

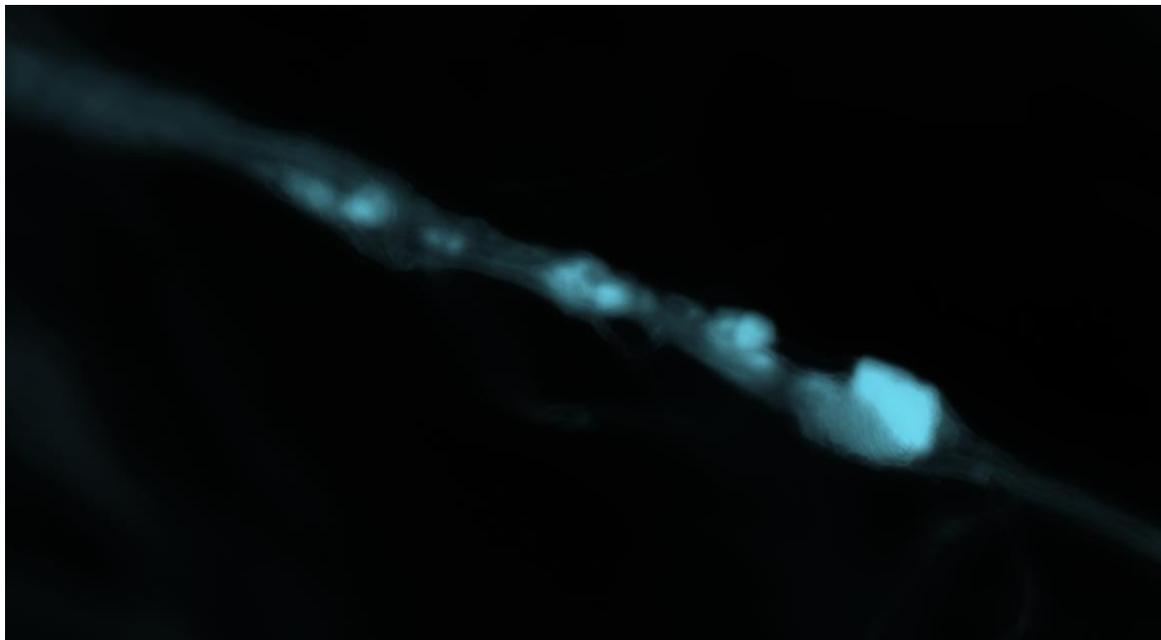


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# Electrospinning of Fibre Embedded Cells as Scaffolds for Muscle Regeneration

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A STUDY OF THE SUITABILITY OF ELECTROSPUN FIBRES FOR TISSUE  
ENGINEERING



Master Thesis 2016-2017

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Nanobiotechnology



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Electrospinning of fibre embedded cells as scaffolds for muscle regeneration

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**Abstract**

Regenerative medicine is an emergent field whose future potential is huge for all sorts of biomedical applications. Despite the progress in tissue engineering has already led to successful clinical trials, great work needs yet to be done in regard of skeletal muscle regeneration. This report gathers the investigation of novel scaffolding techniques for that purpose by the use of coaxial electrospinning. This promising technique offers the possibility to entrap living cells within a core-shell fibre structure to restrict cell growth in a desired direction. This alignment would be a friendly environment for the development of skeletal muscle but requires the assessment of cell viability proliferation and differentiation. The scope of this research aims to address such issues for different cell types, emphasizing on the study of mouse myogenic C2C12 cells, which demonstrated to survive the electrospinning process and remain viable over time. PCL-shell and PEO/alginate-core polymers proved to be the optimal materials for fibre biofabrication the fibrous scaffold. However, further research is required to analyse the regeneration potential, since no evidence of differentiation was found in this project.



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## Preface

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The topic of this master thesis on the nanobiotechnology master's degree at Aalborg University is Electrospinning of fibre embedded cells as scaffolds for muscle regeneration. The supervisors assigned to this project were Peter Fojan and Cristian Pablo Pennisi.

All references have appointed numbers according to their respective sources which are displayed as an exponent at the end of the paragraph, for instance: <sup>2</sup>

References can be placed either before a full stop or a comma, or after a full stop.

The latter indicates that the reference is tied to the entire anterior content, until it is obstructed by the previous reference or section. When placed before, it implies that the reference is either tied to a single sentence, or a specific part of the sentence if invoked before a comma. A reference mid-sentence is meant as a substitution for an article name. If figures display no reference in the caption means they are created by the project student. The sources corresponding to each reference can be found in the bibliography.



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## Contents

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Introduction .....	8
Theory .....	10
Introduction to tissue engineering.....	10
Regenerative medicine .....	11
Role of Stem Cells.....	15
Role of Scaffolds.....	20
Biofabrication Techniques.....	23
Coaxial Electrospinning .....	28
Achievements and Challenges .....	32
Principles of Skeletal Muscle .....	35
Materials and Methods.....	38
Materials .....	38
Methods .....	39
Results and Discussion .....	44
Characterization of Fibres-Scaffold .....	44
Fibre Shell Porosity .....	48
Assessment of Cell Viability .....	50
Proliferation and Differentiation .....	54
Conclusion .....	60
Outlook.....	62
Bibliography .....	64
Appendix .....	68



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## Introduction

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In the past years, pursuit of tissue engineering has followed a rising trend as solution to tackle tissue and organ failure as a result of injury or disease.

