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UNIVERSITÉ DE MONTRÉAL

FABRICATION PAR ÉLECTROFILAGE D'UNE STRUCTURE À ÉLUTION DE FACTEURS DE CROISSANCE POUR CONTRÔLER LA DIFFÉRENCIATION DE CELLULES SOUCHES NEURONALES EN NEURONES MOTEURS

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Ce mémoire intitulé:

FABRICATION PAR ÉLECTROFILAGE D'UNE STRUCTURE À ÉLUTION DE FACTEURS DE CROISSANCE POUR CONTRÔLER LA DIFFÉRENCIATION DE CELLULES SOUCHES NEURONALES EN NEURONES MOTEURS

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RÉSUMÉ

Les blessures médullaires et les lésions étendues des nerfs handicapent des milliers de nouvelles personnes chaque année aux États-Unis seulement. Il n'y a actuellement aucun traitement permettant la guérison de ces blessures. Une approche envisagée est la greffe de structures chargées de cellules souches pour rétablir les fonctions originelles du tissu. Ce travail vise donc à développer une méthode de fabrication d'une structure qui puisse offrir un support mécanique aux cellules, tout en délivrant sur place le cocktail de biomolécules adéquat pour diriger la différenciation des cellules vers le phénotype de neurone moteur. Afin de préparer cette structure, de la gélatine et de l'acide polylactique-L ont été co-électrofilés. L'électrofilage permet la fabrication de fibres de diamètre dépendant de la concentration des solutions en polymère. Les fibres ainsi fabriquées ont été réticulées afin de ralentir et contrôler leur dégradation. Les biomolécules permettant de promouvoir la différenciation des cellules en neurones moteurs, l'acide rétinoïque et la purmorphamine, ont été incluses dans la partie extérieure des fibres, en gélatine. Ces molécules ont diffusé de manière continue à partir des fibres, dans le milieu liquide. Les cellules implantées sur cette structure ont proliféré et se sont différenciées en neurones moteurs. Leur phénotype a été caractérisé par immunofluorescence.

ABSTRACT

Spinal cord injury and extended nerve injury currently have no cure. These pathologies are responsible for the decrease in quality of life of thousands of new people every year in the US only, and are draining huge costs to the healthcare system. Current research in the area focuses on the grafting of an artificial structure loaded with stem cells to restore tissue functions. The objective of this work is to propose a structure that can offer mechanical support to the cells, favor their proliferation, and promote their differentiation into motor neuron, by delivering *in situ* the appropriate cocktail of growth factors. Such structure was prepared by co-electrospinning of poly L-lactic acid and gelatin. Fiber diameter can be adjusted by controlling the polymer concentration. These fibers were crosslinked to slow their degradation. Retinoic acid and purmorphamine were included in the outer layer of gelatin. These two growth factors are known to direct cell differentiation towards a motor neurons phenotype and were continuously released from the fibers in the medium. Cells proliferated on the structure and differentiated into motor neurons. Their phenotype was characterized by immunostaining using sample images.

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BDNF	Brain derived neurotrophic factor
CNTF	Ciliary neurotrophic factor
EGF	Endothelial growth factor
ELISA	Enzyme linked immunosorbent assay
FGF	Fibroblast growth factor
GDNF	Glial cell line-derived neurotrophic factor
HA	Hyalorunic acid
IGF	Insulin-like growth factor
LOCS	Linear ordered collagen scaffolds
MSC	Mesenchymal stem cell
NGF	Nerve growth factor
NSC	Neural Stem Cell
NSLC	Engineered Stem Like Cell
NT-3	Neurotrophin 3
PEG	Polyethylene glycol
PES	Poly ethersulfone
PGA	Poly glycolic acid
PLGA	Poly lactic co-glycolic acid
PLLA	Poly L-lactic acid
RA	Retinoic acid
Shh	Sonic hedgehog
VEGF	Vascular endothelial growth factor

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INTRODUCTION

Une multitude d'accidents peuvent occasionner des lésions des nerfs, voire de la moelle épinière, entrainant une baisse notable de la qualité de vie des patients.

En effet, bien que des nerfs périphériques blessés sur de courtes distances puissent se régénérer, si la blessure est trop importante ou touche la moelle épinière, la régénération du tissu nerveux se fait difficilement. [1] Cette mauvaise régénération du tissu est due à la fois à une activité réduite des cellules souches neuronales, ce qui diminue la plasticité du tissu nerveux, et à la présence de tissu cicatriciel gênant la régénération du tissu originel. [2] Ces raisons font que la thérapie par cellules souches est toute indiquée pour pallier à ce genre de pathologie. En effet, ces thérapies impliquent l'injection de cellules souches pour pallier au manque du patient et restaurer la capacité du tissu à se régénérer.

Les cellules souches ont la capacité de se multiplier et de se différencier, théoriquement, selon les stimuli environnementaux, en n'importe quel type cellulaire. Par conséquent, il est nécessaire de pouvoir contrôler leur différenciation. Les principaux facteurs qui influencent le phénotype vers lequel une cellule souche évoluera sont les propriétés mécaniques de son environnement, la topologie de son support qui doit copier la matrice extracellulaire et les facteurs de croissance qu'elle reçoit. Il est désormais connu que des cellules souches neuronales se différencieront en neurones moteur si elles reçoivent les concentrations adéquates d'acide rétinoïque et de sonic hedgehog. [3] D'autre part, il a été récemment découvert que la purmorphamine a les mêmes effets sur les cellules que sonic hedgehog. [3]

L'utilisation de cellules souches pour de telles thérapies requiert la fabrication d'une structure support qui d'une part offre un milieu aux propriétés mécaniques satisfaisantes guidant l'étalement des cellules et d'autre part évite la migration anarchique des cellules souches injectées. Différentes techniques ont donc été développées pour permettre l'implantation d'une structure chargée de cellules souches dans la zone blessée. Un problème majeur des méthodes proposées jusqu'alors est que la différenciation des cellules souches implantées nécessite la présence de facteurs de croissance qui ne sont pas produits par le patient dans la zone blessée et dont la concentration doit être très finement contrôlée. La majorité des techniques mises au point à l'heure actuelle offrent bien un support mécanique aux cellules injectées, mais ne permettent

pas le contrôle de leur différenciation. Elles requièrent donc en général une injection externe de ces facteurs. [4] La mise en place de telles techniques sur l'homme n'est pas envisageable car elles demandent des opérations chirurgicales répétées beaucoup trop lourdes, et une administration très régulière de ces molécules signal, afin d'obtenir un stimulus contrôlé de leur concentration dans l'environnement entourant les cellules.

Dans ce contexte, les travaux les plus récents réalisés dans le domaine ont comme objectif la fabrication de structures qui puissent à la fois offrir un support mécanique aux cellules afin de permettre leur adhésion, d'organiser spatialement leur croissance, et, le cas échéant, de promouvoir leur multiplication avant implantation, mais qui puissent aussi diffuser directement in situ et à la bonne concentration les facteurs de croissance requis pour contrôler la différenciation desdites cellules. [5]

L'objectif de ce travail est d'utiliser l'électrofilage pour fabriquer une telle structure. Cette technique permet l'obtention de fibres de diamètre variable à partir de différents polymères. Nous proposons d'utiliser l'électrofilage concentrique de deux polymères, la gélatine (couche externe) et l'acide poly-lactique (cœur interne) afin de bénéficier à la fois des propriétés mécaniques des fibres en acide poly-lactique et des bonnes propriétés de contact et de biocompatibilité de la gélatine. En plus d'offrir un bon support mécanique, la structure développée doit pouvoir délivrer des facteurs de croissance.

La première partie de ce mémoire présente une revue de la littérature sur les techniques actuellement étudiées dans le but d'obtenir la régénération de tissus nerveux.

La seconde partie, reprenant les concepts les plus intéressants exposés dans notre revue de littérature, propose, à notre avis, la technologie la plus prometteuse pour fabriquer une structure permettant de promouvoir la prolifération et la différenciation des cellules souches en neurones moteurs. Dans cette partie, les propriétés mécaniques et la dégradation du matériel sont étudiées. Ensuite la vitesse d'élution des biomolécules est évaluée et, finalement, la capacité de ce nouveau matériel à promouvoir la différenciation des cellules souches neuronales en neurones moteurs est validée par l'analyse d'images obtenues par immunofluorescence.

CHAPITRE 1 DÉMARCHE GÉNÉRALE

Ce mémoire présente deux articles. Le premier, intitulé « Approaches for neural tissue regeneration » a été soumis dans *Stem cells reviews and reports* le 17 juillet 2013. Cet article permet d'exposer l'ensemble des techniques étudiées actuellement dans le but d'obtenir la génération de nouveaux tissus à partir de cellules souches. Les connaissances développées dans cette revue permettent de juger des avantages de chacune des techniques connues, et de faire un choix éclairé parmi celles-ci pour répondre à nos objectifs. Le second, intitulé "Differentiation of neuronal stem cells into motor neurons using co-axial electrospun Poly-L-Lacctic Acid/gelatin fibers as an instructive cue-delivering scaffold", a été soumis dans *Biomaterials* le 20 Août 2013. Ces journaux scientifiques ont été sélectionnés pour leur niveau d'impact dans le domaine étudié ; *Biomaterials* étant le journal de référence pour la fabrication de structures en génie tissulaire alors que *Stem cells reviews and reports* publie des protocoles et revues de littératures sur les techniques émergentes dans le domaine.

J'ai réalisé moi-même l'essentiel des travaux expérimentaux, une partie de la caractérisation de la dégradation des fibres a été réalisé sous ma supervision par Charlène Tendey au cours de son stage de fin d'étude. J'ai aussi bénéficié de l'expérience de mes directeur et co-directeurs au cours de ces travaux et de la rédaction des articles.

CHAPITRE 2 REVUE DE LITTÉRATURE

Il existe différentes approches étudiées dans le cadre du génie tissulaire appliqué aux tissus nerveux. Toutes ces approches ont un objectif similaire qui est de promouvoir la différenciation de cellules souches neuronales en un des types de neurones ou en cellules de Schwann. Elles visent généralement des applications soit dans le système nerveux périphérique ou la moelle épinière, afin le plus souvent de réparer des nerfs endommagés, soit dans le système nerveux central avec des applications plus tournées vers les maladies neuro-dégénératives. Bien que les champs d'application varient légèrement, les méthodes envisagées sont très similaires technologiquement, et une revue de l'ensemble de ces approches est proposée ici dans un manuscrit récemment soumis au journal *Stem cells reviews and reports*.

Article 1 – Approaches for neural tissue regeneration

Loïc BINAN, Abdellah AJJI, Gregory DE CRESCENZO, Mario JOLICOEUR

"Approaches for neural tissue regeneration"

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Abstract

There is currently no treatment for neurodegenerative diseases such as Parkinson's or Alzheimer's diseases. While spinal cord injury has no treatment either, nerve injuries are being treated with autologous grafts, a procedure that in turn translates into a loss of function in the donor area. The development of therapies for these pathologies has become urgent as population keeps on ageing. A promising direction of investigation is the use of regenerative techniques to re-grow healthy and functional tissue in the injured area. In this review article, various approaches currently investigated to promote neural regeneration are covered. Those include approaches based on (and many times combining) stem cell therapy, scaffolds made of hydrogel, electrospun fibers and conductive materials as well as the use of soluble or non-diffusible growth factors.

Keywords

Nerve, spinal cord; stem cell, regeneration, biomaterial, scaffold, hydrogel, electrospinning, growth factor.

2.1 Introduction

16 million first-ever strokes occur every year, which makes it the second cause of death worldwide after heart diseases, with associated costs over 65 billion dollars in the United States only. [6] Also, 12,000 new cases of spinal cord injury (SCI) occur every year, with 270,000 people seeing their life quality highly affected. [7] Moreover, every year, 60,000 new cases of Parkinson's disease are diagnosed, for a total of 7 to 10 million people worldwide. [8] However, there is currently no appropriate cure for all these affections and the research in this field is still in its infancy. Pathologies affecting neural tissues can be divided into two classes: those that come from an injury, where the neuronal tissue is physically damaged; and neurodegenerative diseases, where the tissue loses its functions with time, and ultimately dies. For the first class, the use of autologous grafts to repair damaged nerves is now possible in many cases. [9] This

surgical solution consists in taking a nerve, usually with a sensory function from a healthy area, and grafting it in the injured area to serve as a guide for the regrowth of the motoneurons. [9] However, this approach leads to a loss of function in the donor area, and, in most cases, function recovery at the site of implantation does not reach expected levels. [11] For the second class of diseases, there is no actual treatment targeting the cause of the symptoms. Current medical treatments are aimed at slowing down the disease progression and/or minimizing its impacts on patients' quality of life. For instance, dopamine and dopamine agonists are administered to patients suffering from Parkinson's disease in order to compensate for the lack of dopamine inside the brain and thus attenuate symptoms. [12] The difficulties faced when trying to provide an appropriate and complete treatment to those pathologies lie in the fact that it would require the restoration of tissue function, and re-growth of tissue replacing the dead one. By itself, current medication does not appear to be a solution for those two issues since, once those diseases are developed, the site of the pathology becomes hostile to tissue re-growth, which impedes the body regenerating healthy tissue. [13]

It has long been considered that once damaged, a neuronal tissue does not regenerate. Indeed, it was thought that, contrary to other tissues, neuronal tissue did not contain any stem cells, and therefore would not regenerate. Nevertheless, the recent discovery that nervous tissue is actually more dynamic than expected and that it contains stem cells has opened new therapeutic avenues based on a regenerative medicine approach. [14] This opportunity resides on the hypothesis that stem cells may have the same regenerative abilities in neural tissue as in other cases and are able to promote tissue regeneration. A remaining issue that may mitigate the use of stem cell-based therapies for neural tissues is that the number of active neural stem cells is thought to be very low. It is indeed admitted that stem cells do not divide continuously but that they specifically enter cycles of divisions, which prevents cell depletion in the tissue. It is thought that stem cells from the subventricular zone (SVZ) have a longer quiescence sequence between their cycles of proliferation than other stem cells. In fact, it has been suggested by Hwang and colleagues that glial cells might actually be the very stem cells of the nervous system. [15] Future stem cell-based regenerative strategies thus require either the use of stimuli in vivo or the addition of an in vitro step aiming at amplifying the patient's active neuronal stem cells prior to their re-

implantation as cells or tissue. Injuries that could be potentially treated with such a tissue regeneration or replacement technique are those related to nerve or spinal cord damages, or loss of function such as in Parkinson's, Huntington's or Alzheimer's diseases. For neurodegenerative diseases, the strategy may be directed toward the synthesis of a tissue presenting normal activity and thus re-establishing endogenous tissue function. For instance, in Parkinson's disease, the new tissue would be expected to synthesize dopamine up to normal levels in the *substancia nigra* and improve the patient's quality of life. [16] These approaches are particularly promising since damaged areas become hostile to the growth of new tissue, and would not allow for the re-growth of healthy tissue without any intervention except in highly rare cases. [13]

Although the discovery of neural stem cells has paved the way for innovative and efficient regenerative therapies, there are still several issues that remain to be solved before routinely applying such techniques. First, cell implantation and connection with host tissue need to be improved and cell growth and differentiation into desired phenotype(s) must be controlled, more likely through the use of growth factor(s) or related stimuli. Furthermore, structures supporting cell growth, possibly combined to growth factors delivery ability, still need to be developed. This review aims at presenting the various emerging techniques that are proposed for neural tissue regeneration.

2.2 Injection of free cells for in situ tissue regeneration

In 2006, experimental observations reported that stem cells are produced in specific regions of the brain and then migrate to their final destination, allowing for brain plasticity even at the adult stage. [14] This observation led to the conclusion that neuronal tissue is more likely to be able to regenerate, thus opening promising opportunities to cure neuronal diseases. The presence of endogenous stem cells in neural tissue indeed suggests that it should be possible to obtain the same kind of tissue production in other parts of the brain or nerves by providing those progenitors cells. [14] Indeed, the injection of additional stem cells at the site of injury has first been proposed to enhance the regenerative effect of endogenous cells. In that endeavor, mesenchymal stem cells (MSCs) were selected since they are easy to amplify, protect themselves from the immune system, and can differentiate into neural lineage in vitro and in vivo. [17] MSC-derived

neural progenitors were injected directly in the diseased area of the brain in mice that had chronic experimental autoimmune encephalomyelitis. Those injections contributed to maintain normal phenotype of the tissue. [18] The beneficial effect of MSC upon new tissue formation was partly attributed to the extracellular matrix (ECM) produced by the MSCs, which created a reservoir of bioactive substances being responsible for this neuro-supportive environment at the injury site. [17, 19]

Of salient interest, the benefits of stem cell therapy may also be extended to spinal cord injuries as demonstrated by Cummings and coworkers: human neuronal stem cells (hNSCs) cultured as neurospheres were directly injected in spinal cord-injured mice. [20] After 17 weeks, locomotor recovery and remyelination were observed even in myelin-deficient mice. More interestingly, SEM microscopy showed evidence of synapse formation between injected and endogenous cells, and cells migrated in the gray and white matters without participating to the formation of scar tissue in the injured area.

