Politecnico di Torino

Master's Degree in Biomedical Engineering

Bionanotechnologies

Development of electroconductive mats to direct neural cell growthand maturation

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A.Y. 2023/2024

Contents

ABSTRACT

Nowadays, the rapid advancement of manufacturing technologies and the high demand for new drugs and therapeutics make it increasingly essential to advance organ and tissue in vitro models and cell culture systems. Indeed, models such as animal testing or 2D cultures often poorly predict the effects of tested drugs in humans. The significant physiological differences between animals and simplified 2D models, lacking crucial in vivo microenvironmental features, have prompted the scientific community to demand more representative and precise models to obtain reliable results and expedite trial processes, particularly in their early stages, while also adhering to the principles of reducing laboratory animal testing according to the 3Rs (Replacement, Reduction, Refinement). Currently, advanced cell cultures and translational approaches such as microfluidics, organ-on-chip technology, and 3D scaffold-like models are at the forefront of in vitro modeling. They are increasingly necessary for better understanding the mechanics and biophysical behaviors exhibited by cells and tissues in vivo, although their application is still primarily

limited to toxicology and basic research. When focusing on neural tissues, it is imperative to strive to recreate the complex microenvironment that characterizes them and plays a central role in all dynamic cell interactions. For these reasons, a multi-stimulus integrated approach is required to attempt to emulate such a complex system. In this study, we attempted to develop electroconductive PCL-PANI mats with a specific aligned architecture and high biocompatibility to serve as a substrate for undifferentiated neural cells, specifically the NE-4C cell line, capable of differentiating into neurons and astrocytes. The main stimuli provided by the topography and electroconductivity of PANI have been shown to significantly influence NE-4C growth and maturation. The ultimate goal is to incorporate these culture substrates into a new prototype of bioreactor provided by IVTech, which will further integrate a 3D dynamic culture combined with exogenous electrical stimulation to evaluate the effects of these stimuli on the cells, particularly if they work synergistically. By incorporating physiologically relevant stimulation in a controlled environment, populations of neuro-derived stem cells seeded onto these substrates can differentiate and mature into heterogeneous and functional tissues in terms of morphology and marker expression. This marks the first step toward developing a family of next-generation in vitro models, consisting of fully functional, high-throughput platforms that serve as advanced tools for scientific research and drug discovery.

1. INTRODUCTION

1.1. Physiological Background

When we talk about neural tissue and central nervous system (CNS), we must consider the biological complexity of its microenvironment. There is a multitude of cellular phenotypes (figure.1), each with a specific role and a precise metabolic production. These cells have dynamic interconnections regulated by neurotransmitters and electrical phenomena, to which they are very sensible, and generate a structured network of whom dynamics, mechanisms and functionalities are still to be unravelled in detail. A distinction between the different neural cells must be done in order to be clear in the subsequent tractate. In the CNS the adult cells are divided in neurons and glial cells. Neurons are the basic structural and functional elements of the nervous system and consist of a cell body (soma) and its extensions (axons and dendrites). Dendrites transmit electrical signals to the neuron cell body and the axon conducts impulses away. Glial cells, or neuroglia, are support cells that aid the function of neurons and include astrocytes and oligodendrocytes (Silverthorn, U. (2010). Human Physiology), (Schmidt & Leach, 2003). These cells can be obtained from two different kind of stem cells: neural stem cells and progenitor cells. The differences between these two blocks consist in their capability of differentiation and their self-renewal, from them the great part of the cells composing the CNS are derived.

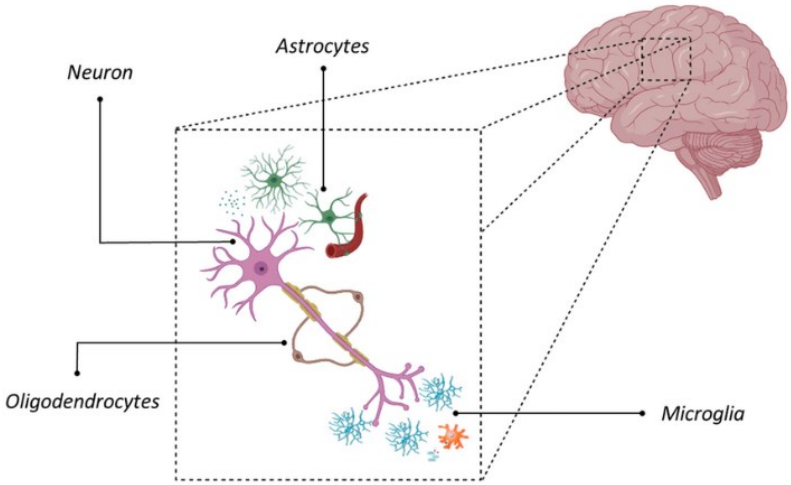

Figure 1. *CNS cellular composition.*

1.1.1 NEURAL TISSUE COMPLEXITY

Neural cells and progenitor cells can differentiate in different kind of cells such as neurons, ependymal cells, oligodendrocytes, and astrocytes. While neural stem cells (NSCs) are multipotent and have a high self-renewal capability, progenitor cells are meant to differentiate to a specific cell lineage, and they are often classified as precursor of a certain adult cell phenotype (Bouhon et al., 2005). It's been demonstrated in different works how NSCs both in adult brain and in the developing can generate mature cells comprehending all the neural lineages such as astrocytes, oligodendrocytes, and neurons with a ratio of 25:5:1 respectively (Chang et al., 2011). These kinds of cells are gaining always more importance in new therapeutic applications to treat neurodegenerative diseases like Parkinson and Alzheimer. The neurogenesis is the process throughout new neurons are generated from immature cells. There are two types of neurogenesis, embryonal development neurogenesis and adult neurogenesis. The latter is a process happening in the mature brain where, in specific region of it, neural and progenitor stem cells are deployed in the formation of new neural tissue. NSCs are located in the anterior sub-ventricular zone (SVZ) of the forebrain and hippocampus, they could be deployed in case of tissue injury, however only a little part of them is recruited and undergo the differentiation needed, for this reason brain tissue is well known to have poor regeneration ability (Bierman-duquette et al., 2022; Temple, 2001). The mature

brain has a large number of specialized neurons that differ in structure, connections, and functionalities, and they are all the results of regulated neurogenesis during embryonic development. Another relevant and parallel process is the gliogenesis for what concerns the glial cells (Qian et al., 2000). The fate of neural stem cells or progenitor cells is highly dependent from different mechanical, chemical and biophysical factors, which can guide the differentiation in a way or another (Götz & Huttner, 2005). Adult neural cells have an intimate connection with their microenvironment and with each other, in fact cell-cell contacts are at the basis of the development of CNS. Immature neural and progenitor cells (Figure 2.) interact with each other through cellular protein-based and adherent junction, and with the matrix surrounding them through receptors and neurotransmitters. The gap junctions between the cells formed by connexin, allow the transport of ions and molecules which have regulatory functions. All the stimuli generated in the brain have the power to up-regulate or sub-regulate the production of the neural tissue. This strict dependence is what we call neural network, and the behaviours and dynamics controlling it are yet to be fully understood. A better comprehension of the mechanisms of neurogenesis and gliogenesis could set new standards for engineering *in vitro* models of functional and mature neural tissue starting from staminal and progenitor neural cells. (Lowry Curley & Moore, 2017)

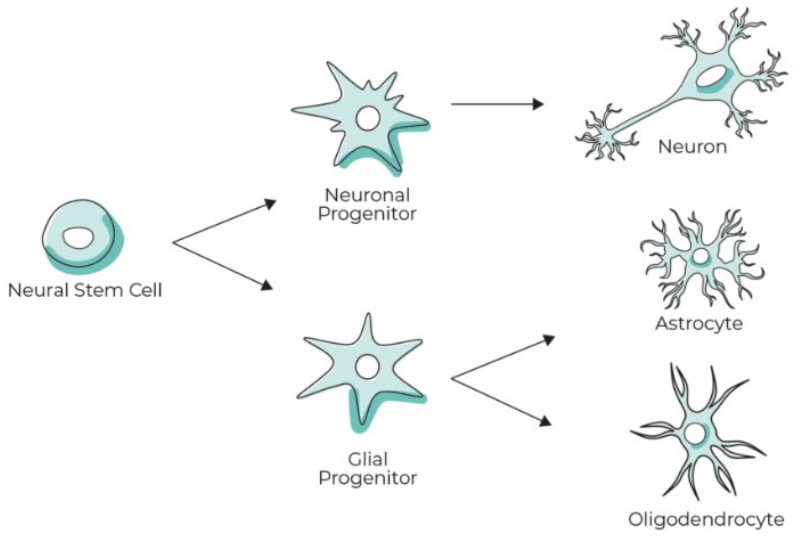

***Figure 2**. Neural Stem Cells differentiation line*

1.1.2. Electroresponsive Properties of Neural And Stem Cells

Neural tissue is considered to be an excitable tissue. It is capable of generating and propagating an electrical signal in response to a stimulus. Endogenous electrical property in neurons is mainly due to the plasticity of the cell membrane's permeability. As a response to chemical signals from cells, the opening of voltage-gated channels alters the permeability of Na+ and K+ ions. This change in ionic flux affects the membrane potential, leading to a sequence of electrical events known as graded potentials and action potentials (spikes). These electrical phenomena cause the propagation of electrical current along the axons (Silverthorn, U. (2010). Human Physiology). These properties are fundamental to have a physiological response fast and precise to external sensory stimuli, thought, language, sleep, and all the functions that trigger the biochemical signalling between cells. Everything in neural networks, from the cell morphology to the different phenotypes features is aimed to make these communications possible and every component of this system plays a key role in neural network dynamics. A clear link between the electrical cues in the neural microenvironment and the features of neural cells has been deeply discussed, the effect of exogenous electrical stimulation at different voltages and frequencies over neural cells has been tested *in vitro*, showing promising results in terms of differentiation, morphology, orientation, and marker expression, suggesting it as a main way to recreate a reliable model *in vitro* (Thrivikraman et al., 2018).

1.2. In Vitro Cells Cultures; Historical Analysis

The importance of new tools in the world of preclinical trials, toxicology, disease modelling and patient specific medicine is pressing. Achieving new standards in mimicking human micro-tissues capable of simulating functional, near-physiological unity, harnessing the knowledge acquired in tissue

engineering and cell cultures is a main topic in drug discovery research, with the aim of successfully addressing and overcoming the limitations related to static 2D cultures (Bédard et al., 2020). Further discoveries in biomaterials interface, microfluidic devices, and human stem cells clinical use, have driven the research to the developing of specific culture systems capable of supporting the self-organization of the tissue and all of the biological cues, providing an adequate substrate where to grow. This step was needed to overcome the issues of the systems currently deployed by industries and researchers, mainly based on 2D static cultures.

1.2.1.Importance Of *In Vitro* Cells Cultures System

In vitro cell cultures are essential in the world of research, we have already seen the complex phases through which the cells undergo *in vivo*: change of morphology, differentiation, production of metabolic, death. All of this happens constantly also *in vitro, and* cell cultures are the only instruments we dispose to better study and acknowledge the behaviour of cells in different circumstances. 2D cell cultures have been for the last decades the only reliable and predictable method to study cellular responses to biophysical and biochemical stimulations, allowing huge discoveries and achievements in different fields. Even if this method has been for years the gold standard and is, these days, well accepted and standardized, there are many aspects in which 2D static models cannot be considered a good model to mimic *in vivo* situations (Culture et al., 2022). It has now become clear how much the cell context matters in terms of biological activities, gene expression and spatial distribution. Hence, the translation to more complex culture system which includes active substrates, three-dimensional structures for the cells to grow in, different phenotypes co-culture and multi-stimuli arrangement, is now become

of primary relevance (Kelm et al., 2018) to better mirror what happens in living human organisms. Moreover, the development of reliable *in vitro* models is essential to reduce the time-cost ratio of preclinical trials involving animals, of which failure percentage is above 90% in the early stages (Schlander et al., 2021). Despite these new generation systems have proven to be more predictive and functional, their peculiar complexity brings with it new challenges such as tissue-tissue interface, mechanical microenvironment, interaction with biomaterials of different nature and spatiotemporal distribution of nutrient and wastes. A whole dimension of different signalling which affect the cells has been ignored for years for simplicity, but technology and knowledge are now mature to move onto the next stage. (Abbott, 2003)

1.2.2. Traditional And Advanced In Vitro Systems For Neural Cell Cultures

For what concerns neural cell cultures, the gap between a simple petri dish and a fully engineered neural tissue *in vitro*, is wide and full of different aspects to highlight. At the basis of a neural cells culture there is the necessity to study the behaviour of neural cells, investigating their principal traits under precise conditions. The main problem involving neural mature cell cultures lies in their sensitivity to the passing process, which requires the detachment of delicate cells from a substrate where they adhered, leading to the death of the majority of the cells or the loss of their function. This phenomenon has increased the use of primary neural cells still undifferentiated, capable of differentiating in various phenotypes and more resistant to the rough process they have to go through during the culture. On the other hand, less differentiated cells could go through an undesired differentiation which can negatively affect the validity of the cultures for what it was conceived (Bierman-Duquette et al., 2022). To solve this major problem neural cells, have to be guided into a correct and

specific differentiation that can include different phenotypes, and in order to accomplish that, the environment surrounding the cells is crucial. As we have seen *in vivo,* neural cells inside the niches are totally affected by their environment, the stimuli provided by the contact with extracellular matrix (ECM), the structure of the space they reside, the chemical and physical factors they perceive; all of these variables can impact their fate, controlling the kind of mature cells they would eventually become. It's clear how the development of new systems which allow the integration of different structural and exogenous stimuli could bring neural cells culture to another level of details and behaviour comprehension. The advancement could involve several factors not necessarily related or interdependent, one of those is certainly the culture substrate. It's been largely demonstrated how giving cells a 3-dimensional structure or a specific nanopattern where to grow and maturate can help them to develop a more natural morphology and indirectly interact with the differentiation. Another element often applied in the innovative culture system is the choice of active biomaterial. In the specific case of neural cells, which are extremely sensitive to the surrounding conditions, interfacing them with a conductive surface could orientate and support neural cell's growth (Saberi et al., 2019). If the target is getting closer to the *in vivo* conditions, the application of a dynamic flow to the culture cannot be neglected, as the continuous replacement of the culture media and the stress linked to the flow has an enormous impact on cell development. One more way to try to positively affect and control a culture system is to give exogenous physical and chemical stimulation. In the case of neural cells, electrical stimulation has been largely studied because of the nature and the electroactivity of these kinds of cells. Introducing or combining one or more of these upgrades to the neural cell cultures has shown outstanding results and deepen the knowledge behind these features along with the standardization of new protocols and the availability of these new technologies on larger scale will progressively lead to the replacement of traditional cultures wherever a closer mimicking is required (Fatehullah et al., 2016).