Nowadays, the range of available treatments includes transplantation, surgery, artificial prostheses or drug therapy. Unfortunately, major damages cannot be effectively repaired exclusively by conventional methods which, in addition, entail a certain number of drawbacks or secondary effects. Therefore, regenerative medicine is a promising research field to overcome these difficulties, by improving or replacing damaged tissue by organ mimics that can work either from the beginning or be developed into fully functional constructs. <sup>1</sup>

In order to develop this novel approach, it is crucial to engineer bioactive scaffolds to provide temporary support for the cells. Artificial fibrous architectures have been generated for this aim, serving as a substitute for the extracellular matrix (ECM) until the native one is created. Furthermore, the features of such structures will determine the proliferation and final differentiation towards certain cell type. Apart from specific mechanical properties that mimic those presented in the natural tissue, a suitable material for this purpose should also be biocompatible, sterilized and biodegradable. Besides, given the necessity of oxygen and nutrient transport throughout the fibres a semi-permeable porous membrane with high surface area is required to ensure the survival of the cells. <sup>2</sup>

Regarding the current available techniques to generate fibrous scaffolds, cross-linking coupled with extrusion is likely among the simplest. However, the resulting fibres containing entrapped cells do not show the ability to scale-up the regenerative process. A more efficient fibre production has been displayed through electrospinning, which uses electric force to draw the acquired charge of a liquid to obtain large quantities of nanofibers. The potential difference between two electrodes allows a continuous fibre generation from the conducting needle where certain solution flows out to the collection plate. <sup>3,4</sup>

In particular, coaxial electrospinning presents the advantage of one-step encapsulation of cells, into core–shell nanofibers. Hence it eliminates damages as consequence of the direct contact of the cells with organic solvents or harsh conditions. In this system the shell solution serves as confinement for the cells to favour an optimal disposition towards the desired tissue. <sup>4</sup>

This project has chosen this technology due to its great controllability, allowing the alignment of the fibres during their generation and deposition. The focus of this research relies on coaxial cell electrospinning to investigate the suitability of

polymeric fibres to produce such scaffold and explore the engineering of skeletal muscle.

A wide variety of materials have been successfully electrospun into fibrous scaffolds, ranging from collagen and alginates to synthetic materials. The co-electrospinning experiments reported here were carried out with polymeric solutions, namely polycaprolactone (PCL) for the shell and polyethylene oxide (PEO) combined with sodium alginate (NaA) for the core solution in which the cells are embedded. <sup>1,4</sup>

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# I. Theory

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## 1.1 INTRODUCTION TO TISSUE ENGINEERING

Earlier in history, biomedicine has been focused on the biological understanding and regulation of diseases. However, nowadays the first goal tackled by this field of research is directed to apply that information to develop new and better therapies.<sup>5</sup> According to their molecular complexity, therapies are classified in three main categories.

The simplest strategy regards chemical products; comprised essentially by organic molecules with low molecular weight. These compounds are capable to interact with biomolecules inside the human body to provoke a response, as it would be the case of well-known molecules.<sup>6</sup>

A higher level of elaboration allows the creation of medical drugs called biopharmaceuticals; whose manufacturing involves biotechnological procedures, such as recombinant proteins. They are either produced by biological sources or extracted from them.<sup>6</sup>

At last, the most ambitious strategies are advanced therapies. Due to the complexity of their development they are usually still in experimental and preclinical stages. Depending on what they employ as therapeutic product, they are grouped as gene therapy, cell therapy or tissue engineering, though these applications often tend to overlap.<sup>6</sup>

Gene therapy is based on the transfer of genetic material targeted to correct or improve at the molecular level certain cellular function or structure that is damaged inside the patient. It can be performed following two strategies: *in vivo*, by direct distribution of vectors into the organism; or *ex vivo*, along with the employment of cells, bringing together both approaches.<sup>7</sup>

Cell therapy is the science that utilizes somatic cells as therapeutic vehicles. After collection, said cells can be modified in culture and then introduced into the patient to provide a therapeutic effect, even if this only refers to the release of certain agents that turn to be beneficial.<sup>6</sup>

On the other hand, tissue engineering does not rely on cells alone, but applies life sciences toward the development of functional tissues capable to work as biological substitutes.<sup>7</sup>

The particular advantage of this strategy over more conventional therapies, such as pharmaceutical drugs, is the potential ability to provide a permanent solution to the problem of organ failure.<sup>8</sup>

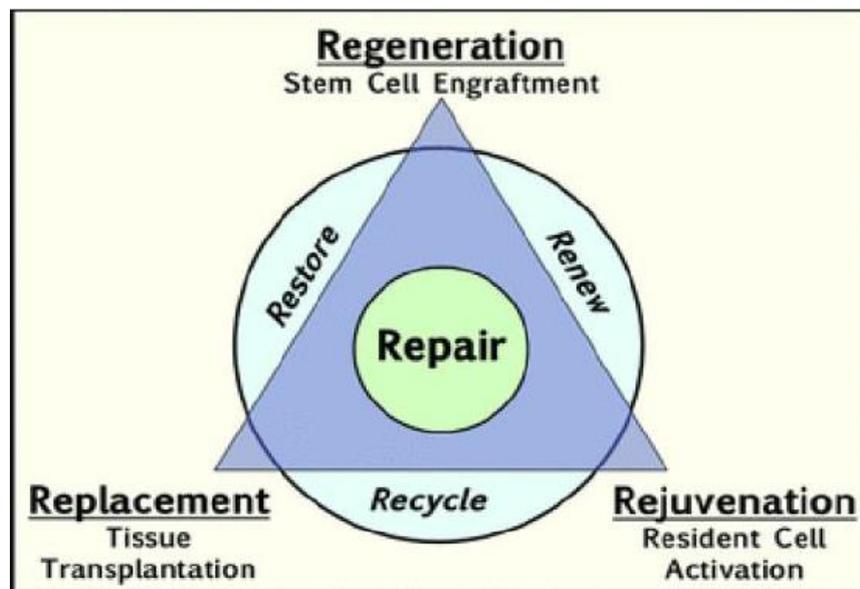
## 1.2 REGENERATIVE MEDICINE

The term regenerative medicine was first coined in 1999, bringing together the fields of cell transplantation, stem cell biology and tissue engineering in one unifying concept. <sup>5</sup>

