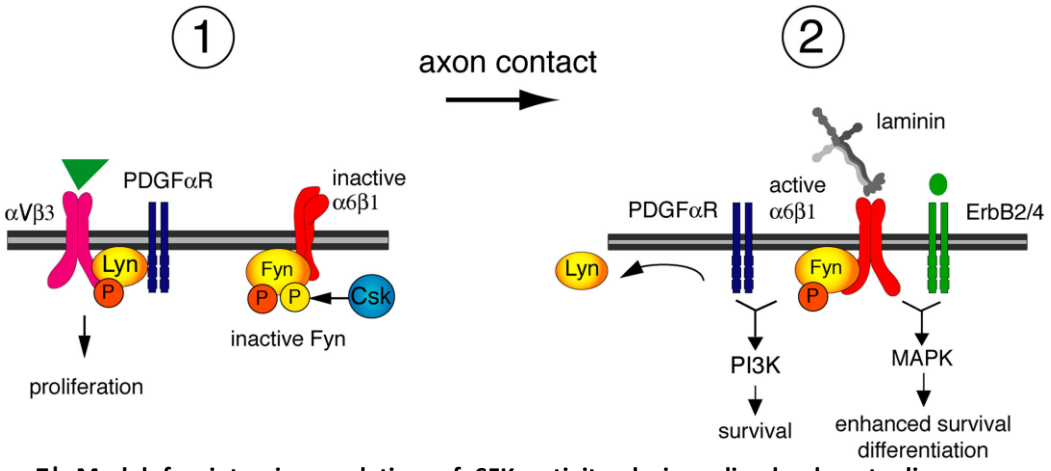
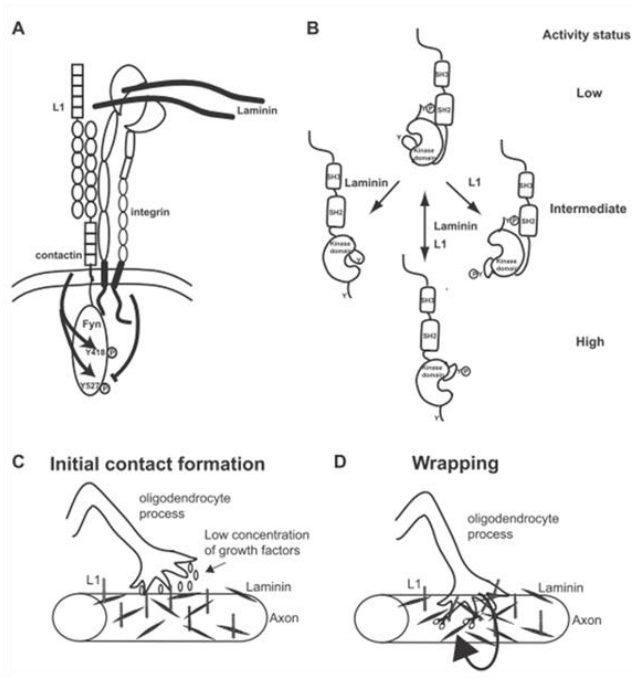


Figure 7| Model for integrin regulation of SFK activity during oligodendrocyte lineage progression (Adapted from Colognato *et al*. 2004).[22]

Active Fyn phosphorylates heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2) and promotes the local translation of myelin basic protein (MBP) mRNA at OL processes. Furthermore, it has been reported that Fyn activity leads to enhanced transcription of the MBP gene. Moreover, Fyn plays an important role in myelination and its absence in the CNS reduces levels of MBP and leads to hypomyelination.[23] Fyn is also a regulator of the cell shape, through modulation of Rho family GTPases (Rho, Rac1 and Cdc42) that regulate the polymerization of actin and thus control cellular cytoskeletal structure and shape[25] (Figure 9). Fyn activation via α6β1-integrin regulates the activity of Rac1, Cdc42 and Rho. Rac1 and Cdc42 are activated [bind guanosine-5'-triphosphate (GTP)] by Fyn, while Rho is inactivated [bind guanosine diphosphate (GDP)] by this kinase.

GTP-bond Cdc42 and Rac1 promote the formation of filamentous actin, while GDP-bond Rho inhibits depolymerisation of actin filaments and the actomyosin contractility, thus, these proteins have an impact on cell morphology. Integrins signalling has proven to play a role in GTPase-mediated oligodendrocyte morphological differentiation. Depletion of activated Fyn in oligodendrocytes depletes GTP-bond Rac1, GTP-bond Cdc42 and GDP-Rho. This altered GTPase activity is accompanied by morphological defects, highlighting the importance of these proteins in oligodendroglial process extension. Ablation of Cdc42 or Rac1 also results in aberrant myelination[26].

8.I



8.II

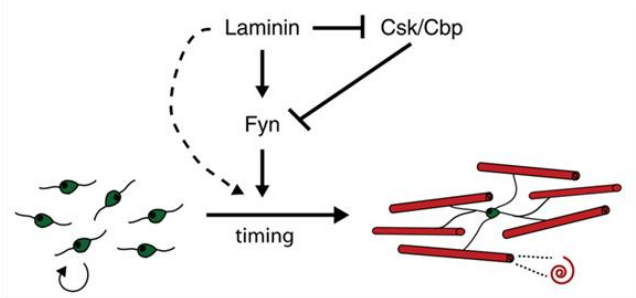


Figure 8| Schematics of Fyn activation. 8.I) Fyn activation is differentially regulated by phosphorylation after stimulation with axonal L1 and laminin. L1 induces phosphorylation of both Tyr-420 and Tyr-531, whereas laminin induces dephosphorylation of Tyr-531[24]. Addapted from (Laursen *et al.* 2009)[24] 8.II) Laminin and oligodendrocyte progenitor development[23]. Addapted from (Relucio *et al*. 2009).[23]

One substrate of Fyn is p190RhoGAP (p190RhoGTPase-Activating Protein), that becomes tyrosine phosphorylated after OPCs differentiation. p190RhoGAP activates the GTPase activity of Rho, thereby promoting the formation of Rho-GDP, leading to its inactivation. Rho family GTPases regulate the polymerization of actin and thus control cellular cytoskeletal structure. Constitutively active RhoA stimulates myosin II-mediated actomyosin contractility, inhibiting branching of OLs and MBP expression decrease, in agreement with the observation that Fyn-mediated inactivation of RhoA promotes OLs differentiation[27,28]. On the other hand, Fyn-mediated activation of Rac1 and Cdc42 stimulates OLs differentiation and promotes actin polymerization, mediating the formation of filopodia and lamellipodia[21].

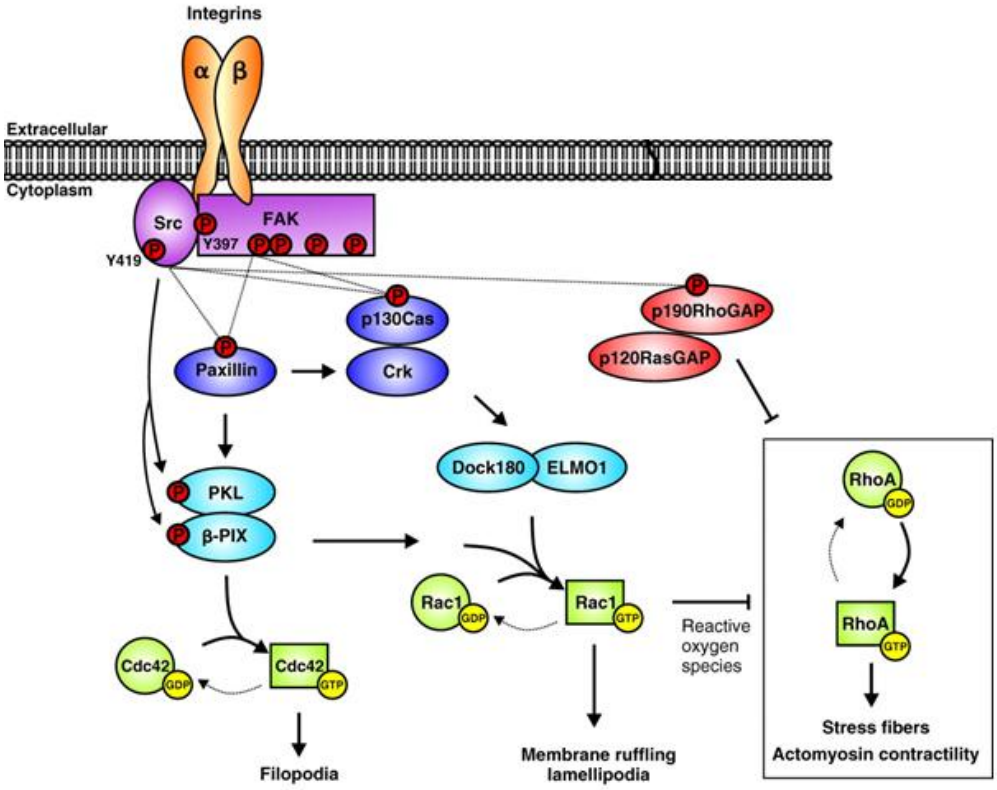


Figure 9| Integrins and Src pathway. Regulation of Rho proteins activity by integrins and SFKs (for example Fyn). Cdc42 and Rac1 activation leads to filopodia and lamellipodia formation, respectively. In addition Rho A inactivation inhibits actomyosin contractility, promoting OLs differentiation. (Adapted from Huveneers *et al*. 2009).[29]

Organization of the cytoskeleton plays an important role in oligodendrocytes differentiation. During the first stage of myelination, oligodendrocytes are extended and branched. The formation and extension of processes in oligodendrocytes, require the full reorganization of the cytoskeleton with the formation of filopodia and lamellipodia mediated by actin polymerization, and the subsequent invasion of microtubules (MTs)[30](Figure 10). Inhibition of the motor protein myosin II, a key regulator of actin cytoskeleton dynamics, potentiates OLs branching, myelination and increase the cell surface area[31].

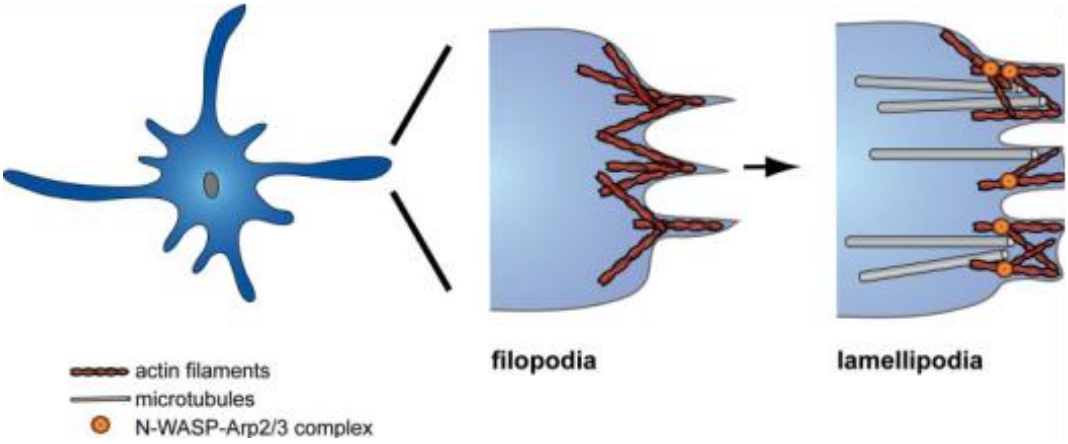


Figure 10| Oligodendrocyte process formation. Extracellular signals mediate polymerization of actin filaments, resulting in the protrusion of the plasma membrane and the formation of filopodia. Actin branching, mediated by the N-WASP/Arp2/3 complex, causes filopodia to extend. Microtubules migrate into the enlarged membrane protrusions and convert them into lamellipodia, ultimately leading to process outgrowth. (Addapted from Bauer *et al.* 2009).[30]

I.2 - Mechanotransduction

Mechanotransduction is the integration of cellular attachment to ECM with mechanical forces sensed and generated by cells and its conversion into intracellular signalling. Cells can sense and respond to external factors, not only biochemical, but also physical, resulting in changes in the cell's morphology, dynamics, behaviour and eventually fate. As an illustration of such influence, neural stem cells cultured on distinct substrates differentiate either into neuronal or glial lineages depending on the substrate elasticity[32]. Mechanical forces may induce nuclear deformations and alter genomic structure and alter transcription factors binding to specific target genes.[17]

I.2.1 Stiffness

The materials stiffness is measured by the relationship between forces applied and the resulting stretch of a material. In a biological context, stiffness or elasticity are referred to as Young's modulus or Elastic modulus *E* and may be defined as the amount of force per unit of area needed to deform the material by a given fractional amount without any permanent deformation (high Elastic modulus corresponds to high stiffness and low deformability) [33]. To quantify it, the Young's modulus *E* and the shear modulus *G* are commonly employed. For the elastic modulus (*E*), the force is applied perpendicular to the material's surface, whereas for shear modulus (*G*), the force is applied parallel to the surface (Figure 11). *E* and *G* are related by the following function: $E = 2G(1+ v)$, where *v* is the Poisson ratio. For materials that do not change volume under stretch, as in case of hydrogels, the Poisson ratio is 0.5. As a consequence, the elastic modulus will be three times its shear modulus. The units for rigidity are force per area, with SI unit being Pascal.[16]

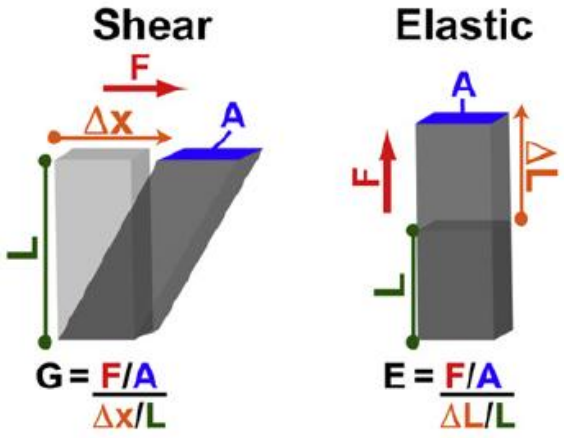


Figure 11| Rigidity Moduli. Stress is the amount of force applied per area (F/A) and strain is the displacement in the direction of applied force relative to initial length (Δx/L or ΔL/L). While both elastic and shear moduli are the ratio of stress over strain, there is a difference in the direction of the applied force. (Adapted from Moore *et al*.2010).[16]

Cells generally reflect better *in vitro* behaviour when cultured on materials with stiffness similar to that of their native microenvironment. The elastic moduli of

mammalian tissues range from 0.1 to 30,000,000 nN/μm^2 (equivalent to KPa) and the *E* of the brain ranges from 0.1 to 10 nN/μm^2 (Table i). The stiffness of tissues is not static, for example the stiffness of brain may change with age or during a pathology, such as MS.[16]

Table i| Elasticity of mammalian tissues. Adapted from Moore *et al*. 2010[16]

Tissue type	Elastic modulus (nN/μm^2)
Brain	0.1-10
Muscle	12-100
Fat	20
Artery	100 -3,800
Areolar connective tissue *(fibroblasts in collagen)*	600 - 1,000
Bone	17,100,000 – 28,900,000

I.2.1.1 - Stiffness and cell fate

The elasticity (or stiffness) of a cellular substrate has effects in cell spreading, morphology and function, in particular in stem cells. Stem cells show lineage-specific differentiation when cultured on substrates matching the stiffness corresponding to their native tissue. For example, when mesenchymal stem cells (MSCs) are cultured on substrates with stiffness mimicking neural, muscle or bone tissue, these cells acquire a neurogenic, myogenic or osteoblast phenotype, respectively (Figure 12)[34].

Saha and others showed that neural stem cells (NSCs) cultured on soft substrates (100-500Pa) are induced to differentiate into neurons, whereas harder substrates (1000-10,000 Pa) induce glial differentiation (astrocytes)[32]. The in vitro differentiation of oligodendrocytes (OLs) is also influenced by substrate stiffness, and culturing this type of cells on very soft substrates leads to a lower relative membrane surface than on stiffer substrates (around 7,000 Pa)[35]. Substrate stiffness has an important role not only in cell differentiation, but also in cell proliferation and self-renewal.[17]

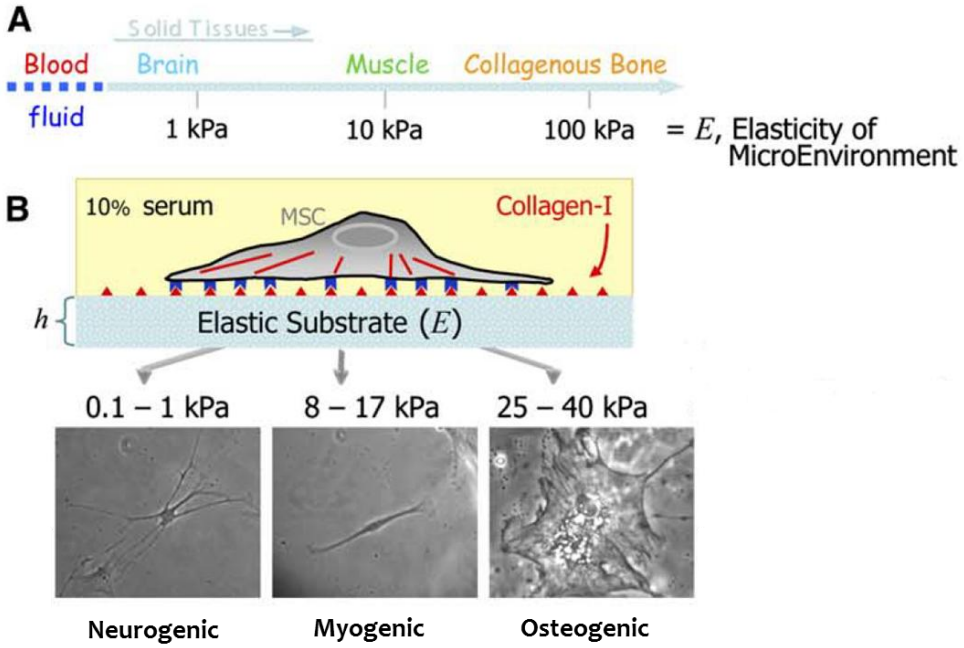


Figure 12| Tissue Elasticity and Differentiation of MSCs. An *in vitro* hydrogel system allows to control *E* and cell adhesion by covalent attachment of ECM proteins. (Adapted from Engler *et al.* 2006).[34]

I.2.1.2 - Mechanisms of mechanotransduction

Mechanical stimuli influence many cellular functions, however it remains largely unknown how mechanical stimuli are transduced into biochemical signals. It is generally believed that similar to soluble ligand-induced signal transduction, mechanotransduction initiates at focal adhesions by inducing local conformational changes, followed by signal transduction cascades inside the cytoplasm. In response to a mechanical stimulus, many enzymes change their kinetics: kinases, phosphatases, adenylate cyclases and GTPases. For instance, focal adhesion kinase (FAK), an enzyme that phosphorylates tyrosine residues on several focal adhesion proteins, including paxillin (a key focal adhesion adaptor protein), is regulated by mechanical stimuli. FAK does not bind directly to integrins (Figure 13A), but its kinase activity increases with mechanical force, but is also dependent on ECM proteins.[16]