1.3. Scaffold-Based Systems For Neural Cells Maturation And Differentiation

When we talk about constructs that have to interact with biological elements, we must evaluate the characteristics and features of the native tissue we want to recapitulate. Engineering substrate for advanced cells culturing involves customizing their physicochemical (hydrophilicity, stiffness, electroconductivity), structural (topography, 3D-structure) and biological properties. In particular, Neural tissue has some peculiar properties that have to be considered in order to engineer a good construct. One of these unique characteristics is the ECM composition, extremely low in components such as fibronectin, laminin and collagen compared to the other main tissues. This composition translates into specific mechanical properties, in fact, the stiffness and low Young modulus directly affect the cell behaviour in terms of differentiation and morphology. *In vivo* neural cells tend to differentiate in glial cells in substrates with a higher stiffness and elastic modulus, on the contrary neuron, differentiation is favoured by lower stiffness substrates. Matrix porosity is another primary element that affects neural tissue fate *in vivo*. Neural cells have a high interconnection and metabolic interchange and, for this reason, a good substrate to mimic neural tissue *in vitro* should have an adequate pore size to guarantee optimal cell migration and metabolic efficiency. Moreover, neural cells have a strong response to the topological features, shape control and orientated pathway are crucial as *in vivo* as *in vitro* to regulate tissue morphology and functionality. During the projecting phase of a construct with the target of representing a good substrate to perfectly mimic neural tissue *in vitro* it's crucial to take into account all of these properties and try to apply the knowledge acquired in the design and fabrication strategies (Zhuang et al., 2018).

In order to obtain these characteristics *in vitro,* scaffold-based systems have been extensively explored. These substrates could be composed of synthetic or natural biomaterials, engineered to supply a 3-dimensional environment for the growth and differentiation of neural cells *in vitro.* These substrates provide a complex physiological structure rather than the bidimensional counterpart, which are not capable of reproducing the same conditions. These substrates are commonly called “scaffolds”. In the term scaffold are included a wide gamma of complex manufactures, made from different biomaterials such as natural polymers, hydrogels, animals derived extracellular matrix (ECM) and synthetic materials among which the most common poli(glicolide-co-ε-caprolactone) (PCL) and Poly(lactic-co-glicolyc acid) (PLGA). The application of these kinds of structures opens to several opportunities for the *in vitro* modelling of neurological diseases, supporting the development of new regenerative therapies and drug testing platforms. Thus, the processing of biocompatible materials with different fabrication techniques, is fundamental to replicate complex biological structures. Indeed, it has been shown that a well-designed microenvironment could promote cell proliferation, migration, ECM production and differentiation, giving a better reproduction of *in vivo* conditions which is the final aim of new generation cell cultures (Culture et al., 2022). Despite the progress regarding the technology and the use of the techniques, manufacturing a functional 3D scaffold remains a challenge. Indeed, the separate control of the principal elements such as biochemical properties, microporosity and matrix elasticity is a difficult goal to achieve. the most common techniques currently used are conventional methods like:

- Polymer phase separation;
- Porogen leaching;
- Solvent casting;
- Freeze drying;
- Electrospinning

and rapid prototyping methods such as:

- 3D printing;
- Stereolitography;

- Fused deposition modelling (FDM).

These methods allow to obtain prefabricated structures with pores, channels, and defined microstructures but unfortunately the conditions to create these scaffolds are often too harsh in terms of pressure and solvent used, hindering the inclusion of cells during the process. Hence, cells have to be seeded after the fabrication permitting an internalization of them only by diffusion and bringing to a poor scaffold cellularization (Eltom et al., 2019). To overcome these critical issues solutions like bioprinting, hydrogel-based systems and building blocks scaffold have been recently investigated. These techniques allow the use of biopolymer and less aggressive solvents, aiming to provide full control over porosity thanks to the rapid prototyping fabrication and mechanical properties through polymer crosslinking. Scaffolds made using these methods have easily tunable biochemically and mechanical properties, but on the other hand materials used does not permit a full control over micro-topography and, in particular the mesh size obtained with the fabrication tends to be too small for an optimal cellularization and a subsequent matrix production (Warren et al., 2021; Zhuang et al., 2018).

1.3.1. Nanofibrous Scaffold For Neural Cell Cultures And Differentiation

Topographical cues play a key role for cell culture substrates to accurately mimic the ECM and give cells a familiar environment where to grow in. It has been shown how cells tend to attach to and to organize around fibers with diameter smaller than their own. Nanoscaled surfaces with dimensions ranging from 20 to 50 nm enhance neural cell adhesion and their protein expression activity. The nanoscaled surface also affects the conformation of adsorbed protein such as vitronectin and the dimension of cell membrane receptors (Mo et al., 2018). The utilization of microscaled polymeric nonwoven mesh in tissue engineering has gained significant popularity due to its extensive surface area

and substantial porosity. Neural tissue is characterized by multiple kinds of anisotropic structure with clear patterns, orientation, and gradients, therefore substrate designed with ordered nanofibers can be a significant tool to correctly guide cells migration and proliferation. The advent of electrospinning technology, enabling the straightforward production of nanoscaled ordered polymeric fibers, has revitalized enthusiasm in the field of biomaterials and neural tissue engineering. (Agarwal et al., 2008; Ma et al., 2005).

1.3.1.1 Electrospinning For Scaffold Fabrication

Electrospinning is an inexpensive and simple polymer-processing technique which has gained a relevant interest in biomedical and scaffold fabrication field due to its versatility to be used with a wide selection of natural and synthetic polymers (Figure 3.). This method consists in the employment of a high voltage electric field to draw or melt a polymer filament from a nozzle to a collector, in a temperature and humidity-controlled environment.

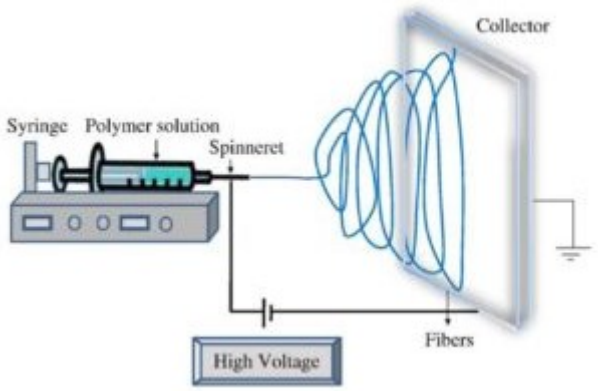

***Figure 3**. Electrospinning process graphic scheme*

The result is a fibrous mat whose properties can be easily guided with a proper choice of the materials used and processing parameters set such as electric field intensity, nozzle-collector distance, flow rate, etc. The sheets obtained range from 2 to 3-dimensional membranes (thickness can vary with processing time), with a microscale topography and a nanoscale fiber diameter. Due to their micro-architecture the nanofibers obtained have enhanced mechanical and stress withstanding properties compared to the bulk material. Different collector and parameter set up allow to prepare random or aligned nanofibrous

mats, with all the benefits derived from that for specific applications. Another meaningful property of electrospinning nanofibers is their remarkable porosity and the subsequent high surface-to-volume ratio, which increase the permeability and the diffusion capacity of nutrients, metabolites and gases inside the structure, all features strongly required in cells interactive application and in tissue engineering (TE). Electrospinning method has consolidated protocols to work with commonly used TE synthetic polymers like poly(ε-caprolactone) (PCL) or poly(lactic-co-glycolic acid) (PLGA), water soluble materials like Poly ethylene oxide (PEO) or Poly vinyl alcohol (PVA), and also a wide range of natural biopolymers like collagen, elastin or silk protein. This technique lends itself to the use with different polymer combinations but the choice of the right solvent which can fit with the polymer chosen and the processing parameters remains the major obstacle to the several possible mix that would lead to new applications. An example of solvent that well fits with synthetic polymer and protein-based solution is 1,1,1,3,3,3-hexafluoro-2-propanol (HFP), which is only a demonstration of the potentiality of this technique. Another important tool of the electrospinning technique is the possibility to produce ordered aligned fibres. There are several methods already standardized but the most common is certainly the use of a rotating collector. A rotating drum collector at a minimum speed which is strongly dependent on the polymer solution used, allows the fabrication of aligned nanofibers at different degrees of orientation. This is determinant for those applications that want to mimic oriented biological structure. (Jiang et al., 2015; Zabielski et al., 2021)

1.3.1.2 Substrate Morphology And Alignment Effect Over Neural Cells Growth

In engineering a substrate that fits and improves a neural cell culture *in vitro*, the architecture is a crucial aspect to care about, in fact it will be decisive for the cellular fate in terms of adhesion, spreading, elongation, and differentiation of stem cells into a specific phenotype. Electrospun fibres have proven to be a good candidate for NSCs cultures, in particular the aligned nanofibrous mats obtainable with this technique have shown over the past years a strong impact

in promoting the NSCs alignment and neurite extension along the direction of the fibres as shown in several studies with different cell sources and polymers used by (Lim et al., 2010; Mahairaki et al., 2011; Yang et al., 2005). Surely there are still poor quantitative studies of the direct effect of alignment over cells function, but it's clear at this point how an aligned substrate affects the morphology and the disposition of them. The impact of an oriented substrate becomes even more evident when functionalized with specific cellular adhesion motives such as LN and RGD. The synergistic action of the aligned fibres and these sequences has been well reported by (Amores de Sousa et al., 2020) in a recent work, where different settings of PCL fibres have been tested to verify the impact of alignment, functionalization, and their synergistic effect. The main driving force of this cellular response to topographical cues like alignment is supposed to be the cell sensitivity to the substrate. Cells are, in fact, capable of recognizing the biomechanical characteristic of the below substrate, translating this information into a biochemical signal to the nucleus which leads to a cytoskeleton remodelling and a subsequent change of morphology of the cell itself. This substrate-induced signalling is still to be clarified especially for what concerns the cytoskeleton-associated molecules responsible for the translation of the biomechanical signal to an intracellular signal. To sum up, the interrogative on how we can control NSCs morphology and differentiation through the topography of the substrate and what kind of impact this can have on neural cell cultures brings renewed interest in these applications. (Lim et al., 2010)

1.3.2. Electroconductive Substrate For Neural Cell Cultures

Due to the natural behaviour of neural tissue to accumulate and transmit electrical signals under the form of potentials generated at the synapses, another key feature to manufact a good neural tissue scaffold is represented by

its electroconductivity. An electrically conductive substrate has been showed to help the neurite growth and promote neural cell proliferation in culture. Exogen electrical stimulation is widely used to enhance NSCs differentiation but in order to be effective it needs an adequate substrate that consents to transfer the electrical cue homogeneously in the tissue and affect positively the expansion and differentiation of the culture. Moreover, electroconductive scaffolds have a role in the cell alignment and neurite formation, they also increase the expression of nerve growth factor (NGF) in neural cells. In the field of conductive substrate, the focus is high on the promising conductive polymers (CPs); these polymers have some interesting characteristics, as they combine optical and conductive properties of inorganic materials like metals and semiconductors with the elevated processability of synthetic polymers. Their conductivity is mainly due to the conjugated double bonds in their molecular structure. Despite the really low conductivity of CPs, the interest in them has exponentially increased thanks to the possibility of doping them with specific salts or by using a redox doping process which strongly enhances the electric charge mobility within the polymer chains. The mix of peculiar physical, chemical, and electrical properties make this class of polymer fascinating for many biomedical and TE applications. To directly stimulate neural cells in a substrate electrically, electroconductive materials are of primary relevance to have a more localized and precise stimulation of the cells adhered and possibly potentiate neural differentiation. A fundamental requirement for an electroconductive materials used in this kind of application is to have minimum electroconductivity higher than the culture media one. This feature is essential to permit an adequate electron flow through the material with no interference by the medium polarization, and to have good biocompatibility. Here are some examples of the main CPs used for biomedical applications:

- poly(3,4-ethylenedioxythiophene) (PEDOT)
- poly(pyrrole) (PPY)

- poly(aniline) (PANI)
- polybenzimidazole (PBI)
- poly (acrylonitrile) (PAN)

Although, the main obstacles for CPs large usage and application are the problematics related to processing, in particular if the aim is to obtain specific 3D or nanostructured architecture. To overcome this barrier, a new road has been taken blending CPs with easier processable synthetic polymers. Obviously, the enhanced processability is obtained at the expense of a higher conductivity but in the optic of fabricating a scaffold capable of satisfying all the project requests, a compromise is needed. (Garrudo et al., 2021; Kaur et al., 2015; Licciardello et al., 2021; Tomaskovic-Crook et al., 2019, 2020; Zhang et al., 2018)

1.3.2.1 PCL-PANI Tuning And Applications

One of the most encouraging polymer blends in the field of CPs, surely is the polycaprolactone (PCL) – polyaniline:camphorsulfonic acid (PANI:CSA), in particular in the form of electrospun nanofibers. In this blend, the PCL confers mechanical properties and processability, it is in fact a common polymer for the electrospinning technique while PANI, opportunely doped with CSA, provide a high electroconductivity. Optimizing the properties and the process parameters could allow the fabrication of a substrate with many characteristics useful for NSCs culture and suitable for the application of an external electric field to further enhance the results wanted in the neural tissue culture.