With the gradual increment of human life expectancy, the number of patients in need of substitutes to repair defective tissues or organs exceeds by far the organ supply. Certain small creatures, salamanders or lizards, have the skill to regenerate a lost limb or tail, whereas higher animals, including humans, are incapable of re-growing anatomical structures of their body. <sup>8</sup>

Despite the complexity of the task, scientists aim to develop alternatives more efficient than donor organs. The final purpose of this field is to induce the regeneration of defective tissues or even organs in vivo, which in turn would restore and maintain the normal function of such structures. This idea is the essence of regenerative medicine, which is represented in Fig 1.1. <sup>6,9,10</sup>

When pursuing the reparation of damaged tissues, three approaches can be followed. Replacement of the faulty organ by a functional one; rejuvenation by the activation of endogenous resident cells; and regeneration by the implantation of stem cells. Advance in all these areas could lead to tackle virtually every defect of the human body by regenerative medicine. <sup>5</sup>



**Figure 1.1.** Therapeutic approaches of regenerative medicine to achieve the goal of reparation: regeneration, replacement and rejuvenation.

### 1.2.1 Applications

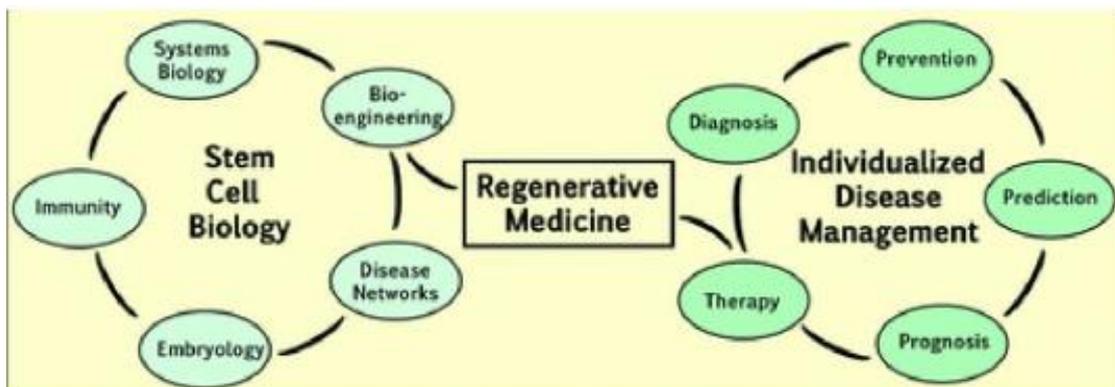
In the past decades, tissue engineering has been used in communion with gene therapy as a hybrid approach. This combination of bioartificial tissue derived from stem cells along with gene therapy may potentially provide regenerative tissue cells within an environment of optimal protein expression and regulation.<sup>5</sup>

Regenerative medicine is an interdisciplinary field whose action plan is summarized in Fig.1.2. By exploiting stem cell technology that focus on the engineering of tissues, treatment modalities could be designed in an individualized manner, thus improving the prediction or prevention of diagnosis and adapting the therapy to a case in particular.

Another relevant application would concern the design of natural-like tissue modelled constructs for basic research. By means of this technology, the utilization of animal models might be dramatically decreased, not only avoiding ethical issues but also discrepancies from human physiology presented even in moderately successful models. If based on human rather than animal cells, in vitro models would provide a more realistic and accurate system in terms of physiologic responses, which could enlighten cellular mechanisms yet unknown.<sup>9</sup>

The engineering of human cell-based in vitro pathological models could symbolize a paradigm shift from conventional monolayer cell cultures. While such technique generally lacks sufficient accuracy or fidelity, engineered biostructures towards more patient-specific approaches may reproduce relevant physiological aspects of the tissue-in question, not observable otherwise.<sup>5,8</sup>

Moreover, the production of three-dimensional 3D tissue-like cell constructs would enable to test the effect of pollutants or pesticides as much as the response induced by pharmaceutical compounds. It would allow high throughput screening for therapeutic drugs thereby decreasing the need for human organ harvest.



**Figure 1.2.** Schematic conceptual correlation between stem cell biology and individualized disease management through the application of regenerative medicine.

In the future, another application could be explored in relation to biotechnological production of meat in vitro to bypass factory farming and the associated problems. In spite of the great knowledge that is yet to be uncovered about such inquiry, the potential impact of this approach is immense. <sup>7</sup>

### **1.2.2 Influential factors**

The basic components of tissue engineering strategies are living cells and biomaterials, including both natural and synthetic matrices. Additionally, growth factors can be considered as well as essential in the process. <sup>5,11</sup>

The introduction of cells is designed to stimulate regeneration, promote vascularization, and supplement the production of hormones and growth factors. The biomaterials constitute an artificial structure known as “scaffold” which bring cells in close proximity so that they can assemble to form tissues. Ideally this structure should be capable of supporting tissue formation in three dimensions (3D) and regulate adhesion, proliferation, phenotypical expression and controlled extracellular matrix deposition. <sup>11,12</sup>

By controlling the adsorption of ions, proteins and other molecules from the surrounding medium, the chemistry of the scaffold surface would indirectly affect cell adhesion, morphology and in turn cellular activity. Whereas its physical structure might control cell function by regulating the diffusion of nutrients, waste products and cell-cell interactions by providing spatial and temporal control of biochemical cues. <sup>13</sup>