Src may also be activated by mechanical stimuli (stress-induced Src activation), since increased tyrosine phosphorylation activity was showed in cells plated on stiff

substrates (8 KPa) but not on soft ones (0.3 KPa). There is evidence that activation of Src kinase by a mechanical stimulus requires the activation of integrins and the integrity of actomyosin cytoskeleton.[36] Additionally, mechanical activation of Src at discrete locations within the cytoplasm appears to emerge from local loading. As focal adhesions emerge, F-actin bundling occurs, allowing the transmition of signals through long distances, resulting in Src activation at remote sites within the cell[16]. These observations highlight the importance of mechanotransduction events for Src signalling.[16]

One mechanism thought to be involved in mechanotransduction is the exposure of protein-protein binding sites upon stretching of certain protein domains. For instance, p130CAS, a downstream target of Src, has been shown to reveal Src phosphorylation sites upon stretching (Figure 13B). Similarly, Talin possesses 11 potential vinculin binding sites, as well as binding sites for actin filaments, integrins, and a number of other proteins which only become available when talin is stretched by mechanical force (Figure 13C), resulting in the reinforcement of interactions established during the early phase of focal adhesions, through the recruitment of additional actin-binding proteins. The talin orientation might ascend from actomyosin pulling of the talin tails relative to the integrin-bond talin heads, with the resulting intramolecular tension and stretching of talin.[16]. Filamins, another familiy of proteins that bridge integrins to actin, have an actin-binding domain followed by 24 immunoglobulin repeats and bind integrins through their C-terminal domain. Within the immunoglobulin repeats, there are protein-binding sites that become available upon stretching of the protein (Figure 13D).[16]

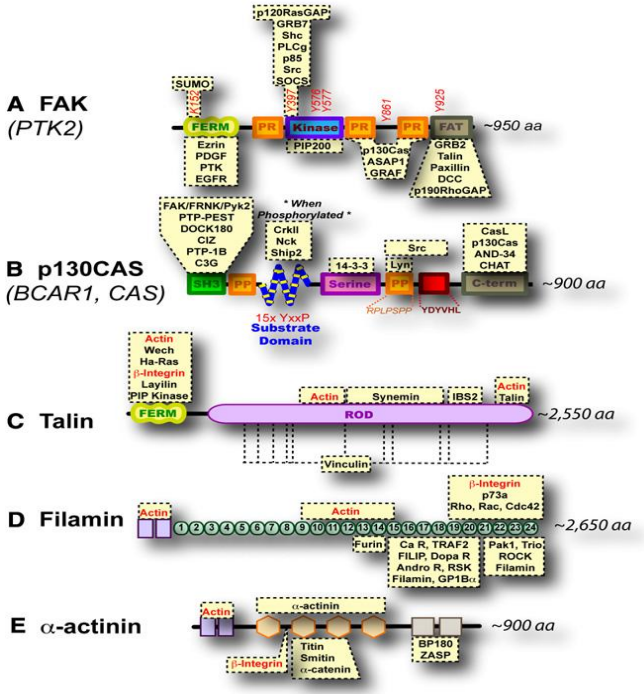


Figure 13| Sensing proteins that bind directly to the depicted domains are highlighted in yellow boxes. (A) FAK does not bind integrins or actin directly, but its kinase activity is regulated by mechanical force, and it has been hypothesized that removal of the FERM domain from the kinase could play a role. (B) The substrate domain of p130Cas contains 15 tyrosine residues that become exposed upon stretching. (C) Stretching of talin's rod domain exposes vinculin binding sites (D) Extension of filamin immunoglobulin repeats (labeled 1–24) regulate the binding of proteins. (Adapted from Moore et al. 2010).[16]

Considering the previous and other observations, one mechanism was proposed to explain rigidity sensing by cells (Figure 14). Integrins bind to ECM proteins and focal adhesions (FAs) initiate. Next, cells exert force on the substrate through actomyosin contraction, resulting in retrograde movement of actin fibers. Depending on the extent of such movement, which in turn depends on the rigidity of the substrate, talin may become stretched or not. In case the substrate is very soft, the matrix deforms in response to the force exerted by the cell and talin does not stretch (Figure 14, bottom left). In case of stiffer substrates, the matrix does not deform extensively and hence, under actomyosin-induced tension, talin (or other mechanosensitive adapter proteins) stretches and enhances its scaffold potential by revealing hitherto hidden domains that interact with other signalling proteins. As a result, stretching of talin reinforces focal adhesions by recruiting vinculin and other FA proteins (Figure 14).[16]

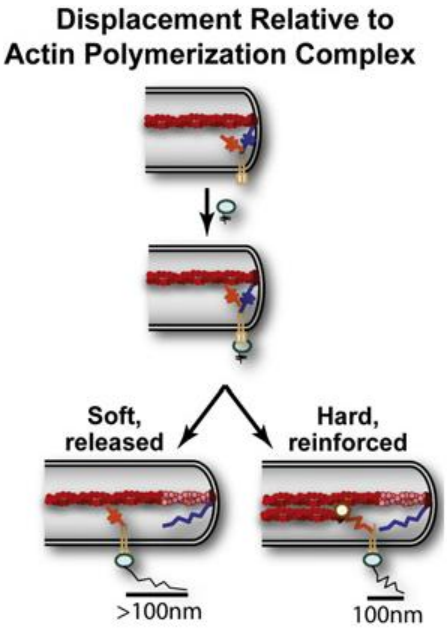


Figure 14| Proposed model for rigidity sensing by cells and focal adhesions reinforcement. (Adapted from Moore *et al.* 2010).[16]

In summary, mechanotransduction is equipped with several mechanisms that allow cells to sense their physical and biochemical environment and respond accordingly. Although some details are beginning to be unravelled[16,17], many aspects of mechanotransduction are still unknown and require further elucidation. Moreover, the role of this type of signalling is still rather unexplored in many cellular types, namely in oligodendrocytes.

I.3 – Objectives

There is a growing number of reports in the literature describing the involvement of mechanical forces and mechanotransduction during the differentiation distinct cells types. For this purpose, several different platforms are being developed that allow to culture cells *in vitro* in conditions that mimic the cellular niches more closely than standard cell culture conditions, attempting to include specific ECM and mechanotransduction elements in the systems. One of the platforms more widely used rely on substrates based on deformable hydrogels.

The main objective of this project was to develop an hydrogel based platform, functionalised with ECM proteins, to be used as a tool to mimic tissue stiffness and ECM repertoire of the brain *in vitro,* aiming to elucidate the role of mechanotransduction and ECM proteins during oligodendrocyte differentiation. Another objective of the project was to establish a method to assess the differentiation state of the cells cultured on the platform developed, taking advantage of maturation markers of oligodendrocytes (namely MBP expression) and morphology analysis by immunocytochemistry.

Chapter II

Materials and Methods

II - Materials and methods

II.1 - Materials

II.1.1 - Cell culture

Cell culture dishes were from Corning-Costar. Cell culture medium DMEM with low glucose (1,000 mg/L) and F-12, fetal bovine serum (FBS), Trypsin, Penicillin/Streptomycin solution and Amphotericin B were from Invitrogen. DMEM with high glucose (4,500 mg/L) was from Hyclone - Thermo. Medium supplements were from Sigma-Aldrich, except growth factors PDGF-AA and bFGF which were from Peprotech and BSA from Calbiochem. Proteins used to promote cell attachment Poly-D- Lysine (PDL), human Fibronectin (FN) isolated from plasma and human Laminin α2 (Merosin) from placenta were from Sigma-Aldrich, Roche and Chemicon (Millipore), respectively.

II.1.2 – Polyacrylamide hydrogels

Acrylamide and bis-acrylamide were purchased from Aplied Chem; Ammonium persulfate (APS), Tetramethylethylenediamine (TEMED), NHS (*N*-Acryloxysuccinimide) and SurfaSil were from Sigma-Aldrich, Fluka, Santa Cruz Biotechnology and Thermo, respectively. Gel polymerization systems were from BioRad. The reagents used to activate coverslips were from Sigma-Aldrich.

II.1.3 - Immunocytochemistry

FITC-labelled agglutinin and goat anti-rat antibodies conjugated with Alexa-488 and ALexa-568 were purchased from Invitrogen. Rat anti-MBP antibody was from Abcam and DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) was from Sigma Aldrich.

II.2 - Methods

II.2.1 - Cell culture

II.2.1.1 - CG-4 cell culture

The Central Glia-4 (CG-4) cell line (kindly provided by Dr. Adil J. Nazarali, College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Canada) is a rat cell line that displays the properties of normal bi-potential oligodendrocyte-type 2-astrocyte (O-2A) cells, with potential to differentiate into either oligodendrocytes or type-2 astrocytes[37]. CG-4 cells were maintained as described in reference 37. In detail, cells were passaged by washing with Puck's solution (80g/L NaCl, 4g/L, KCl, 0.6g/L KH_2PO, 0.9g/L $Na_2HPO_4.7H_2O$ and 10g/L D-glucose)[38] followed by dissociation and detachment using 0.05% trypsin-EDTA. After inhibition of trypsin using serum-supplemented recovery medium (DMEM high glucose supplemented with 2mM sodium pyruvate, 5%FBS, 5µg/mL insulin, 1%Penicillin/Streptomycin and 1% Amphotericin B) cells were centrifuged (201g for 5 min), resuspended, counted and seeded on tissue culture dishes coated with poly-D-lysine (100µg/mL) at a density of 2,500cells/cm^2 in recovery medium. Cells were allowed to attach for 30 minutes inside a CO_2 incubator at 37°C, 5% CO_2/95% air and 95% humidity. After the cells attached, the recovery medium was replaced by CG-4 proliferation medium composed of DMEM high glucose supplemented with apo-transferrin (50µg/mL), biotin (9,8ng/mL), sodium selenite (40ng/ml), 30% of B104-conditioned medium, 1%Penicillin/Streptomycin and 1% Amphotericin B. The cells were maintained in a CO_2 incubator at 37°C, 5% CO_2/95% air and 95% humidity. The medium was changed every other day.[39]

To select the best differentiation media to differentiate CG-4 cells into mature oligodendrocytes, 6,400 cells per cm^2 were plated in 96 well plates with proliferation medium during two days (see above CG-4 cell line culture)[40]. Then, the medium was replaced by differentiation medium and cultured for 3 days. Several differentiation media were tested based on SATO[41], N1 medium[37] and CG-4 differentiation medium[39] . SATO

medium was composed by DMEM high glucose, 100µg/mL apo-transferrin, 100µg/mL bovine serum albumin, 60ng/mL progesterone, 40ng/mL sodium selenite, 40ng/mL thyroxin, 30ng/mL triiodo-L-thyronine, 16µg/mL putrescine and 5µg/mL insulin. Several variations of this medium (SATO1-SATO5) were tested by adding the components described in Table ii (modifications were made according to Czopka *et al*. 2010)[42]. N1 medium was composed by 5µg/mL apo-transferrin, 10ng/mL biotin, 5ng/mL sodium

selenite, 5µg/mL insulin 6.3ng/mL progesterone and 16µg/mL putrescine. Triiodo-L-thyronine (T3) and Thyroxin (T4) were added to the N1 medium (N1+T3+T4) at concentrations described in Table ii. CG-4 differentiation medium (described by the laboratory of Dr. Adil Nazarali)[39] was composed by proliferation medium without conditioned medium: DMEM high glucose supplemented with apo-transferrin (50µg/mL), biotin (9,8ng/mL), sodium selenite (40ng/ml) and FBS (2%)[39].

Table ii| Summary of media components

Media nomenclature	BASE	Additional components
SATO1	SATO	10ng/mL PDGFAA and 10ng/mL bFGF
SATO2	SATO	10ng/mL PDGFAA
SATO3	SATO	1ng/mL PDGFAA and 1ng/mL bFGF
SATO4	SATO	1ng/mL PDGFAA
SATO5	SATO	0.5% FBS
N1 medium	N1	-
N1+T3+T4	N1	30ng/mL T3 and 40ng/mL T4
CG-4 differentiation medium (DM)	CG-4 PM without conditioned medium	2%FBS

For the differentiation experiments performed on coverslips and hydrogels, cells were either differentiated as described, or plated directly on differentiation medium for 2 days, 3 days or 2 days plus one additional day in presence of N1+T3+T4 differentiation medium supplemented with 5% FBS (as indicated in the results section)[37]

II.2.1.2 - B104 cell culture and preparation of conditioned medium

B104 neuroblastoma cells (kindly provided by Dr. Adil J. Nazarali, College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Canada) were grown in B104 proliferation medium [DMEM high glucose:F12 (1:1) containing 10% FBS, 1%Penicillin/Streptomycin and 1% Amphotericin B]. To prepare conditioned medium, cells were detached using 0.05% trypsin-EDTA (trypsin-EDTA was inhibited with B104 proliferation medium), centrifuged (290g for 5 minutes), counted and seeded at a density of 15,000 cells/cm^2 in B104 proliferation medium. On the following day, cells were washed 3 times with Puck's solution and the medium was replaced by defined medium [DMEM high glucose/F12 (1:1), 10µg/mL holo-transferin, 5ng/mL sodium selenite, 16µg/mL putrescine, 6.3 ng/mL progesterone, 1% Penicillin/Streptomycin and 1% Amphotericin B]. The cells were maintained in an incubator at 37°C, 5% CO_2/95% air and 95% humidity. After 3 days, the conditioned medium was collected and added 1µg/ml of PMSF (phenylmethanesulfonylfluoride). Then, the medium was centrifuged at 1,000g at 4°C during 10 minutes and the supernatant filtered (0.22µm) and stored at -20°C[39].

II.2.1.3 - HOG cell line culture

The human oligodendroglioma (HOG) cell line (kindly provided by Dr. Raquel Bello-Morales, Universidad Autonoma de Madrid, Madrid, Spain) was maintained in HOG proliferation medium (DMEM low glucose with 3.57g/L HEPES, 1.5g/L sodium bicarbonate, 10% FBS, 1% Penicillin/Streptomycin and 1% Amphotericin B)[4] in an incubator at 37°C, 5% CO_2/95% air and 95% humidity. To differentiate HOG cells into a mature oligodendrocyte-like state, cells were plated at a density of 6,400/cm^2 in a poly-D-lysine coated plate in HOG growth medium during two days. Two days later, medium was replaced by differentiation medium and several media were tested (Table ii).

II.2.2 - Coatings

A solution of Poly-D-lysine (molecular weight ≥ 300,000) in PBS at a concentration of 100µg/mL was used to cover the bottom of the culture plates and incubated overnight at 37°C. Before using, the plates were washed three times with sterile milliQ (mQ) water, not allowing them to dry. Coverslips used in culture were submerged in 65% nitric acid for 24 hours with agitation, then the acid was removed and coverslips were washed in milliQ water 6 times followed by another wash for 3 hours with agitation. Finally, the coverslips were sterilized with dry heat. The sterile coverslips were placed in a 24 wells plate, and were incubated with the appropriate protein. To coat coverslips with Poly-D-lysine (25µg/mL), Fibronectin (25µg/mL), Poly-D-lysine/Fibronectin (mix 1:1 Poly-D-Lysine at 25µg/mL and Fibronectin at 25µg/mL) or Poly-D-lysine/Merosin (mix 1:1 Poly-D-Lysine at 25µg/mL and Merosin at 25µg/mL) 50 µL of the corresponding protein solution in PBS were added to the coverslips and incubated at 37°C for 4 hours. Then, coverslips were washed three times with PBS. The coated coverslips were not allowed to dry.

II.2.3 - Preparation of polyacrylamide hydrogels

Polyacrylamide hydrogels were polymerized on top of reactive glass coverslips (2x2 cm or 2.5x4 cm), to allow the establishment of chemical covalent links between the hydrogel and the coverslip (according to Cretu *et al.* 2010, with modifications)[43]. First, the coverslips were rinsed with 100% acetone and 100% methanol and then were washed ten times in mQ water. The coverslips were covered with 0.1M NaOH and incubated for 3 minutes, then NaOH was aspirated and coverslips were incubated with (3-Aminopropyl)trimethoxysilame (3-APTMS) for 3 minutes. The coverslips were washed 3 times for 10 minutes per wash and then incubated with 0.5% glutaraldehyde during 30 minutes, then washed 3 times for 10 minutes each (Figure 15). This process was long and not very reproducible in terms of hydrogel adherence to the coverslip, thus we tested 3-(Trimethoxysilyl)propyl methacrylate reagent, because it reacts during only 3 minutes (Figure 16) and also proved to be more efficient at binding the hydrogel to the coverslips.

3-(Trimethoxysilyl)propyl methacrylate was diluted in ethanol (1:200) and just before use was added 3% of diluted acetic acid (1:10 glacial acetic acid: water), this solution was placed on top of cleaned coverslips and allowed to react for 3 minutes, then coverslips were rinsed with ethanol to remove the residual reagent and dried.[44]

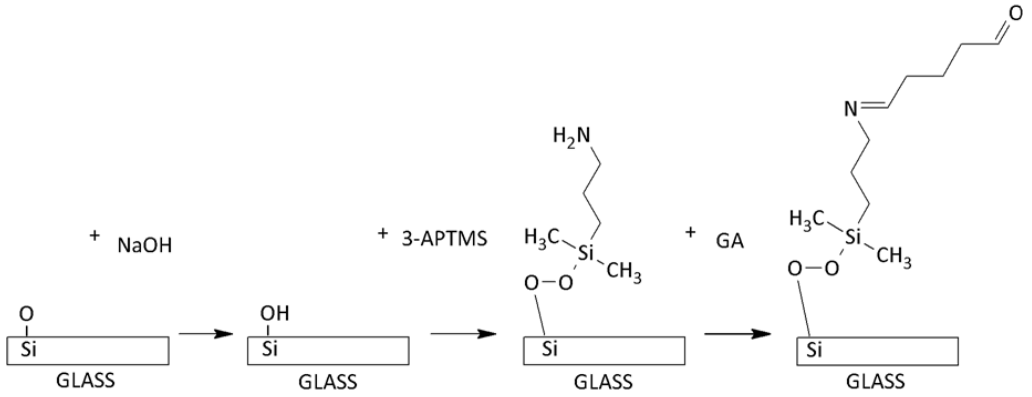


Figure 15| Schematics of the first treatment used to activate the coverslips. Sodium hydroxide (NaOH) creates hydroxyl groups that allow the reaction with (3-aminopropyl)trimethoxysilane (3-APTMS) and then glutaraldehyde (GA) reacts with the NH_2 group.

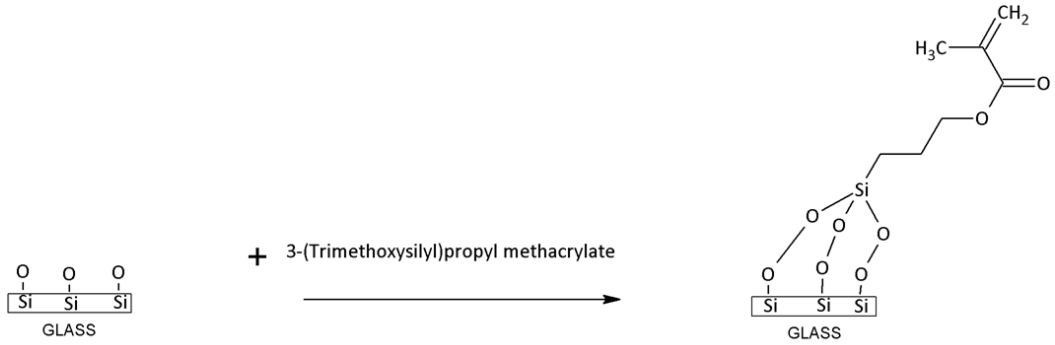


Figure 16| Schematics of the coverslips treatment with 3-(trimethoxysilyl)propyl methacrylate.