Human embryonal carcinoma-derived cells have also been identified as a potential source of stem cells. Their transplantation has been shown to promote the recovery of normal motor capacity in a rat animal model. [21] More specifically, transplantation was performed in rats that demonstrated a deficiency in the passive avoidance test one month after the surgical injury of their neural tissue. Following cell transplantation, a partial recovery of the learning capacity was observed one month after treatment and lasted over 6 months. Heine and colleagues also promoted nerve regeneration in chronically denervated mice. [22] The team used an immortalized neural progenitor mouse cell line (C17.2 cells), to be transplanted into mice having a deficiency in the regeneration of their tibial nerve. Those mice were observed for four months and demonstrated improved physiological recovery when compared to control population. Their nerves presented increased amount of axons and reinervation of the muscular tissue of the foot. The team also reported that the transplanted cells were still present in the nerve region, but they were not presenting neuron phenotype. The latter observation strongly suggested that stem cells participated in the regeneration by providing growth factors to endogenous cells, and that those endogenous cells were the essential component of the newly generated nerve.

Munoz *et al.* injected human MSCs into the dentate gyrus of the hippocampus of mice and obtained a colonization of the whole dorsal area of the hippocampus. [23] Implanted cells were

shown to promote endogenous cell proliferation. New cells were also observed to migrate through the brain for 30 days. After this period, those migrated cells presented markers of oligodendrocytes and mature neurons. In addition, human MSCs were shown to stimulate the production of neuronal survival factors such as nerve growth factor (NGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor-2 (FGF-2) and ciliary neurotrophic factor (CNTF), as well as to promote the proliferation of endogenous cells. Altogether, these studies suggested that the benefic effect of stem cell injections might be attributed to their ability to produce the adequate growth factors on site, these instructive cues leading to the remodeling of surrounding endogenous tissues, rather than to the creation of *de novo* tissue from differentiating stem cells. These conclusions in turn suggest that the injection of the adequate set of instructive cues may be sufficient to obtain similar spectacular effects without stem cell injection.

2.3 Use of growth factors to promote healthy tissue re-growth

2.3.1 In situ growth factor injection

As illustrated in the previous section, stem cell injection has already given very positive results in terms of function recovery. These promising results may however be mitigated as cell injection may result in tumor formation. [24] In addition, most of the effects obtained with free stem cell injection have been attributed so far to the growth factors these cells produced rather than from their ability to differentiate and form new tissue. These conclusions have led to the hypothesis that the delivery of specific growth factors, rather than stem cells, may be favorable for regeneration. In support to this hypothesis is the fact that, in contrast to central nervous system (CNS) neurons, injured peripheral motoneurons are able to regenerate their axons thanks to neurotrophic factors provided by Schwann cells. Their ability to regenerate is however transient, probably because levels of neutrophic factors rapidly decline after injury. Schwann cell capacity to support axonal regeneration was also shown to be enhanced strongly when Schwann cells extracted from six-month chronically denervated sciatic nerve were cultured in vitro in the presence of transforming growth factor β (TGF-β) and forskolin prior to re-implantation. [25] Injection of growth factors and cytokines directly at the injury site has also been tested to

promote tissue regeneration. In that line of thinking, Kobayashi and colleagues demonstrated that specific neurotrophins, when continuously delivered to the damaged area by cannula, prevented the atrophy of axotomized neurons. [26] Growth factor infusion was correlated to the persistence of high mRNA levels for GAP-43, Tα1-tubulin and TrkB (the BDNF receptor). The effect of the treatment was still observed 14 days after the last injection, more likely due to the long-term stimulation of these regeneration-associated genes that acted in synergy with the delivered growth factors. Other synergistic effects mediated by the co-injection of growth factors have also been documented after sciatic nerve axotomy: Boyd and Gordon demonstrated that long-term co-administration of glial cell line-derived neurotrophic factor (GDNF) combined with BDNF efficiently promoted axonal regeneration of motoneurons. [27] When used in combination with BDNF, GDNF was found to increase the number of axons per Schwann cell in vivo. Growth factor effects were however only observable after seven days of continuous injection.

Strategies based on growth factors (**Table 1**) may thus be viable, although the need of continuous delivery by infusion, as originally tested in rat models, may represent a serious impediment for its translation to human. Therefore it appears necessary to develop novel experimental approaches that would allow for the growth factors to be delivered in situ in a spatio-temporally controlled fashion with minimal surgical intervention.

2.3.2 Use of encapsulated growth factors to promote axonal regeneration and replace injured tissue

Classical engineered growth factors delivery systems are based on their diffusion from hydrogel or porous polymeric material, or on the degradation of a polymeric scaffold in which they are freely entrapped or, alternatively, at the surface of which they are grafted. Depending on the design of the system, growth factor delivery can be controlled via pure diffusion through porous polymeric material or through polymer degradation. [28-30] When designing such scaffolds, important characteristics to be taken into account and evaluated are the drug loading capacity of the polymer construct and its degradation time, if needed. Also, the biocompatibility and

biosafety of degradation products are strictly required. Among biodegradable polymers envisaged for brain-specific applications and currently used as delivery systems in vitro or in in vivo animal models, poly lactic co-glycolic acid (PLGA) has been widely studied. [31-33] This Food and Drug Administration (FDA)-approved biodegradable synthetic polymer is mainly used to mimic the extracellular matrix mechanical properties and shape, as well as to strengthen scaffolds originally engineered from natural materials such as gelatin or elastin. [34] VEGF and BDNF encapsulation in PLGA microspheres has been reported by Wang and colleagues using the *water-in-oil-in-water* encapsulation method. [35] The microspheres were then embedded within a crosslinked hyaluronic acid (HA) hydrogel to serve as scaffold for the culture of neural stem cells. The group demonstrated that such a design allowed for a slow and linear delivery of both growth factors for 6 days, with both encapsulated BDNF and VEGF maintaining their respectively known protective and pro-proliferative properties. The same strategy has been used to deliver CNTF from photopolymerizable nanoarray hydrogels. [36] However, a major issue related to the encapsulation of growth factors resides in the difficulty to obtain long-term delivery within a defined concentration range. Hydrogels indeed rapidly release most of the growth factors they contain in an initial burst that is higher than physiological levels, a phenomenon that reduces the duration of the delivery. [37] [38]

The most-often encountered problem is that a longer delivery time requires sufficient loading of the structure, which in turn usually results in unwanted burst of delivered growth factor just after hydrogel injection. In an attempt to address this shortcoming, Betram *et al.* obtained their best results for NGF delivery with microspheres of PLGA-PLLA-PEG. [37] This combination suppresses the burst in secretion observed with PLGA-only hydrogels by controlling growth factor delivery through continuous degradation rather than desorption process. With that enhanced system, NGF was delivered up to 65 days without any observable burst.

An alternative to growth factor encapsulation resides in growth factor direct blending with the polymer that constitutes the scaffold, during its fabrication. The scaffold will then release the included growth factors when degrading in the implantation area. In order to control cell differentiation, retinoic acid (RA)-containing poly(lactic-co-glycolic) acid electrospun fibers have been prepared by its incorporation with the polymer before electrospinning. RA was uniformly

present inside the construct and 80% of the loaded RA was released in 105 days at a constant rate. [39] Johnson *et al.* demonstrated the ability of scaffolds made of fibrin to deliver neurotrophin-3 (NT3) and platelet-derived growth factor (PDGF) in vivo. These scaffolds were prepared by the polymerization of an NT3/PDGF-polymer solution mixed with stem-cell derived neural progenitor cells. The presence of viable cells inside the tri-dimensional structure was confirmed, resulting in enhanced differentiation level of the cells into neurons due to the presence of NT3 and PDGF. In this study, the receptors for epidermal growth factor were neutralized by the addition of a blocking antibody to the structure, thus blocking the inhibitory pathways present in an injured area. Core shell fibers have also been used to deliver NGF for sciatic nerve regeneration in rats. NGF was loaded in the PEG core of the fiber, which was covered by a PLGA shell. After initial release of the NGF that migrated to the shell during the electrospinning process, NGF release was mainly due to diffusion through the shell layer, hence reducing the initial burst phenomenon. Grafting of this conduit for nerve guidance promoted functional recovery with the same efficiency as autografts 12 weeks after implantation. [40] A very similar technique has been used to deliver VEGF using coaxially electrospun fibers of hydroxyl-functionalized poly(ε-caprolactone) as a shell and VEGF-loaded BSA as a core. [41] Erythropoietin was delivered at stroke site using hyaluronan/methyl cellulose (HAMC) hydrogel. The latter was chosen based on its anti-inflammatory properties, mostly attributed to the presence of hyaluronan. Erythropoietin diffused from the implant to the ventricular zone of the stroke, reducing the cavity, and proved to have neuroprotective and neurogenerative properties. [42]

Another advantage of this process may reside in its applicability to perform growth factor gradients. This aspect is particularly appealing since, in the adult nervous system, stem cells migrate to their final location by following growth factor gradients that are present in the tissue. [43-45] Axons are also known to grow along these gradients. More importantly, these gradients have an influence on the differentiation pathway followed by the cells. [46] Therefore, attempts have been made to mimic these natural gradients to spatially localize neurogenesis. [47] Growth factor gradients have been created either by assembling hydrogel pieces filled with growth factors at various concentrations [48] or by growth factor diffusion from wells being positioned within the hydrogel so as to result in the establishment of a gradient upon diffusion. [49] It was shown that gradients of the neurotrophic factors NGF and B27 used synergistically could multiply and

position synapses within the construct. [50] The gradients were created using channels connected with PDMS reservoirs that were filled at different time points to create a diffusion gradient. The synapse density increased proportionally with the NGF/B27 gradients.

2.3.3 Delivery of encapsulated growth factors to the native tissue

Scaffolds could also be applied to the delivery of growth factors in situ without any scaffold colonization by endogenous cells; the structure being used as a reservoir only. This use is believed to be of particular interest in the case of Alzheimer's disease where the patient is unable to maintain the adequate population of cholinergic neurons. That is, since NGF is a growth factor that stimulates the cholinergic function, its delivery to the brain of patients suffering from Alzheimer's disease has been investigated. In this specific case, the NGF be delivered passed the blood brain barrier, and the delivery must be very local to avoid any other regions of the brain to be impacted. Therefore, polymeric devices have been investigated to deliver NGF locally in rat brains. [51,52] While local delivery was successful, remaining limitations were the short half-life of those molecules and the short migration distance, hence the importance of the choice of the implantation site. [51,53] BDNF delivery, via hydrogels implanted into rat brains has also been investigated as BDNF could be used to treat major depression. In these studies, BDNF delivery was found to be successful as an antidepressant effect was observed. Once again, the efficacy of the treatment is closely related to the concentration of growth factors that reaches the targeted cells and the duration of the treatment. [54]

2.3.4 Use of cells to produce and deliver growth factors in situ

As emphasized in the preceding section, growth factor delivery is associated with many hurdles related to adequate spatio-temporal delivery. In order to overcome these issues, in situ synthesis of instructive cues by encapsulated cells has been investigated. In that respect, Shanbhag and colleagues encapsulated two lines of fibroblasts being genetically modified to produce either BDNF or NT-3. [55] The cell-containing scaffolds were demonstrated to enhance cell survival, migration distance of NPCs, and NPCs attachment to the scaffold as well as better survival when

compared to empty scaffolds. NT-3 secretion by modified fibroblasts was shown to promote neuron formation. In their study, Dey and colleagues injected MSCs genetically engineered to secrete BDNF or NGF in YAC 128 mice (a model for Huntington's disease) and measured mice motor abilities. [56] MSCs secretion of BDNF improved neuronal survival and reduced clasping and behavioral deterioration after 9 months. Those results were not observed with NGF-producing MSCs or with pristine MSCs, although mice that had received unaltered MSCs also presented less clasping. This result was attributed to the secretion of anti-inflammatory cytokines by the injected MSCs, once again highlighting the advantages of MSC manipulation for this application. Such a strategy may however be inapplicable due to serious ethical concern.

Cells may also be used as an underlying layer to provide a favorable environment to regenerate neuronal structures *ex vivo*. The experimental approach would then rely on culturing a first layer of cells on top of which the cells of interest would grow. The first cell layer would provide trophic factor(s) to create a niche where the neuronal cells could optimally develop. In that endeavor, the use of a PA6 cell layer (a bone marrow-derived stromal cell line, precursor of adipocytes) was shown to provide an excellent environment in which human embryonic stem cells were able to differentiate in vitro into dopaminergic neurons in a highly specific way (87 %), prior to injection. [57] The exact role of PA6 cells was not fully understood, and was partly attributed to uncharacterized growth factors production. This technique has also been used to deliver growth factors to other cells directly inside the brain: NGF producing fibroblasts were implanted into the forebrains of six patients suffering from Alzheimer's and resulted in significant neuronal growth and slowed down the cognitive decline. [62]

2.4 Use of structures to mechanically support tissue growth

As discussed above, both stem cells and growth factors have shown promising results but their immediate use still presents limitations that prevent their routine implementation to the clinic. While injections of cells and/or growth factors promote tissue regeneration, a structure that offers an adequate mechanical support for the growth of endogenous or injected cells may be required when the injury site is large. [58] Filling the injury cavity with a mechanically relevant scaffold may prevent the formation of scar tissue; the latter being known as a hostile environment for

neurite growth due to its inherent structure and the presence of macrophages and other inhibitory factors. [59] Furthermore, in the case of neurodegenerative diseases, the pathological tissue is no longer able to adequately present the factors promoting proper differentiation of injected stem cells, as already observed when stem cells were injected to treat Alzheimer's disease. [13] [60] A scaffold that would support cell growth and differentiation, offering a protective and stimulating microenvironment may thus be required since the host tissue is no longer able to play that role.

2.4.1 Hydrogels to offer mechanical support to cells

Hydrogels (**Figure 1**) have now emerged as a type of materials of choice for implant design. Hydrogels are usually defined as water-swollen polymer networks, often resulting from the polymerization of hydrophilic polymers. Such polymerization can be chemically driven, through reaction with a crosslinker, [61] or physically driven, through a change in reactivity or conformation that may be induced by pH or temperature changes [62] or even photo-activated. [63] Physically crosslinked hydrogels have the potential to be generated directly in situ; they do not require the use of photo-crosslinkers that are often toxic. However, these hydrogels are usually less stable than their chemically crosslinked counterparts. [62] Hydrogels present many advantages for neural tissue regeneration, as it is relatively easy to create a three-dimensional hydrogel structure harboring adequate mechanical properties. Furthermore, the inherent hydrophilicity of hydrogels - along with their ability to deliver growth factors - make them good candidates for neural cell supportive systems. [64] Amongst them, several polysaccharides including agarose, alginate, methylcellulose, dextran and chitosan as well as polysaccharide blends have been investigated. Soft, positively charged surfaces as those obtained with dextran/chitosan blends were reported to favor neuron attachment by Zudema and colleagues. [65] Hyaluronic acid (HA) was also reported to offer adequate mechanical support as well as good cell adhesion, proliferation and migration properties. [35]

The fact that physiological fluids penetrate hydrogels, combined to their softness and their three-dimensional structures, make them interesting candidates for tissue repair. As already demonstrated for vascular repair applications, hydrogels *per se* are presented by some authors as sufficient to allow neuronal cell growth and tissue formation. [66] On the same note,

differentiation of NSCs has been observed in three-dimensional porous chitosan scaffolds. On their own, these scaffolds were shown to promote differentiation to a higher extend than when soluble NGF was administered to cells cultured on two-dimension surfaces; the combination of three-dimensional scaffold with NGF however gave the best results. [67] The modulus of hydrogels was shown to be a critical parameter: for alginate-based constructs, a lower stiffness was reported to lead to better proliferation and differentiation of NSCs. [68]

Hydrogels have already been tested several times in animal models in vivo animal models for neuronal regeneration. [69-72] Their healing properties have been assayed when used as bridges after spinal cord or nerve sectioning. In such assays, Poly[N-(2-hydroxypropyl) methacrylamide] (PHPMA) hydrogels were successfully used in rats with sectioned spinal cord. [73] PHPMA constructs were reported to be well integrated, to prevent scar tissue formation and to promote axonal colonization. Such success was attributed to the good swelling and elastic properties of this type of hydrogels, combined to their high porosity that allowed neuronal and axonal growth through the structure. [73]