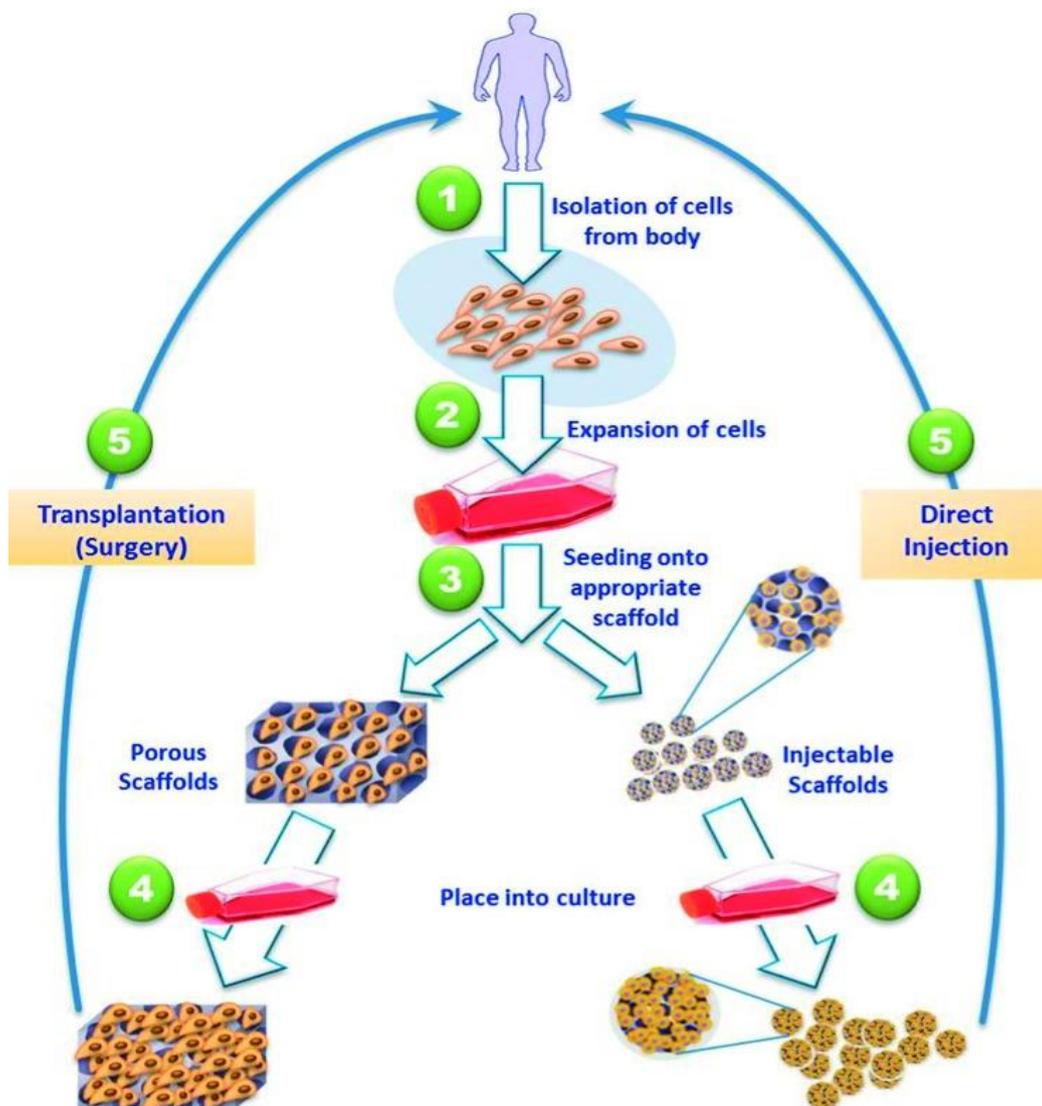
Nevertheless, there are other fundamental factors determining the outcome of new tissue, such as the culturing, concerning the maintenance of oxygen, pH, humidity, temperature, nutrients and osmotic pressure; and the control over growth factors. <sup>5,6</sup>

### **1.2.2 Tissue Engineering Methodology**

Tissue engineering diverge in three main strategies. To begin with, the injection of functional cells, which can either be cell substitutes or previously isolated cells. into a non-functional site aiming to provide cellular replacement and regeneration. Secondly, it is possible to use acellular biomaterials with the ability to induce tissue regeneration. Finally, as a third approach there is the combination of both cells and biocompatible materials, forming what is typically considered scaffolds to allow the creation of tissues de novo <sup>8,9</sup>

There are five main steps, which are illustrated in Fig 1.3, that are required to follow when growing new tissue by applying the mentioned factors:

- 1) Isolation of cells during biopsy by centrifugation or apheresis and later removal of the extracellular matrix holding the cells.
- 2) Cultivation and proliferation of cells to reach a sufficient concentration
- 3) Seeding into the scaffold maintained in a regulated environment.
- 4) Development of tissue promoted by a specific biomaterial, growth factors and hormones as well as mechanical stimuli, either physical or chemical.
- 5) Once the tissue is mature, it is ready to be implanted into the living body. <sup>9</sup>



**Figure 1.2.** Essentials of Tissue Engineering. Cells are isolated and expanded in a petri dish in laboratory. When the number of cells suffice, they are seeded on a polymeric scaffold material, and cultured in vitro in a bioreactor or incubator. Once the construct is matured, it could be implanted in the damaged area of the patient.<sup>6</sup>