Polyacrylamide hydrogels are capable of modelling different stiffness varying the percentages of acrylamide and bis-acrylamide. First, a saturated solution of NHS (*N*-Acryloxysuccinimide) in toluene was prepared and kept covered to prevent evaporation. Then, acrylamide, bis-acrylamide, water and TEMED (Tetramethylethylenediamine) were mixed according to Table iii and afterwards solution of NHS and ammonium persulfate (APS) were added to the hydrogel solutions and vortexed briefly.[43]

Table iii| Hydrogels formulations composition [percentage of acrylamide (AC) and bis-acrylamide (BAC)] volume added (µL) per millilitre of solution

	Gel number 1 10%AC 0.3%BAC	Gel number 2 5%AC 0.2%BAC	Gel number 3 4%AC 0.2%BAC	Gel number 4 3%AC 0.2%BAC	Gel number 5 3%AC 0.05%BAC
AC 40%	250	125	100	75	75
BAC	150	100	100	100	25
NHS	220	220	220	220	220
APS 10%	3	3	3	3	3
TEMED 99%	1	1	1	1	1
Water	376	551	576	601	676

The hydrogel was casted on a reactive coverslip in a platform for gel polymerization with 1 mm spacers (Mini-protean 3, Bio-Rad), flanked by 1 mm Teflon slides or glass slides as shown in Figure 17. In this platform the outer glass was siliconized with a solution of 10% SurfaSil in chlorophorm. After polymerization (30 minutes), hydrogels on treated coverslips (Figures 18 and 19) were washed three times with PBS, on a rocker, five minutes per wash. Gels were sterilized by exposure to UV light during 30 minutes in an air flow cabinet. The hydrogels produced using this method were approximately 1mm thick.

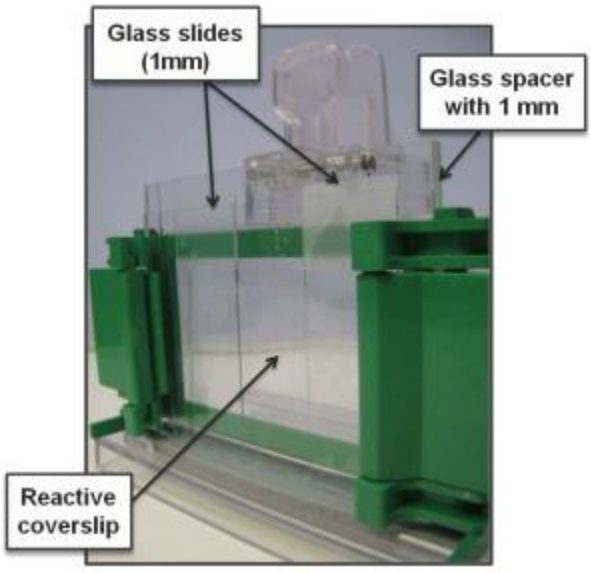


Figure 17| Platform used for the preparation of hydrogels

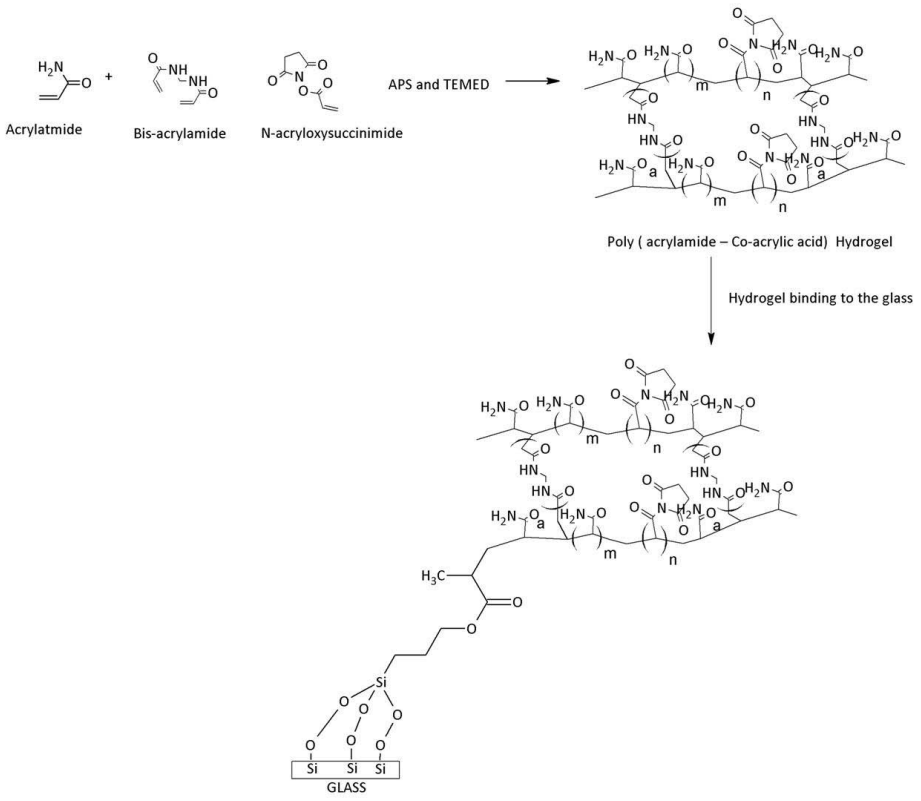


Figure 18| Schematics of the hydrogel polymerization on the reactive coverslip with the first treatment described and the functionalization.

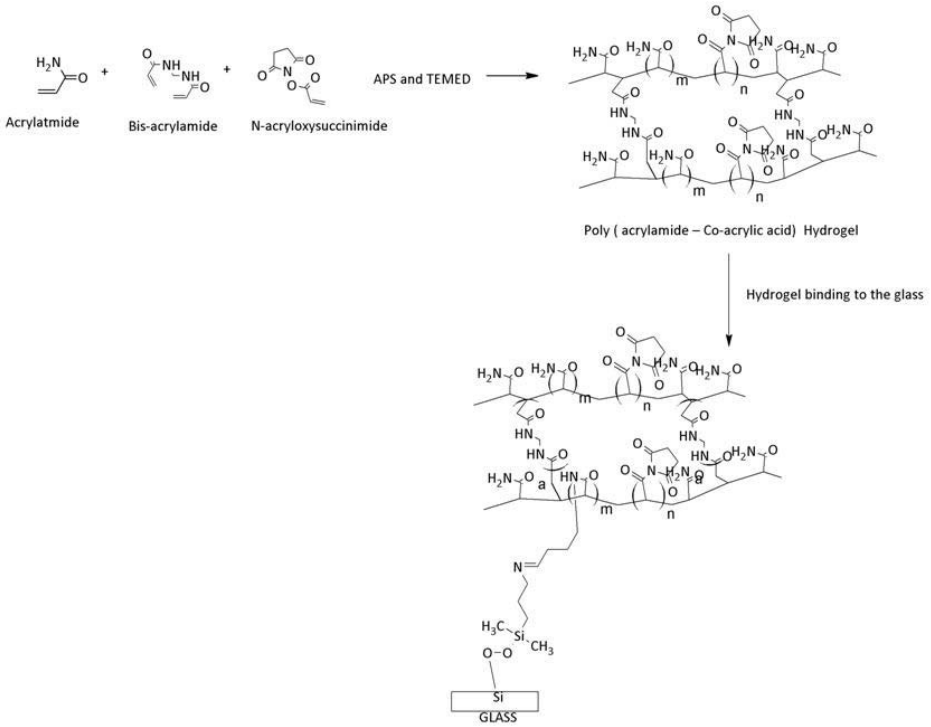


Figure 19| Schematics of the hydrogel attachment to the coverslip treated with 3-(Trimethoxysilyl)propyl methacrylate.

Since cells do not adhere to polyacrylamide hydrogels, these were functionalised with proteins that allow cell attachment, to mimic the extracellular matrix (ECM). The proteins were covalently bond to the top of the hydrogels by reaction of primary amines with the *N*-Acryloxysuccinimide (NHS) on the hydrogel. The schematics of the protein (represented by Lysine for the sake of simplicity) reaction with NHS is depicted on Figure 20 . Merosin (MN), Fibronectin (FN) and PDL were diluted in PBS at a concentration of 25µg/mL,. Combination of PDL/MN or PDL/FN were prepared 1:1 using each solution at a concentration of 25µg/mL.

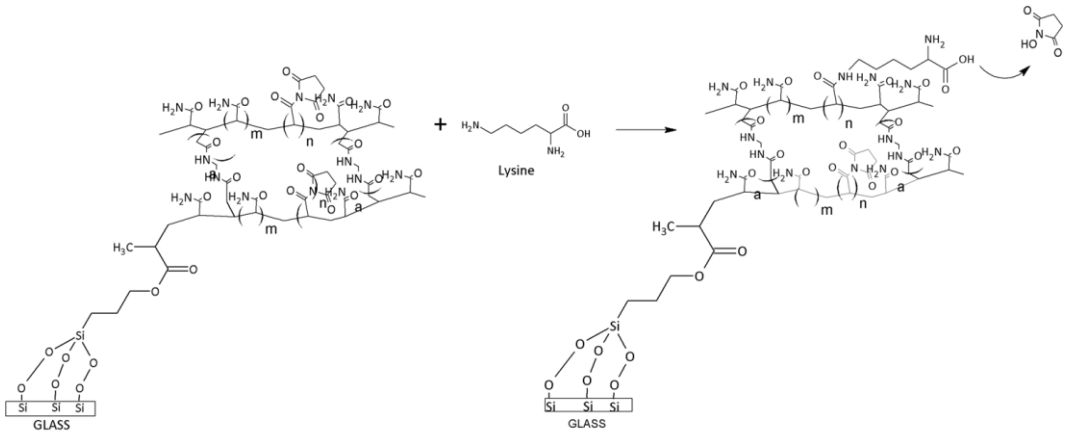


Figure 20| Schematics of the hydrogel polymerization on the reactive coverslip and the functionalization. The amino acid lysine (for simplicity reasons) is covalently bond to the gel, by the reaction of the amine group with the NHS (representative bond of the proteins to the hydrogel).

To functionalise the complete surface of an hydrogel its top was covered with protein solution, using 800µL or 300µL for an area of 12 cm^2 or 4cm^2, respectively. To have more than one protein in each gel, it is possible create spots with different proteins, thus 5µL of protein solution at 25µg/mL were added on top of hydrogels in each spot. The hydrogels were incubated ON at 4^oC to promote the crosslinking of the proteins with NHS and then were washed once with PBS. The unreacted NHS was blocked with 1mg/mL heat-inactivated fatty-acid free BSA (bovine serum albumin) in DMEM low glucose for 30 minutes. BSA solution in PBS (20 mg/ml) was inactivated in a 68°C water bath for 30 min. Hydrogels were rinsed once with PBS and placed in a plate with DMEM low glucose 4h to equilibrate. Hydrogels were washed with PBS once before the cells were seeded.[43]

II.2.3.1 - Rheological characterization of polyacrylamide hydrogels

The stiffness of hydrogels was determined by rheology using a Kinexus Pro rheometer and rSpace software (Malvern). The hydrogels were prepared and polymerized using a vertical electrophoresis system with a 1mm spacer (Mini-Protean 3, BioRad) and equilibrated overnight in PBS, following a similar protocol as for those used for cell culture, except that the gels were not linked to coverslips nor functionalised with protein. After zeroing the rheometer, each gel was loaded and trimmed on the bottom plate. Then, the gap (distance between the top and bottom plates) was defined as 1mm and fine-tuned to a distance at which the gel was applied a normal force of 0.1N. Frequency sweeps were performed from 10 to 0.1Hz (3 reads per decade) with a deformation of 2mstrain (amount of deformation, has no units), at 37^{o}C. The elastic modulus (E', also known as Young's modulus) was calculated using the formula E'= 2G'(1+v), where G' is the complex storage modulus measured by the rheometer at 1 Hz and v is Poisson's ratio, assumed to be 0.5 for materials that do not vary its volume upon stretch, according to the literature[16,32].

II.2.3.2 - Cell culture on hydrogels

Hydrogels covalently bond to glass coverslip with 4cm^2 were inserted in a 21cm^2 culture dishes (3 hydrogels per plate) and CG-4 cells were plated at a density of 15,000cells/cm^2 and were allowed to attach for 1h. Then, the medium was replaced by proliferation medium or differentiation medium, as detailed in section II.2.1.1.

II.2.4 - Immunocytochemistry

Immunocytochemistry was performed on cells cultured on 96 well plates, coverslips or on functionalised hydrogels. The medium was removed, CG-4 cells were washed once with Puck's solution (HOG cells were washed once with PBS) and fixed with

4% paraformaldehyde for 15 minutes at room temperature (RT). The fixing reagent was removed and cells were washed with HBSS three times. To evaluate morphological features, cells were stained with FITC-labelled agglutinin (5µg/mL) for one hour. To stain cells using antibodies, cells were further permeabilized with PBS-Triton 0.1% for 20 minutes and for 5 minutes with PBS-Tween 0.1% and then blocked with 1% BSA in PBS fort 30 minutes. The primary antibody (rat anti-MBP) was diluted in PBS 1%BSA at the appropriated dilution (1:200) and incubated overnight (ON) at 4^{o}C in a humidified atmosphere. The secondary antibodies [goat anti-rat Alexa-568 (1:200) or goat anti-rat Alexa-488 (1:200)] used to label the primary antibody were diluted in PBS with 1% BSA and incubated for 1h at room temperature (RT). For nuclear staining, the cells were incubated with 200ng/ml of DAPI for 5 minutes at RT. Stained samples were visualised using a Zeiss Axiovert 200M fluorescence microscope using AxioVision release 4.8 software (Zeiss) for image acquisition and the Image J software was used to analyse the images.

II.2.4.1 - Fractal dimension analysis

Fractal dimension analysis[45,46] was performed using the Image J software. First the images were converted to an 8-bit image, then using the crop tool one cell was selected and the threshold was adjusted. Finally the cell image was outlined and in the 'analyse' option the 'tools' command was selected and then the 'fractal box count' was selected. This procedure was performed on five replicates per condition for each independent experiment (n).

II.2.5 - Statistical analysis

Statistical analysis was performed using the software Graph Pad Prism by Kruskal-wallis test followed by Dunn's multiple comparison test when the variances were not homogenous (as in case of Figure 22) and by one way ANOVA followed by Tukey's multiple comparison test when variances were homogenous (as in case of Figure 28). To test the

homogeneity of variances a Bartlett's test was performed (* $p < 0.05$, ** $p < 0.01$ and *** $p<0.001$ for statistically significant differences).

Chapter III
Results

III - Results

III.1 - CG-4 cells differentiation

To differentiate CG4 cells, several conditions were tested namely using different media and timings, 3 (Figure 21) or 6 days (data not shown, because cells died at the end of differentiation). Cells were differentiated in different media based on SATO, N1 medium and CG-4 cells differentiation medium (Table ii). Cell differentiation was evaluated by agglutinin staining to assess the cellular branching and morphological changes and MBP expression (marker of mature oligodendrocytes).

Cells cultured in SATO1 and SATO 2 media (Table ii) exhibited few branches, and low cellular survival (Figure S-1). When cells were differentiated in SATO3 (Table ii) the oligodendrocytes showed an increased branched morphology and survival (Figure 21-SATO 3). In Figure S-1 cells cultured in SATO4 did not display a mature oligodendrocyte morphology. SATO 5 promoted a morphological differentiation, however MBP expression was low. N1 and proliferation media without growth factors, did not promote morphological differentiation nor expression of MBP. The best differentiation medium tested was N1+T3+T4, since cells displayed a complex branched morphology and MBP expression was high (Figure 21).

In order to obtain a quantitative measurement of the morphological features of the cells we did a fractal dimension analysis[45,46]. The fractal dimension is a measurement of branching complexity, where 1 corresponds to a low complexity and 2 to a high complexity. Notably, the N1+T3+T4 and the SATO3 media induced an increase of the branching complexity of the cells as compared to those cultured in proliferation medium. There were statistically significant differences between proliferation medium (Figure 22) and SATO3, N1+T3+T4 and CG-4 differentiation medium (DM). These differences are in agreement with the expression of MBP observed and the visual morphological changes (Figure 21). Additionally, N1+T3+T4 conferred a statistically significant higher branched morphology when compared to the N1 medium, indicating that T3 and T4 have an important role in oligodendrocytes differentiation.

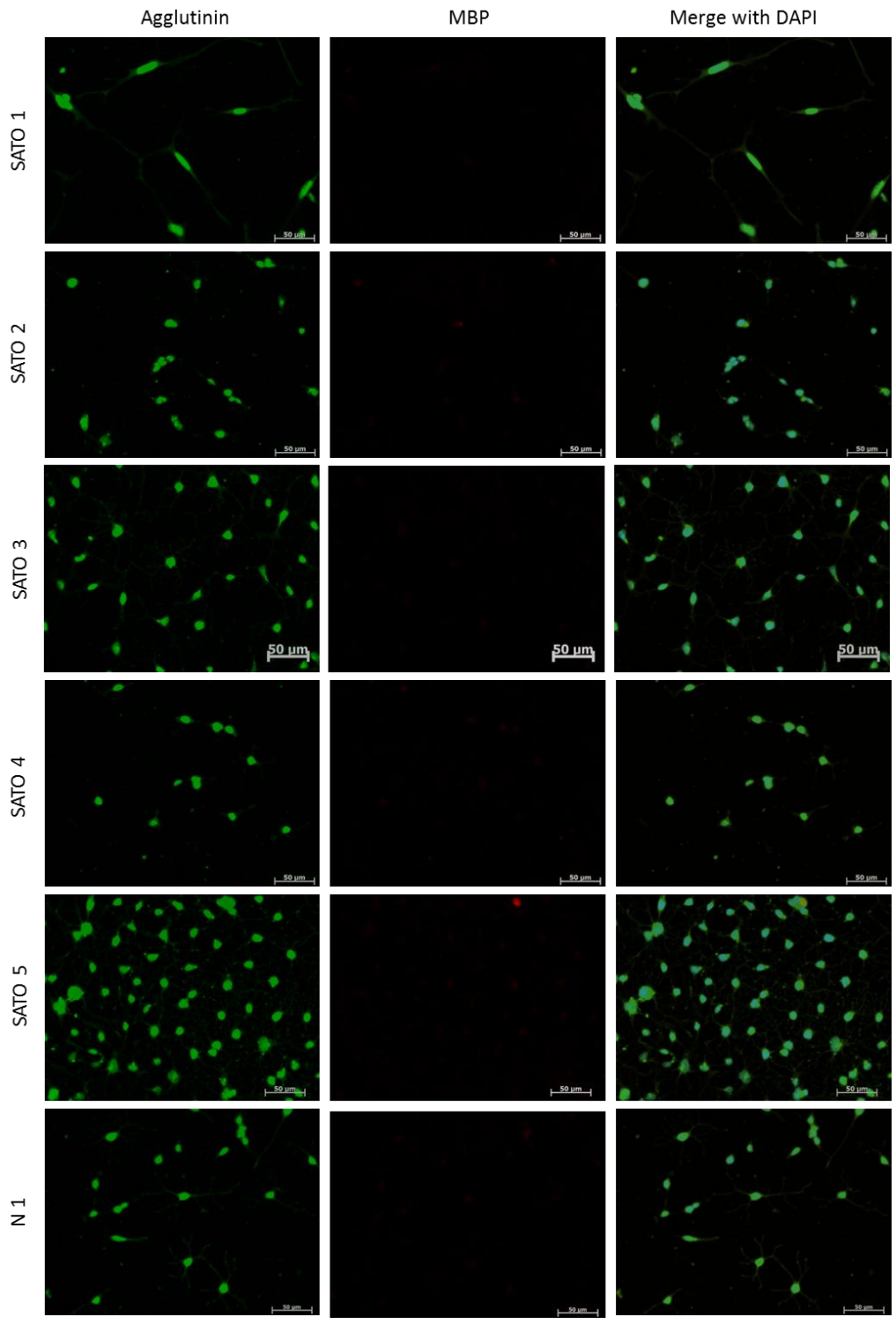


Figure 21| CG4 cells differentiation in different media. Cells were previously cultured for 2 days in proliferation medium (PM) and then cells were induced to differentiate in the presence of the corresponding differentiation medium for 3 days (n=3). Bar corresponds to 50µm**.**

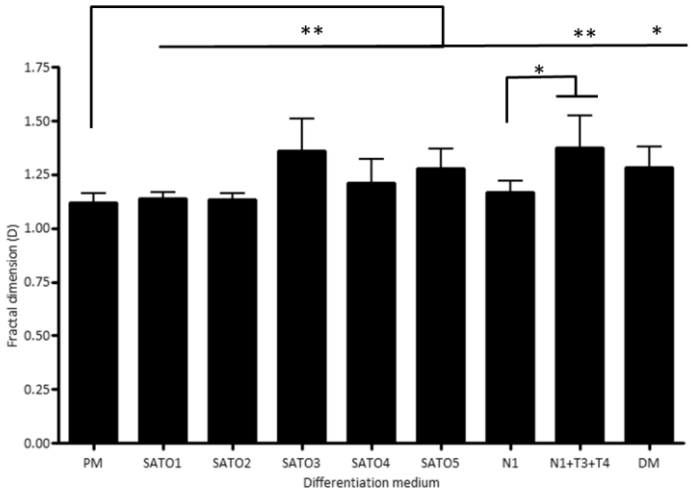


Figure 22| Graphical representation of the fractal dimension analysis. The fractal dimension was calculated for all tested media. Values are mean ± SD (*n*=3). ** $p< 0.01$ and * $p< 0.05$ when compared with the proliferation medium (PM) or between indicated conditions by Kruskal-Wallis test followed by Dunn's multiple comparison test.