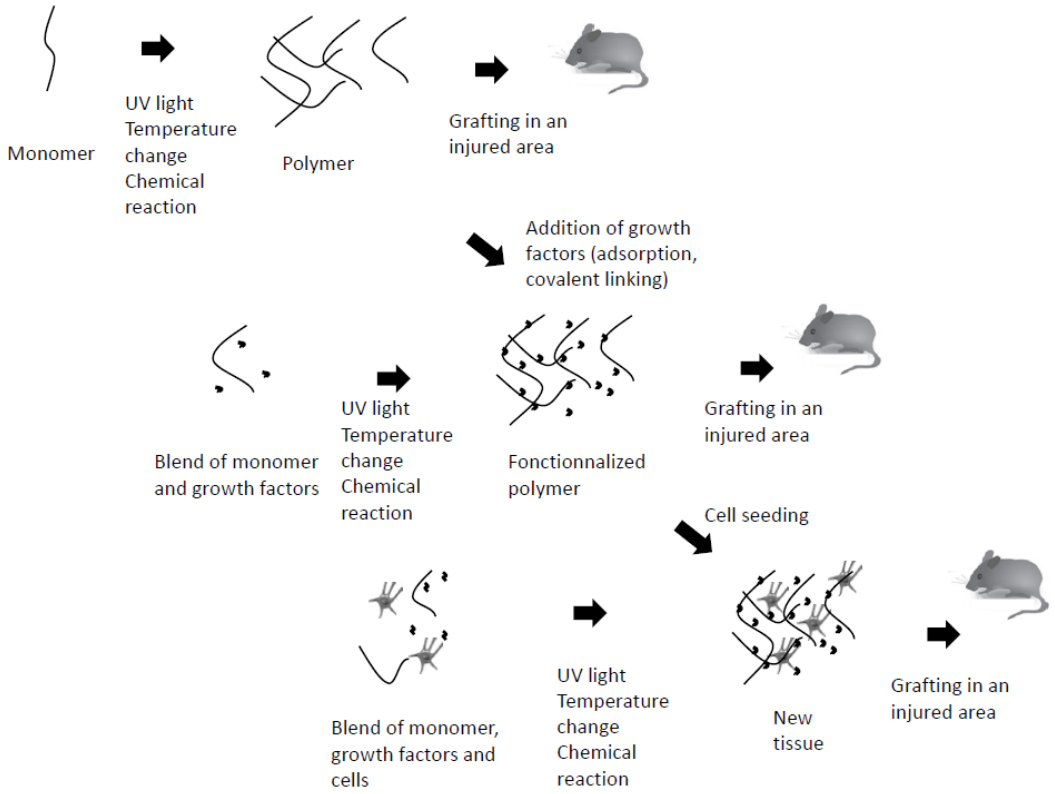

Fig 1. Hydrogel fabrication and application

2.4.2 Electrospun fibers as a mechanical support for cell development

Another fabrication technique, namely electrospinning, has also been demonstrated to be successful for the generation of three-dimensional scaffolds made of micro- to nano- size fibers with controlled properties. This technique was originally developed to create textiles and filters and was then prop osed for wound dressing material. [74] In this process, a polymer solution is pushed through a metallic needle (**Figure 2**) while a strong electric field is imposed between the needle and the collector, charging the surface of the droplet at the extremity of the needle with static electricity (**Figure 3**). The polymer solution is then attracted by the electric field, and forms nanofibers (200-1500 nm of diameter) that dry during their flight between the needle and the collector. [75] This method is well adapted for neuronal scaffold production as fibrous scaffolds with high surface-to-volume ratios and structurally mimicking the extracellular matrix can be generated. [76] Furthermore, various polymers of interest, including biodegradable ones, can be used to create electrospun fibers. Those include poly lactic acid (PLLA), poly glycolic acid (PGA) and their copolymers, PLGA, poly(ether sulfone) (PES). [77-80] This high flexibility in terms of material selection, fiber diameter and orientation, porosity and mechanical properties offers a wide choice of possibilities so that biocompatible mats with adequate degradation rate and mechanical properties can be designed. [81] Examples are given in **Table 2.**

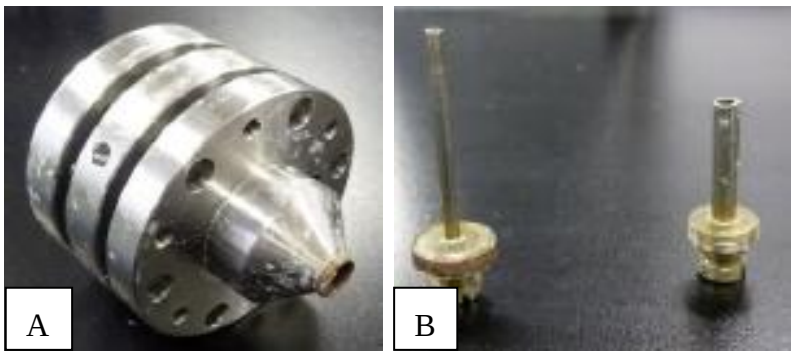

Fig 2. Co-electrospinning needles: device allowing electrospinning of 3 different solutions with a layered core shell structure (A). Needles used with this core shell structure. (B) Diameters are respectively 1.83 and 3mm.

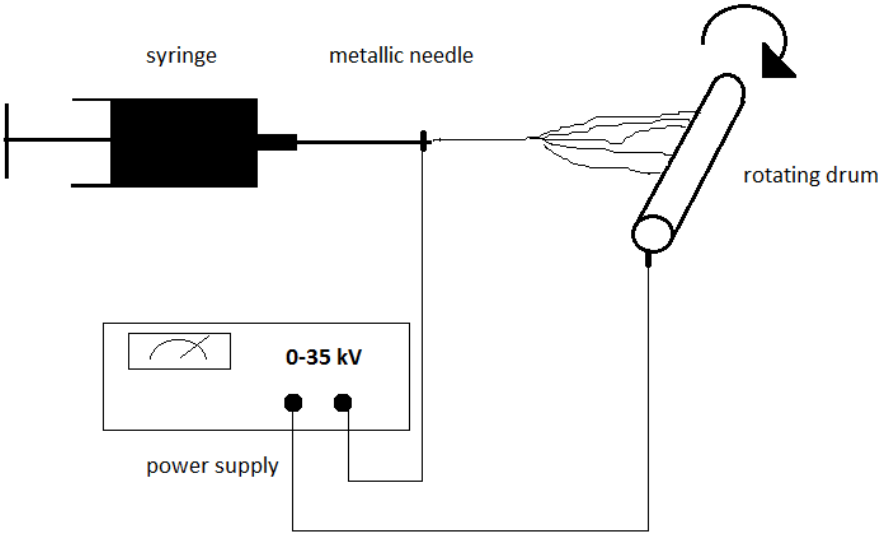

Fig 3. Electrospinning setup

Christopherson and coworkers investigated the influence of electrospun fiber diameter on rat NSCs, while the cells were cultured on PES fibers coated with laminin for cell attachment. [82] Of interest, different phenotypes were obtained with different types of fibers: fibers of small diameter (283 nm) were shown to promote NSCs differentiation into oligodendrocytes whereas larger fibers (749-nm diameter) promoted their differentiation into neurons. Wang *et al.* also showed that aligned tussah silk fibroin (TSF) fibers had a significant beneficial effect on neural differentiation of hESC. [83] They demonstrated cell migration along the fibers, and neurite growth with neurites following the fiber direction. In comparison, the cells cultured on random fibers migrated randomly with a shorter migration range. Neurites were longer on 400-nm aligned fibers, when compared to 800-nm ones. The authors nevertheless mentioned that, in some experiments, neurites happened to grow perpendicularly to fiber direction. They showed a 6% increase in the neuron amount on 400-nm aligned nanofibers when compared to 800-nm aligned fibers. Altogether, neurites were significantly longer and followed the material direction. In comparison, 800-nm random fibers showed a decrease both in number and total neurite length.

Lower cell migration and proliferation were observed on these structures when compared to 2-D culture conditions. Cells were also shown to grow towards the fiber pattern: the thinner fibers directed the elongation of the cells, most likely explaining the increased level of differentiation into neurons. Of interest, cell culture on PLLA fibers was reported to abrogate the need of calf

serum in the culture medium. In the absence of calf serum, neurite alignment was increased. [84] Finally, the implantation of these structures in the area of sectioned nerves or degenerated tissues, as a support for the re-growth of host tissue, has been investigated. Nisbet *et al*. implanted electrospun fibers of poly(ε-caprolactone) in rat brains. [85] 60 days after implantation, scaffolds were not encapsulated in scar tissue, despite a recruitment of microglia and astrocytes. The structure promoted tissue growth as demonstrated by the presence of neurites inside the construct. Intriguingly, cell growth occurred perpendicular to fibers rather than in a parallel direction and randomly aligned fibers showed a better colonization by endogenous cells. The first observation was contradictory to experiments performed in vitro; further investigation is thus required to better understand and master this therapeutic approach.

Co-electrospinning may answer the dual needs of controlling the porosity and mechanical properties of materials, as well as allow drug delivery abilities of materials created with other techniques. In this approach, a core shell structure is created using a double coaxial needle. The core is made of the same polymeric materials as previously evoked. The outer part can be made of various materials such as laminin, which has been shown to favor cell attachment to the fibers. [86] Other proteins such as gelatin may also be used. [87] In such system, the core brings porosity and stiffness while the outer part of the fiber allows for a receptive contact of the cells on the scaffold surface; the outer layer may also be used for in situ growth factor delivery. As an alternative to co-electrospinning, electrospun fiber modification by hydrogel polymerization may be applied to create an outer shell to enhance cell contacts. Using this approach, Poly(ε-caprolactone) electrospun fibers were covered with poly(ethylene glycol)-poly(ε-caprolactone) (PEGPCL) hydrogel, to support neural growth and deliver NGF in situ. [88] This construct was used to coat implanted electrodes. The mechanical support offered by the fibers reduced the degradation rate of the hydrogel, resulting in the elution of NGF, which increased the proximity of neurons and electrodes. An interesting technique has been developed by Huang et al. who electrospun fibers through a hole in a charged mask to create fiber patterns. Using this technique, fibers were patterned on a cell repellant hydrogel; regulating the voltage applied to the mask controlled the size and density of the fiber spots. This technique opens new avenues to control cell colonization through the creation of cell-receptive or -repulsive areas. [89]

Hydrogels and electrospun fibers have thus been demonstrated to favor stem cell growth and differentiation without any addition of growth factors; a very appealing property for the treatment of nerve injuries. However, in the case of degenerative diseases, the patient's cells are usually unable to produce the required set of growth factors promoting regeneration; their inclusion in the implanted scaffold may thus be mandatory. Those factors may also be needed to counter the effects of molecules promoting the disease or, simply to promote the growth of functional tissue. [13] In addition to appropriate growth factor inclusion within a given scaffold, providing healthy stem cells to create tissue with restored functions may be desirable, as endogenous cells have lost this ability.

2.4.3 Use of structures to display bound growth factors in situ

As an alternative to growth factor diffusion from hydrogels or release from electrospun structures, growth factor immobilization through covalent grafting or non-covalent but stable tethering, has also been reported for both hydrogels and electrospun fibers. Covalent grafting of integrin binding peptides inside a collagen structure has been shown to enhance cell attachment to the substrate and improve cell viability [90]. Also, the stable tethering of BDNF onto collagen scaffold has also been assayed. The latter was achieved by designing a chimeric protein corresponding to BDNF fused to collagen binding domain. Scaffolds decorated with chimeric BDNF and seeded with dorsal root ganglia cells were implanted in rats and resulted in good recovery after spinal cord transection. [91] On the same note, the production of EGF fused to collagen binding domain has also been documented; its use for stem cells encapsulation within EGF-decorated collagen-based hydrogel resulted in better cell survival than in control collagen scaffold, while cells were observed to differentiate into various neural phenotypes. [92] Human recombinant BDNF and human recombinant NT-3 were also covalently immobilized on gelatin-based scaffolds using photo-polymerization: photocurable styrene derivatized gelatin was used to produce conduits on which extracellular matrix molecules along with rhBDNF and rhNT-3 were photo-co-immobilized. Dorsal root ganglia showed larger neurite extensions on structures displaying both neurotrophins than on structures displaying only one of the two proteins. [93]

NGF immobilization on chitosan scaffolds using genipin crosslinking was also applied to generate conduits later implanted in denervated rats. Such strategy was shown to lead to nerve reconstruction in vivo. [94]

Electrospun fibers were also used as displaying structures. [95] Amine terminated poly(ethylene glycol) and poly(ε-caprolactone) allowed Cho and colleagues to prepare amine functionalized electrospun fibers on which NGF was subsequently grafted. This strategy permitted to eliminate initial burst in released molecules, while increasing cell differentiation into neurons. Of salient interest, growth factor immobilization onto fibers led to more pronounced results than those related to cell culture on fibers in the presence of soluble cues. [96] In another work, covalent grafting of BDNF was performed on ethylene diamine-modified poly(ε-caprolactone) fibers. Cells proliferated more on these displaying structures even with half the amount of soluble BDNF. [97]

Spatially varying growth factor density during immobilization may also be of interest to explore the potential benefits of gradients of non-diffusible growth factor(s). This may be achieved thanks to recent technical developments now allowing for the creation of patterns of non-diffusible proteins: in that respect, laser-assisted protein adsorption by photobleaching is extremely promising. [98]

2.4.4 Use of anisotropic scaffolds to direct tissue growth

A desirable feature for smart engineered grafts would be to mimic patient's tissue spatial organization in order to direct tissue regeneration, as nerve regeneration implies that new axons grow in the same direction. As previously mentioned, in order to meet this goal, growth factor gradients have been proposed, since they are responsible in vivo for directing the genesis of endogenous tissues. However, several attempts to create such gradients highlighted technical difficulties. [43-45] Therefore, as an alternative, the use of hydrogel- or electrospun fiber-based anisotropic scaffolds has been explored. Most hydrogels are obtained from the polymerization of monomers in solution and therefore show isotropy. Although this fabrication process is not adapted to generate structure determinism, hydrogels made of apo-collagen bear the potential to

be shaped to form aligned structures and membranes. [99] These structures, designated as linear ordered collagen scaffolds, were crosslinked with laminin to direct axon growth. [100] Implantation of this material allowed functional recovery in rats, with improved linear orientation of the newly generated tissue.

When topology needs to be controlled, electrospinning appears as the method of choice since the extent to which fibers are aligned can be varied with mandrel rotating speed. Electrospun fiber structures have been demonstrated to be extremely promising to direct axons growth and model newly grown tissue. In a previously evoked study, aligned PLLA fibers were used to grow motor and sensory neurons. Neurons were shown to grow following nano-fiber orientation and neurites grew along the fibers. [84] Aligned electrospun fiber structures may thus be a key to direct axonal growth and cell migration. [101]

2.5 Use of conductive structures to electrically stimulate neuron differentiation

The neural tissue is electrically active, therefore attempts have been made to create conductive structures to allow electrical stimulation of the cells in order to improve the functionality of the graft. Cells that had been stimulated on a cover glass coated with conductive gold nanoparticles showed an increase in neurite length. [102] The design of a 3-D conductive scaffold made of freeze-dried collagen with polypyrrole, and presenting oriented micro channels has been reported. Electrical stimulation thanks to this structure increased neurite outgrowth in the direction of the electrical field in vitro and resulted in a better remyelination of the axons while Schwann cells were observed to migrate towards the anode. [103] In the case of electrospun fibers, conductivity may be obtained through the use of polypyrrole in the fiber core or as an outer layer being polymerized on electrospun fibers. [104]

Conductive electrospun nanofibers were also proved to enhance the rate of neurite growth and their total length. [105] When polypyrrole was polymerized onto PLA and PLGA electrospun fibers, the resulting structure was shown to be effective for dorsal root ganglia cell culture. Electrical stimulation resulted in 40% longer neurites. [105, 106] NGF has been incorporated in

such conductive constructs, in order to benefit from the growth factor effect as well as the stimulation. Longer neurites were obtained using this combination. [107]

Another way to add conductive properties to materials is to include carbon nanotubes or nanoparticles in the structure. [108] Carbon nanotube-mediated electrical stimulation was shown to promote neural maturity, and increase the speed of neurite outgrowth. [109] Carbon nanotubes were integrated in PLA electrospun fibers and conferred conductive properties. [110] Electrical stimulation resulted in increased levels of neuronal markers and stimulated neurons were more aligned along fibers. A difficulty for the use of nanotubes in electrospinning is the difficulty to spin them directly. To overcome this issue, Miao *et al*. co-electrospun multi-walled carbon nanotubes and poly(vinyl pyrrolidone) using co-axial electrospinning. The core of the fibers was composed of nanotubes while the sheath was made of poly(vinyl pyrrolidone) as a way to make the nanotube solution spinnable. [111]

Finally, carbon nanotubes can be used alone as conductive cell support. The main issue resides in their toxicity; their biofunctionalization is thus required. [112] NSCs were cultured on nano-ropes of carbon nanotubes: the structure strongly increased the maturation of NSCs into neuronal cells and promoted neurite elongation following the topography of the rope. [113]

2.6 Conclusion

Current techniques and strategies that may allow for neuronal tissue regeneration or replacement were reviewed. While stem cell injection was proven to promote functional recovery, it is now accepted that the effect is mainly due to growth factors and other instructive cues being secreted by these cells rather than injected cells differentiating into new tissue. These growth factors may thus be injected directly in situ to reach similar results, however, this requires heavy surgery. Their release from a scaffold implanted in the diseased area though diffusion or degradation, or alternatively, their attachment to an implantable scaffold, has been investigated with some noticeable success. Such a strategy is very attractive as it decreases the amount of growth factor needed and limits their effect to the injured area while taking advantage of the mechanical support provided by the scaffold itself.