1.3.3. Brief Summary Of Electrical Stimulation In Vitro

For many years, researchers have investigated the use of electrical stimulation (ES) to manipulate the central nervous system (CNS). Exogenous ES involves artificially inducing electrical charge to cells to stimulate action potentials (APs). This approach, which can be applied both *in vivo* and *in vitro*, offers flexibility, avoids the use of chemicals, and is feasible. Remarkably, mild electrical stimulation has a profound impact on embryonic stem cells, prompting them to adopt a neuronal fate. While these neuronal cells did not exhibit specific terminal differentiation in laboratory culture conditions, they demonstrated the potential to differentiate into various types of neurons when transplanted *in vivo*, particularly in adult mice. Researchers are currently investigating the use of *in vitro* ES to manipulate various cellular functions, such as regenerative potential, migration, and stem cell fate. It is well-established that endogenous electrical signals play a vital role in processes like development, morphogenesis, cellular communication networks, and neuronal and glial cellular signalling. ES has demonstrated promising results in modulating the morphology of neural cells and influencing the fate of neural stem cells (NSCs) (Yamada et al., 2007). Notably, ES leads to a significant increase in the proliferation and differentiation of fetal NSCs into neuronal cells, facilitates guided cell migration and integration, and promotes differentiation of human NSCs and embryonic stem cells. These biophysical changes are initiated at the cell surface, affecting the functions of membrane proteins, including enzyme activity, membrane receptor complexes, and ion-transporting channels, by modifying the distribution of electric charge. Various in vitro studies have highlighted the role of exogenous electromagnetic fields in regulating several cellular events, such as accumulation of cell-surface receptors, reorganization of the cytoskeleton, changes in cell shape, preferential alignment, alterations in intracellular calcium ion levels, and activation of transmembrane channels. Exposure to electric fields also plays a crucial role in eliciting an appropriate response from stem cells. The possible mechanisms by which electric stimulation affects cell differentiation involve the alteration of membrane potential through hyperpolarization and depolarization, modification of ion

channels including receptor density and distribution, activation of calcium channels, and upregulation of the ERK pathway. These applications are limited by the difficulties on well controlling all the parameters and factors in play. The materials used along with the heterogeneous cell's population have slow down the mechanistic and deep understanding of how to modulate and support the cells during the treatment in order to obtain a precise therapeutic target (Bertucci et al., 2019; Zhu et al., 2019). The implementation of an electric field (EF) necessitates a driving force, and in vitro settings have employed various methods to deliver EFs. The conduction of electrical signals occurs both in vivo and in vitro due to the relatively high conductivity of extracellular fluid in vivo (ranging from 3 to 12 mS/cm) and culture media (15 mS/cm). Traditionally, applying an EF to cell cultures has involved transferring charges between a pair of conductive electrodes and the cell media or electrolyte solution. To comprehend how electric potentials are transmitted through the extracellular space and design an electrical bioreactor, it is crucial to first grasp the interface between the electrode and the electrolyte solution. The transfer of electrical charges is facilitated by electrons originating from the power source, passing through the control circuit, and reaching the electrodes at the cell media interface. At this interface, the transduction of charge transitions from electrons to ions within the media. Depending on the electrode material and the stimulation parameters (such as strength and duration), these reactions can be reversible and pose minimal harm to cultured cells, or they can lead to cytotoxic electrode dissolution, water splitting, and changes in pH. In neural tissues, ionic gradients are necessary for propagating waves along an axon, and the significance of oscillating ionic signalling is gaining recognition in both neurons and supportive cells like glia (Chang et al., 2011; Thrivikraman et al., 2014). Direct current (DC) refers to a constant current flow without changes in magnitude or direction over time, whereas alternating current (AC) involves periodic changes in magnitude and direction. Pulsed current (PC) entails a unidirectional or bidirectional current flow for a brief duration. In vitro experiments have replicated continuous DC fields to induce cell migration. The primary method of applying an electric field (EF) to cell cultures involves direct coupling, where the electrode contacts the culture media. Meanwhile, for in vitro

studies, pulsed EFs are commonly used, typically in a monophasic pattern. Pulsed stimulation, which is more physiologically relevant to the adult nervous system, encompasses a wide range of frequencies. In order to stimulate cells in vitro, pulsed electrical stimulation is administered through a pair of electrodes functioning as a common cathode/anode, employing various patterns and waveforms. Typically, square waves with a small duty ratio are utilized for EF application. These patterns are typically characterized by their frequency (measured in Hz) and pulse width (measured in ms). For what concerns the materials used, metals have been commonly employed for the application of electric fields (EFs) *in vitro*, primarily because of their high charge injection limits. However, the specific mode of charge transfer is heavily influenced by the type of metal used. Noble and precious metals such as silver, gold, platinum, iridium, and palladium are excellent choices for stimulating electrodes, thanks to their remarkable corrosion resistance properties (Bertucci et al., 2019; Thrivikraman et al., 2014; Zhu et al., 2019). In Table.1 are briefly summarized the main methods of cell's electrical stimulation.

Electrical field methods	Cell model	Electrodes	Stimulation parameters	Stimulation duration	Main results of study	Potential mechanism	Ref.
DCEFs	Rat NSC	Agar–salt bridges filled with Steinberg's solution gelled with Ag/AgCl electrodes	For migration analysis, 250 mV/mm. For EF polarity reversal 500 mV/mm	Lasted for 145 min or 3 h. Image was obtained every 5 min	Enhance NSC migration toward the cathode	LY294002 and PI3K inhibitors regulate the orientation and parameters related to changes in parameters related to changes in direction, respectively	(Meng et al., 2011)
PCEFs	Human NCSCs	Top electrodes (99.999%) had pure Au wires cultured on electrode plate had a thin layer of PDMS.	Potential of 200 mV/mm potential and duration of potential and duration of 100 μs.	1, 3, or 8 h of pulsatile electrostimulations with 2, 20, and 100 Hz.	Enhance neuronal differentiation and survival		(Du et al., 2018)
Bifasic ES	Mouse NSCs	Biphasic current stimulator chip with ITO electrodes.	100 Hz with the magnitudes of 4, 8, 16, and 32 mÅ/cm2 with the pulses of 50 and 200 ms in a continuous manner.	1,2, 3, 4, and 7 days.	Promote proliferation and neuronal differentiation.	HSP-mediated differentiation	(Chang et al., 2011)
ACEFs	Mouse NSCs	The Ni-coated wire electrodes Were 8.5 cm apart from each other Other at the sides of the cell and 6 cm apart across the cell.	0.1, 0.5, 1, and 10 Hz; field Amplitudes of 2, 4, and 16 V/m.	0, 1, 7, 14, and 21 days	Neural stem cell viability increases, while neuronal differentiation remains unchanged		(Matos & Cicerone, 2010)

Table.1 summary of electrical stimulations methods (Zhu et al., 2019)

1.3.4. Dynamic In Vitro Culture of Engineered Neural Tissue

The introduction of dynamic three-dimensional cell cultures has revolutionized the field of biological research by bridging the gap between in vitro and in vivo systems. There are two types of 3D cell-culture techniques: static and dynamic culture systems. Static 3D cultures fail to accurately replicate the cellular architecture and function found in living organisms. However, the development of dynamic culture systems, such as microfluidic devices and bioreactors, has provided 3D culture models with the potential to enhance the naturalness of cultured cells and thereby increase their relevance for translational research. These systems facilitate the remodelling of the extracellular matrix (ECM) and enable improved intracellular interactions between cells and the ECM, as well as between cells themselves. As a result, cells are able to attach, proliferate, and differentiate more accurately *in vitro*. With enhanced nutrient supply and other relevant physiochemical cues, dynamic cell-culture platforms provide optimal growth conditions, greater heterogeneity, and improved cell-to-cell communication, allowing cells to faithfully mimic the tissue microenvironment (Aishwarya et al., n.d.). In particular, neural differentiation is really sensitive to a dynamic flow, and it's already been demonstrated how a dynamic neural culture brings a major expression of all the principal protein markers of differentiated mature cells. The hydrodynamic effect of a non-static culture carries some benefits also for neurite outgrowth and morphology of the cells within the culture's substrate. The effect of dynamic flux over neural cells cultures could be further enhanced by combining it with other physical stimuli like an exogenous electrical stimulation as shown in their work by (Grossemy et al., 2021), and the validation of new systems allows dynamic cultures and multi-physical stimuli is everyday more concrete and intriguing.

1.4. Thesis Goal And Workflow

In the subsequent tractate, the development of a smart electroconductive nanofibrous scaffold for *in vitro* neural cell cultures will be described to evaluate the synergistic effect of the engineered nanostructured topography and the functional electroactive material over neural cells in terms of morphology, migration, differentiation, and maturation. Furthermore, all the parameters and the setting will be configured for the establishment of a dynamic neural cell culture within a multi-stimuli bioreactor which integrates electrical stimulation and dynamic flow.

Workflow:

1. Fabrication and surface modification of electrospun nanofibrous mats
2. Morphological and mechanical characterization of the membranes
3. Biological characterization of the membranes
4. Setting preparation for culture in bioreactor

2. MATERIALS AND METHODS

2.1. Fabrication of PCL-PANI membranes

The selection of a proper biomaterial was crucial in order to obtain electroconductive membranes capable of supporting cell's growth and supplying

the correct topographical and physiochemical stimuli. Polymer capable of conjugating electroconductive properties, mechanical resistance and thermal stability and again being easy to process through electrospinning was needed. A good compromise has been found in a PCL-PANI solution. Polyaniline (PANI) is a conductive polymer widely used in biomedical applications due to its physicochemical properties and it can be associated in three different forms depending on its oxidation level:

1. Leucoemeraldine (fully reduced)
2. Pernigraniline (fully oxidized)
3. Emeraldine base (semi-oxidized)

The most used form remains the emeraldine base due to its stability in the air. To fully exploit the electroconductivity of PANI emeraldine base it is doped with protonic acids. However, PANI alone is difficult to process and for this reason, blending it with a more common biodegradable polymer is necessary to overcome the processability limit for conventional manufacturing techniques. The most promising material solution which have recently brought to promising results is the PCL-PANI solution which conjugates the characteristic of a conductive polymer (CP) such as PANI and the flexibility and processability of PCL. For instance, this solution has been used to prepare aligned conductive micro-structured fibres for skeletal muscle TE application. PCL-PANI has all the features to be manufactured into electroconductive membranes, but its hydrophobicity makes it inadequate to support a cell culture. To overcome this limit and allow its usage as a neural cell substrate, a plasma argon treatment has been executed on the membranes according to the results obtained by (Licciardello et al., 2021).

2.1.1. PCL-PANI Solution preparation

The solution was prepared by dispersing 19mg of camphor sulphonic acid (CSA, 99% purity, Sigma Aldrich) and 15 mg of PANI emeraldine base (MW= 100 kDa, Sigma Aldrich) in 3 mL of 1,1,1,3,3,3-Hexafluoroisopropanol-d2 (HFP, 99%

purity, Carlo Erba Reagents, Milan, Italy). CSA is a protonic acid commonly used to drug PANI and enhance its electroconductivity. The selected PANI: CSA ratio has been chosen according to previous works (Licciardello et al., 2021)(Qwazy et al.). The solution was sonicated for 4 minutes at 20kHz and then it was stirred for 4 hours at 300rpm. Consequently, 500mg of PCL (80kDa, Sigma Aldrich) was dispersed in 2 ml of HFP (25% W/V). The two solutions were then blended, obtaining a viscous final solution (10% W/V) which was left under a magnetic stirrer overnight.

2.1.2. Electrospinning set-up and process

The membranes were fabricated with a solution electrospinning instrument, NovaSpider v5 (NovaSpider, San Sebastian, Spain). To obtain both random (used as control) and aligned nanofibrous mats, two configurations were used:

- horizontal configuration using a flat collector to fabricate random membranes.
- horizontal configuration using a rotating drum collector to fabricate aligned membranes.

The solution was loaded in a glass syringe equipped with luer lock, lately charged in the utensil support, and run by a volumetric pump operating a flow of 1 ml/h, at a constant voltage of 20 keV and a distance of 15 cm between the needle and the collector. For what concern the aligned membranes two sets of membranes was fabricated using the same parameters with different rotating speed. The first set of membranes was produced at three rotating speeds: 800 rpm, 1600 rpm, and 3200 rpm. The second set was produced at a tighter range at high rotating speeds: 2800 rpm, 3000 rpm 3200 rpm and 3500 rpm. (Figure 4.)

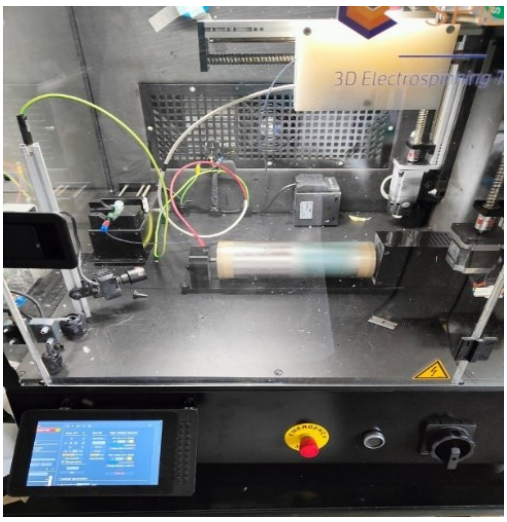

Figure 4. *Electrospinning process using rotating collector*

2.1.3. Plasma treatment of PCL-PANI membranes

In order to enhance the wettability of the membranes obtained, which are strongly hydrophobic due to the nature of the polymers used and to the architecture of the fibres, a surface cold atmospheric plasma treatment was performed on the PCL-PANI membranes. The instrument used is a Stylus Plasma Noble (Nadir S.r.l, Veneto, Italy). The Plasma treatment was deployed both on aligned and random membranes that following have been used as substrates.

5 samples of aligned membranes and 5 samples of random membranes were subdued to plasma treatment following a specific protocol. The samples were laid in a flat support (transwell) which left two sides exposed and treated with a plasma/argon flow for 30 seconds on each side. Cold atmospheric plasma is generated by a high voltage discharge that induces the ionization of argon near the outlet of the gas flow. The treatment was performed by setting the argon flux to 7.5 slm, a high voltage (HV) tension of 10 kVpp and a radiofrequency (RF) power of

9W. Plasma was generated in the stylus that was then manually moved onto the surface of samples.

2.2. Characterization of PCL-PANI membranes

In order to evaluate the properties and features of the fabricated membranes, different tests and analyses have been carried out. The membranes have been characterized in terms of:

- Fibres alignment
- Mechanical properties
- Surface Wettability
- Conductivity

the methods of the test are described in the next chapter.

2.2.1 Alignment analysis

The alignment degree of PCL-PANI membranes has been evaluated qualitatively through image analysis obtained by scanning electron microscopy (SEM) and quantitatively by performing some relevant statistical analysis using ImageJ software and specific distribution and orientation analysis tools.

2.2.1.1 Scanning Electron Microscopy

The PCL-PANI membranes were cut into small samples of 1 cm^2 and attached to the holders. They successively underwent a gold-plating process which prepared the samples for the SEM.

2.2.1.2 ImageJ analysis

Using ImageJ software (National Institutes of Health, Bethesda, MD, USA) with the directionality tool, alignment parameters such as main orientation, distribution, and dispersion were evaluated. This plugin is used to infer the preferred orientation of structures present in the input image. It computes a histogram indicating the amount of structures in a given direction. Images with completely isotropic content are expected to give a flat histogram, whereas images in which there is a preferred

orientation are expected to give a histogram with a peak at that orientation. To highlight the differences images at 800, 1600 and 3200 RPM were considered and processed.