The N1+T3+T4 was selected as the differentiation medium for further differentiation experiments using CG-4 cells

III.2 – Rheological measurements of the polyacrylamide hydrogels

Rheological measurements were done using a rheometer by performing a frequency sweep of 10-0.1Hz at a constant strain (2 mstrain) at 37^{o}C. Figure 23A shows that the complex modulus was linear over a wide range of frequencies (10-0.1Hz) for all hydrogels' formulations. The Young's moduli (stiffness) of the hydrogels were calculated from the measured G' complex modulus values at 1Hz using the following formula: E'=2G'.(1+v), where G' is the complex storage modulus measured by the rheometer and v is Poisson's ratio, assumed to be 0.5.[32] The frequency of 1HZ was chosen, since it was shown to better mimic the physiological frequency[47]. The percentage of acrylamide and bis-acrylamide is correlated with the stiffness of the hydrogels and the increase of either one enhanced the Young's moduli (Figure 23). The hydrogels produced had a range of stiffness between 6632.55±2685.44 Pa (Gel number 1) and 362.27±64.44 Pa (Gel number 5) as shown in Figure 23B, compatible with the range of stiffness described for the brain tissue[16], as intended.

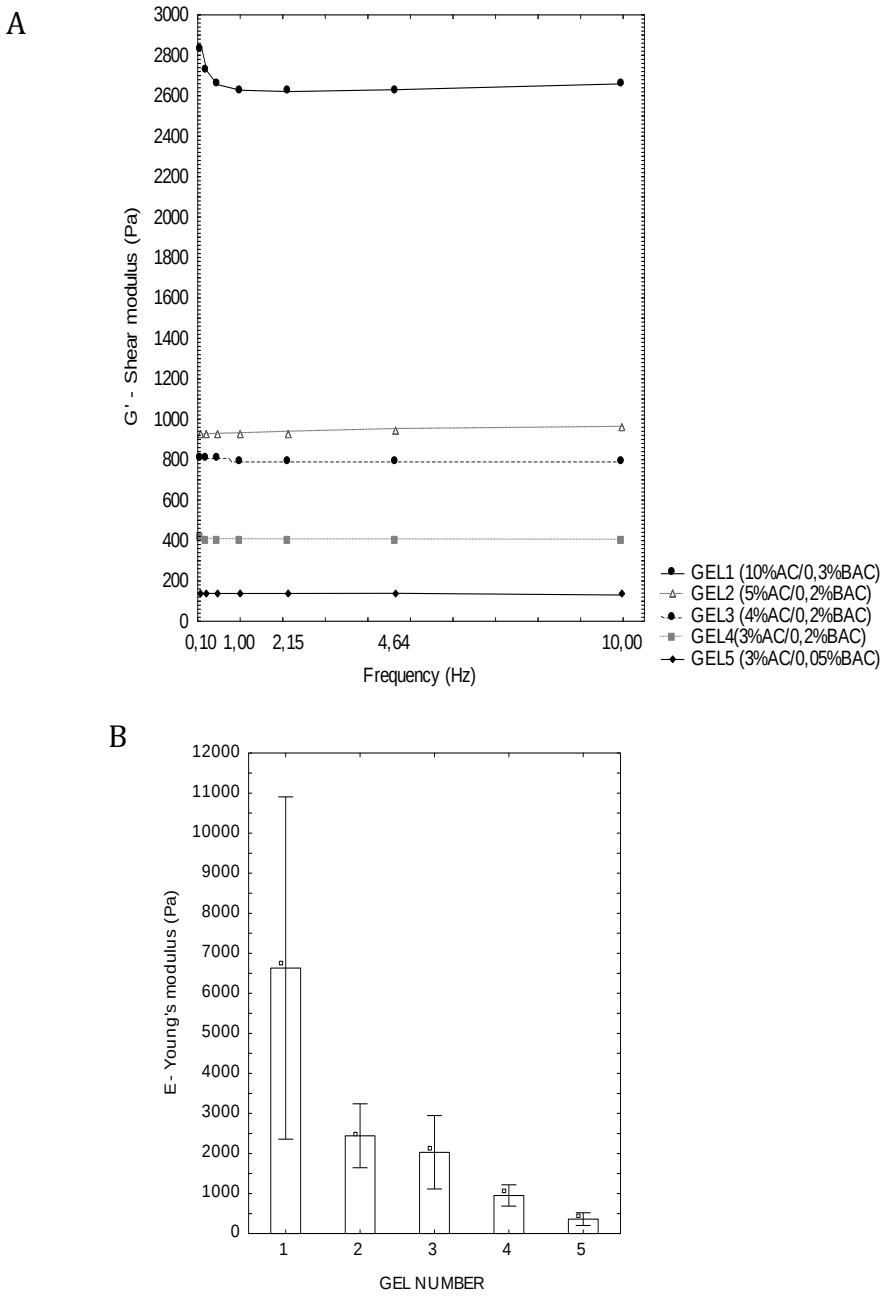


Figure 23| Data of the mechanical properties of the hydrogels by rheometry, produced using distinct formulations. A) Representative rheological measurements of the shear modulus (G') of five distinct formulations of polyacrylamide hydrogels across a frequency sweep (0.1-10Hz) AC: acrylamide; BAC: bis-acrylamide . B) Mean ± standard deviation (SD) of the Young's modulus (amount of force per unit of area needed to deform the material by a given fractional amount without any permanent deformation) calculated from the elastic modulus measured at 1Hz, according to the formula E'= 2G'(1+v), where G' is the complex storage modulus measured by the rheometer and v is Poisson's ratio, assumed to be 0.5.(Young's modulus plot n=3)

III.3 - CG-4 cells culture on hydrogels

III.3.1 - Optimization of the protein concentration

To culture cells on hydrogels, the substrates needed to be functionalised with adhesive proteins or peptides, since cells will not readily adhere to polyacrylamide. Additionally, we were interested in studying the role of distinct extracellular matrix proteins in OLs differentiation using this setup, such as laminin-2 (merosin) and fibronectin since those proteins were shown to play a role in several aspects of OLs differentiation and maturation.[22,23,48,49] For this purpose, the protein concentrations used had to be optimized. The cells adherence to the hydrogel is dependent on the protein concentration, so we functionalised the hydrogels with poly-D-lysine (PDL) merosin (MN) or fibronectin (FN) at different concentrations (10, 25, 50 and 100µg/mL) for 2h at 37^{o}C. We selected 25µg/mL the most suitable protein concentration, since CG-4 cells were adherent and healthy at that concentration, not improving at higher ones, as shown for the case of MN (Figure 24). We hypothesised that the hydrogel could be saturated at protein concentrations higher than 25µg/mL, so we performed a silver staining of the hydrogels functionalised with increasing concentrations of a BSA solution (Supplementary data) of the hydrogel (Figure S-3) to determine the relative amount of protein bond. Although these results are preliminary and not totally conclusive, data indicates that the hydrogels might not be saturated with protein when used at a concentration of 25µg/mL (this issue is further considered in the Discussion section).

III.3.2 – Hydrogels functionalization time

The cells' attachment to hydrogels could not be enhanced by increasing the protein concentration (as shown above), however, the efficiency of cell attachment to hydrogels functionalised with merosin or fibronectin was not as high as observed on coverslips, so we hypothesized that the time of functionalization might not be sufficient to allow the proteins to react with the hydrogel. In the previous procedure (Figure 24) the

functionalization was performed for 2h at 37°C, hence we tested different functionalization times: 2h, 3h and 4h at 37°C and overnight at 4°C, based on Cretu 2010[43].

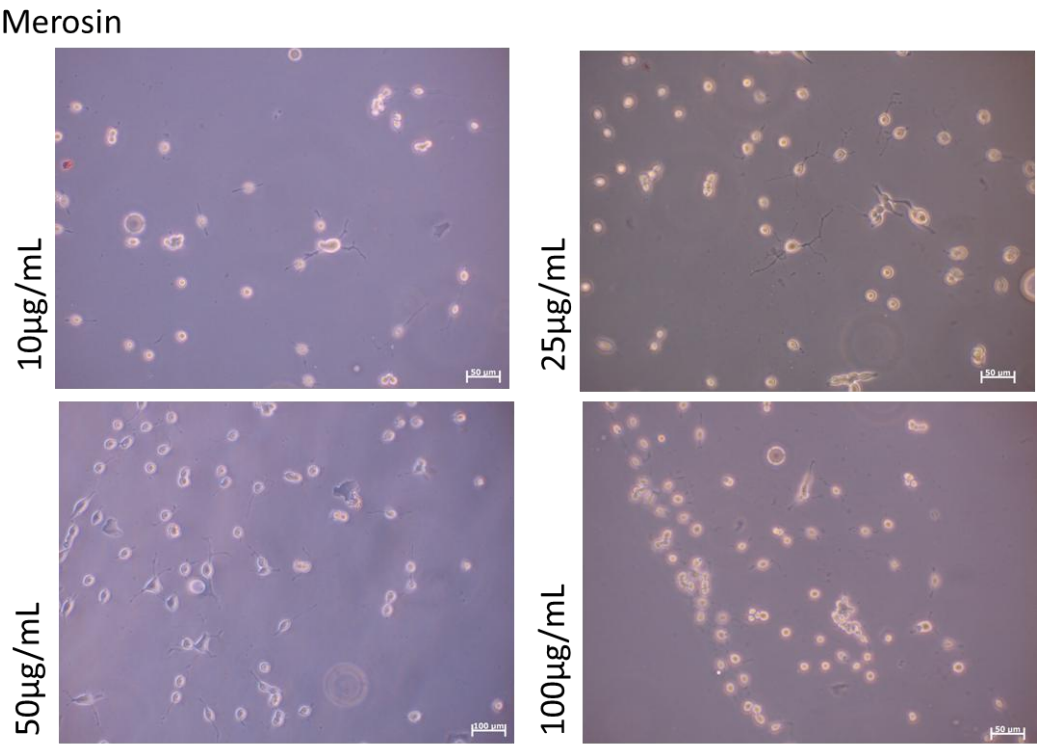


Figure 24| Phase-contrast microscopy images of CG4 cells cultured on hydrogel number 1 functionalised with different concentrations of merosin in proliferation medium (image representative of all proteins tested). The density of adherent, healthy cells was higher at 25µg/mL (n=1). The scale bar corresponds to 50µm.

The density of adherent cells increased when gels were functionalised ON at 4°C (Figure 25-ON). At 37°C the number of adherent cells did not increase with higher reaction time, probably because protein might dry and denature or become degraded (Figure 25-2,3 and 4h).

III.3.3 – Morphological changes of CG-4 cells on hydrogels

After the optimization of the reaction time and the concentration of the protein, cells were seeded on hydrogels with different stiffness degrees functionalised with distinct proteins (Figure 26). On softer hydrogels, cell adherence decreased when compared with stiffer hydrogels and this phenomenon was independent of the protein used. We verified

that the density of the cells attached was higher on PDL functionalised hydrogels than on MN gels (data not shown), so we tested whether mixing PDL and MN (1:1)[48] would enhance cell attachment, and in fact it did (Figure 26). Hence, from this point onwards, all MN coatings were performed as 1:1 mix of PDL/MN (25µg/mL of each protein).

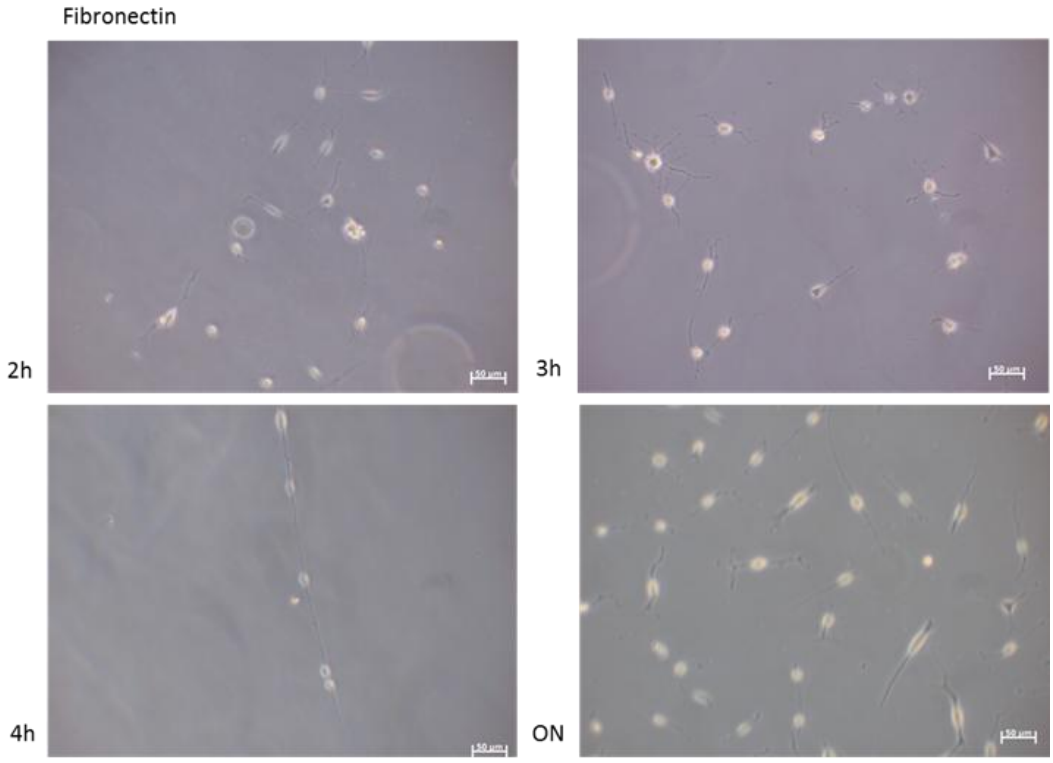


Figure 25| Phase-contrast microscopy representative images of CG-4 cells cultured on hydrogels functionalised with proteins (fibronectin in these images) during different time periods (n=1). The scale bar corresponds to 50µm

CG-4 cells cultured in presence of proliferation medium on hydrogels functionalised with fibronectin showed a clear bipolar morphology, mainly on the stiffest hydrogel (number 1), while in presence of PDL/MN cells were more branched, showing an intermediate morphology on PDL alone (Figure 26). We selected hydrogel number 1 to continue with further experiments because on this gel the cell density obtained was higher and we could obtain OPC-like cells on FN and OL-like cells on MN functionalised matrices (Figure 26), even without any changes in terms of media composition (all performed using proliferation medium). This initial data suggested that CG-4 cells were

indeed responding to mechanotransduction stimuli – mechanical cues and ECM proteins provided by this hydrogel platform.

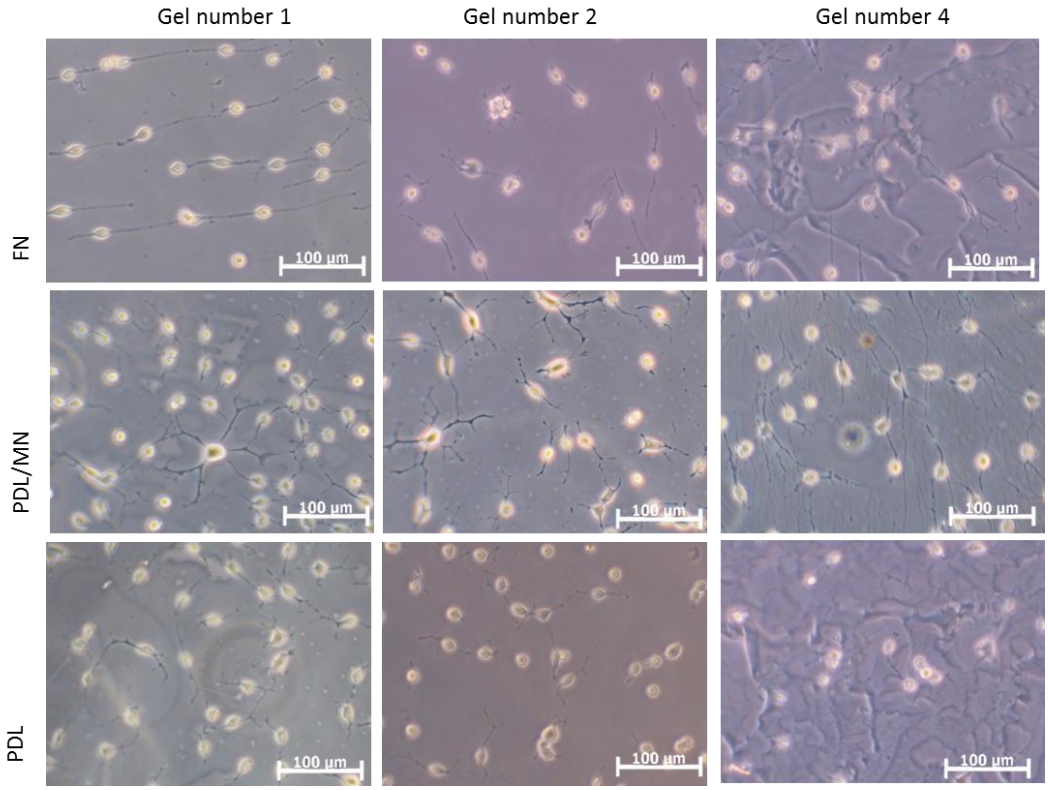


Figure 26| Phase-contrast microscopy images of CG4 cells cultured on functionalised polyacrylamide hydrogels. Cells were cultured with proliferation medium, on hydrogels with formulation numbers 1, 2 and 4 (formulations described on Table iii), bearing different stiffness values: ~6.5KPa, 2.5KPa and 1KPa, respectively. Hydrogels were functionalised with 25µg/ml of distinct ECM proteins: Fibronectin (FN), PDL/merosin (PDL/MN) or Poly-D-lysine (PDL) as control. CG4 cells seem to respond to the different substrate stiffness and ECM proteins, reflected by changes in their morphology. Bar corresponds to 100µm.