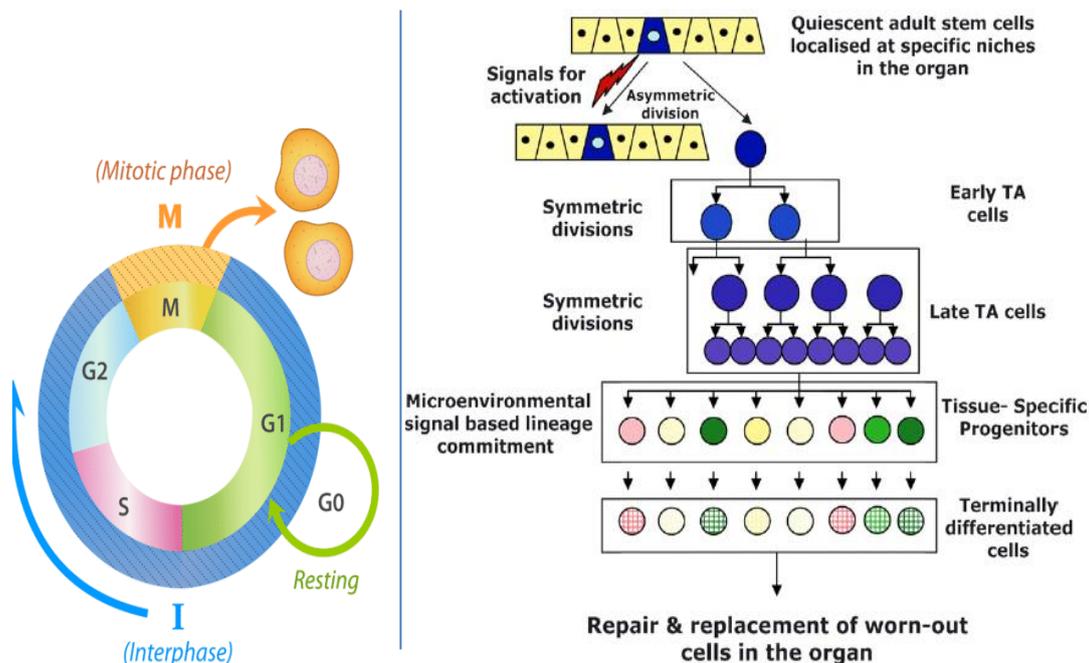
## 1.3 ROLE OF STEM CELLS

The biology of stem cells is closely related to tissue engineering. Their successful isolation and culturing supposed a radical change in the concept of regeneration. In essence, the emergence of stem cell research has made possible regenerative medicine.<sup>14</sup> Not only are stem cells the most recurrent tool concerning cell and tissue therapies, but also they are considered to play a role in regeneration just by their own stimulation, holding promise for the restoration of damages tissues and organs.<sup>5</sup>

In the future, stem cell transplantation might offer an unlimited and minimally invasive source of cells for vital organs replacement. However, previous steps are required to establish such clinical application, starting by the development of a model where functional cells can be differentiated *in vitro*, and followed by the design of a microenvironment for cell long-term maintenance.<sup>10</sup>

### 1.3.1 Cell self-renewal

Cells are the most basic building blocks of living organisms. Given that tissue engineering involves the growth of cells in culture, deep understanding of the cell multiplication process is required. Cell cycle, which is illustrated in Fig. 1.4, is usually coordinated with continuous growth of mass and protein synthesis. Furthermore, it displays an internal control, operating at specific checkpoints around the cycle before proceeding to a different phase, whose purpose is the interruption of the progress unless all appropriate conditions are fulfilled.<sup>15 6</sup>



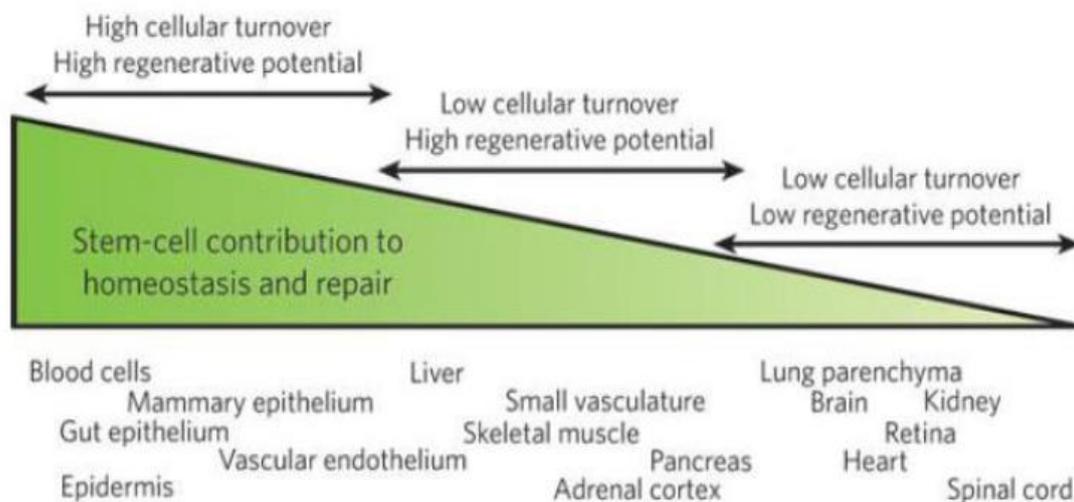
**Figure 1.3.** *Right:* cell cycle scheme.<sup>15</sup> *Left:* stem cell hierarchies in tissue regeneration and symmetry of divisions, for repair and replacement.<sup>16</sup>

In mature individuals, most cells are present in a quiescent state (G<sub>0</sub>) where there is no further division nor preparation for it. This resting phase can be as long as life span. However, this situation may be reverted by the presence of growth factors. The regenerative capacity in any organ or tissue is determined by the activation of quiescent stem cells localized at very specific niches in the organ. Although in vitro cells are often capable of exponential growth, this ability is rarely found in vivo. <sup>10,17</sup>

As cells develop from embryonic and fetal stages to an adult state, they become increasingly restricted to their differentiation. Hardly ever do they reverse this process afterwards. Most cell division in postembryonic life is found among stem cells or their immediate progeny. Stem cells possess the capacity of both self-renewal and generation of a progenitor that will lead to differentiation, known as asymmetric division. Other stem cells divide symmetrically, generating either two equal stem cells, or two differentiated cells for a specific tissue. Fig. 1.4 shows the range of possibilities when it comes to cell division. <sup>16,17</sup>