Altogether, ideal nervous or neuronal implants should present several characteristics. The literature is now consensual upon the fact that, for an implant, mimicking the extracellular matrix features including porosity, pore size, and mechanical properties, while acting as a reservoir of instructive cues and harboring adequate topology for an appropriate spatial organization of the tissue, are all desirable traits. Therefore, despite all promising results presented therein, a significant amount of work remains to be done in order to fulfill all those requirements and enable the long-term success of regenerative medicine approaches. Above all, any technique should cope with surgeons' constraints. In that endeavor, hydrogels present several interesting features as they are well characterized, commonly used as drug carriers, and since they can be prepared with a variety of chemicals. Also, as an alternative, electrospinning was proven to be very promising as it permits to control the mechanical properties of the scaffold while designing conductive materials. Alignment of fibers and incorporation of growth factors were also shown to be feasible for additional tailoring of electrospun scaffold, i.e., to direct tissue growth and control cell phenotype. Many other materials may also lead to the development of constructs with improved regenerative potential: polyurea silica aerogels, [114] phosphate-based glass fibers [115], to name a few, are indeed amenable to produce scaffolds with interesting properties regarding their biocompatibility, nano-porous structure, mechanical strength, directionality or surface functionalization. Matrigel, which is made of extracellular proteins secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma that easily self assemble to form a matrix, has been assayed to regenerate nerve tissue and gave good results regarding the differentiation of dopaminergic neurons. [116] Finally, self-assembled fibers made of spider silk have been reported to spatially support neuronal and astrocytic differentiation. [117] [118]Scaffold design could also benefit from a other fabrication techniques such as oriented growth factors immobilization strategies [119] , layer-by-layer lithography [120], microcontact [121] [122] or inkjet printing [123] [124] to give a few examples of very promising approaches being currently explored. Finally, recent research is now pointing towards alternatives to electrical stimulation to promote cell differentiation: sub sonic vibrations [125], stretching [126], cyclic tensile loading [127] and low level LASER (LLL) stimulation [128] are promising avenues that may impact significantly future research in neural regeneration.

Table 1. Growth factors and their use in the context of neuronal disease treatment

Growth factor	Effect	Enhanced Phenotype / Pathway	Delivery technique	Species	Ref
BDNF	Increases number of neurons in striatum, septum, thalamus and hypothalamus	N/A	Intraventricular administration	Rat	**[129]**
	Promotes myelinisation, prevents migration	TrkB and p75 pathways for cell death, Rho kinase for migration	Injection	Rat	**[130]**
GDNF	Regulates proliferation, differentiation and survival of neurons	Pathways associated to Ret and GDNF family receptor α1 (GFR α1), on the surface of migrating enteric neural crest–derived cells	N/A	Chick	**[131]**
Erythropoietin	Prevents apoptosis, anti-oxydant, promotes differentiation into dopaminergic neurons	Mimics lower O_2 environment	Addition to the medium, *in vitro*	Rat	**[132]**
EGF	Enhances NSC proliferation	Neural stem cell	Released from implanted hydrogel	Mouse	**[133]**
FGF-2	Maintains cell viability, increases cell proliferation	N/A	Addition to the medium, in vitro	Rat	**[82]**
FGF-4	Promotes serotoninergic differentiation	N/A	Addition to the medium, in vitro	Rat	**[134]**
FGF-8	Promotes dopaminergic and serotoninergic differentiation	Effect on antero posterior position	Addition to the medium, in vitro	Rat	**[134]**
	Mitogen, promotes differentiation into mesencephalic precursors	Effect on antero posterior position	Addition to the medium, in vitro	Rat	**[132]**

Table 1. Growth factors and their use in the context of neuronal disease treatment (continues)

Growth factor	Effect	Enhanced Phenotype / Pathway	Delivery technique	Species	Ref
Growth differentiation factor 5 (TGF-β)	Prevents apoptosis, neurotrophic and neuroprotective effects on dopaminergic neurons	Pathways involves serine/theronine kinase receptors, BMPs	Intracerebral injection	Human	**[130]**
IGF-1	Promotes differentiation into oligodendrocytes	Inhibition of BMP signaling	Injection	Rat	**[135]**
	Protects motor neurons, inhibit cell death	MAPK and PI-3K pathways	Addition to the medium, in vitro	Rat	**[136]**
	Increases cell survival, protects cells from epoxomicin effects and from apoptosis	Activation of PI3/AKT pathways, reduce cytochrome c release	Addition to the medium, in vitro	Human (SH-SY5Y)	**[137]**
Interleukin 1-6	Promotes neuronal survival, protects against damage, induces neuronal differentiation, stimulates astrocyte proliferation	Interleukin receptor	N/A	Rat	**[138]**
Mash-1	Promotes neural proliferation and differentiation	X-Notch-1 receptors	N/A	Rat	**[139]**
Neurogenin1 -2	Required for dorsal root ganglia development	trkC+ and trkB+ neurons (ngn2) trkA+ (ngn1)	Knock-out manipulations	Mouse	**[140]**
Neuroreguline 1β	Regulates outgrowth of neurites	erbB receptors phosphatidylinositol 3-kinase (PI3K)	Addition to the medium, in vitro	Rat	**[141]**
Neurturin (NTN)	Helps survival and neuritogenic factor	GDNF family receptor (GFR) α-2 and signal transducing receptor kinase Ret	Injection	Rat	**[130]**

Table 1. Growth factors and their use in the context of neuronal disease treatment (continues)

Growth factor	Effect	Enhanced Phenotype / Pathway	Delivery technique	Species	ref
NGF	Promotes choline acetyltransferase in forebrain	ChAt (motoneurons)	Injection	Human	**[129]**
Retinoic Acid	Promotes differentiation into neurons	N/A	Addition to the medium, in vitro	Rat	**[82]**
	Promotes differentiation into oligodendrocyte	N/A	Addition to the medium, in vitro	Rat	**[142]**
Shh	Promotes differentiation into motoneurons	Ventralization	Injection of shh releasing microspheres	Mouse	**[143]**
TGF-α	Stimulates proliferation	EGF receptor	N/A	Mouse	**[144]**
	Promotes dopaminergic differentiation, mitogen for cells of ectodermal and mesodermal origin, induces neurite outgrowth	EGF receptor	In vitro induction	Human	**[145]**
VEGF	Prevents inflammation, secondary degeneration	Reduces leukocyte infiltration and microglial activation, suppresses inflammatory cytokines	In vivo delivery using an implanted cannula	Mouse	**[146]**

Table 2. Examples of tri-dimensional scaffolds generated by electrospinning

Material	**Solvent**	**Porosity**	**Mechanical characterization**	**Bioproperty**	**Conductivity**	**Ref**
Chitosan/polyethylene oxyde	Acetic acid	70%	Tensile strengh 4MPa, modulus 147 MPa	Non toxic, nonantigenic (chitosan's properties)	10^{-7} S/cm	**[147]**
poly(ethersulfone)	DMF/Toluene	71%			N/A	**[80]**
Poly(ε-caprolactone)	1:1 tetrahydrofuran and *N*,*N*-dimethylformamide	90%	Tensile modulus : 5.0 ± 0.7 MPa, yield stress : 0.55 ± 0.06 MPa	Non toxic by-products	N/A	**[148]**
	chloroform	90%	Tensile modulus : 6.4 ± 0.2 MPa, yield stress 0.55 ± 0.06 MPa	Non toxic by-products	0.0015 $S \cdot cm^{-1}$	**[148, 149]**
polyurethane/collagen	1,1,1,3,3,3,-hexafluoro-2-propanol	62-71%	Tensile stress 4.53MPa	Cell adherent, biodegradable	N/A	**[150]**

Table 2. Examples of tri-dimensional scaffolds generated by electrospinning (continues)

Material	Solvent	Porosity	Mechanical characterization	Bioproperty	Conductivity	Ref
poly(lactide-co-glycolide)	Dichloromethane +N,N-dimethyl formamide (9:1)	75%	Young modulus 134MPa (40 MPA for aligned fibers)	Biocompatible and biodegradable Interesting mechanical properties but no cell recognition site	N/A	**[79]**
poly(L-lactic acid)	1,1,1,3,3,3-hexafluoro-2-propanol	71%			N/A	**[77]**
	2,2,2-trifluoroethanol (TFE)	Pore size : 4.51 µm	Tensile strength 0.063MPa, young's modulus 0.41MPa		N/A	**[151]**
Polyaniline/ camphorsulfonic acid /gelatin	1,1,1,3,3,3-hexafluoro-2-Propanol		Modulus 614 MPa	Conductive : allows electrical stimulation	0.01-0.021 S/cm	**[152]**
polyvinylidene fluoride–trifluoroethylene	methyl ethyl ketone	43-80%	N/A	Piezoelectric : stimulates neurons, neurite elongation	N/A	**[153]**

Table 3. Examples of techniques tested in vivo

	Substance delivered	Advantages	Inconvenients	Species	Ref
Injection of Growth factors	Erythropoietin	Enhances nerve recovery, effect on muscle regeneration	Single injection, no duration, effect on muscle regeneration	rat	**[153]**
	IGF-1, IGF-2	Promotes intramuscular nerve sprouting	Immunoreactivity and proliferation of interstitial cells	Rat, mouse	**[155]**
	BDNF, CNTF	Enhances survival	Effective only before the 5^{th} or 7^{th} week.	rat	**[156]**
Injection of recombinant genes	NGF-β gene	Enhances survival and neurite outgrowth, frees from the numerous injections of GF	Gene manipulation : use of an adenovirus, can be expressed in the liver, need longer investigation	rat	**[157]**
Injection of cells	MSCs	Support axonal regrowth by producing growth factors	Huge variability in effects (length of the axons)	rat	**[158]**
Injection of cells and growth factors	Bone marrow stromal cells and granulocyte colony-stimulating factor	Significant functional recovery, smaller scar, reduced apoptosis, differentiation into CD-4+ cells	Needs improvement, involves several injections of growth factor	rat	**[159]**

Table 3. Examples of techniques tested in vivo (continues)

	Substance delivered	**Advantages**	**Inconvenients**	**Species**	**Ref**
Implantation of artificial matrix for cell support	Polyamidoamine hydrogel	Facilitates nerve regeneration, functional recovery, no inflammation, biodegradability, biocompatibility permeability, non immunogenicity, mechanical strength	Degradation time	rat	**[160]**
Implantation of supportive structure (growth factor delivery system)	Neurotrophin-3 and chondroitinase ABC on electrospun collagen	Improves efficacy of the structure, no repeated injections of growth factors	Fast release at the beginning	In vitro	**[161]**
	BDNF in agarose hydrogel	Fills irregular defects in injured spinal cord, prevents inflammatory response, delivers BDNF in situ	Study with cells is needed	rat	**[162]**
	Hyalorunic acid hydrogel containing BDNF	Locomotor function recovery, prevents inflammation	Study with cells is needed	rat	**[163]**

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2.9 Conflict of interest statement

The authors indicate no potential conflict of interest.

CHAPITRE 3 FABRICATION DES FIBRES ET OBTENTION DE NEURONES MOTEURS

3.1 Présentation

Il apparaît qu'une approche basée sur l'utilisation de fibres électrofilées bénéficie de l'ensemble des avantages mis en évidence dans la revue présentée au chapitre précédent. En particulier, l'utilisation de fibres ayant une structure cœur-couronne nous a semblé idéale. En effet, en utilisant un cœur d'acide poly lactique, un matériau approuvé par la FDA et aux propriétés bien connues, il est possible d'obtenir des fibres ayant un diamètre choisi, un tissu de grande porosité présentant des propriétés mécaniques répondant aux contraintes d'utilisation chez l'humain. En ajoutant à ce cœur une couronne de gélatine, il est possible de rendre le matériau plus favorable à l'attachement des cellules à la surface des fibres. Nous avons donc retenu cette technique de fabrication pour créer notre structure. Cette technique nous a permis d'obtenir une permettant de contrôler la différentiation de cellules en neurones moteurs. Ces résultats ont été soumis dans *Biomaterials* le 20 Août 2013.

3.2 Article 2 – Differentiation of neuronal stem cells into motor neurons using co-axial electrospun Poly-L-Lactic Acid/gelatin fibers as an instructive cue-delivering scaffold

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Abstract

Neural stem cells (NSCs) provide promising therapeutic potential for cell replacement therapy in spinal cord injury (SCI). However, high increases of cell viability and poor control of cell differentiation remain major obstacles. In this study, we have developed a non-woven material made of co-electrospun fibers of poly L-lactic acid and gelatin with a degradation rate and mechanical properties similar to peripheral nerve tissue and investigated their effect on cell survival and differentiation into motor neuronal lineages through the controlled release of retinoic acid (RA) and purmorphamine. Engineered Neural Stem-Like Cells (NSLCs) seeded on these fibers, with and without the instructive cues, differentiated into β-III-tubulin, HB-9, Islet-1, and choactase positive motor neurons by immunostaining, in response to the release of the biomolecules. In addition, the bioactive material not only enhanced the differentiation into motor neuronal lineages but also promoted neurite outgrowth. This study elucidated that a combination of electrospun fiber scaffolds, neural stem cells, and controlled delivery of instructive cues could lead to the development of a better strategy for peripheral nerve injury repair.

Keywords

Electrospinning, drug delivery, motor neuron, nerve, regeneration, differentiation.

3.2.1 Introduction

Traumatic injury to the spinal cord causes cell death and axonal degeneration resulting in functional motor and sensory loss. [163] With 12,000 new cases every year in the US only [164], spinal cord injury results in a dramatic loss in life quality. The process of spinal cord repair is often complex due to cell death during the first two weeks, the fragile nature of the cells, and the appearance of scar tissue that is chemically and mechanically hostile to the re-growth of functional tissue. [165] The lack of treatment is directly related to the lack of grafts. In this context, cell transplantation therapy using neural stem cells (NSC) provides a promising therapeutic potential to replace the damaged cells after SCI. [166] Studies by several groups, have demonstrated the potential of restoring functional motor activity after transplanting motor neurons derived from neural stem / progenitors cells in paralyzed animals. [163, 167, 168] Unfortunately, neural stem cells transplanted into the spinal cord have much tendency to differentiate into either astrocytes or oligodendrocytes and rarely undergo differentiation into neuronal lineages. [169] [170] Therefore expandable sources of motor cells and optimal scaffolds that can promote neural differentiation to specific lineage are needed for effective spinal cord repair.

Various attempts have been made to develop a scaffold to support neural tissue growth, but a remaining limitation is related to the need of a continuous delivery of the appropriate biomolecule(s). Their periodic injection is not a viable option as it requires several heavy surgical procedures, and their concentration should not significantly vary with time. [171-173] Therefore, neural tissue regeneration requires a scaffold permitting the controlled release of specific instructive cues in situ, in addition to harboring adequate mechanical properties. In that context, electrospun fibers have attracted interest for nerve tissue regeneration. The versatility of

electrospinning allows for the fabrication of non-woven meshes of fibers of diameters and mechanical properties that can be adjusted by properly choosing the polymer to be processed and its concentration. [174] Furthermore, it offers a possibility to incorporate instructive cues within the nanofibers, which will serve as controlled delivery system. [175] The high surface-to-volume ratio of the electrospun fiber mats as well as their structure that mimics extracellular matrix, make them well-adapted scaffolds for neuronal tissue engineering. [176] The high number of polymers that can be electrospun also makes it possible to obtain a wide range of material properties. [177-178] However, most of these materials need to be coated to improve cell adhesion. [174] [180] To reduce this limitation, more and more scientific reports explore the use of coaxial electrospun materials where two immiscible polymer solutions are electrospun using concentric needles. [181, 182] Two important uses of this technique can be as follows: one is the use of extracellular matrix as an outer shell to favor cell contact and the other is the use of the inner shell as a reservoir of instructive cues for progressive delivery. [183-185] Another characteristic reported in literature is that fiber diameter itself plays an important role in cell adhesion and differentiation. [186] In fact, according to this study, an ideal fiber diameter for nerve tissue engineering would be between 400 and 600 nm to allow for a maximum neurite outgrowth.

Fibroblast and epidermal growth factors (FGF and EGF) are biomolecules commonly used to favor neuronal stem cell (NSC) proliferation, and a cocktail of retinoic acid and purmorphamine has been widely shown to promote NSC differentiation into motor neurons. [187] These are issued from stem cells by the successive expression of Islet-1 and HB-9 homeogenes. Therefore, since choactase is the enzyme that catalyzes the biosynthesis of acetylcholine, the

neurotransmitter used by motor neurons, it is commonly admitted that cells positive for Islet-1, HB-9 and choactase genes are motor neurons. [188]

In this work, we have combined the co-electrospinning technology to generate fibers with concentric layers of two different polymers, i.e., Poly L-lactic acid (PLLA) as a core and gelatin as a shell, with a drug delivery strategy since the gelatin layer was loaded with RA and purmorphamine.

We thus report the fabrication of co-electrospun fibers with tunable mechanical properties, degradation and drug release rates. The nanofibers matrices were characterized morphologically, physically, chemically, and biologically to evaluate their efficacy to promote cell survival and differentiation towards motor neuronal lineages, which could lead to better strategy for peripheral nervous system repair.

3.2.2 Materials and methods

3.2.2.1 Fabrication and characterization of scaffolds of nanofibers

Fabrication of the fibers

PLLA (Purac, USA) and gelatin (Sigma, Oakville (ON) Canada) were electrospun using a co-axial needle (Linari, Pisa, Italy). Briefly, PLLA and gelatin were each dissolved in trifluoroethanol (Sigma, Oakville (ON), Canada) to final concentrations of 10% and 7% w/v, respectively. Solutions were agitated overnight and the gelatin solution was then heated at 70°C to allow gelatin dissolution. Fibers were collected on a rotating drum set to 1400 rpm with an electrospinning distance of 15 cm and an applied voltage of 24 kV. The flow rate of both solutions was set to 1 mL/h in order to form fibers with an average diameter of 1.6 μm. For the fabrication of loaded fibers, retinoic acid (Sigma, Oakville (ON) Canada, R2625) and purmorphamine (Cayman Chemical Company, Ann Arbor, MI, USA, 10009634) were dissolved in the gelatin solution prior to electrospinning to reach 0.2% w/w purmorphamine and 0.08% w/w retinoic acid in the dry gelatin.