CG-4 cells cultured in proliferation medium on hydrogels or on coverslips showed a different morphology (bipolar or branched), which was dependent on the ECM protein used. Performing fluorescent agglutinin staining we could better observe cells displaying a bipolar morphology like OPCs when plated in presence of fibronectin and that this morphology was more evident on hydrogels than on coverslips (Figure 27). On the other hand, cells in presence of merosin displayed a branched morphology with more processes, and the processes seemed more complex in cells cultured on hydrogels (Figure 27)

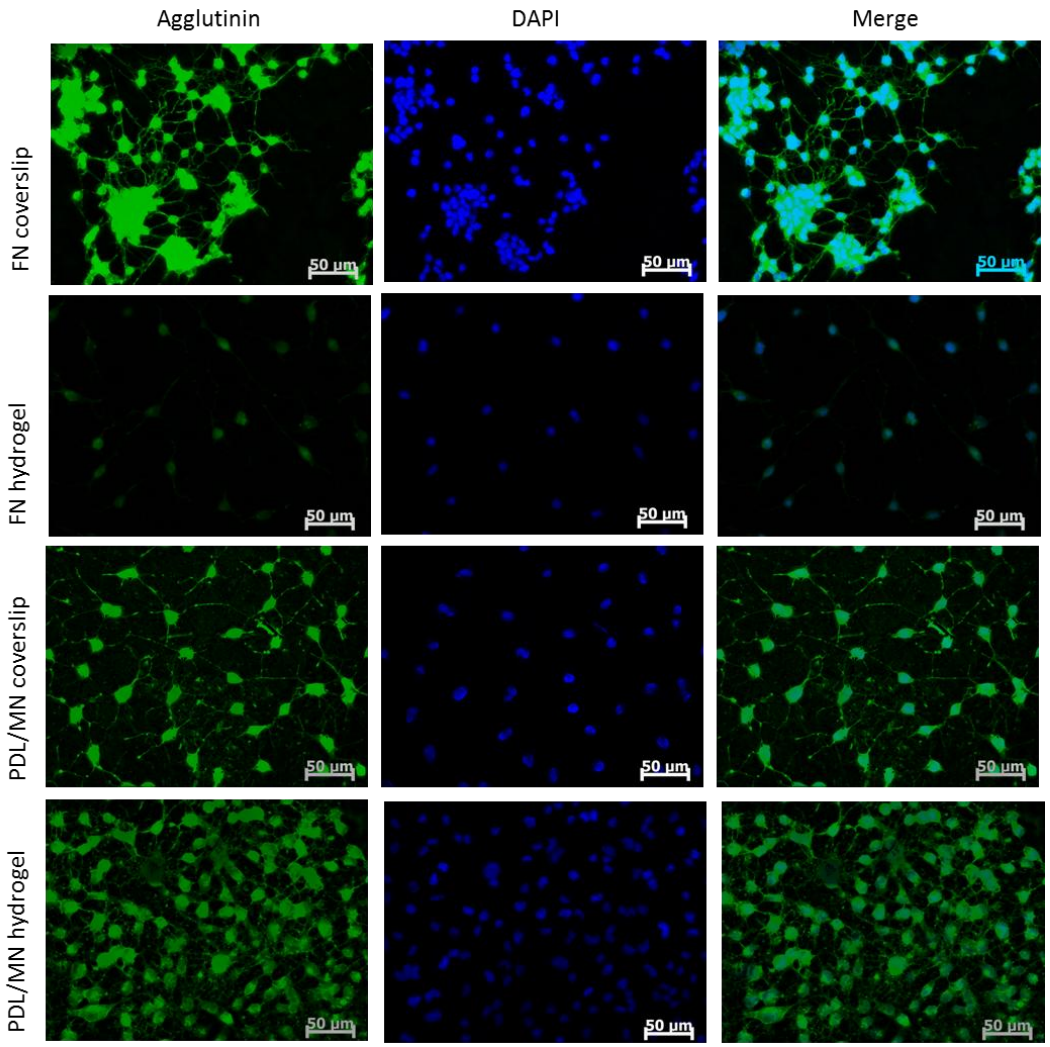


Figure 27| CG-4 cells cultured with proliferation medium on hydrogels and coverslips. Cells were stained with agglutinin (green) and DAPI. (blue). Cells on FN coated coverslips had a more branched morphology than on FN functionalised hydrogels, where cells displayed a bipolar morphology (n=2). In presence of PDL/MN cells showed a more branched morphology when cultured on hydrogels than when cultured on coverslips.

To assess the influence of the proteins and the hydrogel on cell morphology, namely the complexity of branches, we performed a fractal analysis (Figure 28). As referred above, the fractal dimension is a measurement that permits cell morphology assessment by a mathematical method[45,46]. The possible fractal dimension values are between 1 and 2: 1 for low complexity of branch morphology (essentially a one dimensional line) and 2 for high complexity of branch morphology (if fractal dimension reached 2, the branches would be essentially a bi-dimensional planar structure). As shown in Figure 2, cells on hydrogels with FN had the lowest values of fractal dimension (low

complexity – more bipolar morphology) and cells on hydrogels with PDL/MN had the highest values (more branched morphology). When cells were plated on coverslips coated with FN or PDL/MN, there were no significant differences between the proteins (Figure 28).

We observed that mechanotransduction cues provided by the combination of deformable hydrogels and the ECM proteins have an important impact on cell morphology, because cells on hydrogels displayed different morphologies depending on the ECM protein immobilised and this did not happen on stiff glass. Based on this morphological analysis, we propose that CG-4 cells, when culture in proliferation medium, maintain a more progenitor-like bipolar morphology or switch into a more branched and differentiated-like morphology more effectively in presence of fibronectin or merosin, respectively, when cultured on compliant (6.5KPa) hydrogels than on stiff glass coverslips.

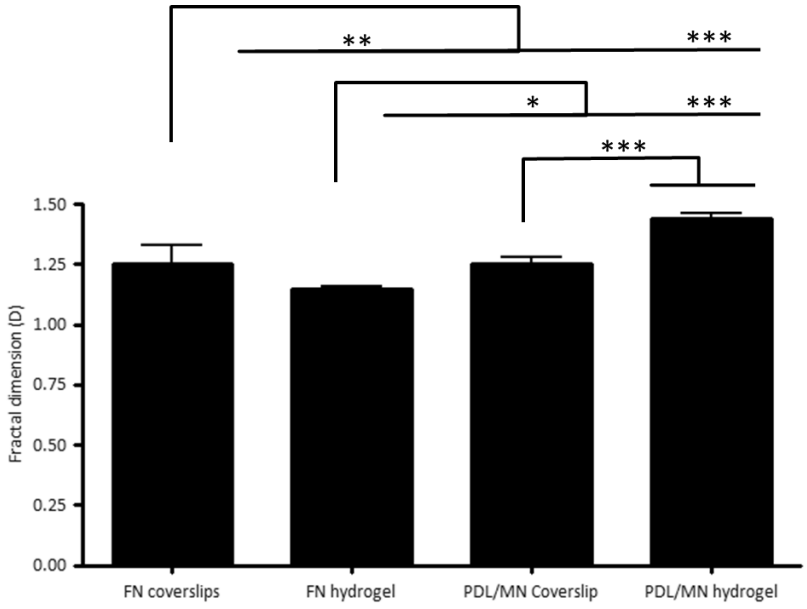


Figure 28|Graphical representation of fractal dimension analysis. CG-4 cells were cultured for two days in proliferation medium on the indicated platforms (hydrogel or coverslips) functionalised/coated with the indicated proteins (FN or PDL/MN). Values represented mean ± SD (*n*=2). Statistical analysis by one way ANOVA followed by Tukey's multiple comparison test (*** P<0.001 ** *p*< 0.01 and * *p*< 0.05).

III.3.4 - CG-4 cells differentiation on hydrogels

Cells were induced to differentiate on hydrogels following the same protocol used to select the differentiation medium. Cells were cultured for two days with proliferation medium and then medium was switched to differentiation medium for three days. The differentiation of cells was performed on hydrogels and coverslips (as a control). To address whether CG-4 cells differentiated into mature oligodendrocytes, cells were stained with agglutinin and for MBP and analysed by immunocytochemistry. In control cells (2 days with proliferation medium) there seemed to be a weak expression of MBP and mostly restricted to the nuclear area (Figures 29 and 30).

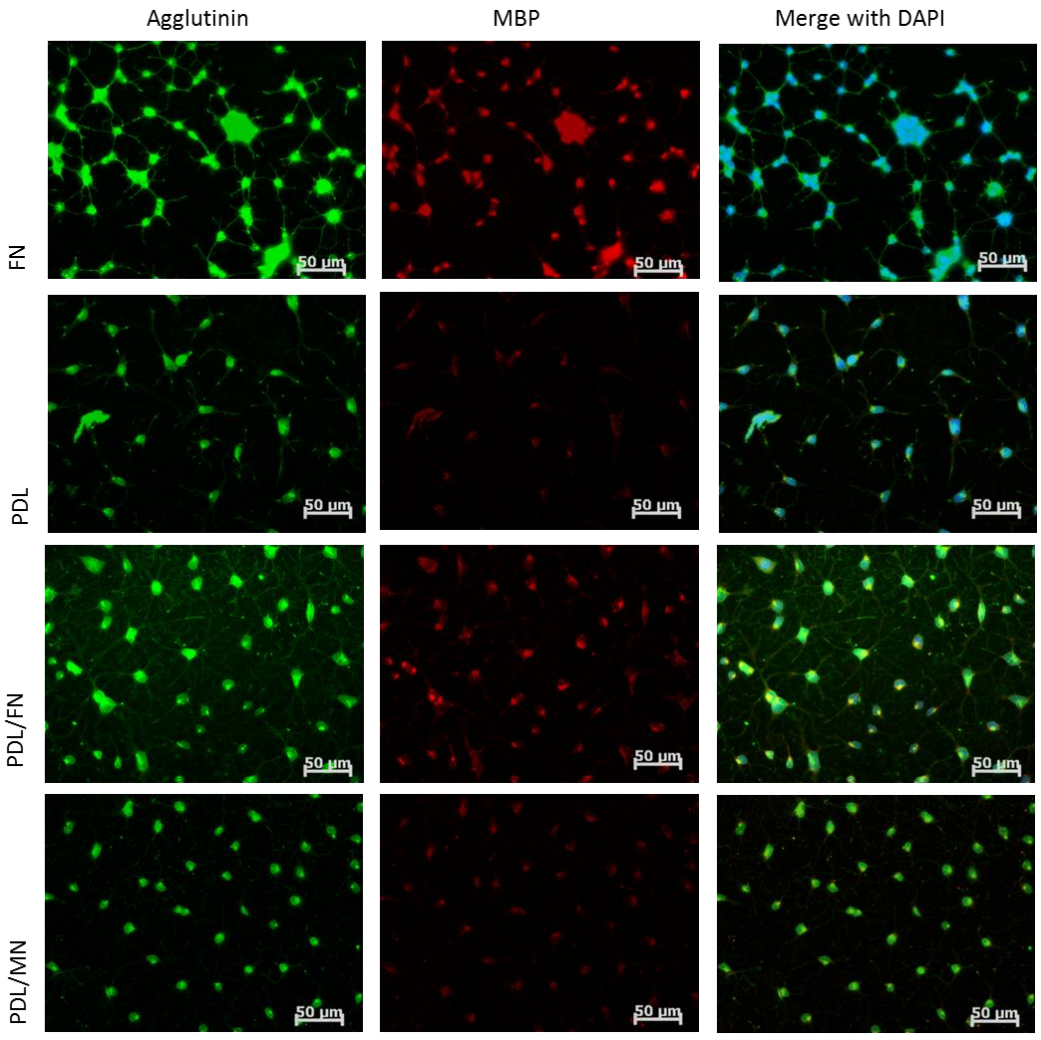


Figure 29| CG-4 cells were cultured on coated coverslips with proteins described in image. Cells were cultured for 2 days with proliferation medium. Cells were stained with agglutinin (green), anti-MBP antibody (red) and DAPI (blue). Two independent experiment. Bar corresponds to 50µm.

This pattern of MBP expression on coverslips and hydrogels was similar for all conditions, except on hydrogels with PDL/MN, where a few cells expressed MBP in branched pattern (Figure 30 - PDL/MN). On hydrogels, it was not possible to perform a triple staining as on coverslips, because hydrogels were auto-fluorescent on the rhodamine filter (red channel).

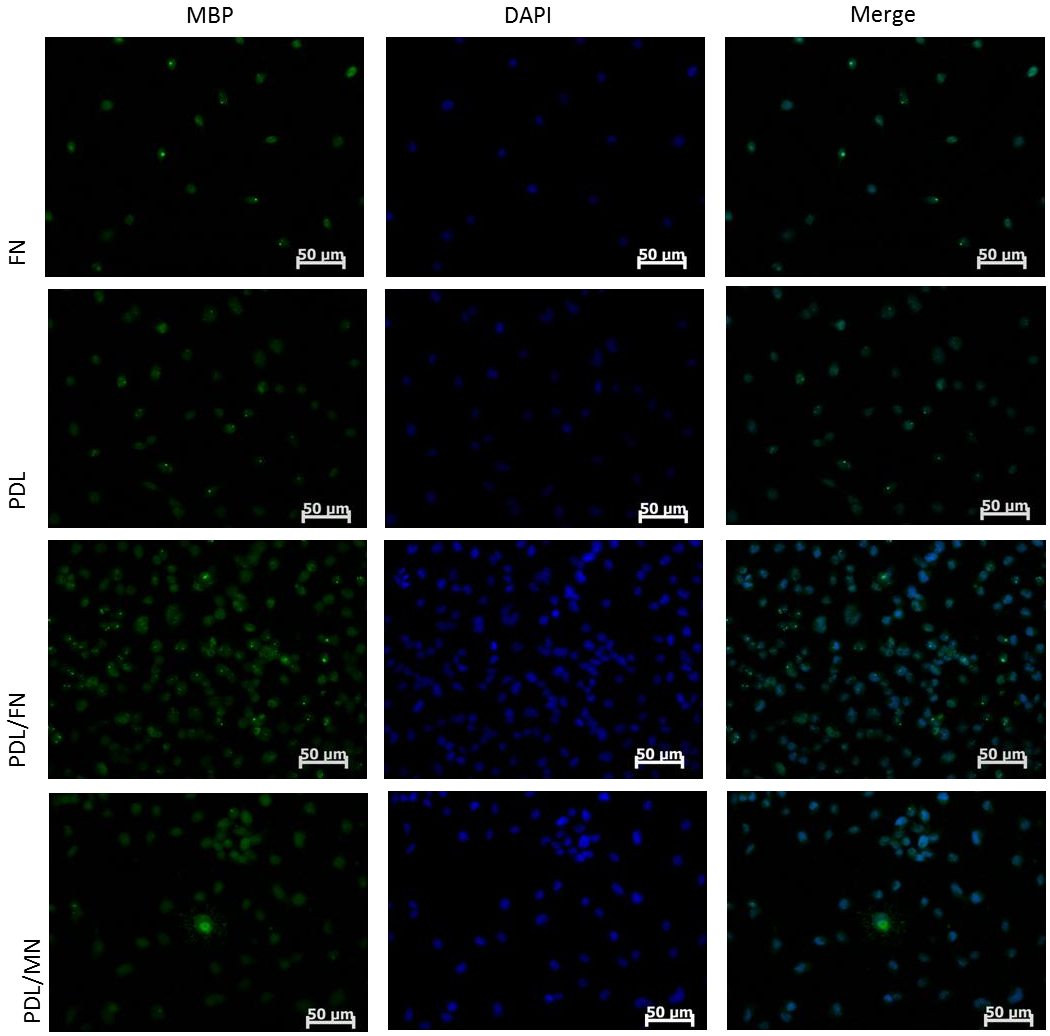


Figure 30| CG-4 cells were cultured on functionalised hydrogels with proteins described in image. Cells were stained with MBP (green) and DAPI (blue). This experiment was performed 2 times. Bar corresponds to 50µm

As expected, after 3 days on coverslips with differentiation medium several cells expressed MBP in a branched pattern and the expression seemed to be more intense (Figure 31) than previously observed for cells on PM (Figure 29). In Figure 31 MBP staining seemed to be increased in cells cultured on double-coated coverslips with PDL/FN or PDL/MN, which could be due to the fact that PDL promotes cell adherence, enhancing the

cell density and putatively contribute to cell survival, increasing the number of MBP positive cells. On hydrogels, many cells died and the remaining living cells did not express MBP with a differentiated pattern (Figure 32), in contrast to what was observed on coverslips. Since in general CG-4 cells die soon after differentiation and we observed that cells cultured on hydrogels functionalised with PDL/MN with proliferation medium seemed to differentiate prematurely (at least partially), as assessed by the morphological analysis shown in Figures 26, 27 and 28), we hypothesized that CG-4 cells cultured with differentiation medium on hydrogels (and putatively expressing MBP) could be dying sooner due to earlier differentiation onset as compared to cells cultured on coverslips. Hence, we decided (i) to increase the cell density to 15,000 cells/cm^2, hopefully enhancing cell survival after differentiation and (ii) after allowing cell attachment to the substrate on recovery medium for 1h (after seeding the cells), the medium was switched directly to differentiation medium, to better control the differentiation timing.

CG-4 cells were cultured on hydrogels and coverslips directly for 3 days with differentiation medium and then cells were fixed and immunostained with anti-MBP antibody and DAPI. In Figure 33, cells on coverslips expressed MBP in a branched pattern and the expression was quiet enhanced as compared to control cells (Figure 29). Furthermore, there were no evident differences between cells differentiated with this procedure (3 days with differentiation medium) or the previous procedure (2 days with proliferation medium followed by 3 days with differentiation medium), indicating that the direct differentiation protocol was equally valid. After 3 days in culture with differentiation medium on functionalised hydrogels many cells died and there was still no evident MBP expression (Figure 34).