2.2.2 Mechanical characterization

To assess the mechanical properties of the membranes and highlight the difference between random and aligned mats, mechanical tests were carried out. Uniaxial tensile test was performed using MTS QTest™/10 uniaxial tensile apparatus equipped with a 10 N load cell (Figure 5.). The samples were firstly cut into bone-dog shapes and six samples for each membrane's kind were prepared for the test. Before every test, the thickness of each sample was measured. The speed used to execute the test was 1 mm/min. Three main parameters that describe the mechanical behaviour of the nanofibrous mats have been chosen:

- Young modulus (E)
- Ultimate tensile strength (UTS)
- Strain at failure (ε%)

the equations used to obtain the parameters are:

$$E = \frac{\sigma}{\varepsilon} \qquad (1)$$

With σ representing the stress and ε representing the strain.

$$\sigma = \frac{load}{area} \qquad (2)$$

$$\varepsilon = \frac{l_f - l_0}{l_0} \qquad (3)$$

***Figure 5**. MTS set up and samples preparation.*

2.2.3 Surface Wettability Analysis

The wettability of plasma-treated and untreated PCL and PCL-PANI samples was measured using the Krüss Drop Shape Analyzer apparatus. Right after the process, a first time point (t0) was taken, and a first measurement of the average contact angle took place calculated for random and aligned samples; all the measurements were repeated with the same number of non-treated samples of the same category. The measurements were carried out at different time steps: 2h, 4h, 8h, 24h. This experiment had the purpose of evaluating the effect and the persistency of the plasma treatment over time.(Aijaz et al., 2019)

2.3. *In vitro* test on conductive nanofiber membranes

The aim of this work is to develop a substrate for neural cell cultures, by guaranteeing all the features needed and resembling neural physiological

environment. To assess this point, *in vitro* cellular tests have been carried out, starting from a neural progenitor stem cell line NE-4C, which undergoes complete differentiation into neurons and astrocytes. The protocols followed for cell expansion and seeding will be described in detail in the following paragraphs.

2.3.1 NE-4C expansion and seeding

The first step consists in an expansion process of the cell line. The culture medium used has the following composition:

- 89 % V/V of EMEM Eagle's Minimum Essential Medium)
- 10 % V/V of fetal bovine serum (FBS)
- 1% V/V of L-Glutamine solution

NE-4C cells were cultured and expanded to confluence in flask, they were later detached using trypsin and visually counted using a cell counter. Once understood the approximative number of cells in the flask, a quote of the solution containing the right number of cells needed for the seeding was withdrawn from the total and then diluted with fresh culture medium to obtain a certain cell density in a total volume. The volume is established ad hoc for the seeding basing on the number of wells required by the experiment. To have a perfectly flat surface to seed, the nanofibrous membranes have been fixed on transwell inserts. The membranes went through a process of preconditioning with the same culture media before being seeded. NE-4C cells were seeded at a density of 20000 cells / cm^2 on random and aligned membranes. Cells seeded on tissue culture plastic were used as controls.

2.3.2 NE-4C proliferation on nanofibrous membranes

The aim of this experiment is to understand the differences in NE-4C viability when cultured on random and aligned fibrous membranes. Moreover, will be clarified also the effect of the plasma treatment over cell attachment and proliferation on the substrates. After seeding the membranes at what, from now on, will be called t0, cell proliferation was evaluated through CellTiter-Blue viability assay. The time points set for this phase were chosen at 24h and 72h. Cell morphology was also

evaluated by performing the fluorescent staining of cell nuclei and cytoskeletons at the same time points.

2.3.2.1 Cell titer blu assay

This test provides a homogeneous method for monitoring cell viability exploiting the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Nonviable cells, that have lost their metabolic capacity, do not generate a fluorescent signal. Culture medium was removed from the samples and a solution of CellTiter Blue reagent (Promega) and EMEM, in a ratio of 1:6, was added to each sample. After incubating for 1 hour at 37°C, 100 µL of each sample were withdrawn and the fluorescence values were detected by using the microplate reader with a 530 nm excitation / 590 nm emission filter set.

2.3.2.2 DAPI/PHALLOIDIN staining

After 7 days of culture, samples were fixed using a 4% Paraformaldehyde (PAF) solution in PBS. To enhance cell membrane permeability, 0.5% v/v Triton X-100 in PBS was added. Following a 10-minute PBS wash, samples were pre-incubated with 1% w/v Bovine Serum Albumin (BSA, from Sigma-Aldrich) in PBS on a rocking platform shaker for 30 minutes, followed by another 10-minute PBS wash. Phalloidin, dissolved in 1% w/v BSA in PBS at a ratio of 1:60, was used for a 40-minute incubation to label the cell cytoskeleton, followed by another 10-minute PBS wash. For cell nucleus labelling, samples were incubated in a diluted solution of DAPI (4,6-Diamidine-2-phenylindole dihydrochloride) in PBS for 10 minutes. To prepare the diluted DAPI solution, initially, 2.1 µL of DAPI stock solution was dissolved in 100 µL of PBS, and then this solution was further diluted at a ratio of 1:1000 in 2 mL of PBS. The confocal microscope was used to observe the structure of NE-4C cells after the staining.

2.3.3 NE-4C Differentiation on nanofibrous membranes

The second phase of the *in vitro* test was the differentiation of the neural cells cultured on both aligned and random nanofibrous membranes. NE-4C is a line

known to differentiate into two different neural cell phenotypes, neurons, and astrocytes, under the right culture conditions. To obtain the results desired, a strict protocol has been followed. The cells have been newly seeded and cultured as described in paragraph 2.3.2 but in a different culture medium, with the addition of a differentiation agent. The time points chosen to fix the samples and check the differentiation state were 7 days and 14 days. To verify the morphology of the differentiated cultures and the expression of protein markers typical of the two differentiated phenotypes, two assays have been performed, DAPI/PHALLOIDIN staining and immunostaining, whose protocols will be described in detail following.

2.3.3.1 NE-4C differentiation

The process of differentiation implies the use of two different culture media, one for the first 4 days of culture and one for the maintaining in the remaining lapse of time.

Differentiation medium:

- 89% V/V of EMEM
- 10 % V/V of FBS
- 1% V/V of L-Glutamine solution
- 20 μM of retinoic acid (RA)

The medium has to be refreshed every 2 days.

Maintaining medium:

- 98% V/V of EMEM
- 1% V/V of FBS
- 1% V/V of L-glutamine

2.3.3.2 Immunostaining protocol

To demonstrate the differentiation of the seeded cells into the different phenotypes desired, a protocol of immunostaining has been carried out. The aim was to visualize the presence of specific proteins or RNA (antigens) expression in

fluorescence, marking the cells with a sequence of primary antibodies that bond and retrieve the antigen, and secondary antibodies that bond with the primary and are marked with a fluorescent staining element thus allowing their visualization. In this specific case, the protein expressions of MAP2 (microtubule-associated protein) related to the presence of differentiated neurons, and GFAP (glial fibrillary acidic protein), a protein fundamental element of astrocyte filaments, were evaluated.

The immunostaining protocol was executed to the culture fixed at 7 and 14 days to understand the differentiation evolution. To proceed the PBS was removed and a solution of TRITON-X at 0.2% v/v in PBS was added to the culture, this step is to permeabilize the cell membrane. The solution was incubated for 10 minutes. The solution was then removed and substituted with another solution of BSA at 2% w/v in PBS and incubated for 1 hour. The next step was the incubation of the samples in the solution of primary antibodies in BSA at 1% w/v and tween 20, a tension-active agent, at 0.1% v/v. The solution was incubated overnight at 4°C. Then the solution was rinsed and samples were incubated with secondary antibodies in the same composition solution for 1h at room temperature. The samples were finally rinsed in PBS. The immunofluorescence imaging was performed by using the confocal microscope.

2.4 Bioreactor system set-up and preparation

The following phase consists in the set-up of a bioreactor system to further implement a dynamic culture system. The bioreactor was supplied by IVTech s.r.l and it consists of a new developed prototype of their LB1 (live box 1) implemented with an external station which generates an electrical stimulation.

2.4.1 PCL Ring Fabrication

During this work, the main target was to make possible the positioning of the membranes inside the culturing chamber of the bioreactor. To accomplish this task,

a custom-made PCL ring has been designed and fabricated ad hoc to guarantee the stability and the fixation of the membrane on site once under flow condition, and to avoid leakage of medium which can enter in direct contact with the electrical sensor.

The steps to obtain the PCL ring were:

1. The ring was designed with a 3D CAD software, with an inner diameter of 14,5 mm and a square section of 1 mm.
2. The 3D model was then printed with a Rokit Healthcare IN VIVO printer (Figure 6.), entirely in PCL.
3. The ring was tested to fit in the chamber and to keep the membrane in its housing as prevented.

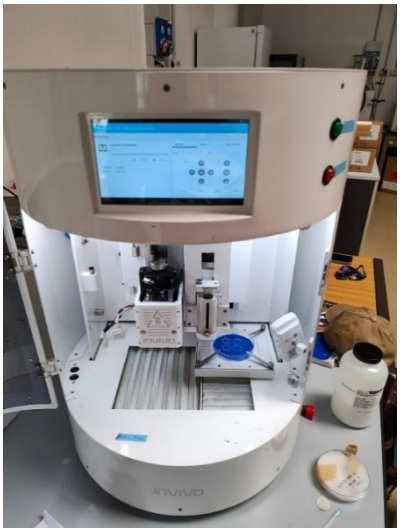

***Figure 6**. Rokit Healthcare IN VIVO printer*

2.4.2 Indirect Cytotoxicity Test For Electrical Sensors

To assess if the sensor that was going to be used as actuator of the electrical field could have a potential toxic effect over the cell culture, an indirect cytotoxicity test

was executed to assess the absence of cytotoxic substances release. The support was immersed in the culture medium for 24h and the medium was then used to culture NE-4 cells. The cell viability was evaluated at 24h after treatment with CellTiter blue assay as already described. The results were compared with a control treated with fresh medium.

2.4.3 Electrical parameter set-up

Even though the electrical stimulation phase has not been reached in this work, a robust work of literature research and data collection regarding all the main electrical parameters for *in vitro* electrical stimulation has been carried out, discussing, and comparing the actual state of art with the possibilities of our accommodation and establishing a consistent protocol of potential testing that could be carried forward in future works.

3. RESULTS AND DISCUSSION

3.1. PCL/PANI membrane characterization

The primary aim of this thesis work was to develop a functional substrate suitable for interacting with neural stem cells and capable of promoting their growth. We focused on two key factors:

- Architecture and topography of the substrate: We aimed to create a nanostructured and precisely patterned scaffold, which could serve as an ECM-like interface to guide and orient cellular growth and interconnections.
- Electroconductivity of the material: Previous research has demonstrated that neural cells respond to electrical stimuli. To achieve this, we chose a PCL-PANI blend, a polymer combination known for its electroconductivity and mechanical properties. This blend is also suitable for various fabrication methods, and we opted for electrospinning to produce aligned fibrous membranes.

By integrating these features, we aimed to develop a substrate that would effectively support NE-4C cell growth and enhance their functionality.

3.1.1. Membranes alignment and directionality

After fabricating the membranes as described in the second chapter, we proceeded to evaluate their alignment and directionality. Our objective was to validate the electrospinning protocol by demonstrating the impact of rotation speed, expressed in rpm, on fiber alignment, as documented in various literature sources[ref]. We aimed to select the most homogeneous and aligned batch of membranes to extract the desired characteristics. The SEM images (Figure.7) indicate that alignment improved as the rotation speed increased. There was a noticeable contrast in terms of randomness when comparing with mats fabricated using a flat collector, which

resulted in entirely random fiber arrangements, with those produced using a rotating collector. The rotating collector led to progressively more aligned polymer sheets.

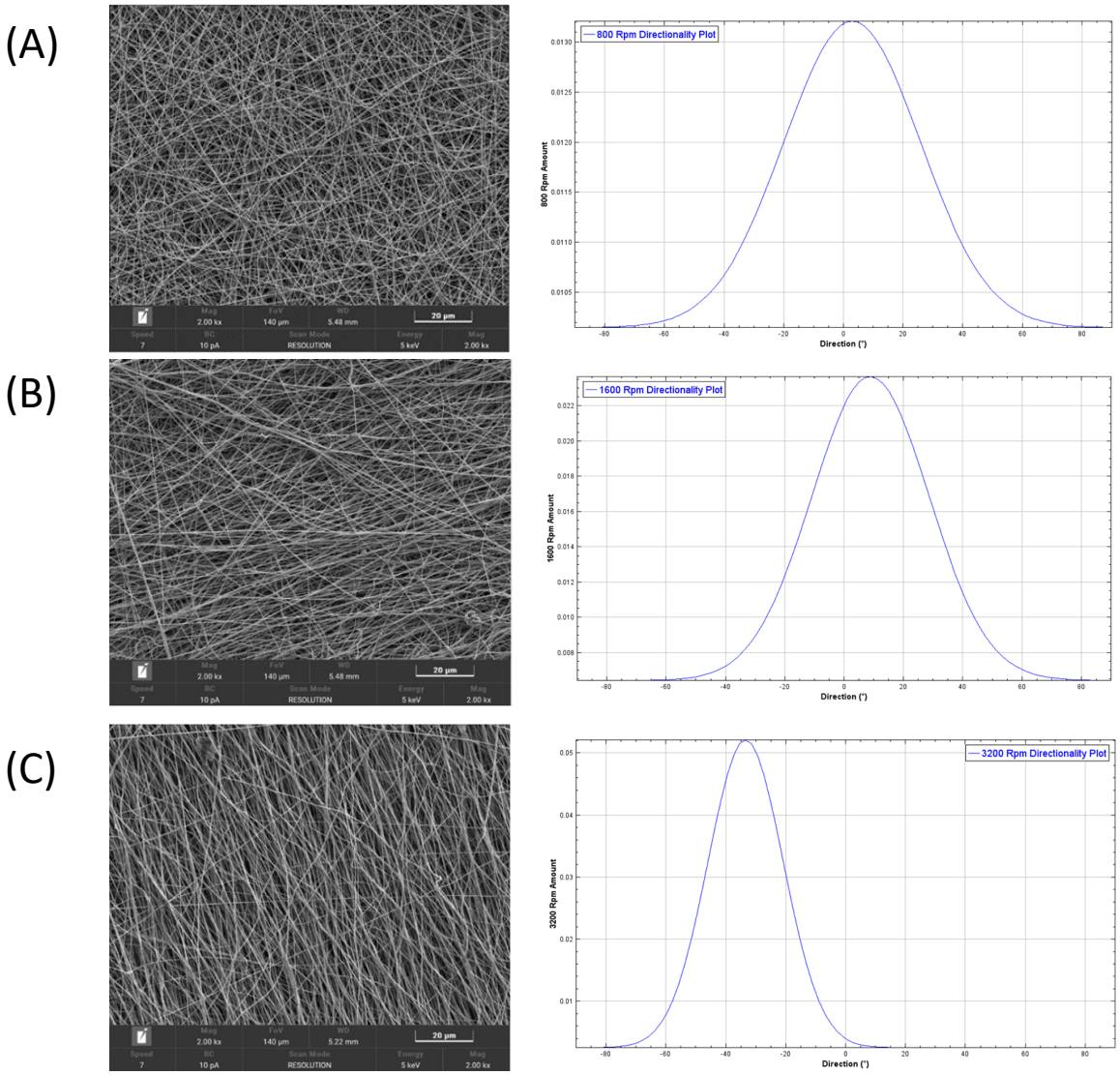

Figure 7. *SEM image of a membrane spinned at 800 (A), 1600 (B) and 3200 (C) rpm and its directionality Plot*

In addition to qualitatively assessing alignment, we also evaluated other parameters such as gaussian goodness, fibers amount and dispersion (Figure 8.), which were crucial in determining the appropriate fabrication speed to achieve the desired level of alignement. Amount is a measure of the preferential direction angle in terms of numbers of fibers meanwhile dispersion indicates how centered is the gaussian to a close range of values. Among the speeds tested, the mats fabricated at 3200 rpm exhibited not only a good level of alignment but also the lowest dispersion. This indicated a larger proportion of fibers oriented in a single direction angle and a lower standard deviation of the Gaussian distribution. Membranes obtained at 800

rpm started to show an orientation but it was not sufficient for the purpose, the 1600 rpm membranes had a good orientation but it resulted too dispersed and inconsistent in different sheet's portions. In contrast, the membranes obtained at 3500 rpm exhibited a more pronounced alignment peak but were more dispersed and uneven across the entire sheet. Furthermore, the operational conditions of the electrospinning instruments were not as reliable and repeatable at 3500 rpm as they were at 3200 rpm. The data collected were compared with other studies in the literature, and they demonstrated consistency in terms of alignment and directionality parameter trends (Mo et al., 2018).