This arrangement establishes a well-defined hierarchy, on the top of which are quiescent or low proliferative stem cells. These will result in the transient amplification of early and late precursors capable of a higher proliferation, which in turn will produce lineage committed progeny. The division of the later will finally result into non-cycling terminally differentiated cells. <sup>16</sup>

This scheme applies to the majority of tissues, whose self-renewal capacity differ widely depending on the cellular turnover, as seen in Fig.1.5. For instance, epidermis and blood cells present great regenerative potential, whereas kidney and spinal cord cells are among the lowest. <sup>17</sup>

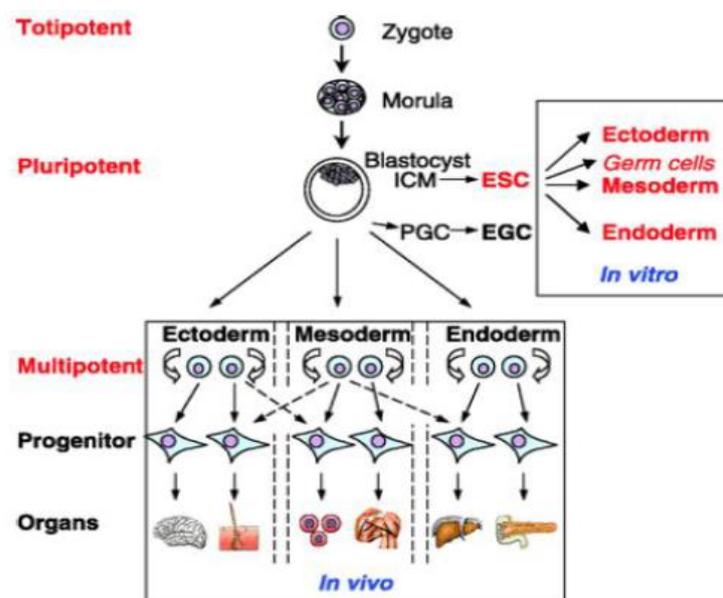


**Figure 1.4.** Stem cell homeostasis and repair. Cellular turnover and regenerative potential

### 1.3.2 Stem cell potentiality

Based on the ranged of differentiation potentials stem cells can classify as totipotent, pluripotent, multipotent, and unipotent. These classification is displayed in Fig. 1.6.

The zygote and blastomeres until three days old post-fertilization are totipotent cells with the ability to generate all types of cells. Cells derived from the inner cell mass of blastocyst, until fourteen days after oocyte fertilization, are pluripotent. This means they are capable to produce all cell types from all three embryonic germ layers (i.e., ectoderm, mesoderm and endoderm), excluding the extra embryonic lineage. On the other hand, multipotent stem cells possess the ability to differentiate into all cell types within one particular lineage whereas unipotent stem cells have the competency of differentiating into only one lineage.<sup>18</sup>



**Figure 1.6.** Classification of stem cells according to their potentiality and their source of origin.

### 1.3.3 Stem cell sources

Besides, stem cells can also be classified based on their source of origin.

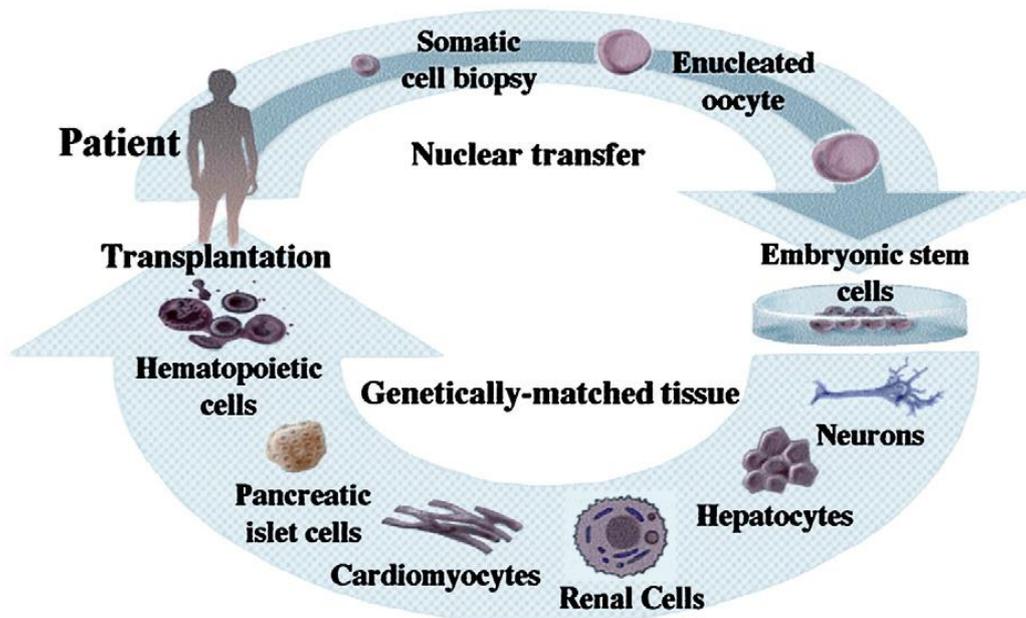
In first place, embryonic stem cells (ESCs), which derived from the inner cell mass of preimplantation embryos. They exhibit the properties of proliferation to an undifferentiated but pluripotent state, and differentiation into many specialized cell types.<sup>18,19</sup> Five days post-fertilization, during the blastocyst stage, isolation of ESCs can be taken by aspirating the inner cell mass from the embryo. They are capable to differentiate *in vitro* into cells from all three germ layers. Nonetheless, since ESCs represent an allogenic resource, clinical application is limited due to the potential to evoke an immune response. Moreover, when implanted they have been shown to form teratomas.<sup>9,18</sup>