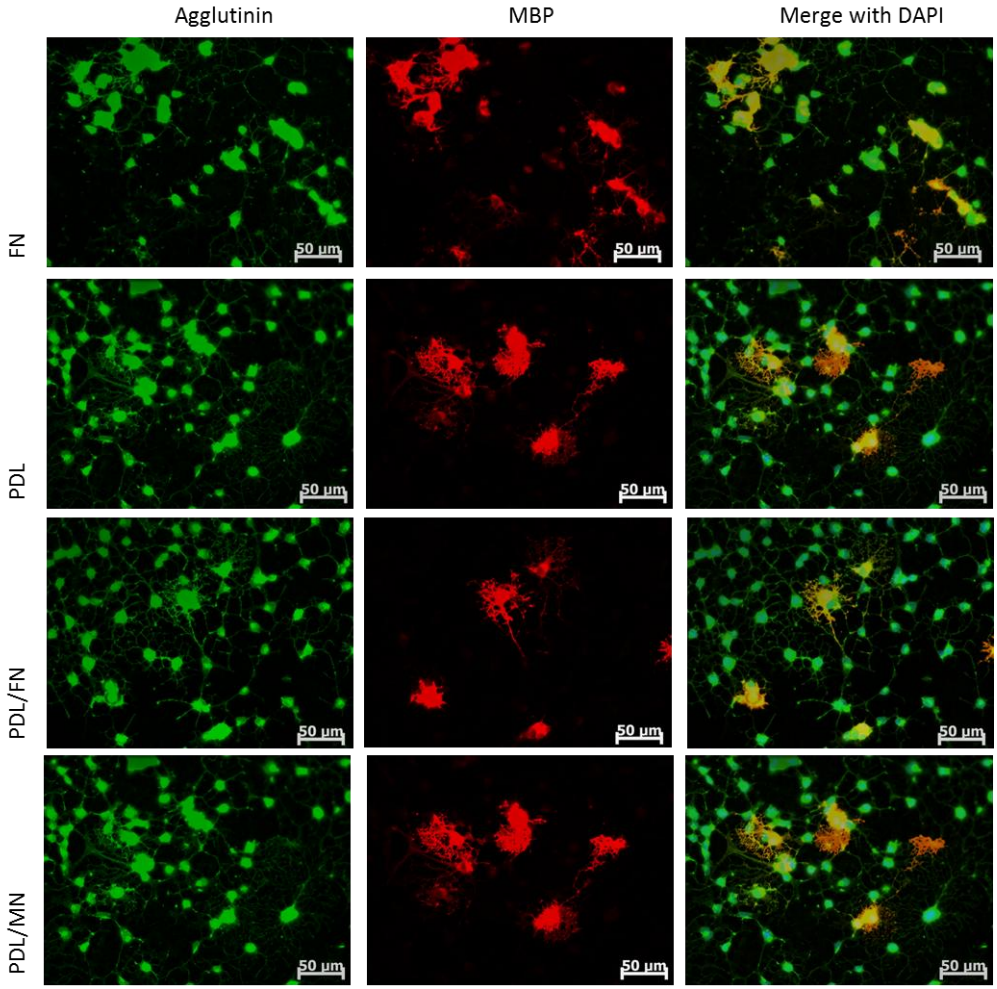


Figure 31| CG-4 cells were cultured on coated coverslips with proteins described in image. Cells were cultured for 2 days with proliferation medium followed by 3 days with differentiation medium on coverslips coated with the indicated proteins (n=2). Cells were stained with FITC-agglutinin (green), anti-MBP antibody (red) and DAPI (blue) Bar corresponds to 50µm.

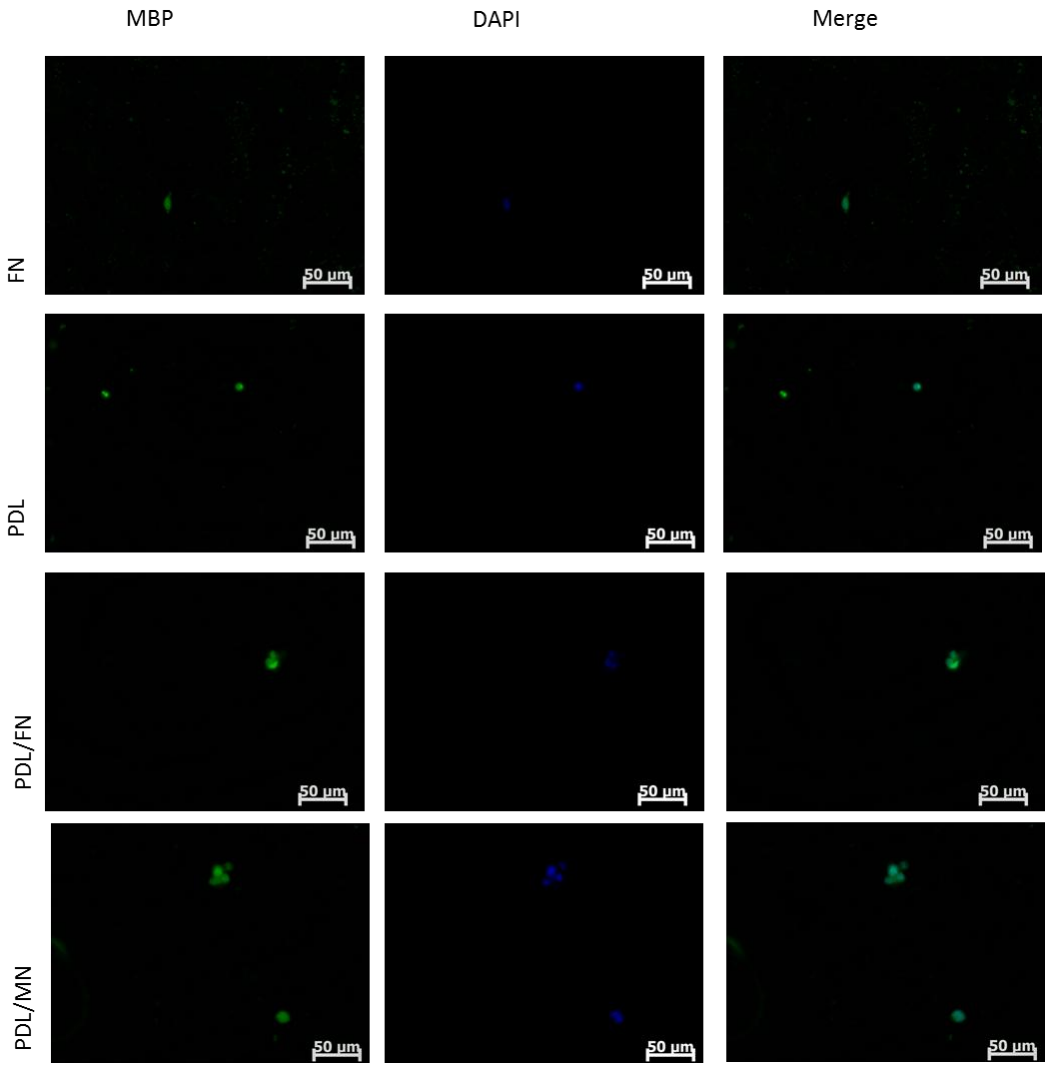


Figure 32| CG-4 cells were cultured on functionalised hydrogels with proteins described in image. Cells were cultured 2 days with proliferation medium followed by 3 days with differentiation medium (n=2). Cells were stained with anti-MBP antibody (green) and DAPI (blue).Bar corresponds to 50µm.

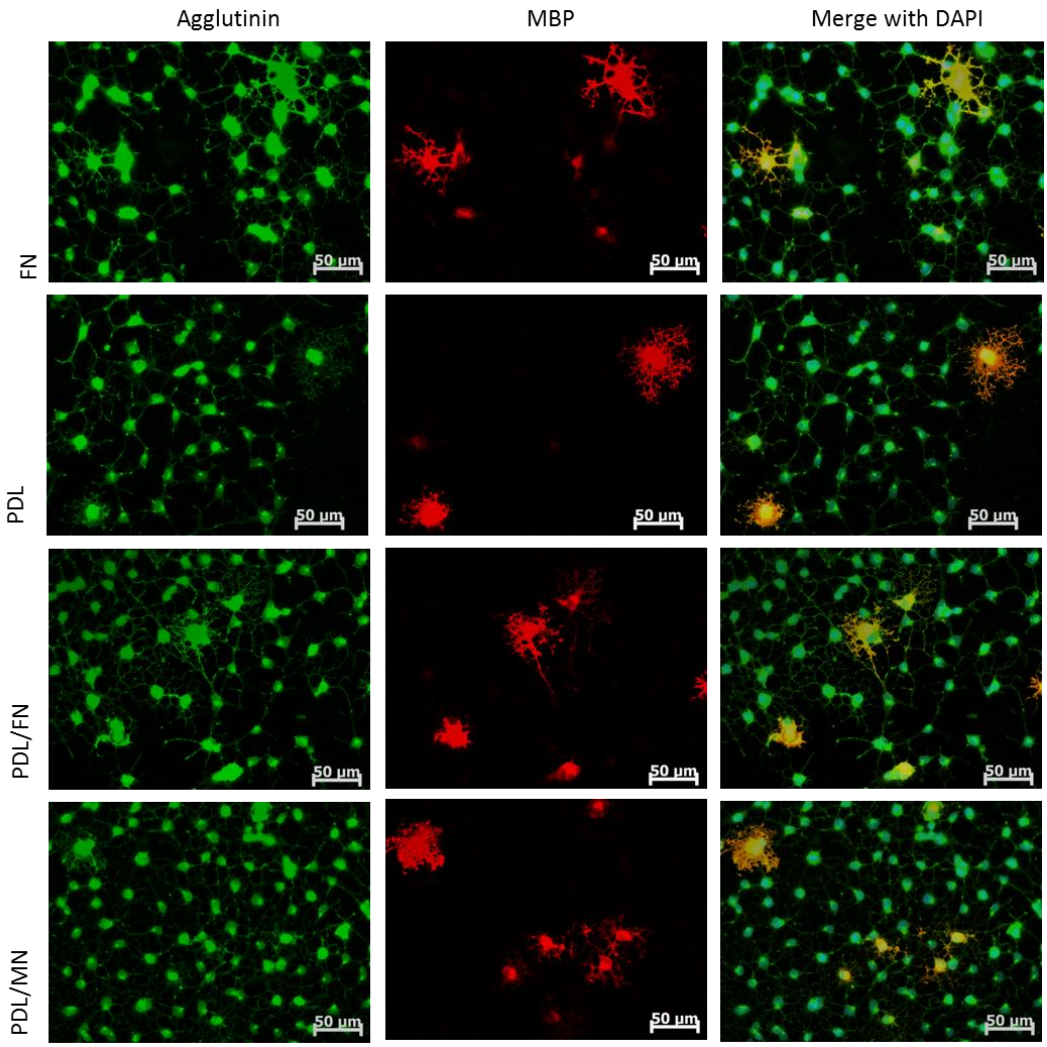


Figure 33| CG-4 cells were cultured on coated coverslips with proteins described in image. Cells were cultured directly 3 days with differentiation medium on coverslips coated with indicated proteins. Cells were stained with FITC-agglutinin (green), anti-MBP antibody (red) and DAPI (blue). This experiment was performed only once. Bar corresponds to 50µm.

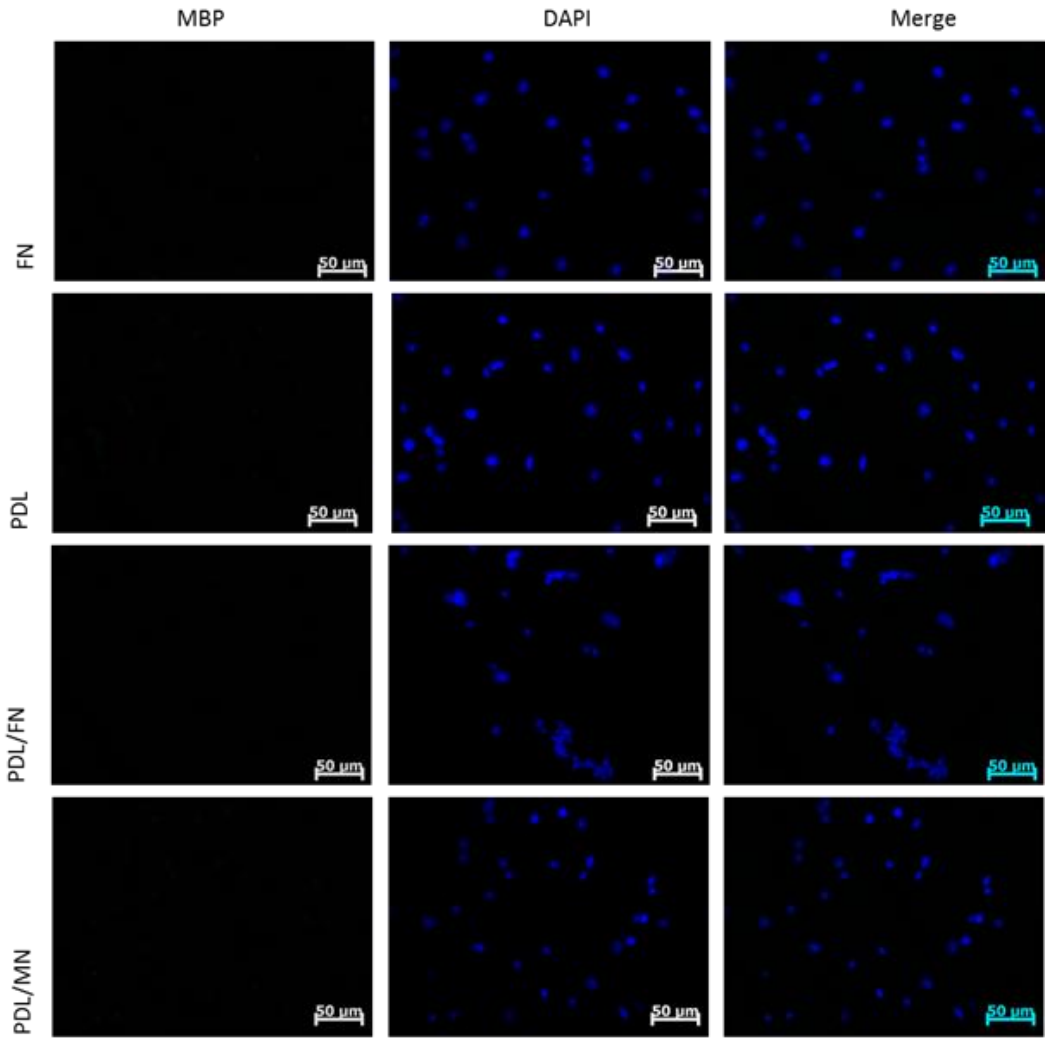


Figure 34| CG-4 cells were cultured on functionalised hydrogels with proteins described in image. Cells were cultured for 3 days with differentiation medium. Cells were stained with anti-MBP antibody (green) and DAPI (blue). This experiment was performed only once. Bar corresponds to 50µm

Further testing our hypothesis that cells may differentiate faster on hydrogels than on coverslips and based on the literature, we inferred that the differentiation medium used in these experiments might not be sufficient to maintain the differentiated oligodendrocytes alive for more than one day after MBP expression onset, so we decided to test a direct differentiation protocol using differentiation medium for only 2 days. Moreover, it was shown that the addition of 5% FBS after MBP expression onset maintained cells alive, in a differentiated state and expressing MBP for several days[37]. Hence, we decided to perform direct differentiation experiments for only 2 days with the selected differentiation medium (N1+T3+T4) and in parallel, allow the cells to remain for one additional day in the presence of differentiation medium supplemented with 5%FBS, similar to what was described in the literature[37]. In summary, 15,000 cells/cm^2 were seeded on hydrogels with recovery medium to allow cell attachment (for 1h), then recovery medium was switched to proliferation medium (control) or differentiation medium. Two days later, the cells cultured with proliferation medium and part of the cells cultured with differentiation medium were fixed and stained as described in the Materials and Methods. The remaining cells cultured with differentiation medium were changed to differentiation medium supplemented with 5% FBS for further 24 hours.

The differentiation of CG-4 cells during 2 days on coverslips seemed to be insufficient time to allow MBP expression (Figure 35) as compared to the direct differentiation 3 days protocol (Figure 33).

CG-4 cells that were induced to differentiate on hydrogels for 2 days were also stained with anti-MBP antibody and DAPI and it was observed that some cells expressed MBP and displayed a branched pattern, especially when cultured on hydrogels functionalised with PDL/FN or PDL/MN (Figure 36). For cells on hydrogels functionalised with PDL or FN it seemed that there was no branched pattern of MBP expression (Figure 36). These results further indicate that CG-4 cells differentiated faster on hydrogels and then died, since after 3 days in culture on hydrogels with differentiation medium there were only few living cells, which did not express MBP (Figures 32 and 34), while at least some cells on hydrogels with differentiation medium for 2 days expressed MBP in a branched pattern (Figure 36).

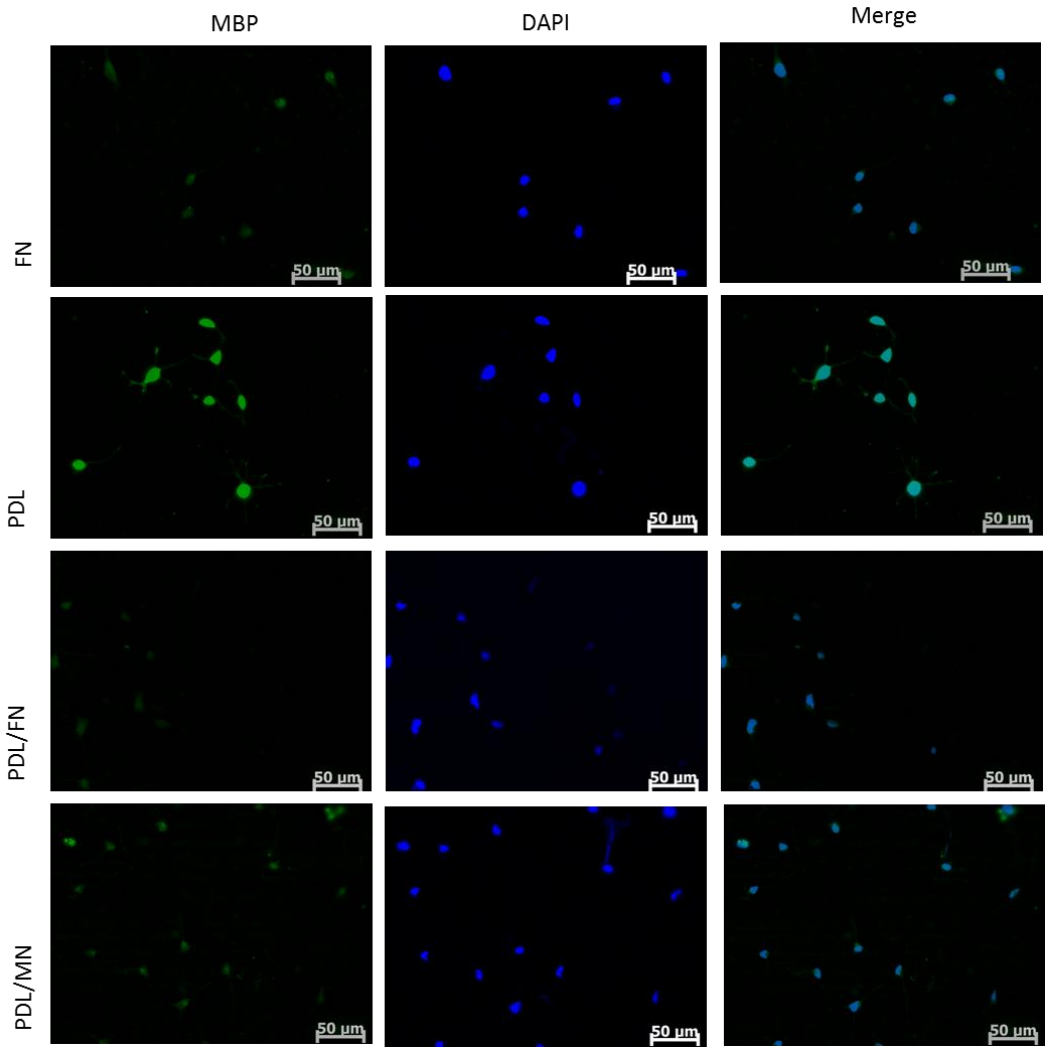


Figure 35| CG-4 cells were cultured on coated coverslips with proteins described in image. Cells were cultured for 2 days with the differentiation medium. Cells were stained with anti-MBP antibody (green) and DAPI (blue). This experiment was performed only once. Bar corresponds to 50µm.