Crosslinking of the fibers

In order to control the degradation rate of the gelatin outer shell, a crosslinking step was performed following fiber fabrication. Co-electrospun fiber mats were cut to fit in a 24 well plate containing 0.5% or 0.7% w/w glyceraldehyde in 70% ethanol. Samples were left 19 hours in these solutions at room temperature according to the protocol by Sisson et al. [189]

Scanning electron microscopy (SEM)

Samples were imaged after with a JSM-7600TFE microscope equipped with a field emission gun from JEOL, after their fabrication and after cell culture. For sample preparation, fibers were dried using successive dilutions of ethanol (50%, 75%, 90%, 100%) to avoid shrinkage, and stored under vacuum before imaging. All samples were coated with gold (2 times for 15 s). Observations were performed with a LEI (low secondary electron image) detector and an acceleration voltage of 2kV.

Transmission electron microscopy (TEM)

TEM was performed with a JEM-2100F microscope equipped with a field emission gun from JEOL, to further confirm the core/shell structure of the fibers. Fibers were co-electrospun directly on a copper grid and observed at 200kV for bright field imaging.

Diameter measurement

Mat samples were prepared as described for cell culture, and placed in culture medium (50% DMEM; 50% F12 during 7days) in an incubator (37°C) to assay for their degradation in culture conditions. Fiber diameter was measured with Photoshop software on SEM images at 70 different locations for each mat sample for statistical analysis.

Degradation rate of the gelatin outer shell

The degradation of the gelatin shell was assayed at different time points by placing fiber mat samples in medium in an incubator at 37 °C. Gelatin amount on fiber mats was determined by measuring the amount of free amine groups of the gelatin by adapting the Orange II dye protocol already published. [190] Fiber mat samples (approx. 3 mg) were incubated 30 min in 1 mL of a

solution of 40 mM Orange II dye (pH 3). Unbound Orange II dye was removed by three 1 mL washes (pH 3). Remaining dye was then desorbed by immersing the samples in a NaOH solution (pH 12, 15 min). Supernatants were acidified with 1% v/v of pure HCl. Eluted Orange II dye concentration was then measured by spectrophotometry (480 nm). The amount of eluted Orange II dye was normalized relative to the sample weight in order to derive the amount of remaining gelatin per g of fiber.

Mechanical testing

Mechanical testing was performed using an INSTRON instrument (Norwood, MA, USA). Fiber mat samples (2 cm x 5 mm) were crosslinked as for cell culture. Samples were then placed between pneumatic grips so that 5 mm of sample remained free. The Young's modulus was calculated as the slope of the initial linear part of the curve of the tensile stress (in MPa) plotted against tensile extension.

Enzyme-linked immunosorbent assay (ELISA)

To determine the release profile of retinoic acid from the fibers, the meshes were cut into round patches (16 mm of diameter) and a time dependent release of RA was performed by ELISA. The concentration of RA in medium was determined by ELISA assay (MyBiosource, San Diego, USA, MBS705877) following the manufacturer's instructions. Briefly, the samples were placed in medium (50% DMEM, 50% F12 with antibiotics) and samples of 25µl of liquid medium were taken periodically and placed at -80°C. Taken medium was replaced by fresh, and the suspension medium was changed every 3 days. Samples and standards were added to the 96-well plate and incubated for 1h at 37°C, which was followed by the addition of TMB substrate (20 min at

37°C). Optical density was determined using a plate reader set to 450 nm with a correction at 540 nm.

3.2.2.2 NSLC Culture and differentiation

Cell culture

Engineered Neural Stem-Like Cells (NSLCs) provided by New World Laboratories Inc. (Laval (QC), Canada) were cultured in neural progenitor medium (Lonza, Waskerville, USA, CC-4461) supplemented with laminin (Sigma, Oakville (ON), Canada, L2020-1MG) (5mg/L). For proliferation studies, cells were cultured on tissue culture plate or fiber mats, with EGF and FGF (Lonza, Waskerville, USA) in the medium. For the characterization of NSLC differentiation, cells were cultured on fibers loaded with purmorphamine (0.2% $w/w_{gelatin}$) and retinoic acid (0.08% $w/w_{gelatin}$) without any growth factor in the medium.

Fiber mat sample preparation and cell seeding

Fiber mat samples (10% Plla, 7% gelatin) were cut to the size of a well from a 24 well plate (16 mm) using a punch. Samples were considered sterile after the crosslinking step during which they stayed overnight in 70% ethanol. Samples were then rinsed 3 times with PBS (1mL) and cells were seeded at 45,000 cells/well in 2 mL of neural progenitor maintenance medium (Lonza, Walkerville, MD, USA, CC-4461). Cells were trypsinized and spun down at 1000rpm for 5 min and the resultant pellet was re-suspended and dissociated in fresh growth medium. The cell suspension ($45x10^3$ cells/well) was seeded on nanofibers with and without the presence of purmorphamine (0.2% $w/w_{gelatin}$) and RA (0.08% $w/w_{gelatin}$). Cells were incubated at 37°C, 5% CO2 for 3 weeks and maintenance medium was changed every three days.

Immunofluorescent staining and microscopy

To assess cell phenotype, samples were rinsed three times with 1 mL PBS and fixed in 1 mL formaldehyde (3.7%, Sigma) during 30 min. Samples were permeabilized in Triton (0.5%, 20 min), blocked with donkey serum 5% (Sigma, D9663-10ML, 1 h) and immunostained at 4°C overnight with different primary antibodies: goat anti-actin, -nestin, -choactase, -HB-9, -Islet-1 (sc-1616, sc-21248, sc-19057, sc-22542, sc-23590, all from Santa Cruz Biotech, Dallas, TX, USA) or mouse anti-TUJ-1 (Neuromics, Edina, MN, MO15013). Primary antibodies were dissolved in 0.1% Triton, 1% donkey serum in PBS, to reach a concentration of 3 µg/mL. After 3 additional washes, samples were incubated with secondary antibodies: donkey anti-goat or goat anti-mouse (sc-362265, sc-362257, Santa Cruz Biotech, Dallas, TX, USA) in 1% donkey serum for 1 h. Additional SYTOX green (Invitrogen, Burlington, ON, Canada, S7020) was added during 15 min at 1 µM to stain nuclei. Samples were then observed under fluorescent light (488 nm for TUJ-1 and sytox stainings and 555 nm for other stainings) with a Zeiss microscope.

Cells were counted on four SYTOX stained 5x images from four different samples with the software ImageJ and counts were averaged. Neurite outgrowth was measured on TUJ-1-stained 40x images. Fluorescence staining was required for these studies due to the opacity of the fibers, which prevented normal light observation. 40x images cover a 350µm x 350µm area; 5x images cover a 2100µm x 2800µm area.

Statistical analysis

Data were analyzed by performing student tests. Results are presented as means ±standard deviation.

3.2.3 Results

At first, mats made of co-electrospun fibers consisting of poly L-lactic acid as core and gelatin as outer shell loaded with RA and purmorphamine were fabricated and characterized. Then, their use to culture NSLC and differentiate them into neurons was investigated.

3.2.3.1 Mat fabrication and characterization

Fabrication of mats with controlled fiber diameter

The co-electrospinning technology was used for the fabrication of fibers with a core/sheath structure made of PLLA and gelatin, respectively. Our first attempts were conducted with low polymer solution concentrations, i.e., 7% PLLA and 5% gelatin, in an effort to generate sub-micron diameter fibers since these were reported to harbor a more receptive surface for cell contact, attachment and phenotypic differentiation. [174, 186] As expected, fiber diameters of 0.7 μm were generated, however corresponding mats were extremely fragile and sensitive to static electricity. We therefore produced fibers with an average diameter of 1.6 μm, varying from 0.8 to 2 μm (**Fig 4**) by increasing PLLA and gelatin concentrations up to 10 and 7%, respectively. These fibers were easier to manipulate and mechanical properties of the resulting mats were compatible with practical work. Mat thickness of ~ 50 μm was obtained after 20 min of electrospinning, this thickness was proportional to fiber winding duration on the rotating cylinder. Of interest, while varying polymer concentrations resulted in varying fiber diameters, fiber morphology and mat structure were identical for the polymer concentrations that were used (**Fig 4**, compare panels A and B). As expected, the co-electrospun fibers possessed a core/sheath structure, as evidenced by TEM imaging. Indeed, as it can be seen in **Figure 4** (panel D), fibers displayed a well-delimited inner darker area resulting from the different properties of the two

polymers constituting the fibers. The loading of the fibers with RA and purmorphamine did not affect the morphology of the fibers or that of the mat as deduced from SEM imaging (**Fig 1,** compare panels A and C).

Fiber crosslinking increased mat stiffness and stability of the gelatin outer layer.

The fibers were then treated with two concentrations of glyceraldehyde to crosslink the gelatin following Sisson's work on crosslinking methods. [189] The impact of glyceraldehyde crosslinking upon mat mechanical properties is reported in **Figure 5 A**. The Young's modulus of the mat significantly increased upon glyceraldehyde treatment, varying from 0.14 +/- 0.08 MPa (untreated fibers) to 1.23 +/- 0.1 MPa for 0.7% w/w glyceraldehyde crosslinking solution. Of interest, loading RA in gelatin prior to crosslinking did not affect mat mechanical properties (**Fig 5 A**). In parallel, glyceraldehyde-mediated crosslinking induced a loss of elasticity of the mat, as evidenced by the decrease in the tensile strain at break of the mats when increasing glyceraldehyde concentration (**Fig 5 C**), while the tensile stress at break increased with the crosslinking level (**Fig 5 B**).

Note that, overall, the glyceraldehyde-mediated crosslinking did not affect fiber shape according to SEM imaging (**Fig 6**)

The impact of the crosslinking procedure upon fiber degradation, when fibers were incubated into culture medium, was first evaluated by monitoring fiber aspect (**Fig 6**) and diameter (**Fig 7 A**) changes using SEM images. As can be seen in **Figure 6**, progressive signs of a new layer covering the fibers were observed, a phenomenon that may be attributed to the degradation and re-deposition of polymer fragments. Non-crosslinked fibers had a significantly smaller diameter at day 0 that their glyceraldehyde-crosslinked counterparts (**Fig 7 A**), a result that was more

likely due to the swelling and drying of the fibers with ethanol (a required step for rinsing and sterilizing fibers before cell culture), which might have reduced the amount of gelatin at the fiber surface. (**Fig 7**) This single wash thus appeared to be sufficient to remove a significant, although limited, portion of the gelatin on pristine fibers. For pristine fibers, fiber diameter was observed to decrease for 5 days to reach an asymptotic limit, more likely due to almost complete dissolution of the gelatin layer up to the PLLA core. However, this decrease in fiber diameter was significantly reduced by the glyceraldehyde crosslinking treatment of the fiber mats. Indeed, for the 0.5% glyceraldehyde treatment, the crosslinked fibers had the same diameter at day 7 as that of freshly rinsed non-crosslinked fibers (day 0) (**Fig 7 A**). In complement to SEM imaging, the degradation kinetics of the fiber gelatin outer layer was also evaluated using a dye assay allowing for the quantification of free amine groups (Orange II dye). Since, for our fibers, amine groups are only present on gelatin, the Orange II dye assay allowed for a quantification of gelatin that remained on our fiber mats. For non-crosslinked fibers, results of the Orange II dye assay strongly suggested that gelatin was totally dissolved within the first 5 days in culture medium (**Fig 7 B**), in excellent agreement with conclusions drawn from diameter measurements. In contrast to pristine fibers, the 0.5% glyceraldehyde treatment drastically reduced the degradation rate of gelatin as the latter was still detectable at day 5. Of salient interest, fibers treated with 0.7% glyceraldehyde harbored more than 80% of their initial gelatin after 25 days of incubation.

Retinoic acid release was also affected by the gelatin outer layer stability, as revealed by in-solution RA measurements by ELISA (**Fig 8**). Indeed, in the case of fibers crosslinked with 0.5% glyceraldehyde, an important initial burst in the release of the RA was observed; such a burst was not observed in the case of 0.7% glyceraldehyde crosslinked fibers. Apart from this initial burst, RA concentrations in the medium were very similar for the two treatments. These concentrations

were relatively stable over time with values around 50 +/- 1.8 nM, then reducing to ~25+/-3 nM after 6 days in culture medium, to reach ~15+/-1.9 nM on day 10 and 13. The amount of retinoic acid present in the medium was quite stable in spite of medium renewal performed at days 3, 6, 9 and 12. This may be explained by the short half-life (around 17 h) of RA in culture medium. [191] Of interest, these RA concentrations in culture medium were within the range of those proposed by Hu and Zhang for best neural stem cell differentiation. [188] This fiber mat was thus selected to culture and differentiate neuronal stem cells

3.2.3.2 Use of the mat to create an interconnected motor neuron network

NSLCs proliferated onto the fibers and penetrated within the scaffold

The mat of electrospun fibers promoted NSLCs proliferation (**Fig 9**), although at a slower rate than those cultured in control wells (Cellbind, data not shown). However, NSLC cultures on fiber mats presented fewer aggregates than in control wells, these aggregates being largely interpreted in the literature as a symptom of poor surface contact and biocompatibility. Interestingly, NSLC cultures on fibers that had not been loaded with any instructive cues reached their maximum cell number and plateaued from day 7. Cells then remained quiescent whereas in tissue culture plate NSLCs rapidly formed aggregates that detached from the plate around day 14 to form neurospheres (Supplementary data, 14). Surprisingly, with instructive cue-loaded fibers, a significant ($p<0.005$) increase of cell number at day 14 was observed, i.e. 4.2 times higher than that observed with unloaded fibers (**Fig 9**). However, cells cultured on these fibers died at day 21. Another interesting behavior of the cells cultured on fiber mats being loaded with instructive cues was that they also penetrated inside the construct. This phenomenon was revealed by the

observation of different focal planes within the same microscopy image (Supplementary data, 16). Also, SEM images indicated that cells were able to penetrate inside the fiber mesh and that their extensions grew along the fibers (**Fig 13,** G and H).

NSLCs differentiated into motor neurons when cultured on fibers loaded with instructive cues

Cells cultured on unloaded fibers remained positive for nestin (a marker of pluripotent potential), whereas cells cultured on RA- and purmorphamine-loaded fibers were nestin-negative from day 2 (**Fig 10**). These cells were characterized by larger diameters and presented more neurite extensions (**Fig 12**). The number and size of neurites increased with culture time; this was in stark contrast with cells cultured on unloaded fibers, which presented reduced size and number of neurite extensions (**Fig 12**). During their differentiation into motor neurons, NSLCs successively expressed the homeogenes Islet-1 and HB-9 and became positive for choactase, the enzyme responsible for the biosynthesis of the neurotransmitter used by motor neurons. Cells cultured on unloaded fibers remained negative for these markers during the whole experiment, while remaining nestin positive. On the contrary, cells cultured on instructive cue-loaded fibers were positive for islet-1 from day 2 (**Fig 11 A**). The other homeogene tested in our study, HB-9, was expressed later, i.e. around day 7, and was present only in nuclei as shown by its systematic co-localization with SYTOX green staining of the nuclei (**Fig 11 E**). At last, the cells started to produce choactase from day 7, while creating a network of choactase-positive cells around day 14 (**Fig 11 I**). Cells being positive to islet-1, HB-9 and choactase were considered to be differentiated motor neurons, as largely accepted in the literature. The increase of neurons

population on loaded fibers was accompanied by a decrease of nestin, a multipotent markers, indicating a loss of multipotency phenotype and the differentiation of the cells.

Cells grow neurite extensions on loaded fibers

Cell only presented slight changes in their morphology when cultured on unloaded fibers, while those cultured on instructive cue-loaded structures were characterized by a significant increase of their neurites length from day 7 (**Fig 12 and 13**). Cell aggregates were also observed from day 7 (**Fig 12**) in cultures with RA- and purmorphamine-loaded fibers, while those aggregates were shown to be interconnected in the sample imaged at day 14.

3.2.4 Discussion

Various strategies have been explored to create adequate scaffold for nerve regeneration. [192-194] Hydrogels have been well characterized and offer a 3-D structure for cell colonization. Their swelling properties and mechanical properties make then good candidates to easily fill an injury cavity without further damaging surrounding tissue. [195] These hydrogels have been used to deliver instructive cues [196] in situ but the fine control of their drug release rate is a daunting task. [197] A limitation to their use is the difficulty to obtain anisotropic structures in order to control the topology of the new tissue. [198] Electrospinning is a technique that opens these avenues. It is indeed possible to align fibers by increasing the collector rotating speed. Such alignment fits the needs for nerve supporting structure development. [199] The co-electrospinning of gelatin and PLLA has already been shown to be promising for the fabrication of fiber mats that closely mimic the architecture of the extracellular matrix. [200] As further highlighted in our current study, such an approach provides a material with a biocompatible

surface ensuring a good cellular contact via gelatin and decent mechanical properties thanks to the PLLA core. Our mats were shown to favor neural stem cell proliferation, colonization, and differentiation; furthermore, NSLC were able to penetrate within the mat (Supplementary data, 16), a very important asset for the generation of a three-dimensional functional biological structure.