Secondly, adult stem cells, scattered in various tissues and organs, capable to produce at least one type of differentiated functional progeny. One of the limitations of cell-based approach for organ replacement is the inherent challenge of growing specific cell types in large quantities in vitro. For this aim, acquiring native targeted progenitor cells from the organ of interest would signify a considerable advantage. Said progenitor cells do permit the possibility to be expanded, and implanted in the same person without rejection.<sup>20</sup>

Another approach is the reprogramming of somatic cells, which are already differentiated, to a pluripotent state, which would avoid ethical or allogenic issues. Until recently, there was only two main strategies to pursue that goal, either by nuclear transfer into oocytes or by fusion with ESCs.<sup>21,22</sup>

Fig. 1.7 represents the procedure by which the nucleus from a somatic cell isolated from the patient is transfer to a previously enucleated oocyte, in order to generate embryonic stem cells with the same genetic charge as the patient. Said stem cells are then differentiated into the corresponding tissue that needs to be transplanted and the immunogenic reaction of the organism will be avoided.<sup>9</sup>

Seeing as such techniques suggested that unfertilized eggs and ESCs harbour certain factors to confer pluripotency to adult cells, a decade ago the essentials of the process were uncovered by Takahashi and Yamanaka. They fist characterized the induced pluripotent stem cells (iPSCs), achieved through the presence of only four transcription factors, which has become one of the most impactful discoveries in tissue engineering and regenerative medicine.<sup>23</sup>



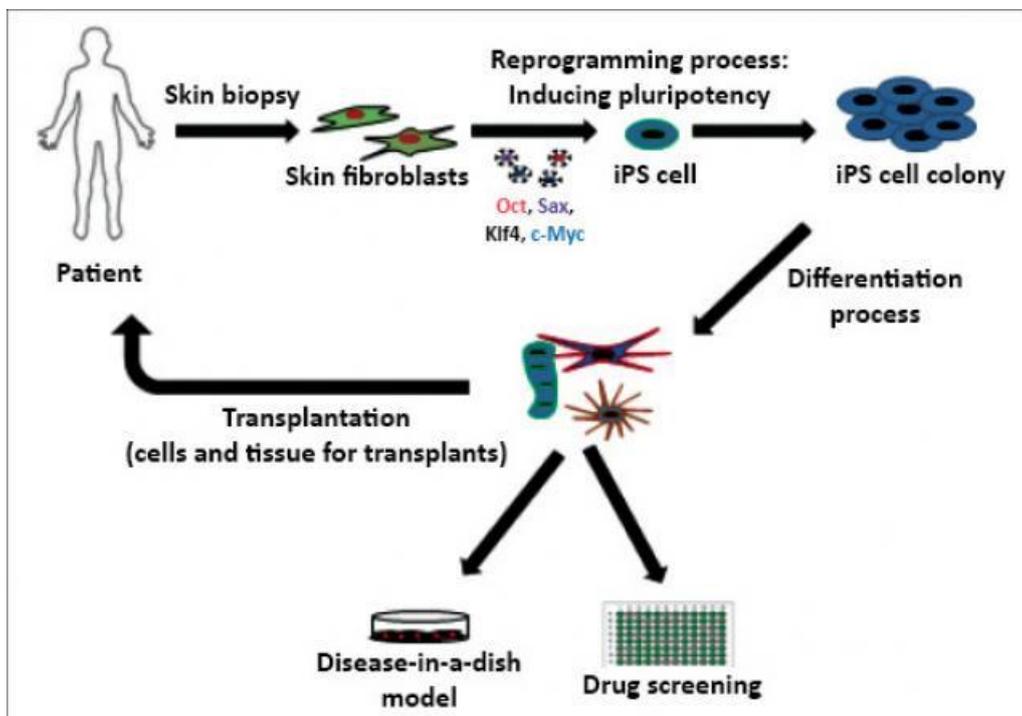
**Figure 1.7.** Strategy for therapeutic cloning by nuclear transfer and engineering of matched-tissue as biological substitute.<sup>9</sup>

The original “cocktail” to generate iPSCs requires only the transcription factors Oct3/4, Sox2, c-Myc and Klf4, depicted in Fig. 1.8. Despite the simplicity, current research investigates a further optimization of the process. Novel strategies focus on such as the expression of specific micro-RNAs (miRNAs) to replace the reprogramming factors; or direct reprogramming by retroviral injection, avoiding the pluripotent stage thereby reducing the risk of tumour formation. <sup>20,23,24</sup>

During the process, the addition of reprogramming factors leads to numerous changes, involving physical structure, gene expression and proliferative capability. Functional genes from the somatic cell are switched off to activate the endogenous stem cell-associated genes. All these variations establish the induction of pluripotency and generation of embryonic stem-cell-like iPSC. <sup>25</sup>

The key advantage of iPSC over other stem cells is that are generated from a patient’s own cells and thus, specific and immuno-compatible. Moreover, they can be grown in infinite amounts and could circumvent ethical and religious controversies associated with ESC. <sup>10,26</sup>

The target cells offer the possibility of conducting ‘clinical-trials-in-the-dish’, for disease models, providing a platform for drug screening, and gene/cell manipulations and therapy in pre-clinical investigation, as well as to bulk up cells to be transplanted into the cell donor. <sup>5 25</sup>



**Figure 1.8.** Medical and research potential of iPSC technology. The reprogramming process of a somatic cell into an iPSC and back to a differentiated state only requires the uptake of transcription factors and the newly differentiated cells have great medical applications. <sup>25</sup>