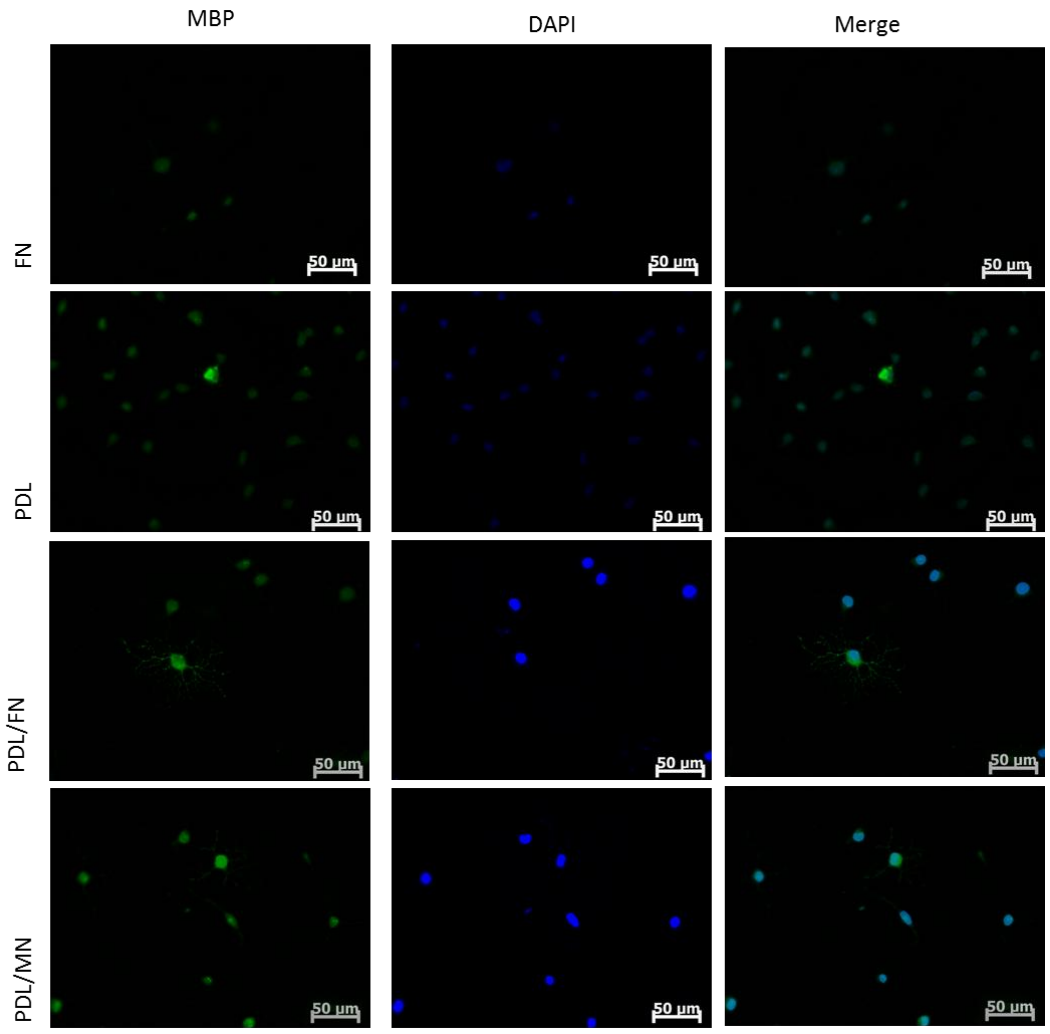


Figure 36| CG-4 cells were cultured on functionalised hydrogels with proteins described in image. Cells were cultured for 2 days with differentiation medium. Cells were stained for MBP (green) and DAPI (blue). This experiment was performed only once. Bar corresponds to 50µm.

In order to attempt an improvement in maintaining differentiated oligodendrocytes alive[37], cells were cultured for 2 days with differentiation medium plus one additional day in presence of differentiation medium supplemented with 5% FBS. The MBP expression and the branched pattern of CG-4 cells cultured on coverslips seemed to increase slightly when cells were differentiated under these conditions (Figure 37) comparing with only 2 days with differentiation medium (Figure 35).

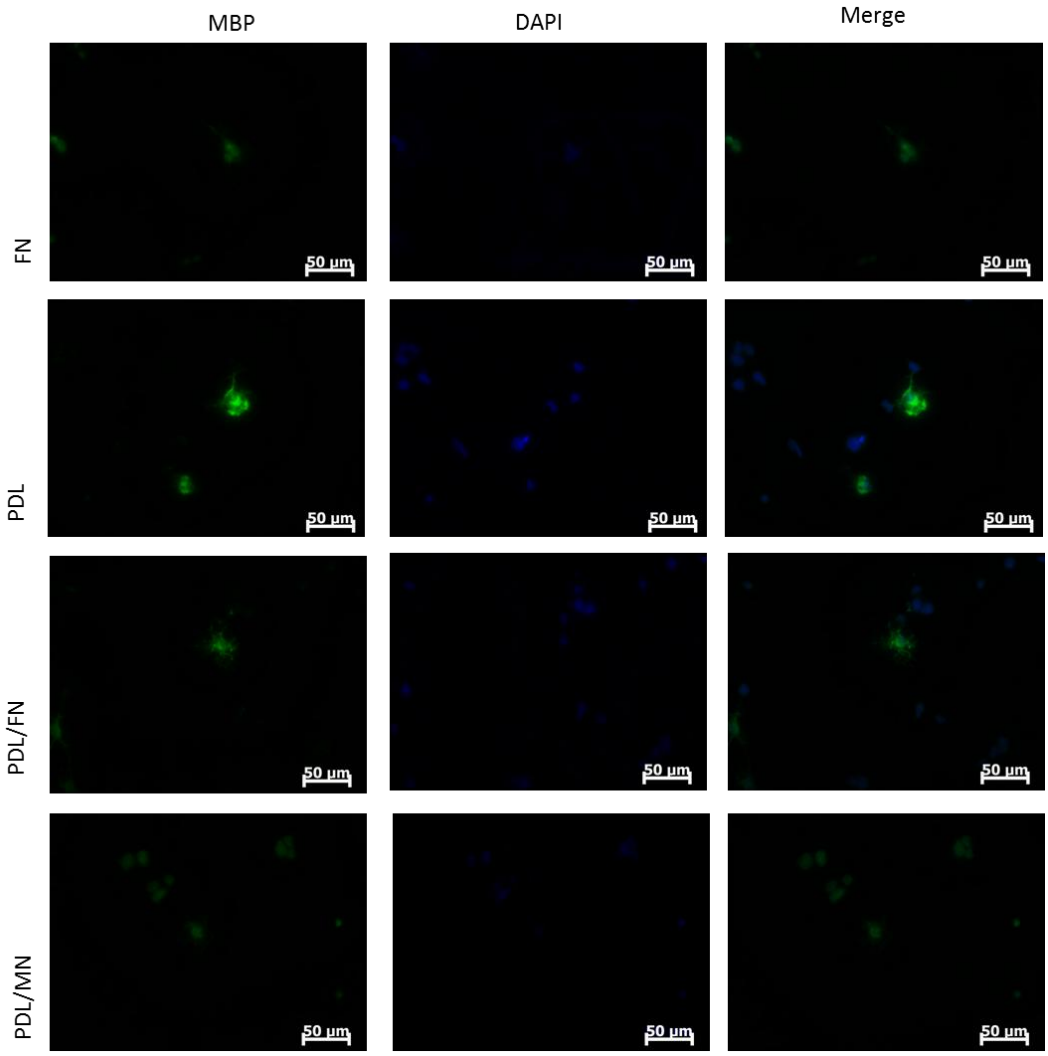


Figure 37| CG-4 cells were cultured on coated coverslips with proteins described in image. CG-4 cells were cultured for) 2 days with differentiation medium plus one additional day with differentiation medium supplemented with 5% FBS. Cells were stained with anti-MBP antibody (green) and DAPI (blue) (n=1) . Bar corresponds to 50µm.

This effect was much more evident for cells cultured on hydrogels, as depicted on Figure 38, comparing to cells cultured with differentiation medium (N1+T3+T4) only for 2 days (Figure 36), 3 days (Figure 34) or 3 days after 2 days in PM (Figure 32). Hence, using the 2 days differentiation medium plus 1 day differentiation medium supplemented with 5% FBS protocol led to more evident MBP expression, branched morphology and higher cell density at the end of differentiation, mainly on hydrogels functionalised with PDL/FN or PDL/MN (Figure 38). On hydrogels functionalised with PDL or FN, MBP expression was weak, mainly in FN, however this was expected because FN has a key role in maintenance

of OPCs. Although these are preliminary results (n=1), they seem promising, suggesting that with this procedure, MBP expression seems to be higher and morphological maturation of the cells seems to occur faster when cells were cultured on PDL/MN-functionalised compliant hydrogels than on similarly coated coverslip (Figures 37 and 38).

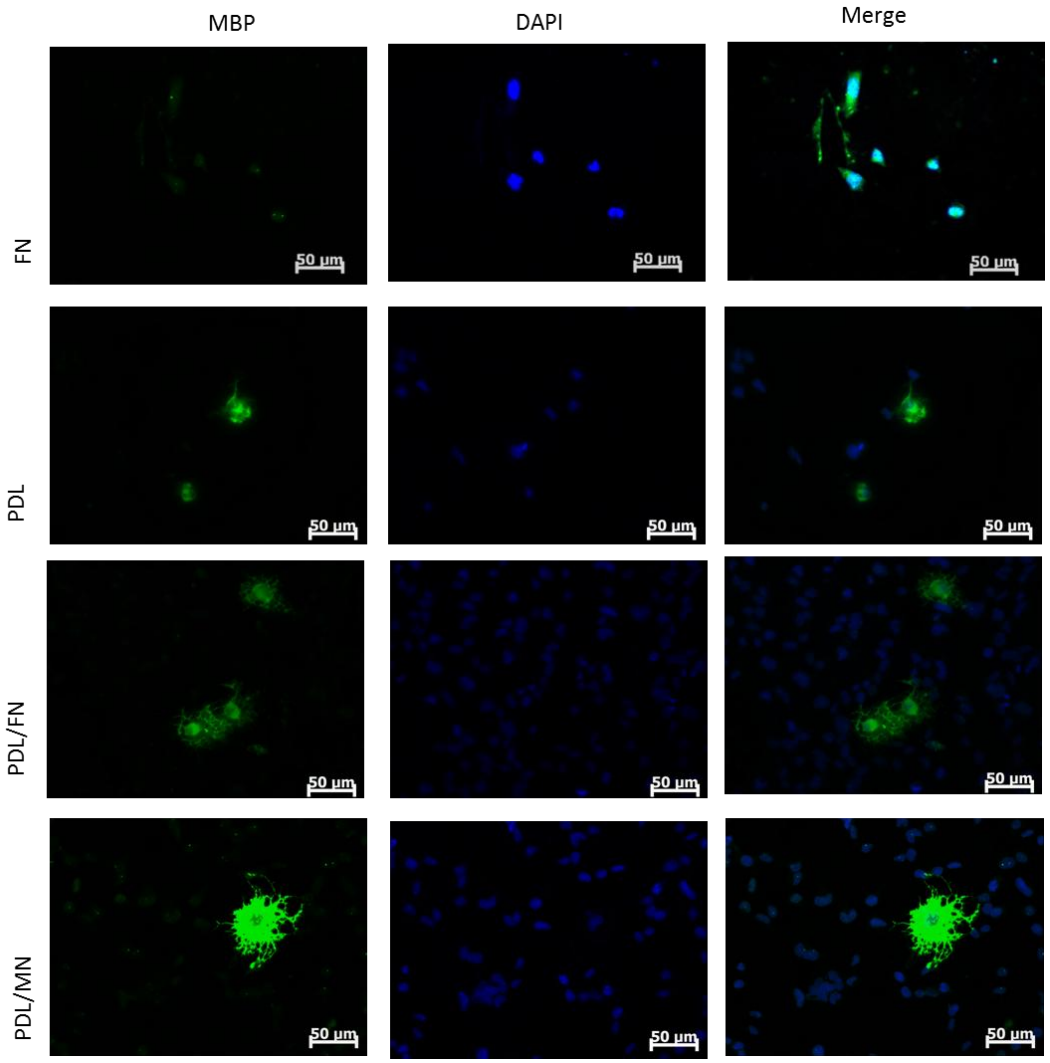


Figure 38| CG-4 cells were cultured on functionalised hydrogels with proteins described in image. CG-4 cells were cultured for 2 days with differentiation medium plus one additional day with differentiation medium supplemented with 5%FBS. Cells were stained with anti-MBP antibody (green) and DAPI (blue) (n=1). Bar corresponds to 50µm.

Chapter IV
Discussion

IV – Discussion

The scope of using *in vitro*-cultured cells for purposes of tissue engineering, regenerative medicine or *in vitro* screening assays is limited by the conditions used to culture such cells. Additionally, the understanding of the basic principles that govern cellular differentiation and other cellular functions is hampered by the experimental artifacts that may emerge due to particular *in vitro* culture conditions. In order to maintain cells *in vitro* in a way that more closely resembles the *in vivo* situation it is important to reproduce the cellular milieu to the highest and reasonable extent possible, using not only soluble factors, but also ECM elements and mechanical cues which mimic the biochemical and biophysical microenvironment of the cells. Using hydrogels, it is possible to mimic the stiffness of distinct tissues (namely of the brain, in the context of this thesis). For instance, polyacrylamide hydrogels are capable of modelling different stiffness degrees by simply varying the percentages of acrylamide and bis-acrylamide, and although polyacrylamide hydrogels are not biocompatible, they are rather to useful to screen the best conditions to induce proliferation or differentiation of cells *in vitro,* making way for the future use of adequate biocompatible materials.

During this work, three important components were optimized to differentiate CG-4 cells into mature oligodendrocytes: soluble factors, ECM proteins and mechanical environment. These elements were expected to trigger signal transduction pathways in cells at multiple layers, namely those initiated by growth factor receptors, integrin receptors and mechanotransduction sensors, respectively. Our approach was to identify conditions that provided measurable and relevant phenotypic changes during oligodendrocytes differentiation (namely MBP protein expression and morphological changes) using CG-4 cells, and in future studies to dissect the pathways and mechanisms involved in such events.

Initially, CG-4 cells were induced to differentiate in conventional polystyrene culture plates, varying only the soluble factors and their concentrations. The soluble

factors play an important role in regulating cell differentiation and we found not only morphological differences but also differences in MBP expression between the several media tested (Figures 21, 22 and S-1). We could conclude that medium N1 supplemented with T3 and T4 provided the best differentiation conditions among the several tested media. We could also substantiate the idea that T3 and T4 play an important role in oligodendrocyte differentiation, and in particular for CG-4 cells since their differentiation using N1 medium in the absence of the thyroid hormones was not as effective (Figures 21 and 22)

The two other components explored in this work (ECM proteins and mechanical environment) were tested using PDL, FN and MN (and combinations) immobilised on coverslips and polyacrylamide hydrogels.

The mechanical aspects were approached having in mind that the brain stiffness ranges from 0.1 to 10KPa (Table i)[16], so our aim was to obtain hydrogels with stiffness degrees ranging between these values and compare its effect on CG-4 cells differentiation with standard cell culture conditions using highly stiff polystyrene plates and glass coverslips. We obtained formulations that produced hydrogels presenting Young's moduli ranging from 0.1 to 6.5KPa (Figure 23), well within the desired target range. Initial studies indicated that the most promising stiffness for the objectives of this study was the 6.5KPa hydrogel (hydrogel number 1, Figure 23), based on morphology of the cells cultured on distinct hydrogels (Figure 26). For reason of time constrain, it was not possible to fully evaluate further the other formulations and the 6.5KPa gel was selected for the remaining studies. In the future, to better screen the best stiffness conditions we will create new hydrogels formulations to obtain a wider range of stiffness degrees and test together with the ones already obtained.

Since cells do not adhere to the polyacrylamide hydrogels, these were functionalised with proteins that allow the cell attachment. Moreover, this provides an opportunity to test the impact of distinct ECM proteins in the biology of oligodendrocytes. The cell attachment to the functionalised hydrogels was dependent on the protein concentration (Figure 24) and functionalization time (Figure 25). In order to increase the density of adherent cells, we tested four concentrations of proteins (10, 25, 50 and

100µg/mL) and the selected concentration was 25µg/mL, since there was no visible improvement of cell attachment using higher concentrations. However, this optimization was performed only for functionalization at 37^{o}C during 2 hours and tested only using cells on proliferation medium (Figure 24), so it would be interesting to address longer functionalization times (e.g. overnight at 4^{o}C, as in Figure 25 ON) and test the possible impact that the utilization of higher concentrations might have in a context of cellular differentiation. This idea is supported by the observation that apparently the hydrogels should not be saturated with protein at a concentration of 25µg/mL, as assessed by silver staining using BSA as a test protein (Figure S-3). Nevertheless, it is also reasonable to hypothesize that, at higher concentrations, bulky proteins like FN and MN might hinder further formation of covalent bonds between additional protein molecules and the hydrogel (somehow similar to the 'hook effect' observed in ELISA assays) and not further enhance or even inhibit cell adherence and differentiation.

To avoid this effect and attempt to enhance the density of adhesion epitopes on hydrogels, we propose the future use of smaller biologically active groups, like oligopeptides (short amino acids sequence) instead of the whole proteins. Active fragments of ECM proteins were analysed and certain short amino acids sequences were identified which appear to bind to and activate receptors on the cell surface, such as integrins and mediate cell adhesion and signal transduction. For example, the RGD (Arg-Gly-Asp) peptide is the cell binding domain of fibronectin; laminin also has several sequences of adhesion peptides: YIGSR, SIKVAV and RNIAEIIKDI which are involved in integrins activation and mediate cells adhesion[50]. In the literature, it was described that the synthetic functional peptides activity was similar to the proteins and their use in polymer surfaces functionalization was described[51,52]. So, the use of the functional peptides may enhance the amount of functional groups binding to the hydrogels and improve the maintenance or differentiation of the cells.