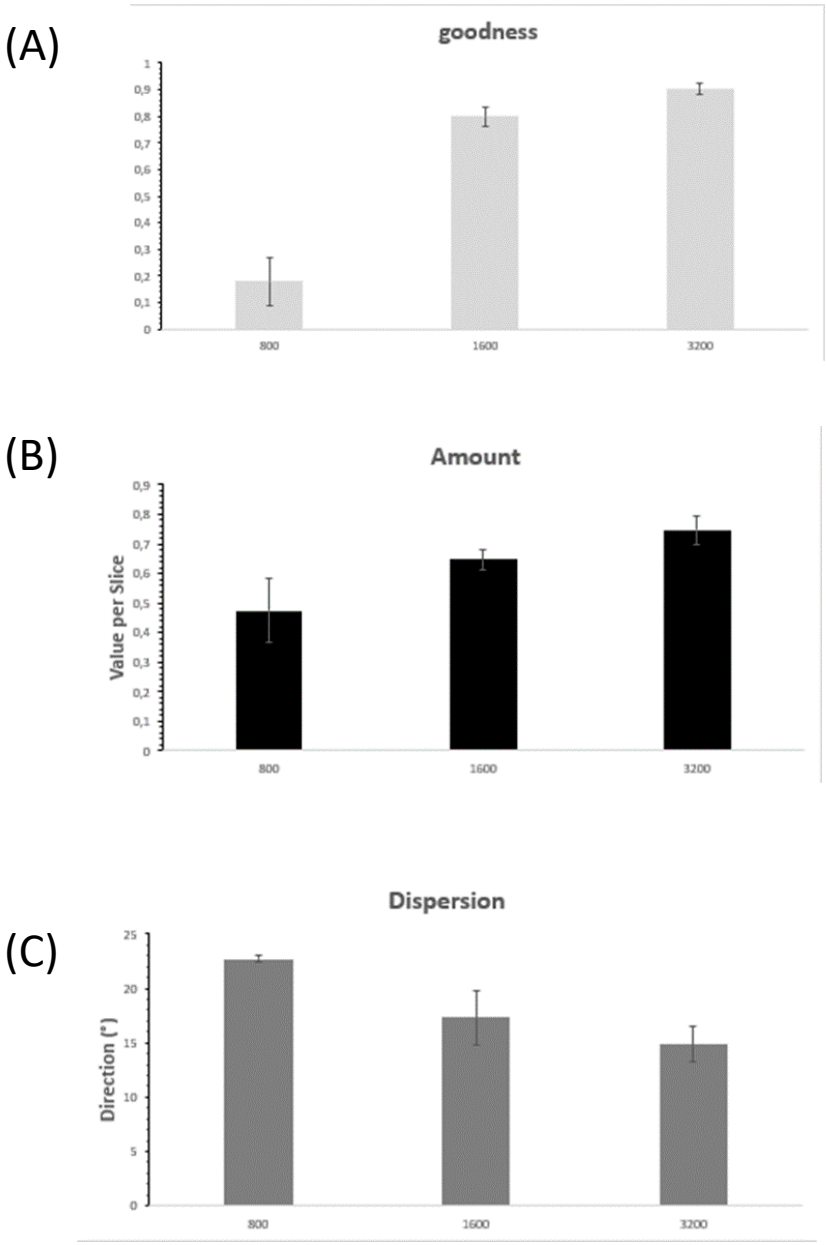

***Figure 8**. Gaussian goodness (A), fibers amount (B) and fiber dispersion (C) comparison between 800, 1600 and 3200 rpm*

3.1.2. Mechanical characterization

In order to ensure stability and mechanical support throughout the entire duration of the cell culture, it was essential for the obtained membranes to possess suitable mechanical properties. To assess these properties, mechanical tests were conducted on both random and aligned samples of PCL-PANI membranes (Figure 9.). The samples underwent characterization through uniaxial tensile tests, following the protocol outlined in Chapter 2. These tests aimed to determine key mechanical parameters, including ultimate tensile strength (UTS) (Figure 11.), strain at failure and Young's modulus (Fig. 10). Given that the primary influence on the samples' mechanical properties was expected to be the membrane's architecture, particular attention was focused on observing how the mechanical behavior would change with alignment. As clearly demonstrated in the results, the strong anisotropy of the aligned samples resulted in significantly greater resistance to traction. This was reflected in an average Young's modulus nearly three times higher than that of the random samples, as well as a higher UTS. As anticipated, the deformation at the point of failure was slightly greater in the random samples, indicating a more ductile behavior. It's important to note that all the data collected were statistically evaluated based on a large number of tested samples.

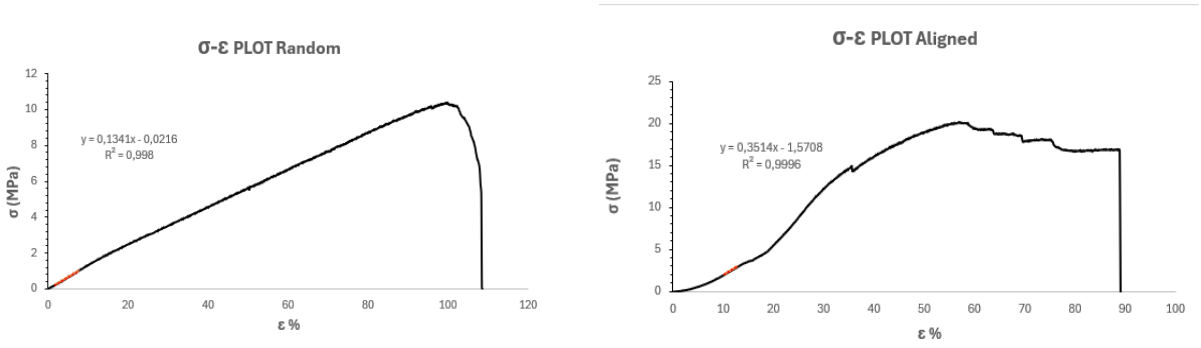

Figure 9. *Average strain stress curves for random and aligned samples*

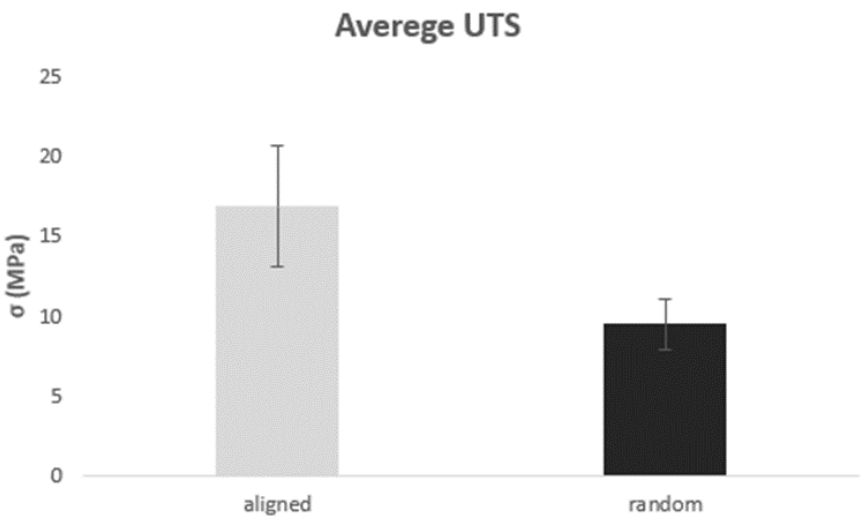

***Figure 10**. Average UTS comparison of random and aligned fibers*

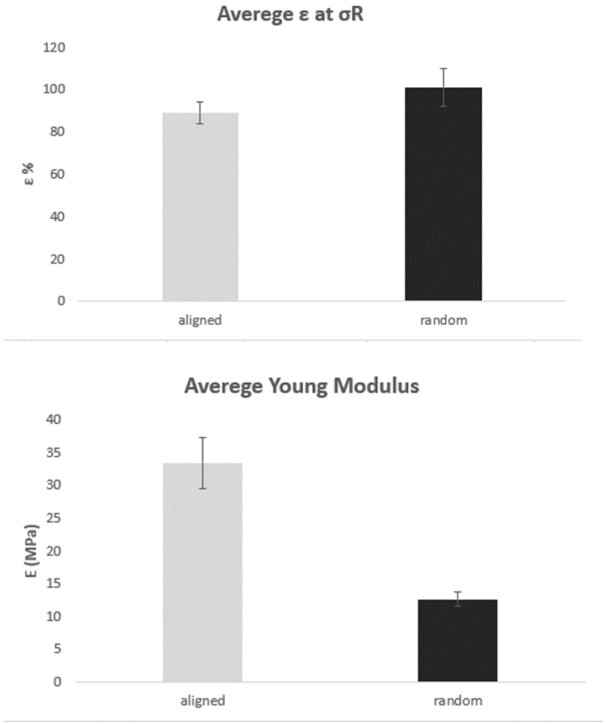

***Figure 11.** Average deformation at break and Young modulus comparison between random and aligned fibers*

3.1.3. Surface wettability characterization

As is well-established in the literature, the PCL-PANI polymer blend is inherently hydrophobic (Yoshinari et al., 2009) (Balint et al., 2013). To render the membranes suitable for cell cultures, it was imperative to enhance their surface wettability. To achieve this characteristic, a plasma-argon treatment, as previously described in the preceding chapter, was conducted. This plasma-argon treatment in an atmospheric environment was essential for exposing the COOH groups of the polymer, thereby rendering the surface more hydrophilic. The objective of this characterization was twofold: firstly, to assess the effectiveness of the plasma treatment, and secondly, to discern the differences in terms of contact angle and wettability between the aligned and random membranes. Contact angle images are reported (Figure. 12) as well as contact angle quantification (Figure. 13). Additionally, we sought to evaluate the durability of the treatment over a short period (Figure. 14). The samples were subjected to testing using a sessile-drop procedure following the treatment. The initial results were immediately apparent, with a significant reduction in contact angle observed between the treated and untreated membranes at T0, indicating the successful execution of the treatment. Notably, there were differences in contact angle values when comparing the random and aligned membranes. The aligned topography exhibited higher contact angle values both before and after the treatment when compared to the random samples. This surface behavior can be attributed to the oriented structure, which reduces outer porosity and asperities, resulting in a more homogeneous substrate (Balan et al., 2022). Nevertheless, after the plasma treatment, the membranes achieved an acceptable threshold of wettability, making them suitable for serving as a substrate for neural cell culture (Garrudo et al., 2019). Furthermore, the effects of the plasma treatment were evaluated over time at different intervals for 24 hours. The treatment, under the proposed conditions, exhibited a progressive increase in contact angle for both aligned and random membranes. After 24hour, the contact angles nearly returned to the conditions observed in non-treated samples. While this outcome was not as hoped for, it is important to consider that various factors may have influenced the procedure, and refining this protocol in the future may yield more robust results.