Moreover, the mechanical properties of our co-electrospun fiber mats were easily tailored by varying gelatin and PLLA concentrations as well as the extent of gelatin crosslinking via glyceraldehyde treatment. The Young's moduli of the mats included in this study were comprised between 0.1 and 1.2 MPa, depending on the crosslinking treatment applied to the mat (**Figure 5 A**), while values around 0.45 MPa have been reported for peripheral nerves. [201] Therefore the mechanical properties of our material closely match those of peripheral nerve tissue and can be adjusted around this value for specific use. This is a very important asset that contrasts with Young's moduli of most electrospun mats being significantly higher than that of nerve tissue. [202, 203] In addition, the strong elasticity of our material, which can withstand up to 80% elongation before rupture, facilitates its manipulation. (**Figure 5 C**)

Another advantage of our mats resides in their ability to serve, via their gelatin sheath, as a reservoir of instructive cues whose release in situ must be controlled both spatially and temporally for tissue engineering purposes. Retinoic acid (RA) and sonic hedgehog are known to promote NSLC differentiation into motor neurons. [204] Purmorphamine has also been recently demonstrated to activate the sonic hedgehog pathway [205] and thus provoke the differentiation of NSLCs into motor neurons when used in combination with RA, hence motivating our choice of instructive cues. In that endeavor, fiber loading with selected concentration of retinoic acid did

not affect fiber morphology or Young's modulus (**Fig 5**), an observation that contrasts with previous reports. [206] Tuning the gelatin layer stability via glyceraldehyde crosslinking was a key to eliminate significant initial release burst while maintaining a concentration of retinoic acid in the medium at about 50 nM. (**Fig 8**) Such a value corresponded to the concentration suggested by Bao-Yang Hu and Su-Chun Zhang to promote NSLC differentiation. [188] After a few days of culture, the concentration of retinoic acid decreased around 25 nM, which was still sufficient to promote cell differentiation. The release of retinoic acid was then constant for up to 14 days, a salient difference with more simple mats that commonly allow constant delivery for less than a week. [197, 207] Such a drug release study was not conducted for purmorphamine due to the technical difficulties associated with its detection.

NSLCs cultured on RA- and purmorphamine-loaded fibers differentiated in 14 days. Cell progressively differentiated as deduced from the successive expression of 2 homeogenes (Islet-1 and HB-9) (**Fig 11**) prior to the detection of choactase-positive cells (**Fig 11**). Most of the cells appeared to be motor neurons, and cells started differentiating before day 2, as they were already negative for nestin and positive for Islet-1 at this stage. The expression of the HB-9 gene product was observed later, i.e. at day 7. We also observed that choactase was expressed only after 14 days of culture under these conditions and was present all along the neurites and in the nuclei. At this time, cells were positive for Islet-1, HB-9 and choactase, unambiguously confirming their motor neuron phenotype. [188]

Throughout this study, NSLC proliferated well on the fiber mats, although their proliferation was faster on tissue culture plate. This observation can be related to the three-dimensional nature of the mat allowing for cells migration; this process consumes energy and may thus decrease cell

proliferation when compared to growth on the two-dimensional culture plates. Cell number significantly increased on RA- and purmorphamine-loaded fibers at day 14. (**Fig 9**) This is more likely due to the mitogen activity of the sonic hedgehog pathway that was activated by purmorphamine. [208] Also, cells were shown to aggregate from day 7 (**Fig 12**) and to die before the 21st day of culture on these fibers whereas no sign of aggregation was observed on fibers without any instructive cues loaded. These differences suggest that NSLCs, once differentiated into motor neurons may require additional biochemical and/or electrical stimulation to remain viable. The culture protocol we applied may not provide adequate conditions to prolong cell viability. This may not have any negative impact if these cell-bearing structures are implanted early.

At last, of salient interest is the fact that neurite length increased significantly on our instructive cue-loaded fiber mats, (8-fold longer at 14 days of culture, **Fig 13**) whereas a round phenotype was observed on pristine fibers. Therefore we observed the creation of extended, mechanically connected networks of motor neuron cells in the newly generated tissue in vitro, an extremely promising result in the context of the regeneration of an in vivo nerve network.

3.2.5 Conclusions

We here report the fabrication of mats made of co-electrospun fibers of PLLA and gelatin to deliver retinoic acid and purmorphamine in situ. This material presented adequate mechanical properties and was able to progressively deliver loaded instructive cues. NSLCs cultured on these mats successfully proliferated and differentiated rapidly into motor neurons. We thus believe that this novel structure can offer mechanical and chemical support for the fabrication of motor nerve grafts from NSLCs, either in vitro or in situ. This work may also provide a starting point for further fabrication of any kind of neural tissue by loading appropriate instructive cues.

3.2.6 Acknowledgment

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3.2.7 References

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3.2.8 Figures

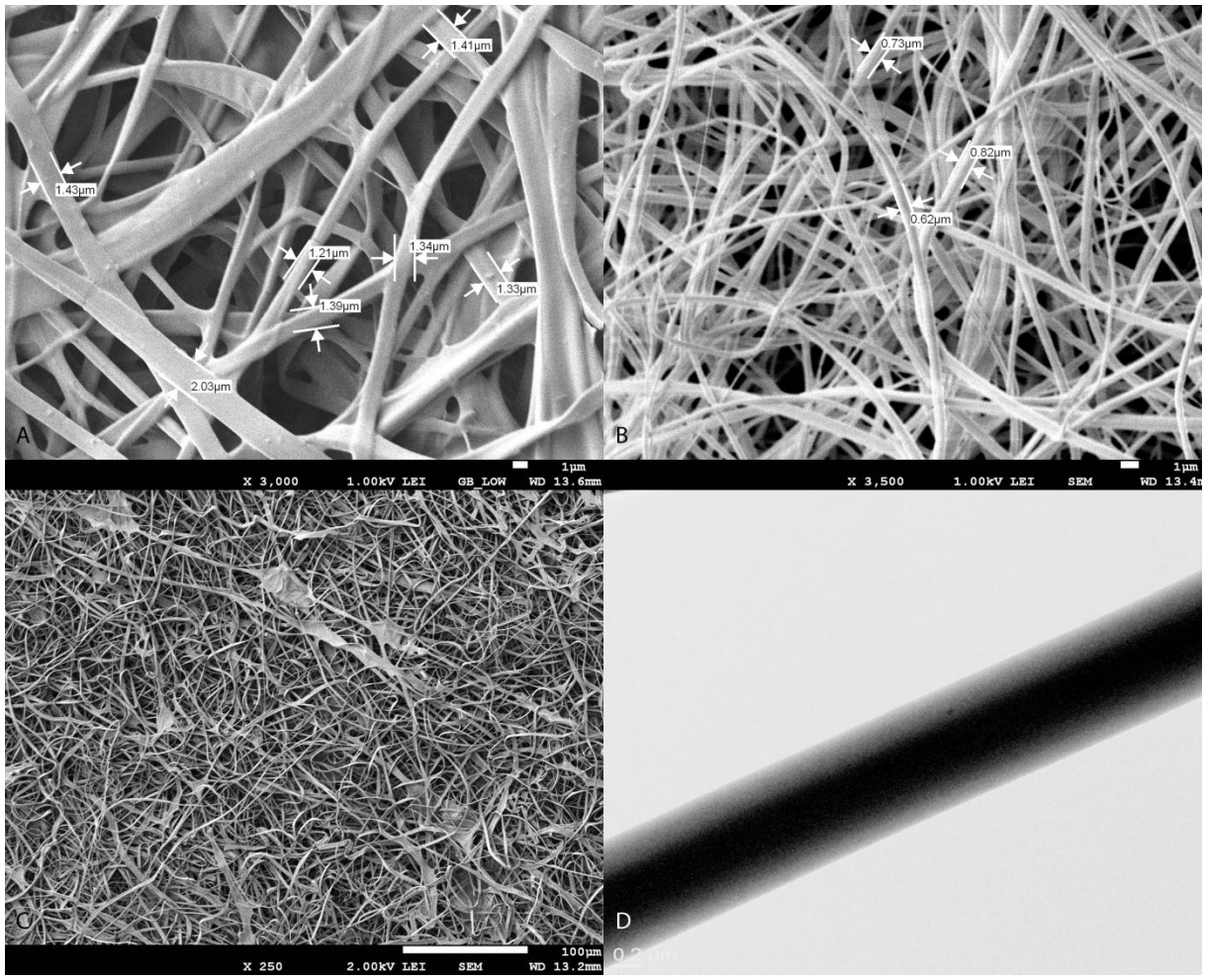

Fig 4. Fiber morphology*:* SEM images of co-electrospun fibers corresponding to 10% PLLA (core) and 7% gelatin (outer shell) (A, C) or 7% PLLA and 5% gelatin (B) without any instructive cues (A, B) or with retinoic acid and purmorphamine in the gelatin (C) and TEM image showing the core/shell structure of the fibers (D)

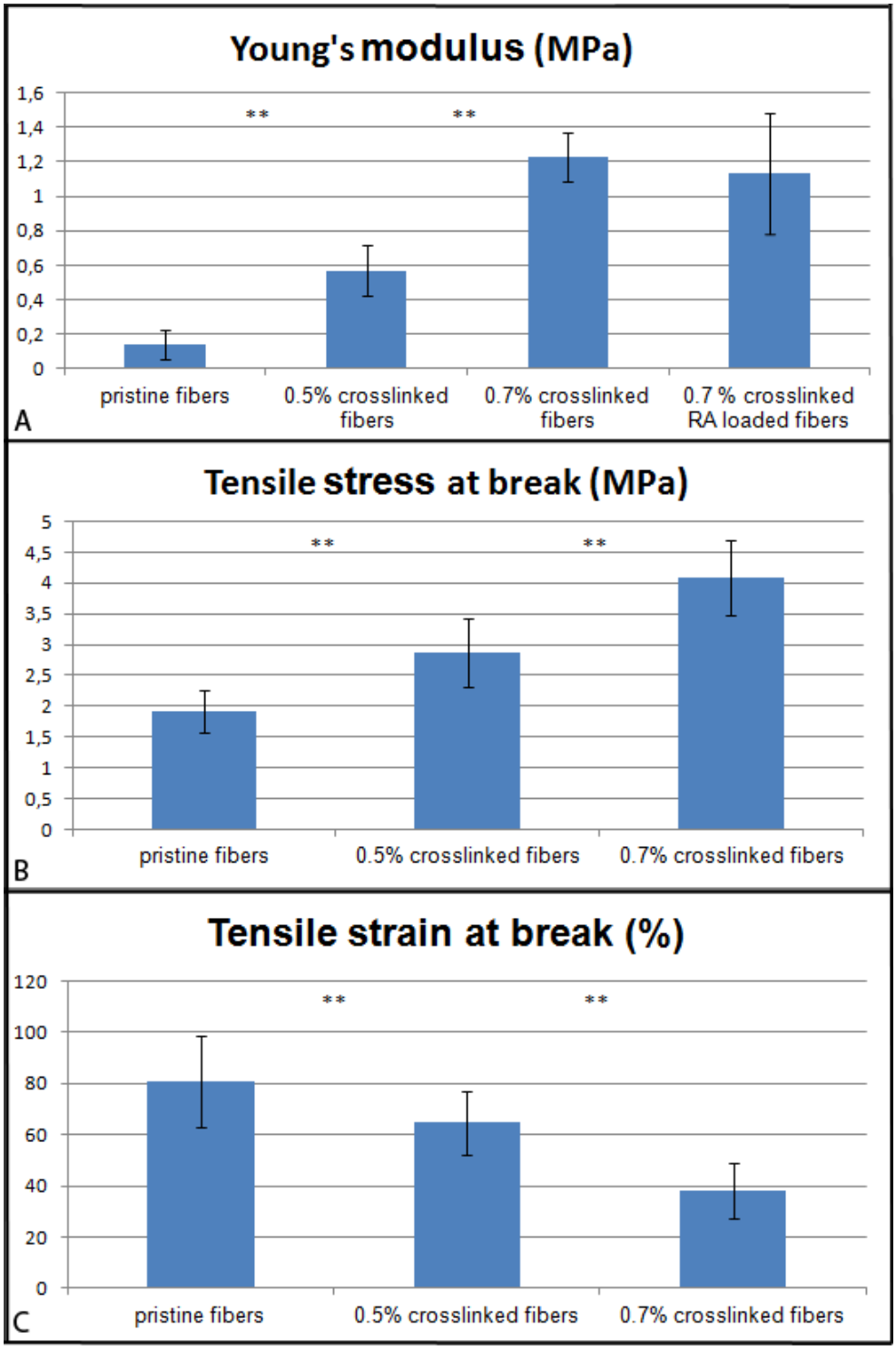

Fig 5. Young's modulus (**A**), Tensile stress (**B**) and strain (**C**) at break of fibers crosslinked with different concentrations of glyceraldehyde.

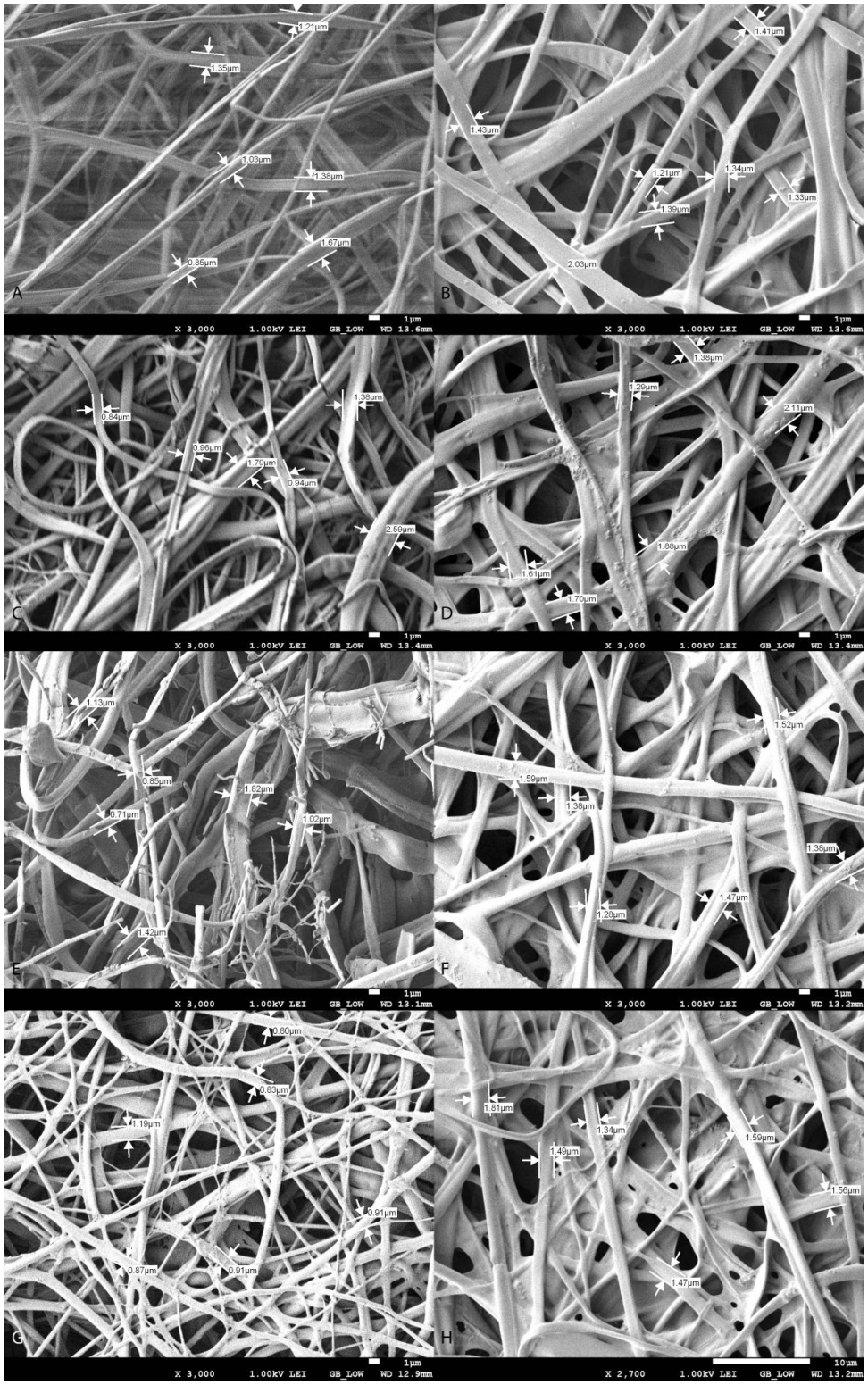

Fig 6. SEM images of untreated (A, C, E, G) and crosslinked (B, D, F, H) fibers at different degradation times: day 0 (A, B), day 3 (C, D), day 5 (E, F) and day 7 (G, H)