Deformable hydrogels transmit mechanical cues to cells[16,34] and CG-4 cells seemed to respond to those mechanical cues, since cells cultured with only proliferation medium showed already morphological differences comparing to those cultured on coverslips (Figures 27 and 28). For cells to sense mechanical cues and alter their morphology it is

crucial to activate integrins by binding of ECM proteins[29]. So, when FN was bond to the hydrogels probably there was activation of αVβ3 integrins[22] and CG-4 cells displayed a bipolar morphology consistent with OPCs, to a higher degree than the same cells on FN coated coverslips (Figure 27 and 28). To confirm whether the bipolar morphology corresponds to the OPC stage, it will be important to stain the cells with OPCs markers, such as A2B5. On the other hand, in the presence of MN, cells cultured in PM already displayed a branched morphology (probably involving activation of α6β1 integrins[22]), characteristic of more mature oligodendrocytes, and again, at higher degree when cells were cultured on MN-functionalised hydrogels as compared to MN-coated coverslips (Figures 27 and 28). These cells were stained for MBP (late stage marker) and its expression seemed to increase when cultured on PDL/MN in comparison with other adhesion proteins tested (Figure 30). To confirm these results, similar experiments should be performed, staining cells for MBP and A2B5 to verify whether there is a switch of the expression of these proteins. In parallel, studies for the specific activation of integrins should also be performed.

Our experiments suggested that CG-4 cells differentiation seems to occur faster on hydrogels than on coverslips (Figures 31 -38), but the procedure to differentiate cells might still be improved with slight modifications to the differentiation medium, for instance by adding different percentages of FBS or PDGF-AA/bFGF at low concentrations (between 0.1 to 1ng/mL, reported to enhance survival of differentiated oligodendrocytes)[42] and at different differentiation time-points. Also, it seems that hydrogels functionalised with PDL/MN are the most favourable ones in inducing CG-4 cells differentiation, as anticipated (Figure 38). Nevertheless, due to issues of time constrain, some of the final experiments were not performed 3 times (as indicates in the results section) and require further validation.

At this moment, as a result of this work, it is possible maintain and differentiate CG-4 cells on hydrogel number 1. Other hydrogels will be tested, as well as other cell types as well, such as other cell lines and primary oligodendrocyte precursor cells. We have already begun preliminary experiments using the human oligodendroglioma cell line HOG (Supplementary data, Figure S-2), which will be soon tested on hydrogels. Primary cells

present several advantages, such as not being genetically modified, being more similar to the *in vivo* cells and their use approximates *in vitro* to the *in vivo* responses. So, rat OPCs will be used on hydrogels to screen the best conditions (ECM proteins and stiffness) to maintain their progenitor state and also to differentiate into mature OLs with an *in vitro* model closer to the *in vivo* situation.

Chapter V

Conclusion

V – Conclusion

In conclusion, we optimized a differentiation medium (N1+T3+T4) to differentiate CG-4 cells into mature oligodendrocytes. This medium was formulated based on the literature, but this exact composition has not been described yet, to the best of our knowledge.

As depicted on Figure 39 we can concluded that CG-4 cells cultured on proliferation medium respond to mechanotransduction cues as we observed cells more similar to OPCs or more similar to mature oligodendrocytes, when plated on hydrogels functionalised with FN or MN, respectively (Figures 27 and 28).

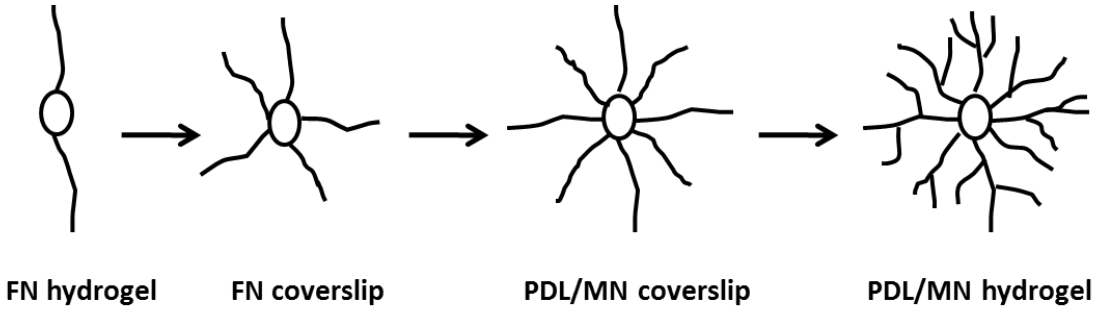


Figure 39|Schematics of the CG-4 morphology. This figure summarise the morphological changes observed when cells were cultured on the indicated platforms and proteins with proliferation medium.

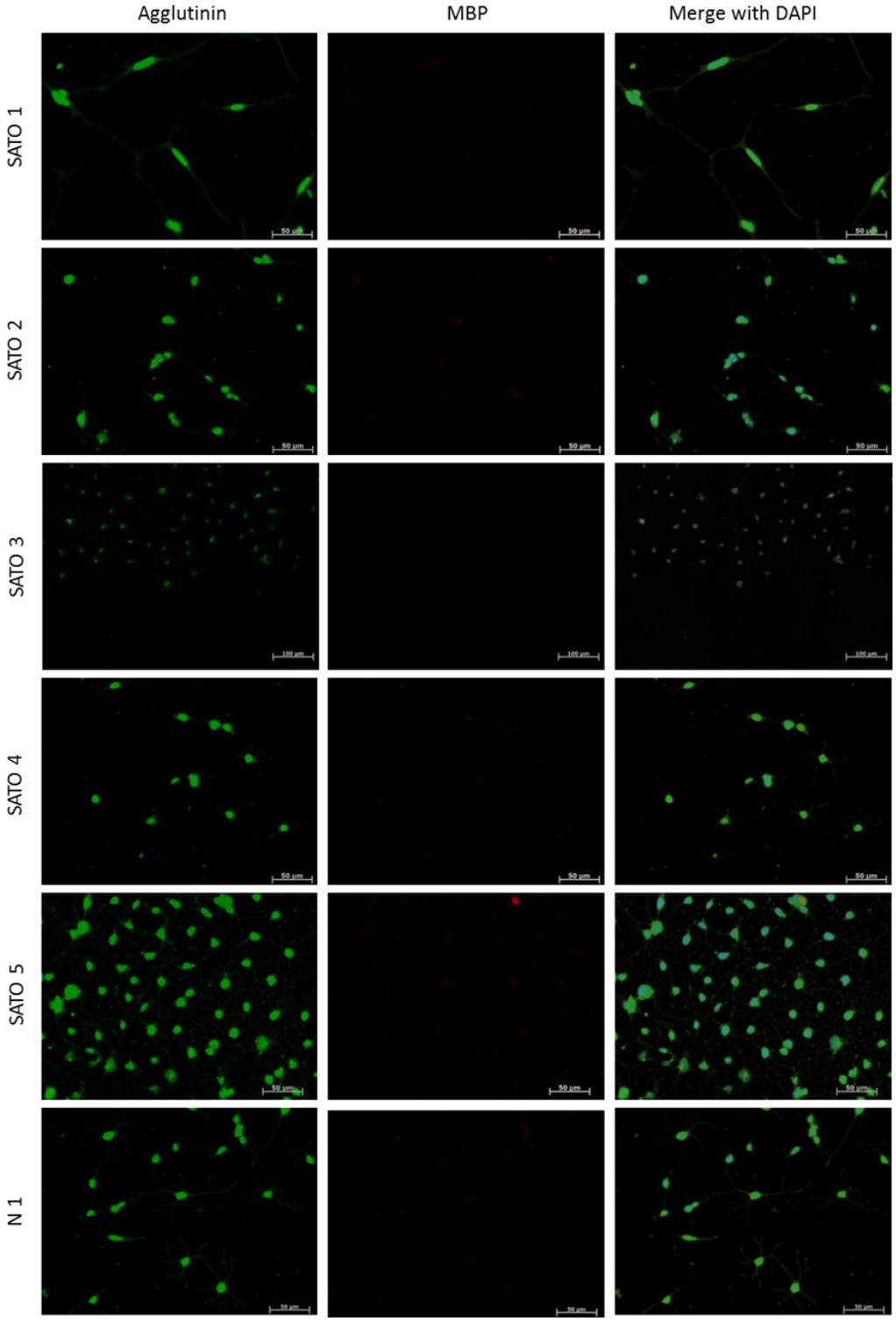
Finally, we obtained differentiated oligodendrocytes which were MBP positive and showed a mature morphology when cultured on differentiation medium on hydrogels and on conventional platforms (coverslips and culture plates). However, the differentiation process seems to be faster when cells were cultured on compliant hydrogels.

The added value of this work was the establishment of an innovative platform to study the maintenance and differentiation of oligodendrocytes *in vitro* , and to dissect the involvement of several important players, such as soluble factors, ECM proteins and mechanotransduction elements.

Supplementary data

Supplementary data

1 - CG-4 differentiation



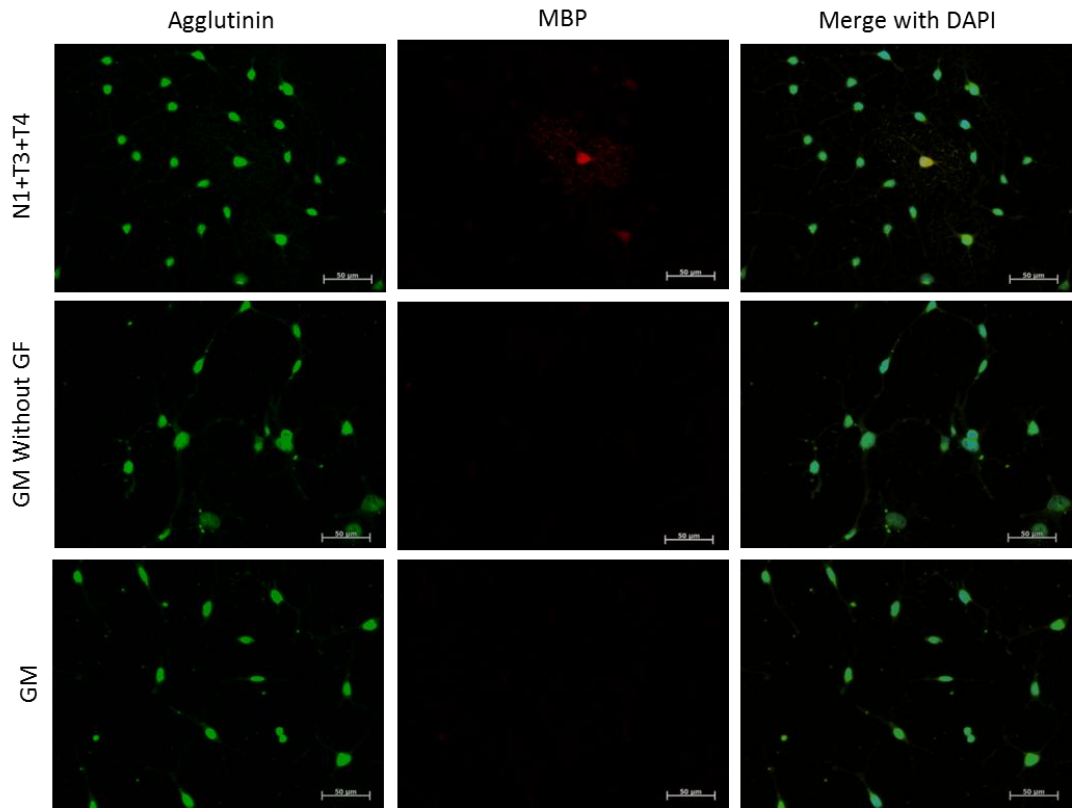
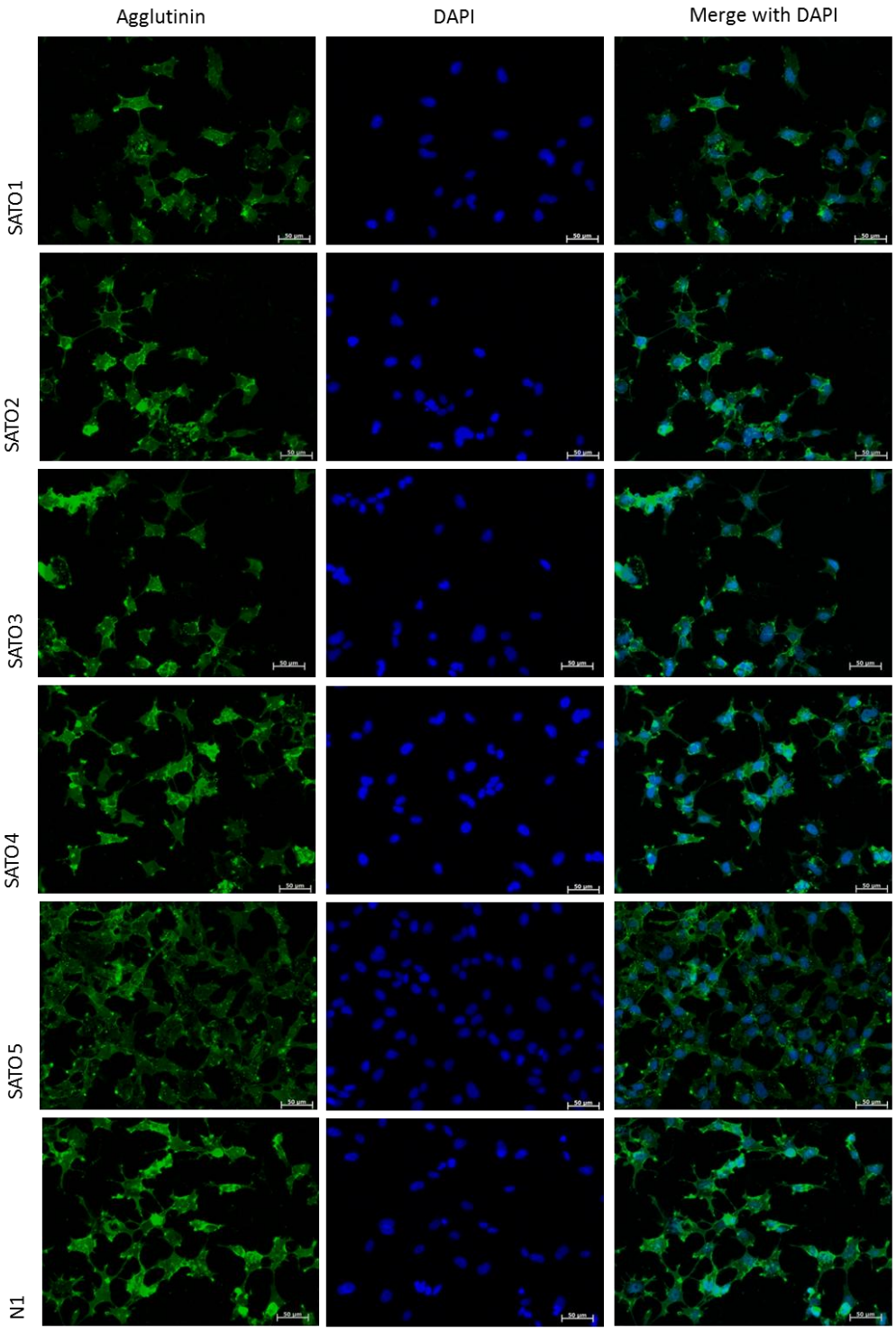


Figure S 1| CG4 cells differentiation in different media. Cells were previously cultured for 2 days in proliferation medium (PM) and then were induced to differentiate in the presence of the corresponding differentiation medium for 3 days (n=3).

2 - HOG differentiation

The differentiation of the HOG cell line was tested with several differentiation media and assessed by agglutinin staining. The tested media were the same used for the CG-4 cell line. We observed more processes when cells were cultured in N1 medium, while using other media, the cells showed very few processes. In general, the literature has shown that processes in HOG cells are small[4,53] and typically do not display such a developed a branched morphology like CG-4 cells, as expected (Figure S-2)

This cell line was not yet tested on hydrogels, because priority was given to the CG-4 cell line, which is a better cellular model (the maturation expression markers and morphology are more robust). However, the HOG cell line is a human cell line model that will hopefully be useful to screen the best conditions to differentiate human oligodendrocytes.


Agglutinin
DAPI
Merge with DAPI
SATO1
SATO2
SATO3
SATO4
SATO5
N1

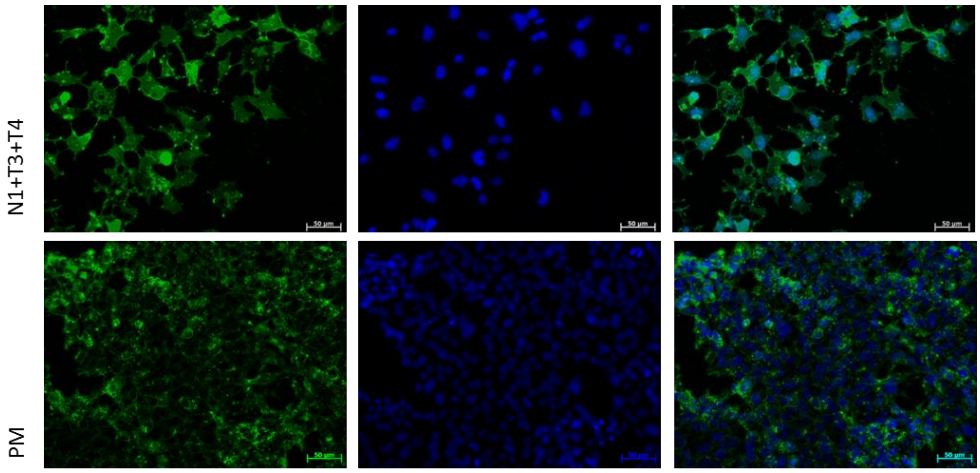


Figure S 2| HOG cells differentiation in different media. Cells were previously cultured for 2 days in proliferation medium (PM) and then were induced to differentiate in the presence of the corresponding differentiation medium for 3 days.

3 - Silver staining of the hydrogels

Hydrogels (hydrogel number 1, 6.5KPa) were functionalised using different concentrations of BSA (1000, 500, 250, 100 and 10µg/mL) for 2h at 37^{o}C to assess which would be saturating concentration of protein. To stain the hydrogels proteins were fixed for 30 minutes with a solution with 25%methanol and 5% acetic acid. Then, gels were dehydrated during 10 minutes with each percentage of ethanol 50% and 30%, respectively. After dehydration a solution of sodium thiosulfate (0.2g/L) was added during one minute. This step was followed by 3 washes of 5 minutes each. Then silver nitrate (2g/L) was added for 20 minutes. The developing solution was constituted by 37% formaldehyde, 30 g/l sodium carbonate anhydrous and 10 mg/l sodium thiosulfate and was incubated for 15 minutes. In the last step (to stop the development) a solution of 50g/L Tris and 2.5% acetic acid was added.

The hydrogels did not seem to be saturated at 100µg/mL BSA (Figure S-3). The highest concentration of PDL, FN and MN tested for cell adherence.

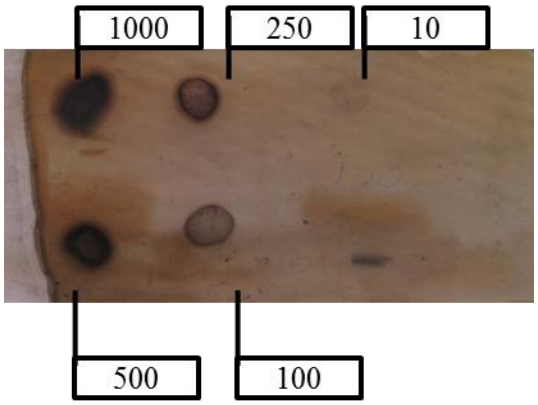


Figure S 3| Hydrogels silver stained. The hydrogel was functionalised in distinct spots with a BSA solution of 1000, 500, 250, 100 and 10 µg/mL for 2 hours at 37^{o}C.

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