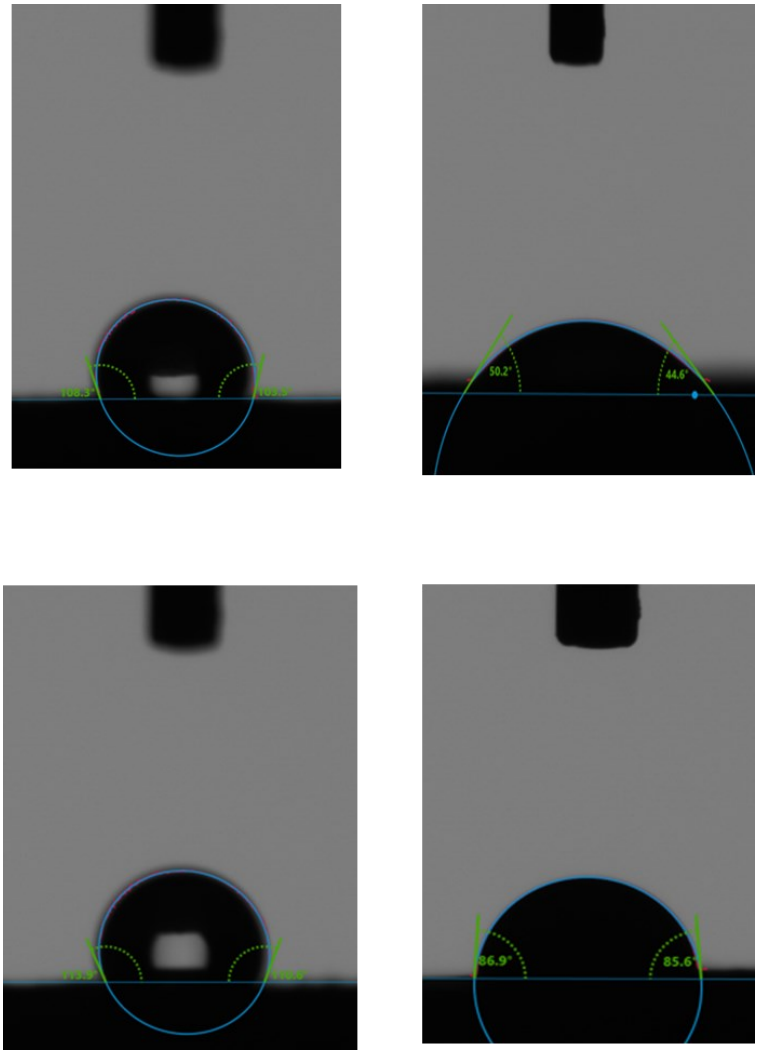

***Figure 12**. Random and aligned fibers samples before and after the plasma treatment images*

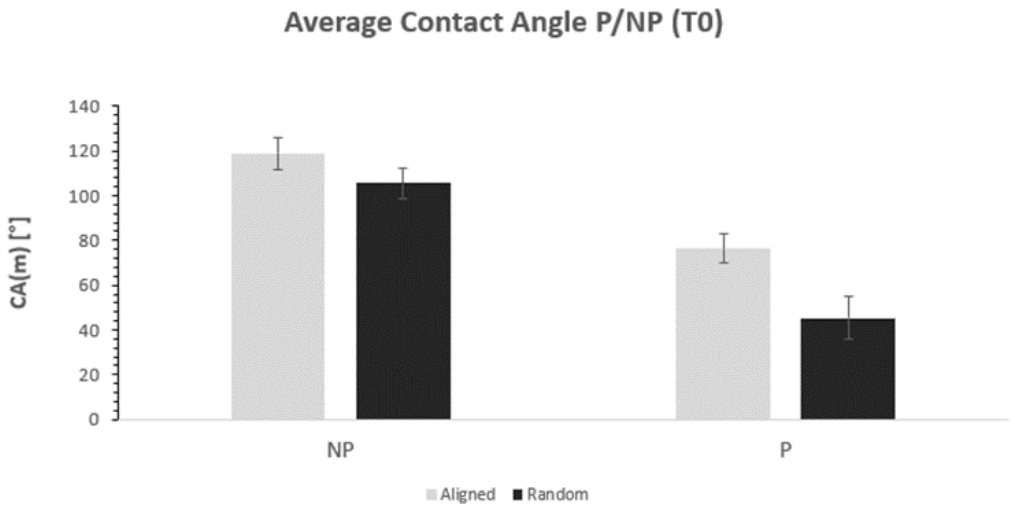

***Figure 13**. Average contact angles between treated and not treated membranes comparison*

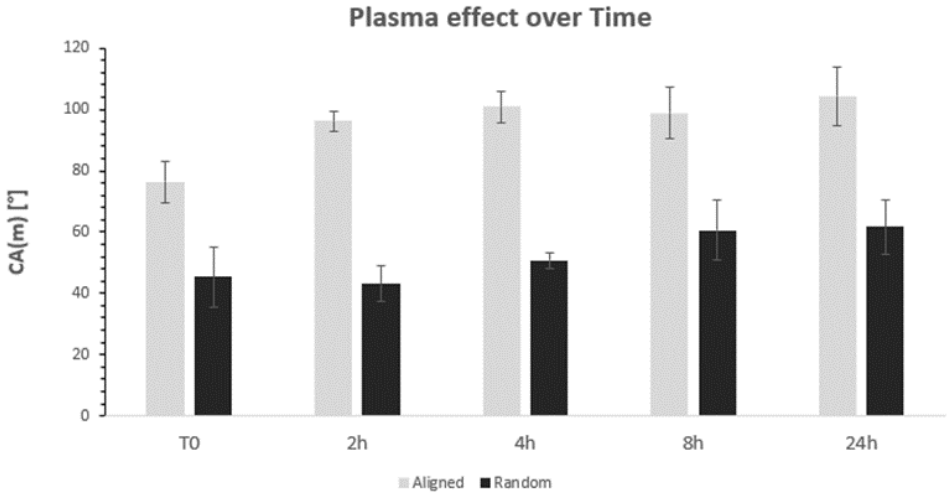

***Figure 14**. Comparison between aligned and random fibers contact angle trend after different time steps*

3.2. Biological *In Vitro* tests

Having prepared and characterized the membranes, the primary objective of this thesis work continues to be the establishment of a functional NE-4C culture capable of proliferation and differentiation into specific cell phenotypes, ultimately developing organized tissue by harnessing the features of the fabricated and functionalized substrate. In the following sections, the results of the various steps were summarized to demonstrate, from a biological perspective, the feasibility and validity of the work undertaken.

3.2.1. NE-4C Viability on PCL-PANI membranes

The proliferation assay was conducted on the membranes with the purpose of highlighting the differences in viability between plasma-treated and untreated membranes, as well as between random and aligned membranes (Figure. 15). The interaction between cells and biomaterials is strongly influenced by the surface chemistry of the substrate, which can regulate protein adsorption and subsequently affect cell behavior. When the substrate comes into direct contact with the culture media, protein adsorption plays a pivotal role in the initial cell attachment process (Sperling et al., n.d.) (Manuscript, 2021). The plasma treatment applied to the membranes allowed us to create two distinct and opposing conditions for comparison: untreated membranes, which exhibited pronounced hydrophobicity, and treated membranes with significantly lower contact angles. The results of the CellTiter-Blue assay, conducted after 24 hours and 72 hours, revealed a higher proliferation rate in plasma-treated membranes compared to their non-plasma-treated counterparts of the same type. This outcome aligns precisely with expectations based on the well-established link between surface wettability and cell attachment, as documented in the literature.(Bouhon et al., 2005)

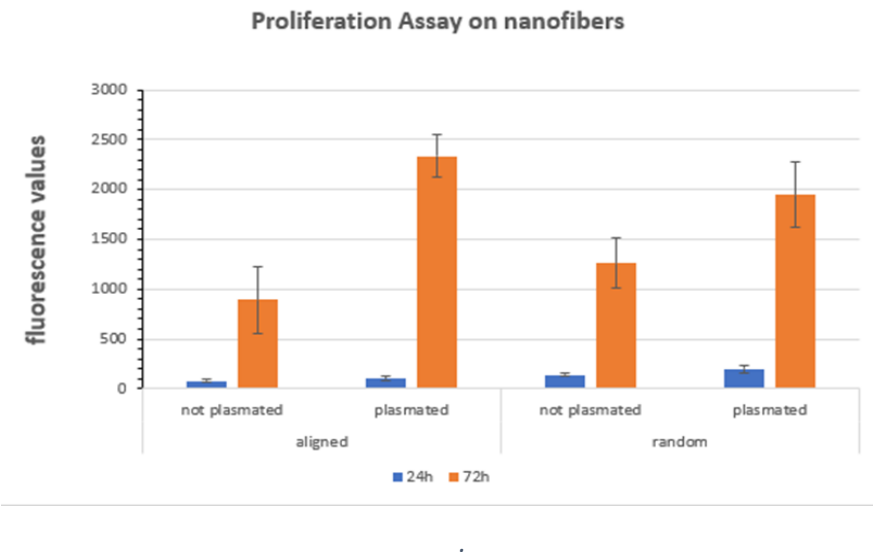

Figure 15. Plot of cell viability in untreated and treated conditions for aligned and random membranes

Another aspect of our investigation was to examine the variations in viability between the two types of membranes: random and aligned. Within the first 24 hours of culture, cells exhibited higher proliferation rates in random membranes. This is likely attributable to their greater porosity, which allows for improved nutrient transfer and, consequently, an initial boost in viability. However, this trend reversed after 72 hours of culture. Despite their lower porosity, the aligned mats, once colonized, facilitated better cellular interconnection. In fact, cells tended to align themselves along the fibers' orientation, enhancing cellular crosstalk, which significantly impacted proliferation. This behavior becomes even more evident when examining confocal images (Figure. 16), where it is apparent that the two cultures, at the same time point, differ in substrate colonization and cluster orientation, with a notably higher level of organization observed in the aligned membranes.

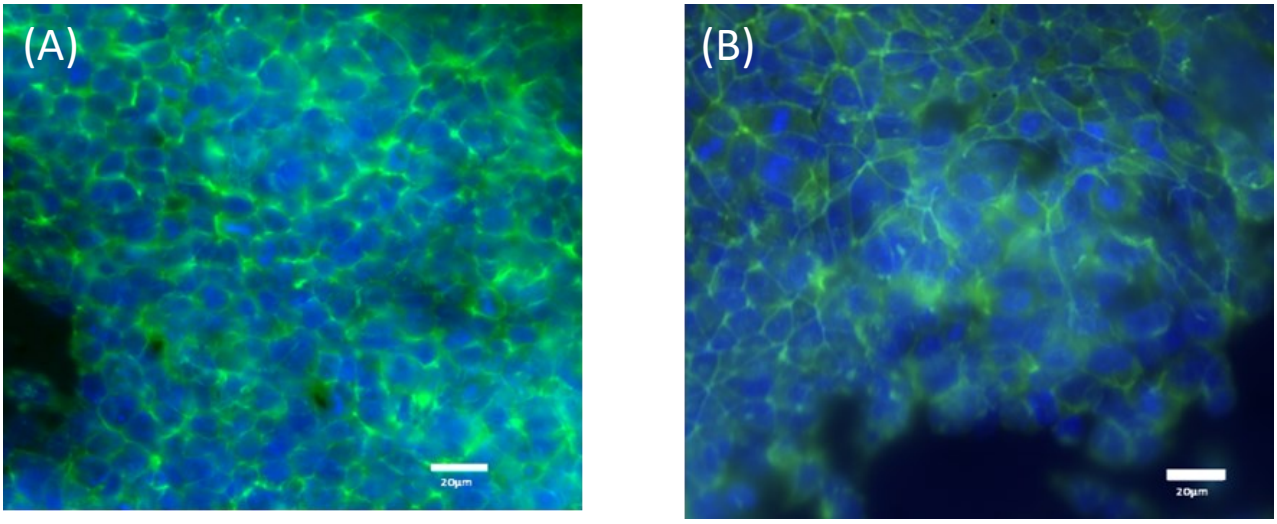

***Figure 16**. Confocal images of aligned (A) and random (B) membranes at 72h. in blue is represented the dapi staining of cell nuclei, in green the phalloidin staining of cytoskeletons (Scalebar = 20 µm).*

3.2.3. Differentiation of NE-4C on nanofibrous mats

NE-4C cells are known to differentiate into two distinct cell phenotypes: astrocytes and neurons. The differentiation protocol was executed using aligned nanofibrous mats to gain insights into the interactions and effects of a nanostructured, anisotropic, and oriented substrate, such as the fabricated membranes, on the

differentiation process. Differentiation is a delicate process influenced by various environmental factors. One crucial aspect is comprehending how the substrate's topography impacts cell behavior in terms of orientation and distribution. The challenge lies in directing and guiding neural cell differentiation along specific pathways. An aligned substrate, like the membranes we fabricated, is expected to significantly enhance neurite outgrowth and guide neural differentiation into specific pathways. Cellular directionality is particularly critical, especially for neurons, whose primary function is to transmit signals over long distances in the body. Axons must be stretched and oriented, mirroring the natural state within the human body. The primary reasons why neurite alignment is promoted by the substrate can be attributed to two essential factors: mechano-sensing and cytoskeleton dynamics. Cells undergo shape remodeling when they come into contact with the natural extracellular matrix (ECM), and axonal alignment results from focal adhesions and the reorganization of microtubules and F-actin (Fan et al., 2019).By examining the confocal images obtained through brightness and fluorescence cytoskeleton staining overlays (Fig. 17), it becomes evident that the cells are well-oriented along the direction of the fibers (B) compared to the unconditioned sample. This qualitative result demonstrates how the micro-architecture and orientation of the membranes mimic, to some extent, the characteristics of the ECM, leading to cytoskeletal dynamics that resemble those observed *in vivo*.

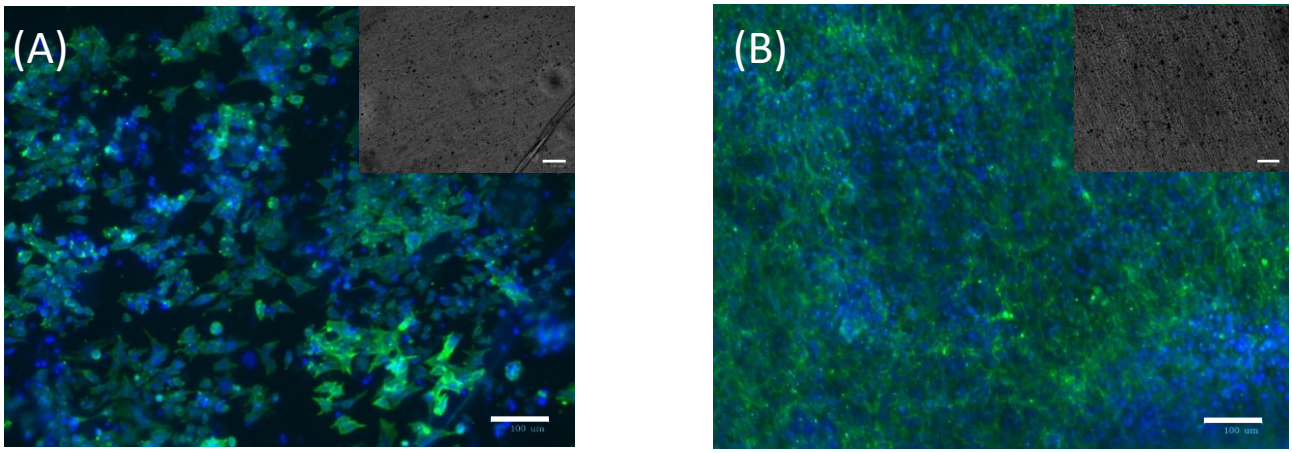

Figure 17. *Confocal images of 14 days culture compared with brightfield images of unconditioned (A) and conditioned(B) membranes at the same lenght scale (Scalebar = 100 µm)*

*A*s evident from the confocal images observed at 7 days (Figure. 18) and 14 days (Figure. 19), two cultures were compared—one representing a control group exposed to a simple proliferation medium and the other conditioned with a specific differentiation medium. After 7 days, with DAPI/Phalloidin staining, cells in the differentiated group appeared more clustered and morphologically developed compared to the control group likely due to the initial stages of differentiation. Upon reaching the 14-day mark, the differentiating culture displayed clear cellular differentiation and, in comparison to the control, which appeared to have slowed in growth, exhibited structured interconnections and a tissue-like behaviour (Liu & Hu, 2018).