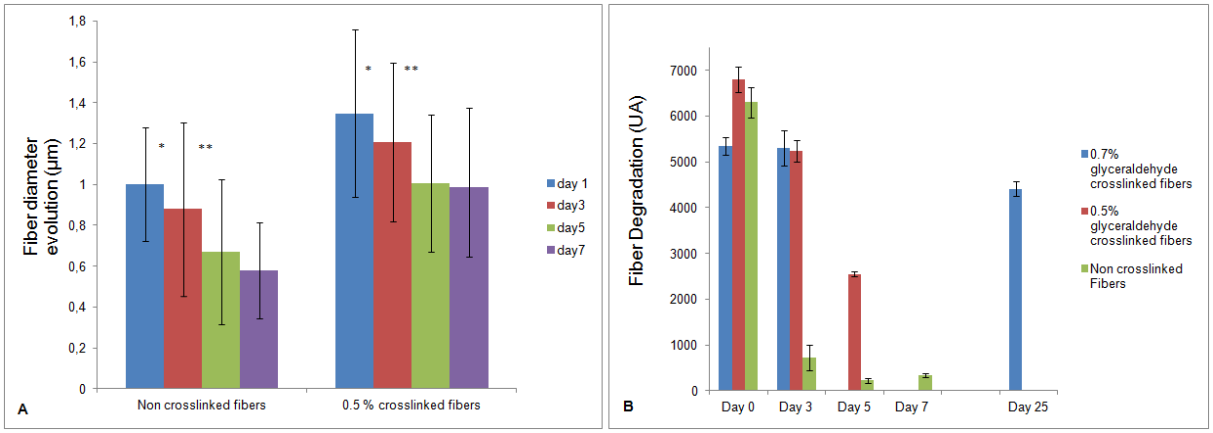

Fig 7. Evaluation of gelatin layer degradation by (A) fiber diameter measurements from SEM imaging and (B) Orange II dye assay after various glyceraldehyde crosslinking treatments. (For panel A, * Indicates student test $p<0.05$, and ** indicates $p<0.001$)

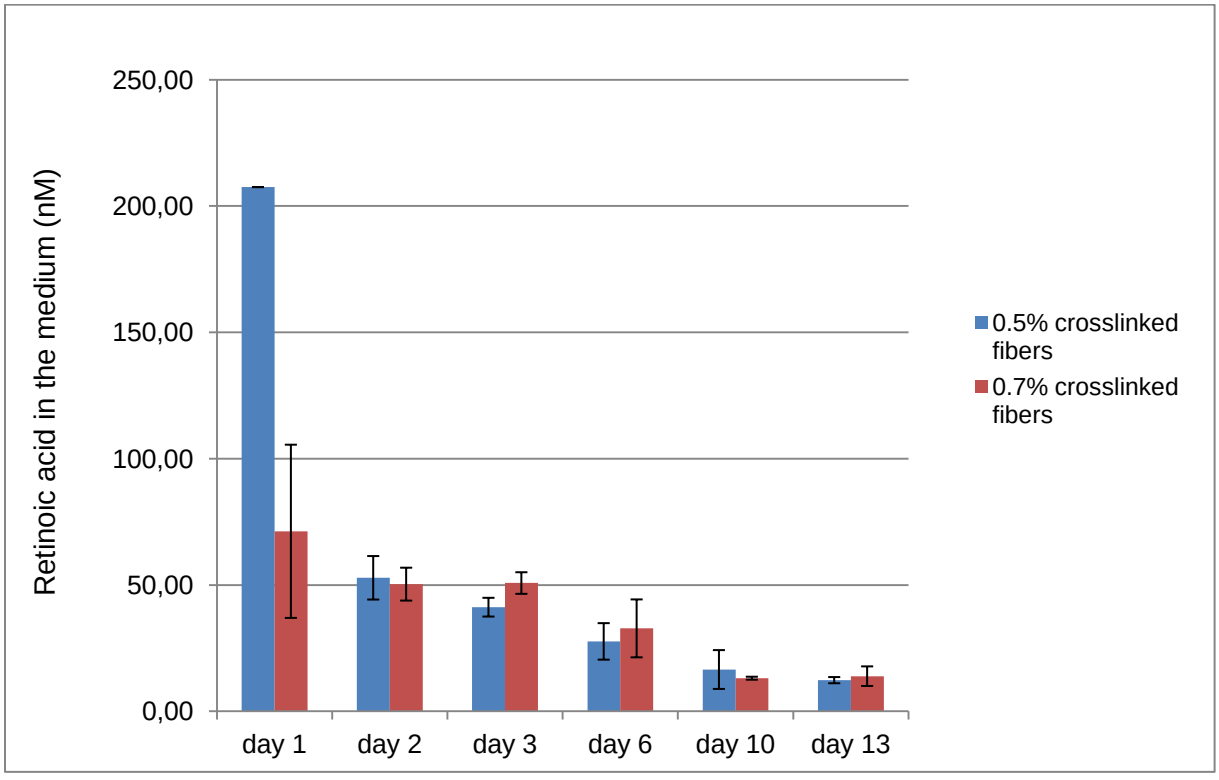

Fig 8. Concentration of retinoic acid released from the fibers in the culture medium. Medium was changed at days 3, 6, 9, and 13.

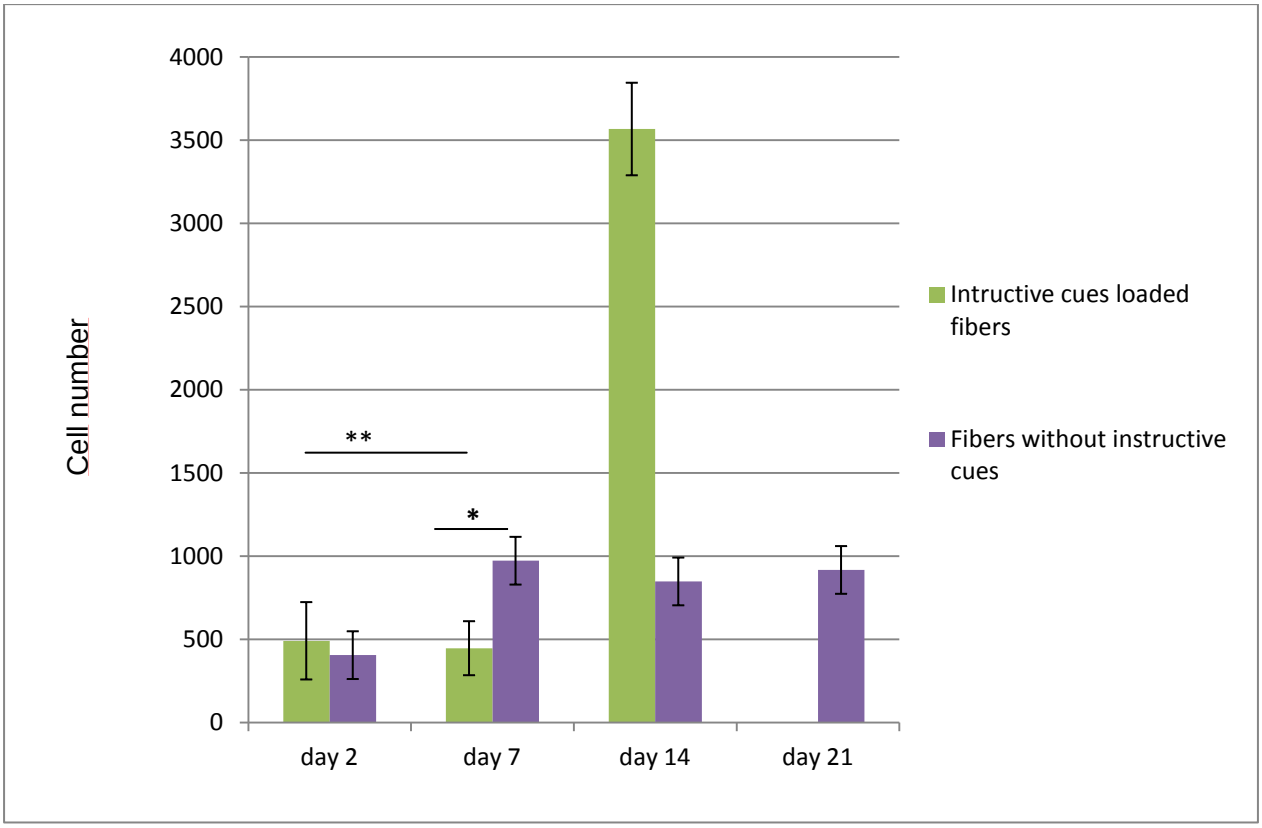

Fig 9. Cell counts on RA- and purmorphamine-loaded fibers or control fibers

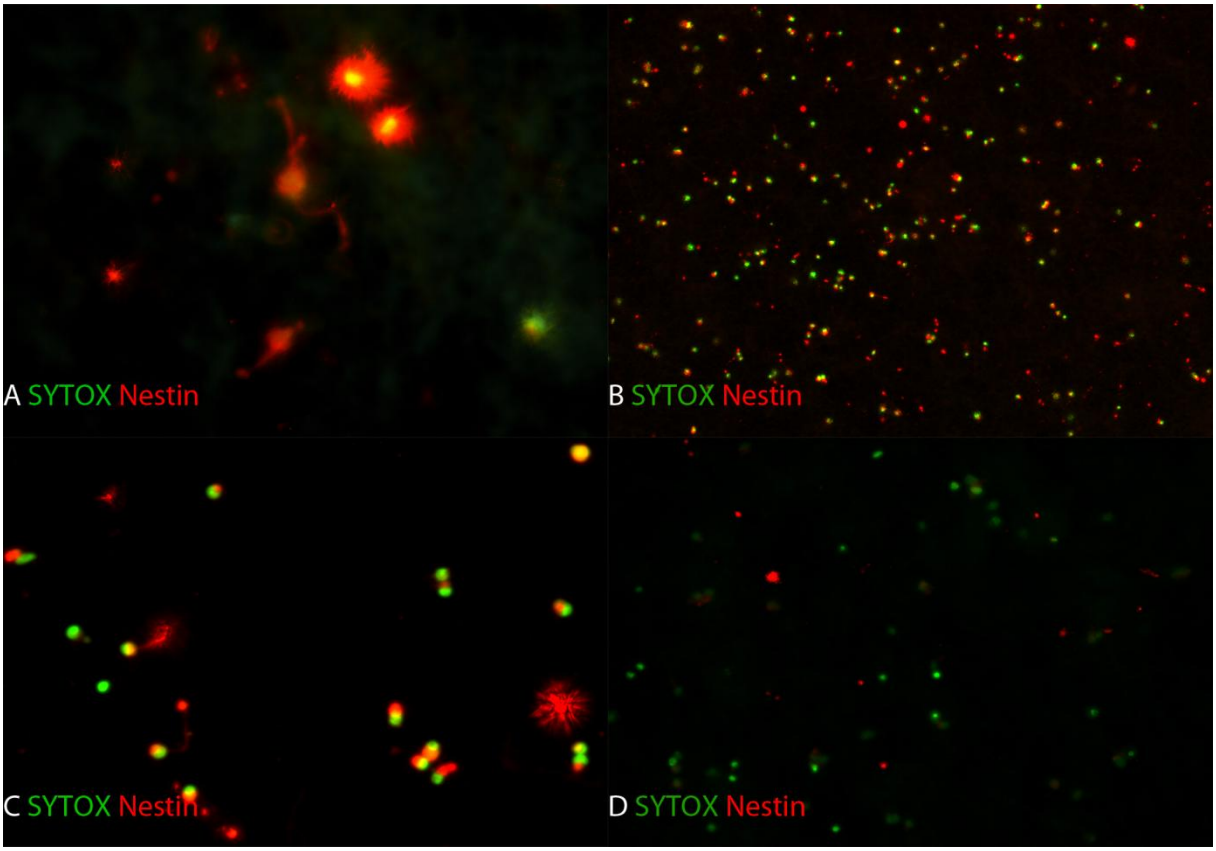

Fig 10. Nestin and SYTOX staining of cells cultured on control fibers at days 2, 7 and 14 (A, B, C) or RA- and purmorphamine-loaded fibers at day 2(D). Images are 40x (A, C, D) or 10X (B)

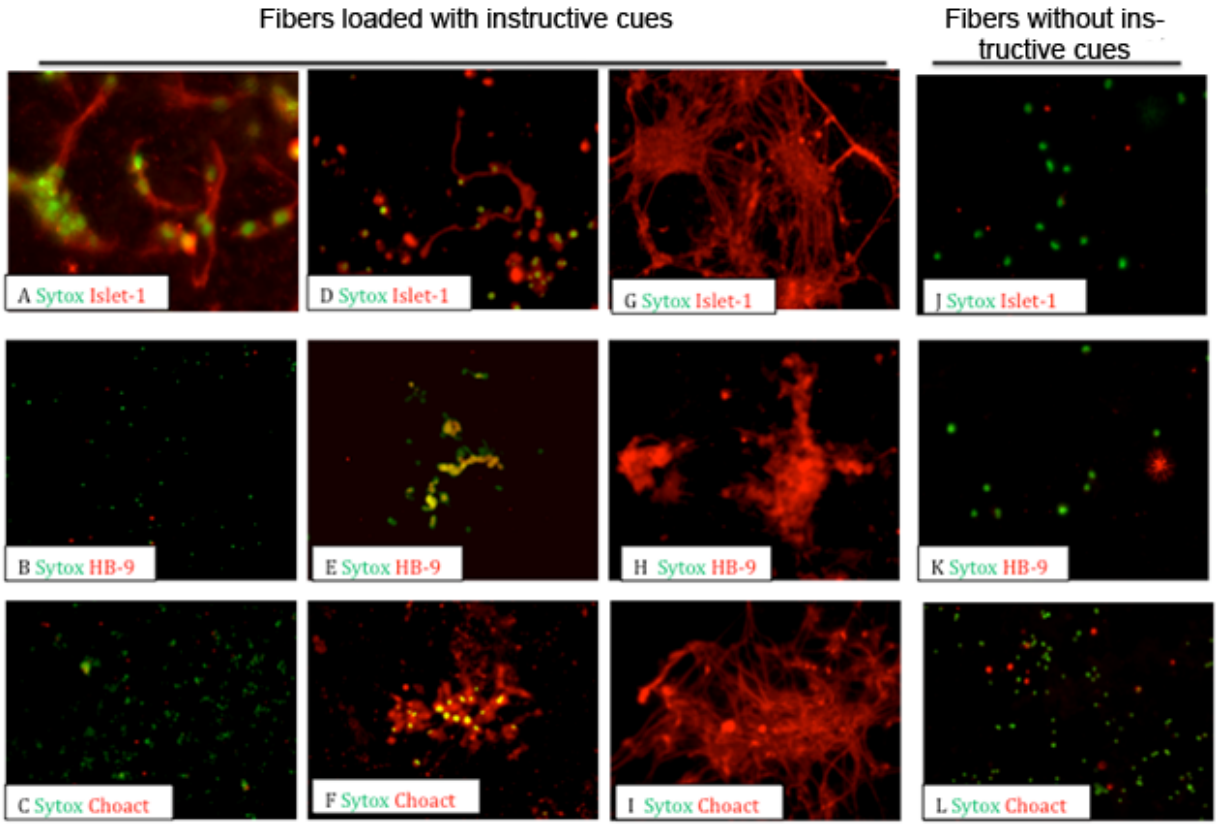

Fig 11. Representative images of NSLCs at different time points in culture on RA and purmorphamine loaded fibers (from A to I) and fibers without instructive cues (J, K, L, only at day 14). Cell morphology on 40x images obtained with Cells stained for Islet-1 at day 2 (A), 7 (D), 14 (G), for HB-9 at day 2 (B), 7 (E), 14 (H), and for Choactase at day 2 (C), 7 (F), and 14 (I).

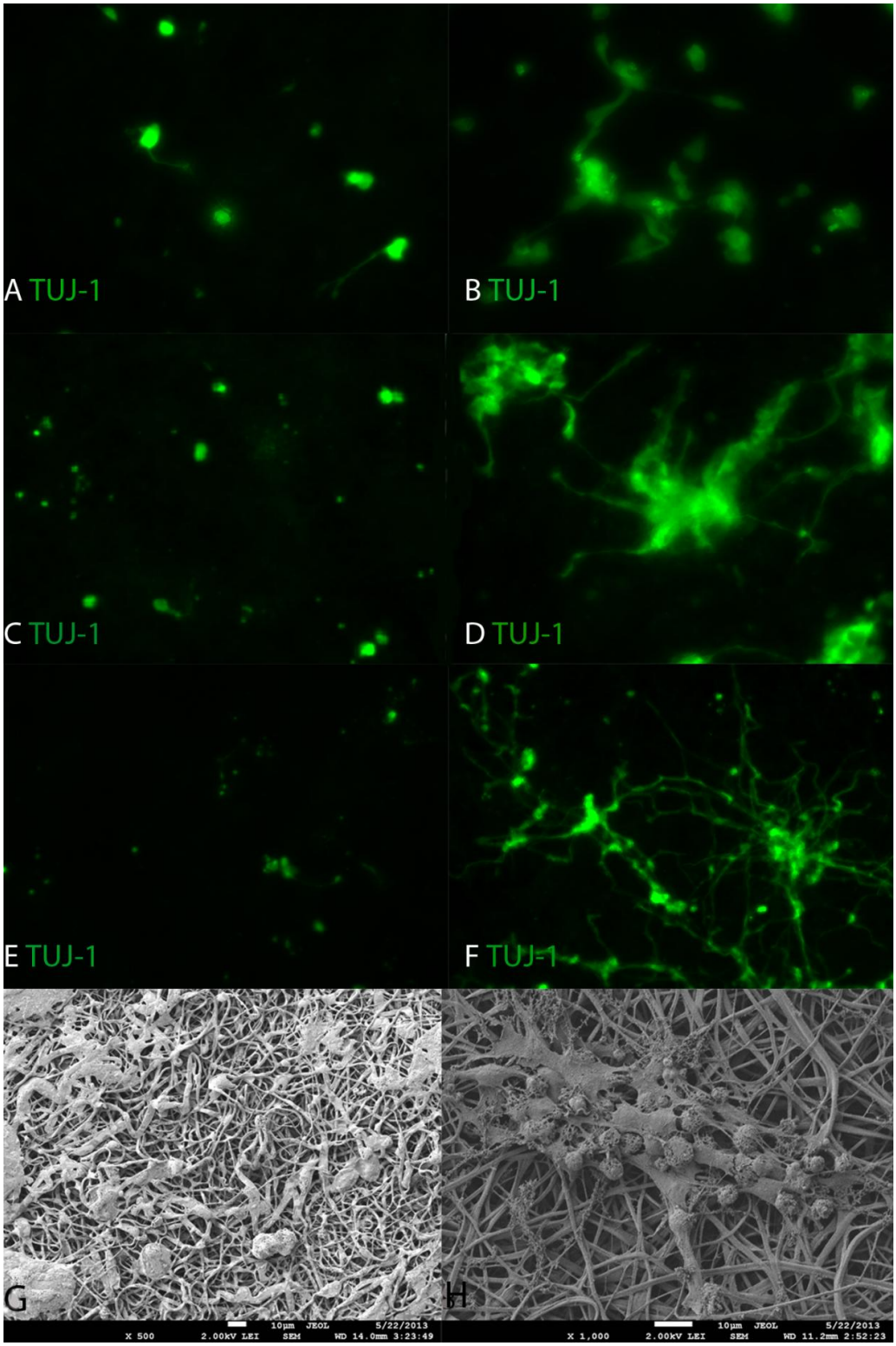

Fig 12. Morphological evaluation: cell morphology on 40x images obtained with TUJ-1 staining and SEM images. Cells cultured on pure fibers (A, C, E, G) or RA- and purmorphamine-loaded fibers (B, D, F, H) at day 2 (A, B, G), 7 (C, D), and 14 (E,F, H).