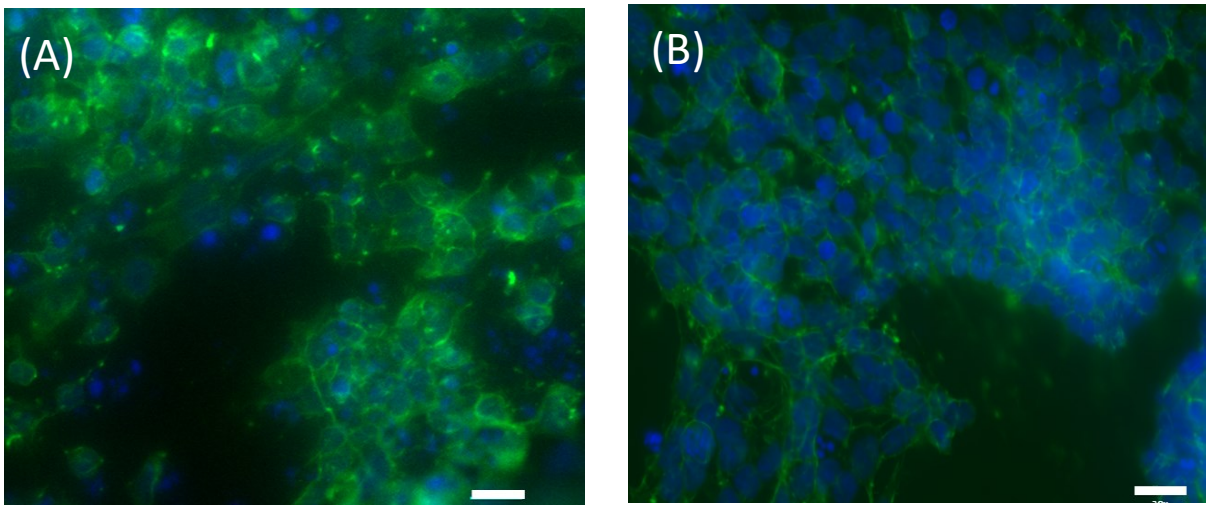

***Figure 18**. Morphology after 7 days of culture for control (A) and conditioned (B) culture (Scalebar = 20 µm).*

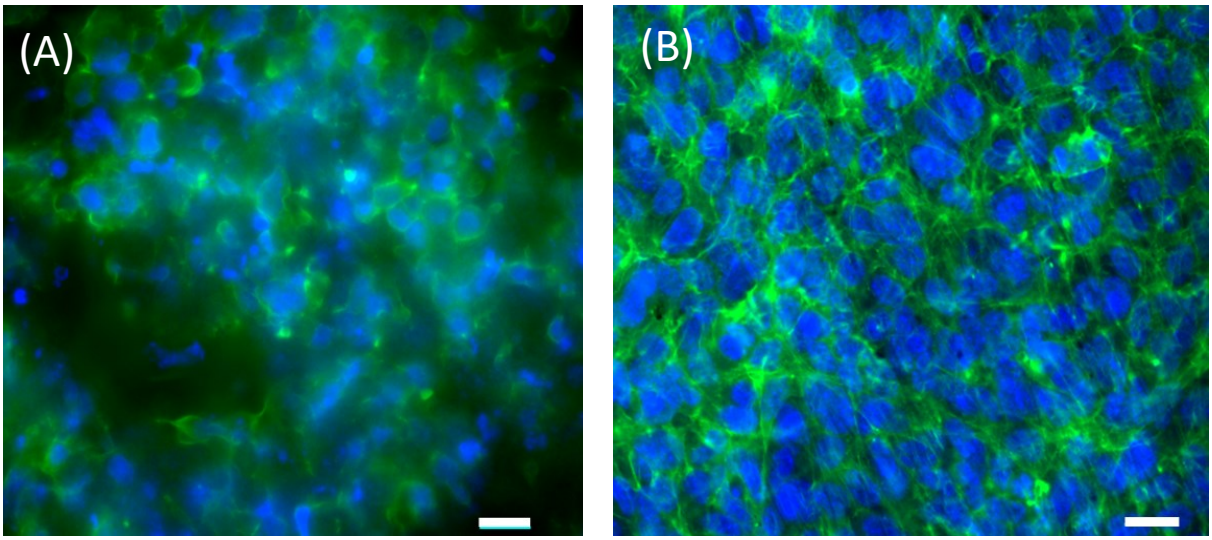

***Figure 19**. Morphology after 14 days of culture for control (A) and conditioned (B) culture (Scalebar = 20 µm).*

3.2.3.1. Protein expression in differentiated culture

The differences observed between the control culture and the conditioned one are likely attributed to the interaction between astrocytes and neurons. Astrocytes play a pivotal role in the neurogenesis process as they can exert regulatory control over neuronal expression and redirect stem cells towards adopting a neuronal fate. While the cells in the control culture may have autonomously differentiated primarily into neurons, as is typically the case, the culture conditioned with the differentiating medium provided the necessary stimuli to introduce a significant astroglia component. This, in turn, resulted in a more uniform and synergistic differentiation process (Sirkkunan et al., 2022). To confirm this hypothesis, immunostaining tests were conducted to comprehensively analyse the protein markers expressed by the culture. The conditioned culture exhibited a pronounced presence of both GFAP, the protein associated with astroglia cells, and MAP2, the protein marker associated with neurons at 14 days after differentiation induction (Figure. 20 and Figure. 21). These results not only demonstrate the complete differentiation of the culture into the two target cell phenotypes but also confirm how the presence of astrocytes enhances overall differentiation due to astroglia-associated neurogenesis(Song et al., 2002) (Környei et al., 2005).

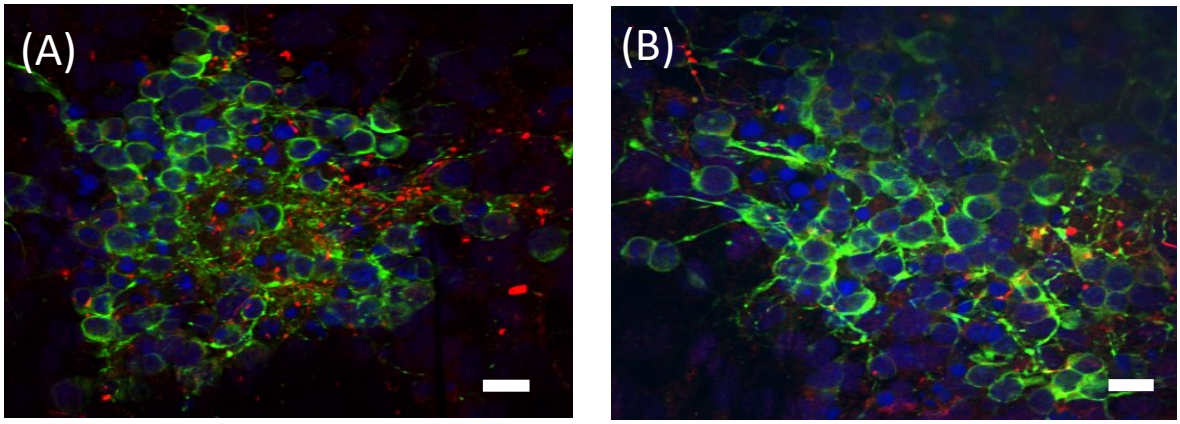

***Figure 20**. Marker expression of differentiated NE-4C after 14 days of culture. In blue DAPI, in green MAP2, in red GFAP (Scalebar = 20 µm).*

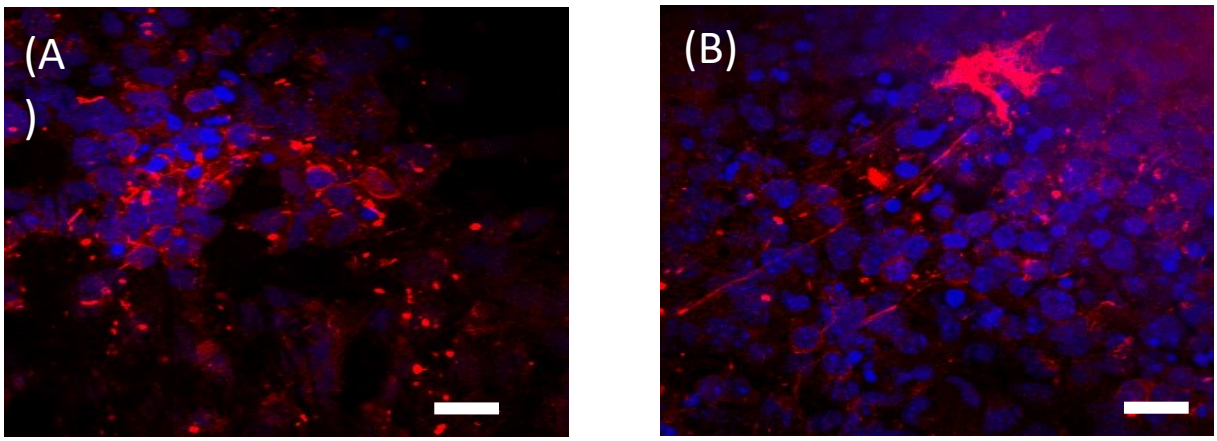

***Figure 21.** Marker expression of differentiated NE-4C after 14 days of culture. In blue DAPI, in red GFAP. (Scalebar = 20 µm).*

3.3. Set up for bioreactor dynamic culture

The main purpose of this thesis work remains to prepare a functional substrate which could have been integrated to a bioreactor, with the aim to put together the substrate features like nanostructured aligned topography and conductivity, with the advantages of a dynamic culture and the possibility to electrically stimulate the cells. A bioreactor capable of conjugating these features was supplied by IVTech S.R.L.

Although, a major challenge was to adapt the membranes fabricated to make them suitable for the culturing chamber of the bioreactor.

3.3.1 how to integrate the engineered substrate in a more complex culture system

The major issue was to find a system that allows to maintain the membranes in their lodge not moving during the dynamic flow, not to permeate fluid from the top to the bottom of the chamber and guarantee a contact surface to transmit the electrical stimulation. The custom-made ring designed and printed ensures the fixation of the membranes and avoids lateral leakage, nonetheless it leaves the majority of the membrane's surface free so that it can be adequately stimulated and reached by the medium.

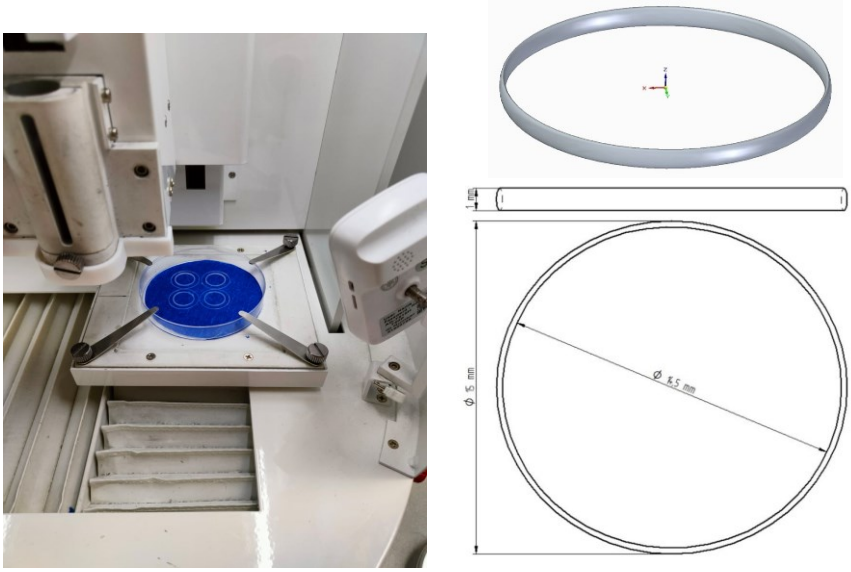

***Figure 22**. PCL custom-made ring CAD model and fabrication*

4. Final thoughts

4.1 Conclusions

Looking at the aims and objectives of this work, it can be concluded that overall good results have been obtained upon analysing each step individually, from assessing the need that gave rise to this idea, to fabricating the mat, characterizing it, and the culture phase. The main objective was to demonstrate how substrate alignment, material conductivity, and wettability, when combined, have a positive effect on neural cell culture, based on existing literature on the individual strategies. Fabricating a conductive biomaterial like PCL-PANI blend through electrospinning required extensive optimization but led to the creation of not only a highly aligned mat but also a diverse range of membrane alignments, which were further explored for their effects and clearly showed a tangible impact over neural cell behaviour. The use of a conductive material was crucial to enhancing this culture system with electrical stimulation, and plasma-argon functionalization to modify wettability was necessary and highly effective in making PCL-PANI mats suitable for neural cell culture. Mechanical and physical characterization results were comparable to literature and aligned with expectations based on the technologies and materials used. The final construct, along with the strategies adopted to integrate it into a dynamic environment, proved to be an ideal substrate for the culture phase. Cell growth and differentiation occurred as expected, with clear and distinct differences between the engineered system and the control group. It's evident that all processes can and should be refined and expanded upon, but it's not presumptuous to assert that the final result meets initial expectations and provides a solid foundation for future development.

4.2 Future perspectives

At present, it has become exceedingly relevant in an increasingly demanding sector to develop reliable and efficient systems that can serve as real alternatives to those currently in use. Achieving this goal relies on deploying a wide range of technologies, understanding biological structures, and fostering interdisciplinary collaboration. In this study, we have constructed a viable and efficient system for culturing neural cells, with enhanced performance compared to static and 2D methods. It is important to note that this is a prototype, and all processes and steps can be further optimized and developed. This model has been thought to be implemented in a dynamic and actuated systems such as a bioreactor and all the set up in terms of lodging and electrical parameters research have been prompted, the natural step forward will be a complete assessment of the model in a bioreactor device, and this should be the next achievement. Although the many limitations and needs of improvements, examples like this one should pave the way forward. Understanding how living biological compounds respond to different stimuli and architectures is crucial. The variability in behaviour must be considered when dealing with the complexity of cells and tissues; nothing can be neglected or taken for granted. The only way to understand and discover is through experimentation. This study involved a combination of different stimuli, fabrication technologies, and material properties, all of which were shown to aid in tissue growth. However, many other alternatives can be explored, and the methods used can be perfected. The ultimate goal is to achieve results that are not only significant for scientific research but also competitive as translational devices in the drug and medical testing markets.

Bibliography

Abbott, A. (2003). *Biology ' s new dimension*. *424*(August).

Agarwal, S., Wendorff, J. H., & Greiner, A. (2008). Use of electrospinning technique for biomedical applications. *Polymer*, *49*(26), 5603–5621. https://doi.org/10.1016/j.polymer.2008.09.014

Aijaz, M. O., Karim, M. R., Alharbi, H. F., & Alharthi, N. H. (2019). Novel optimised highly aligned electrospun PEI-PAN nanofibre mats with excellent wettability. *Polymer*, *180*(July), 121665. https://doi.org/10.1016/j.polymer.2019.121665

Aishwarya, P., Agrawal, G., Sally, J., & Ravi, M. (n.d.). *Dynamic three-dimensional cell-culture systems for enhanced in vitro applications*.