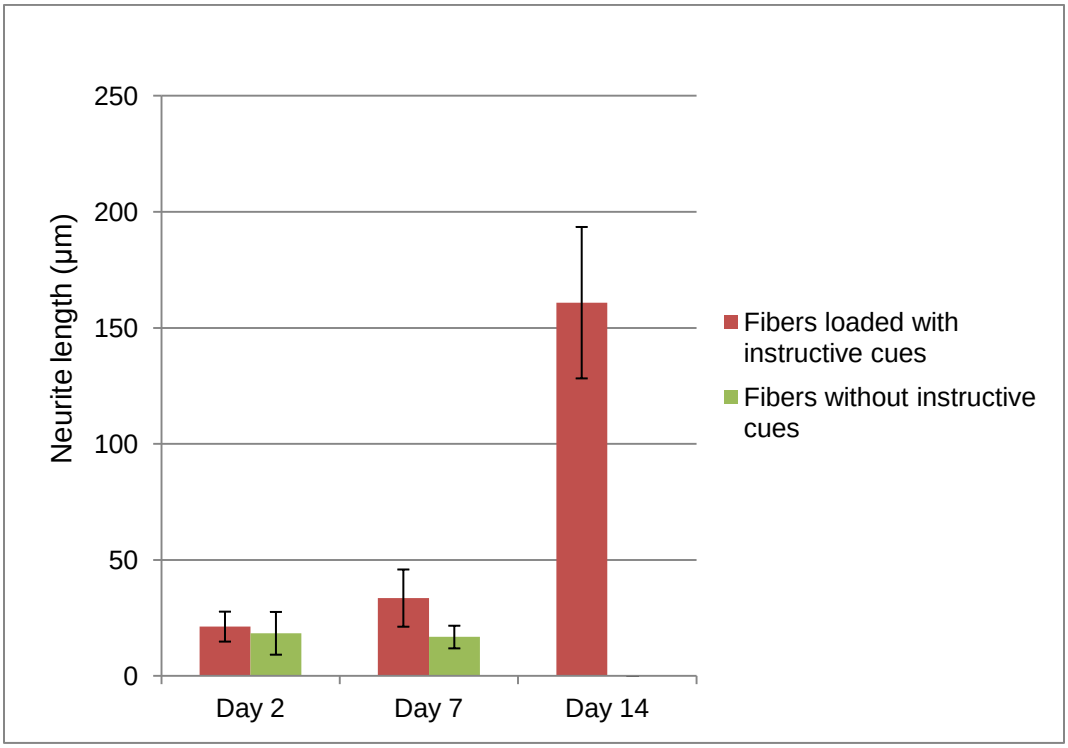

Fig 13. Neurite length on fibers with or without RA and purmorphamine. (The value at day 14 for pure fibers is under detection limit capacity).

3.2.9 Supplementary data

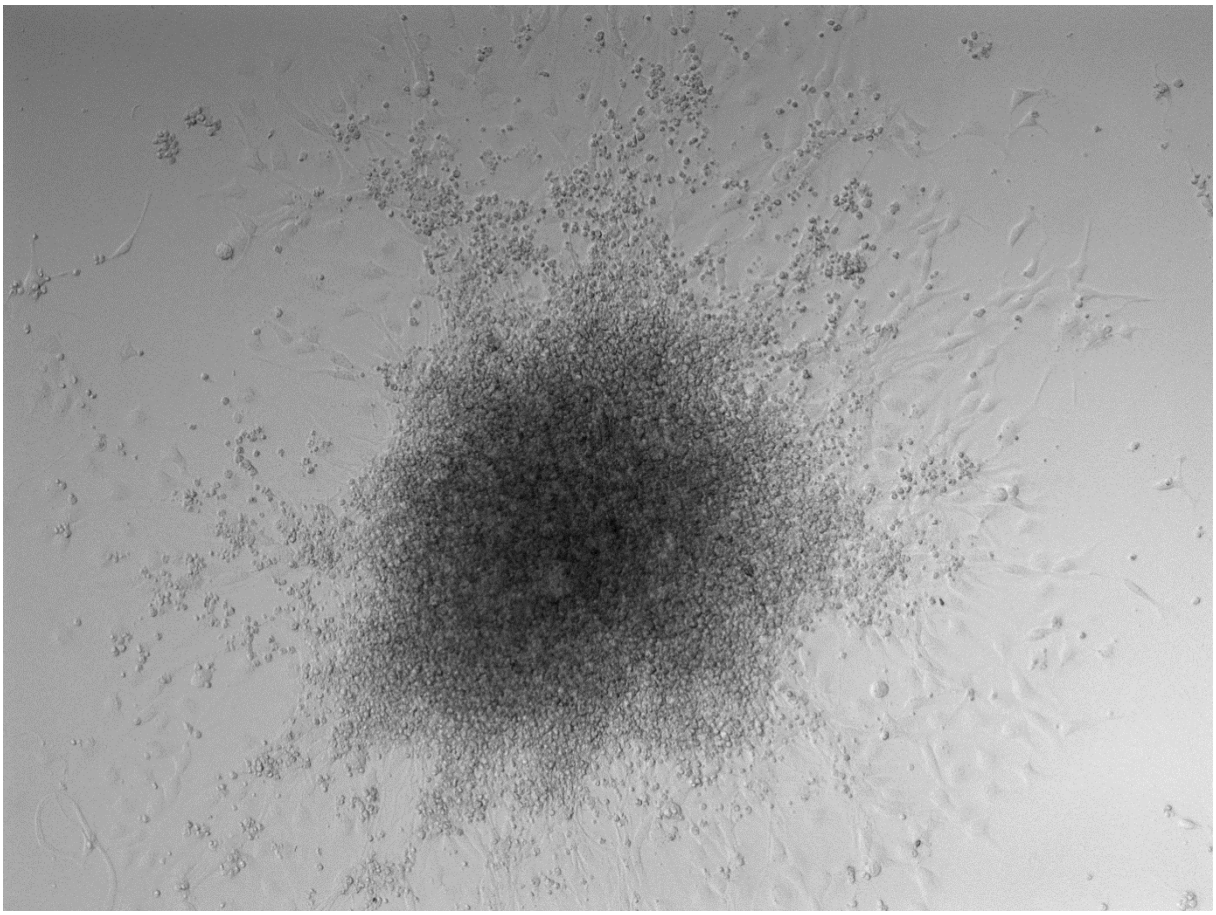

Fig 14. Formation of neurospheres on tissue culture plate after seven days of culture.

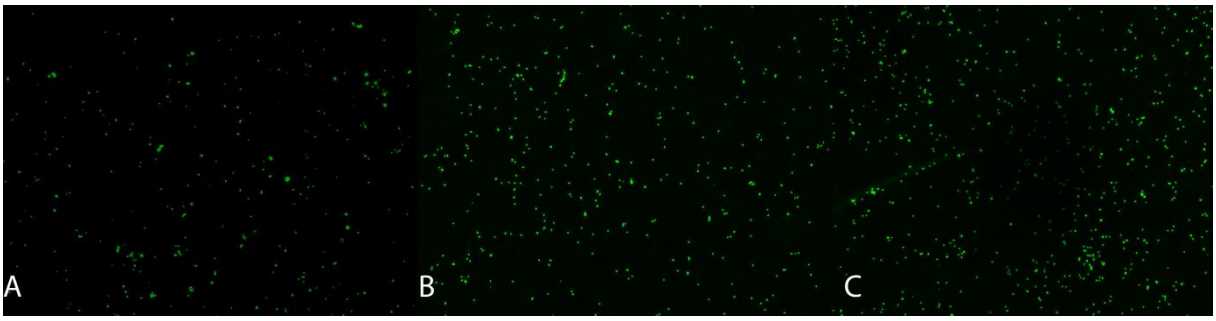

Fig 15. Images of cells cultured on fibers and stained with SYTOX green for nuclei at day 2 (A), day 7 (B) and day 14 (C).

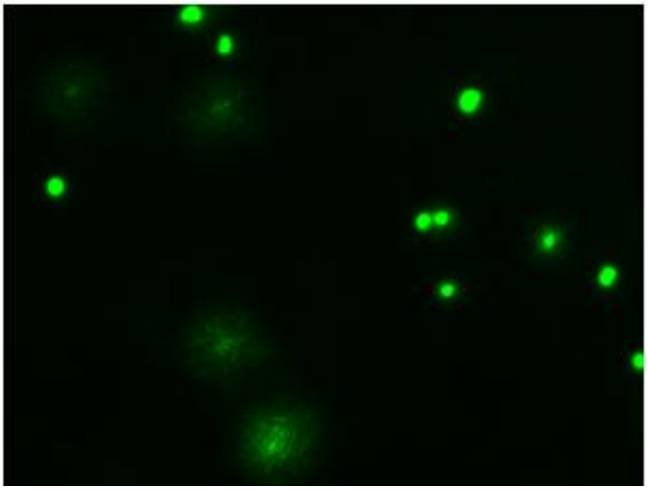

Fig 16. Cell penetration: at least two different focal planes are present on this 40x image obtained with nucleus staining.

CHAPITRE 4 DISCUSSION GÉNÉRALE

Ce travail a permis de développer une technique de fabrication de fibres permettant la livraison de biomolécules in situ afin de favoriser la différenciation de cellules souches neuronales en neurones moteurs. Afin de pouvoir juger des différentes techniques et des matériaux à disposition, une importante revue de littérature a été réalisée. Elle a permis de définir les caractéristiques importantes qu'une structure doit avoir pour aider à la différenciation de cellules souches. Les plus importantes sont une topologie et des propriétés mécaniques proches de celles de la matrice extracellulaire, une surface favorable au contact et à l'attachement des cellules, et la capacité de relâcher progressivement des molécules dans son environnement. Ainsi, nous avons pu sélectionner une méthode essentielle : le co-électrofilage. En effet, cette technique nous a semblé la plus prometteuse puisqu'elle répond extrêmement bien aux critères de mimétisme de la matrice extracellulaire et qu'il nous a semblé possible d'y apporter des modifications pour qu'elle satisfasse aux autres critères. Ainsi, nous avons choisi d'ajouter une couche externe de gélatine aux fibres afin d'en améliorer les propriétés de contact avec les cellules. L'application d'une étape de réticulation de la gélatine permet d'ajuster la vitesse de dégradation et les propriétés mécaniques du matériau pour les adapter en fonction de l'utilisation de la structure fabriquée. Ces propriétés constituent un avantage important de la méthode proposée puisqu'ils la rendent flexible tout en assurant sa totale autonomie : comme souhaité, le matériel fabriqué ne nécessite aucune injection ou manipulation pour que les cellules se différencient après leur implantation sur la structure. Ce nouveau matériau a permis d'obtenir la différenciation efficace de NSCs en neurones moteurs en 14 jours. Ce travail propose une solution pour la fabrication de greffons de nerfs moteurs à partir de cellules souches. Ceux-ci pourront alors être implantés chez le patient. Une limitation qui est apparue est la mort des cellules pour des temps de culture trop long, probablement faute de stimuli complémentaires, bien que cette limitation nous parait liée au travail en puits de culture et devrait être supprimée par une implantation sur le patient.

La grande flexibilité de la méthode fait qu'il serait envisageable de l'utiliser pour générer d'autres phénotypes neuronaux, en changeant simplement les molécules délivrés et éventuellement en choisissant d'autres propriétés mécaniques.

CONCLUSION

Ce travail propose une méthode efficace de fabrication d'une structure à greffer dans une zone blessée de la moelle épinière, ou dans une section blessée d'un nerf, afin de rétablir les fonctions du tissu natif. Dans une première partie, ce rapport propose une revue de la littérature, soumise au journal *Stem cell : reviews and reports*, permettant de faire le point sur l'ensemble des méthodes envisagées actuellement pour répondre à la problématique de la régénération de tissu nerveux. Dans un second temps, ce rapport expose une nouvelle méthode de fabrication d'une structure qui permet de faire proliférer et de différencier des cellules souches neuronales en neurones moteurs. Cette méthode de fabrication a été soumise au journal *Biomaterials.* L'utilisation du co-électrofilage du PLLA et de la gélatine, couplé à différents degrés de réticulation, ont permis d'obtenir des fibres aux propriétés mécaniques et à la vitesse de dégradation modulables. Ces fibres ont permis de délivrer des signaux moléculaires pour obtenir la différenciation de cellules souches neuronales en neurones moteurs. L'intérêt de cette méthode est de permettre la fabrication d'une structure qui est implantable et qui, une fois implantée, permet par elle-même de promouvoir la prolifération et la différenciation de cellules souches neuronales en nerf moteur, sans avoir recours à de multiples chirurgies ou à de lourdes méthodes de livraison de facteurs de croissance dans le site blessé.

À l'avenir, il serait intéressant de tester l'applicabilité de cette méthode pour l'obtention de nerf *in vivo*. D'autre part, cette technique pourrait pouvoir permettre l'obtention de bien d'autres phénotypes, en changeant simplement les biomolécules utilisées en fonction du type de cellule que l'on souhaite remplacer chez le patient, capacité qui demande des travaux supplémentaires.

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ANNEXE 1 – Protocole Orange II

Préparation des solutions :

- Solution T_3 : pH = 3
  - T_3 : 20 µL de HCl pur (12M) dans 50 mL MilliQ
- Solution T_{12} : pH = 12
  - NaOH (1M) : 0,8 g dans 20 mL MilliQ
  - T_{12} : 2,5 mL de NaOH (1M) dans 50 mL
- Solution orange : 40 mM de orange II dans la solution T_3, i.e. 14 mg/mL

Protocole

- Adsorption : Les échantillons sont plongés dans la solutions de orange II (1 mL dans mon cas) pendant 30 min) à 40°C.
- Lavage : Les échantillons sont rincés trios fois avec la solution T_3 puis séchés à l'air pendant 10 min
- Désorption : Les échantillons colorés sont immergés dans 1 mL de la solution T_{12} pendant 15 min à température ambiante
- Mesure de l'absorbance à 483 nm
  - Les surnageants sont acidifies par l'ajout de 1% v/v de HCl pur (12M).
  - Calibration
    - Préparer une première solution à 1 mM : 50µL de la solution à 40mM de Orange II + 1,95 mL T_{12}
    - 1^{er} point : Dilution 1/10 : 1350µL T_{12} + 150µL 1 mM Orange + 15µL HCl pur
    - Points suivants : (5 ou 6) dilutions successives 1/3 : 1000 µL T_{12} + 10µL HCl pur + 500µL de la solution précédente
    - On obtient $\varepsilon \approx 23$ UA/mM.

ANNEXE 2 – Protocole Immunocoloration

Les échantillons sont rincés trois fois avec du PBS, puis fixés avec une solution de formaldéhyde 3.7% (1mL par échantillon) pendant 30 minutes. Après trois rinçages au PBS, les cellules sont perméabilisées avec 0.5 mL de Triton X100 (0.5%) pendant 20 minutes. Après trois lavages additionnels, les échantillons sont placés dans du sérum de singe (5%) pendant 1 heure.

La solution contenant les anticorps primaires est faite de 1% de sérum de singe, 0.1% Triton dans du PBS. 15 µL d'anticorps primaires à 200µg/mL sont dilués dans 1mL de la solution précédente. Les échantillons sont placés une nuit à 4 degrés dans 500µL de solution d'anticorps I^{res}.

Après 3 nouveaux rinçages au PBS, les échantillons sont placés dans une solution contenant les anticorps secondaires (1% de sérum de singe, 15µL d'anticorps à 200µg/mL) pendant une heure.

Après 3 rinçages additionnels, les échantillons sont placés entre deux lames de verre et imagés avec un microscope ZEISS, en lumière fluorescente.