Amores de Sousa, M. C., Rodrigues, C. A. V., Ferreira, I. A. F., Diogo, M. M., Linhardt, R. J., Cabral, J. M. S., & Ferreira, F. C. (2020). Functionalization of Electrospun Nanofibers and Fiber Alignment Enhance Neural Stem Cell Proliferation and Neuronal Differentiation. *Frontiers in Bioengineering and Biotechnology*, *8*(October), 1–16. https://doi.org/10.3389/fbioe.2020.580135

Balan, P., Indrakumar, J., Solaimuthu, A., Murali, P., & Korrapati, P. S. (2022). Bioinspired Nanofibers: Preparation and Characterization. *Handbook of Research on Nano-Drug Delivery and Tissue Engineering: Guide to Strengthening Healthcare Systems*, *5169*(1), 253–296. https://doi.org/10.1201/9781003161196-10

Balint, R., Cassidy, N. J., Cartmell, S. H., Chen, M. C., Sun, Y. C., Chen, Y. H., CHECHEL'NITSKAIA, S. M., BAIGULOVA, S. A., Dan, L. I., Huang, J., & Kaner, R. B. (2013). Polyaniline nanofibers: a unique polymer nanostructure for versatile applications. *Acta Biomaterialia*, *9*(3), 2341–2353. https://doi.org/10.1021/ar800080n

Bédard, P., Gauvin, S., Ferland, K., Caneparo, C., Pellerin, È., Chabaud, S., & Bolduc, S. (2020). Innovative human three-dimensional tissue-engineered models as an alternative to animal testing. *Bioengineering*, *7*(3), 1–40. https://doi.org/10.3390/bioengineering7030115

Bertucci, C., Koppes, R., Dumont, C., & Koppes, A. (2019). Neural responses to electrical stimulation in 2D and 3D in vitro environments. In *Brain Research Bulletin* (Vol. 152). Elsevier Inc. https://doi.org/10.1016/j.brainresbull.2019.07.016

Bierman-duquette, R. D., Safarians, G., Huang, J., Rajput, B., Chen, J. Y., Wang, Z. Z., & Seidlits, S. K. (2022). *Engineering Tissues of the Central Nervous System : Interfacing Conductive Biomaterials with Neural Stem / Progenitor Cells*. *2101577*, 1–29. https://doi.org/10.1002/adhm.202101577

Bouhon, I. A., Kato, H., Chandran, S., & Allen, N. D. (2005). Neural differentiation of mouse embryonic stem cells in chemically defined medium. *Brain Research Bulletin*, *68*(1–2), 62–75. https://doi.org/10.1016/j.brainresbull.2005.08.022

Chang, K. A., Kim, J. W., Kim, J. a., Lee, S., Kim, S., Suh, W. H., Kim, H. S., Kwon, S., Kim, S. J., & Suh, Y. H. (2011). Biphasic electrical currents stimulation promotes both proliferation and differentiation of fetal neural stem cells. *PLoS ONE*, *6*(4). https://doi.org/10.1371/journal.pone.0018738

Culture, C., Duval, K., Grover, H., Han, L., Mou, Y., Pegoraro, A. F., Fredberg, J., & Chen, Z. (2022). *Modeling Physiological Events in 2D vs .* 266–277. https://doi.org/10.1152/physiol.00036.2016

Eltom, A., Zhong, G., & Muhammad, A. (2019). Scaffold Techniques and Designs in Tissue Engineering Functions and Purposes: A Review. *Advances in Materials Science and Engineering*, *2019*. https://doi.org/10.1155/2019/3429527

Fan, J., Zhang, Y., Liu, Y., Wang, Y., Cao, F., Yang, Q., & Tian, F. (2019). Explanation of the cell orientation in a nanofiber membrane by the geometric potential theory. *Results in Physics*, *15*(July), 102537. https://doi.org/10.1016/j.rinp.2019.102537

Fatehullah, A., Tan, S. H., & Barker, N. (2016). Organoids as an in vitro model of human development and disease. *Nature Publishing Group*, *18*(3), 246–254. https://doi.org/10.1038/ncb3312

Garrudo, F. F. F., Chapman, C. A., Hoffman, P. R., Udangawa, R. W., Silva, J. C., Mikael, P. E., Rodrigues, C. A. V., Cabral, J. M. S., Morgado, J. M. F., Ferreira, F. C., & Linhardt, R. J. (2019). Polyaniline-polycaprolactone blended nanofibers for neural cell culture. *European Polymer Journal*, *117*(February), 28–37. https://doi.org/10.1016/j.eurpolymj.2019.04.048

Garrudo, F. F. F., Mikael, P. E., Rodrigues, C. A. V., Udangawa, R. W., Paradiso, P., Chapman, C. A., Hoffman, P., Colaço, R., Cabral, J. M. S., Morgado, J., Linhardt, R. J., & Ferreira, F. C. (2021). Polyaniline-polycaprolactone fibers for neural applications: Electroconductivity enhanced by pseudo-doping. *Materials Science and Engineering C*, *120*(November). https://doi.org/10.1016/j.msec.2020.111680

Götz, M., & Huttner, W. B. (2005). The cell biology of neurogenesis. *Nature Reviews Molecular Cell Biology*, *6*(10), 777–788. https://doi.org/10.1038/nrm1739

Grossemy, S., Chan, P. P. Y., & Doran, P. M. (2021). Enhanced Neural Differentiation Using Simultaneous Application of 3D Scaffold Culture, Fluid Flow, and Electrical

Stimulation in Bioreactors. *Advanced Biology*, *5*(4), 1–8. https://doi.org/10.1002/adbi.202000136

Jiang, T., Carbone, E. J., Lo, K. W. H., & Laurencin, C. T. (2015). Electrospinning of polymer nanofibers for tissue regeneration. *Progress in Polymer Science*, *46*, 1–24. https://doi.org/10.1016/j.progpolymsci.2014.12.001

Kaur, G., Adhikari, R., Cass, P., Bown, M., & Gunatillake, P. (2015). Electrically conductive polymers and composites for biomedical applications. *RSC Advances*, *5*(47), 37553–37567. https://doi.org/10.1039/c5ra01851j

Kelm, J. M., Sittampalam, G. S., & Ferrer, M. (2018). SC. *Drug Discovery Today*. https://doi.org/10.1016/j.drudis.2018.07.007

Környei, Z., Szlávik, V., Szabó, B., Gócza, E., Czirók, A., & Madarász, E. (2005). Humoral and contact interactions in astroglia/stem cell co-cultures in the course of glia-induced neurogenesis. *Glia*, *49*(3), 430–444. https://doi.org/10.1002/glia.20123

Licciardello, M., Ciardelli, G., & Tonda-Turo, C. (2021). Biocompatible electrospun polycaprolactone-polyaniline scaffold treated with atmospheric plasma to improve hydrophilicity. *Bioengineering*, *8*(2), 1–18. https://doi.org/10.3390/bioengineering8020024

Lim, S. H., Liu, X. Y., Song, H., Yarema, K. J., & Mao, H. (2010). Biomaterials The effect of nano fi ber-guided cell alignment on the preferential differentiation of neural stem cells. *Biomaterials*, *31*(34), 9031–9039. https://doi.org/10.1016/j.biomaterials.2010.08.021

Liu, Z., & Hu, Z. (2018). Aligned contiguous microfiber platform enhances neural differentiation of embryonic stem cells. *Scientific Reports*, *8*(1), 1–12. https://doi.org/10.1038/s41598-018-24522-9

Lowry Curley, J., & Moore, M. J. (2017). 3D neural culture in dual hydrogel systems. *Methods in Molecular Biology*, *1612*, 225–237. https://doi.org/10.1007/978-1-4939-7021-6_17

Ma, Z., Kotaki, M., Inai, R., & Ramakrishna, S. (2005). Potential of nanofiber matrix as tissue-engineering scaffolds. *Tissue Engineering*, *11*(1–2), 101–109. https://doi.org/10.1089/ten.2005.11.101

Mahairaki, V., Lim, S. H., Christopherson, G. T., Xu, L., Nasonkin, I., Yu, C., Mao, H., & Koliatsos, V. E. (2011). *Nanofiber Matrices Promote the Neuronal Differentiation of Human Embryonic Stem Cell-Derived Neural Precursors In Vitro*. *17*. https://doi.org/10.1089/ten.tea.2010.0377

Manuscript, A. (2021). *Materials Chemistry B*. *10*(207890), 4118. https://doi.org/10.1039/D1TB00789K.Volume

Mo, X., Sun, B., Wu, T., & Li, D. (2018). Electrospun nanofibers for tissue engineering. In *Electrospinning: Nanofabrication and Applications*. Elsevier Inc. https://doi.org/10.1016/B978-0-323-51270-1.00024-8

Qian, X., Shen, Q., Goderie, S. K., He, W., Capela, A., Davis, A. A., & Temple, S. (2000). Timing of CNS Cell Generation. *Neuron*, *28*(1), 69–80. https://doi.org/10.1016/s0896-6273(00)00086-6

Saberi, A., Jabbari, F., Zarrintaj, P., Saeb, M. R., & Mozafari, M. (2019). Electrically conductive materials: Opportunities and challenges in tissue engineering. In *Biomolecules* (Vol. 9, Issue 9). https://doi.org/10.3390/biom9090448

Schlander, M., Hernandez, K., Chih, V., Cheng, Y., & Ferrandiz, J. M. (2021). How Much Does It Cost to Research and Develop a New Drug ? A Systematic Review and Assessment. *PharmacoEconomics*, 1243–1269. https://doi.org/10.1007/s40273-021-01065-y

Schmidt, C. E., & Leach, J. B. (2003). Neural tissue engineering: Strategies for repair and regeneration. *Annual Review of Biomedical Engineering*, *5*, 293–347. https://doi.org/10.1146/annurev.bioeng.5.011303.120731

Sirkkunan, D., Pingguan-Murphy, B., & Muhamad, F. (2022). Directing Axonal Growth: A Review on the Fabrication of Fibrous Scaffolds That Promotes the Orientation of Axons. *Gels*, *8*(1). https://doi.org/10.3390/gels8010025

Song, H., Stevens, C. F., & Gage, F. H. (2002). Astroglia induce neurogenesis from adult neural stem cells. *Nature*, *417*(6884), 39–44. https://doi.org/10.1038/417039a

Sperling, L. E., Pranke, P., & Reis, K. P. (n.d.). *This article is protected by copyright. All rights reserved.* 1–27.

Temple, S. (2001). The development of neural stem cells. *Nature*, *414*(6859), 112–117. https://doi.org/10.1038/35102174

Thrivikraman, G., Boda, S. K., & Basu, B. (2018). Unraveling the mechanistic effects of electric field stimulation towards directing stem cell fate and function: A tissue engineering perspective. *Biomaterials*, *150*, 60–86. https://doi.org/10.1016/j.biomaterials.2017.10.003

Thrivikraman, G., Madras, G., & Basu, B. (2014). Intermittent electrical stimuli for guidance of human mesenchymal stem cell lineage commitment towards neural-like cells on electroconductive substrates. *Biomaterials*, *35*(24), 6219–6235. https://doi.org/10.1016/j.biomaterials.2014.04.018

Tomaskovic-Crook, E., Gu, Q., Rahim, S. N. A., Wallace, G. G., & Crook, J. M. (2020). Conducting Polymer Mediated Electrical Stimulation Induces Multilineage Differentiation with Robust Neuronal Fate Determination of Human Induced Pluripotent Stem Cells. *Cells*, *9*(3), 1–14. https://doi.org/10.3390/cells9030658

Tomaskovic-Crook, E., Zhang, P., Ahtiainen, A., Kaisvuo, H., Lee, C. Y., Beirne, S., Aqrawe, Z., Svirskis, D., Hyttinen, J., Wallace, G. G., Travas-Sejdic, J., & Crook, J. M. (2019). Human Neural Tissues from Neural Stem Cells Using Conductive Biogel and Printed Polymer Microelectrode Arrays for 3D Electrical Stimulation. *Advanced Healthcare Materials*, *8*(15), 1–10. https://doi.org/10.1002/adhm.201900425

Warren, D., Wallace, G. G., & Crook, J. M. (2021). *Engineering in vitro human neural tissue analogs by 3D bioprinting and electrostimulation*. *020901*, 1–26. https://doi.org/10.1063/5.0032196

Yamada, M., Tanemura, K., Okada, S., Iwanami, A., Nakamura, M., Mizuno, H., Ozawa, M., Ohyama-Goto, R., Kitamura, N., Kawano, M., Tan-Takeuchi, K., Ohtsuka, C., Miyawaki, A., Takashima, A., Ogawa, M., Toyama, Y., Okano, H., & Kondo, T. (2007). Electrical Stimulation Modulates Fate Determination of Differentiating Embryonic Stem Cells. *Stem Cells*, *25*(3), 562–570. https://doi.org/10.1634/stemcells.2006-0011

Yang, F., Murugan, R., Wang, S., & Ramakrishna, S. (2005). *Electrospinning of nano / micro scale poly (L -lactic acid) aligned fibers and their potential in neural tissue engineering*. *26*, 2603–2610. https://doi.org/10.1016/j.biomaterials.2004.06.051

Yoshinari, M., Wei, J., Matsuzaka, K., & Inoue, T. (2009). Effect of cold plasma-surface modification on surface wettability and initial cell attachment. *World Academy of Science, Engineering and Technology*, *58*, 171–175.

Zabielski, K., Sajkiewicz, P., Niemczyk-soczynska, B., & Zaszczy, A. (2021). *Hydrogel , Electrospun and Composite Materials for Bone / Cartilage and Neural Tissue Engineering*. 1–23.

Zhang, Q., Beirne, S., Shu, K., Esrafilzadeh, D., Huang, X. F., & Wallace, G. G. (2018). Electrical Stimulation with a Conductive Polymer Promotes Neurite Outgrowth and Synaptogenesis in Primary Cortical Neurons. *Scientific Reports*, *8*(1), 1–10. https://doi.org/10.1038/s41598-018-27784-5

Zhu, R., Sun, Z., Li, C., Ramakrishna, S., Chiu, K., & He, L. (2019). Electrical stimulation affects neural stem cell fate and function in vitro. *Experimental Neurology*, *319*(April), 112963. https://doi.org/10.1016/j.expneurol.2019.112963

Zhuang, P., Xuyang, A., An, J., Kai, C., & Yian, S. (2018). Biomaterials 3D neural tissue models : From spheroids to bioprinting. *Biomaterials*, *154*, 113–133. https://doi.org/10.1016/j.biomaterials.2017.10.002