### **1.3.4 Applicability of stem cells**

The field of regenerative medicine shows great interest in stem, progenitor, and differentiated cells, derived from both adult and embryonic tissues. Despite the broad investigation in all of them, nowadays tissue-derived cells represent the dominant strategy when it comes to approved clinical therapies.<sup>20</sup>

The inclination towards this cell type in special can be explained by both their ready availability and perceived safety. Current research is focus on seeking stem cell populations in locations that are yet to be identified and obtaining greater numbers of stem cells from adult tissues.<sup>19</sup>

Both ESCs and iPSCs are potentially infinite sources of cells for tissue engineering and are rapidly progressing towards a clinical application.<sup>5,26</sup> Nonetheless, due to the risk of formation of tumours by ESCs and iPSCs, a tight control over cell fate in every one of them is essential for the safety. For this purpose, high-throughput screening of iPSCs can define the optimal dosages of developmental factors in order to obtain lineage specification and minimize persistence of pluripotent cells. Finally, the same basic principles applied to the engineering of cellular grafts from differentiated cells are being leveraged to create suitable microenvironments for reprogramming.<sup>10,27</sup>

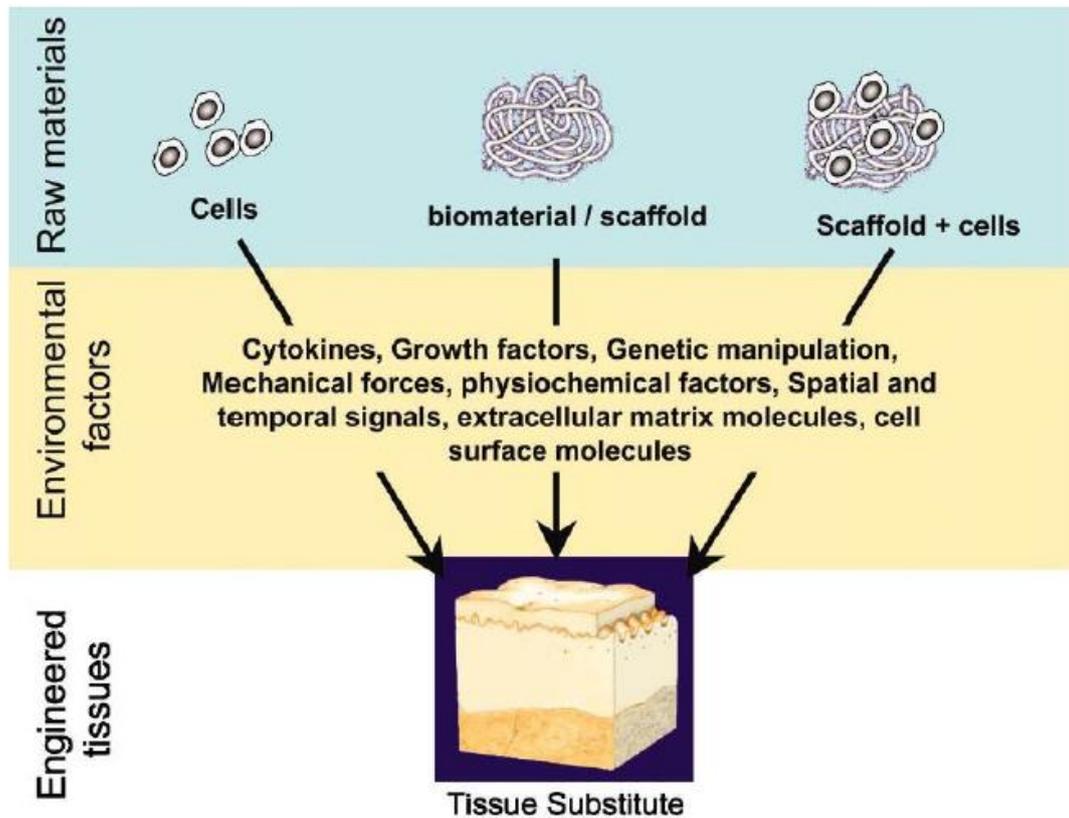
## **1.4 ROLE OF SCAFFOLDS**

Scaffolds can be natural, manmade, or a composite of both. Biomaterials forming the scaffold are essential to guide the direction of new tissue growth as well as provide an optimal spatial environment to restore the structure and function of certain tissue. In addition, bioactive molecules, growth factors or cells from the organism may be attracted by such biomaterial once this is implanted.<sup>6</sup>

In summarize, the key functions scaffolds are to deliver the seeded cells to the desired site in the receptor body, enhance cell-biomaterial interactions and cell adhesion and provide an adequate transport of nutrients, growth factors and gases to ensure cell survival, proliferation, and differentiation. Besides, scaffolds should also confer negligible inflammation or toxicity in vivo, and control the structure and function of the engineered tissue.<sup>28,29</sup>

Even more, it is also possible to implant certain biomaterial without cells, in this case the purpose of the procedure is to encourage the body's natural ability to repair itself.<sup>8</sup>

The scaffolding components, processing and the environmental factors involved, such as cytokines, growth factors, forces, ECM and surface molecules to produce an engineered graft or tissue, are depicted in Fig. 1.9.



**Figure 1.9.** Basic scheme of scaffolding techniques and components involved in the process <sup>8</sup>

There are three classes of biomaterials that are regularly used for engineering tissues and organs.

The first type are naturally derived materials, such as collagen, chitosan and algae derived alginate. These hydrogels are composed largely of water and are often used to fabricate scaffolds due to their compositional resemblance to living tissue. <sup>12,30</sup>

The second class regards synthetic polymers, such as polycaprolactone (PCL), polyglycolic acid (PGA), polylactic acid (PLA), polylactic-coglycolic acid (PLGA), and poly(ethylene glycol) among others. <sup>31</sup>

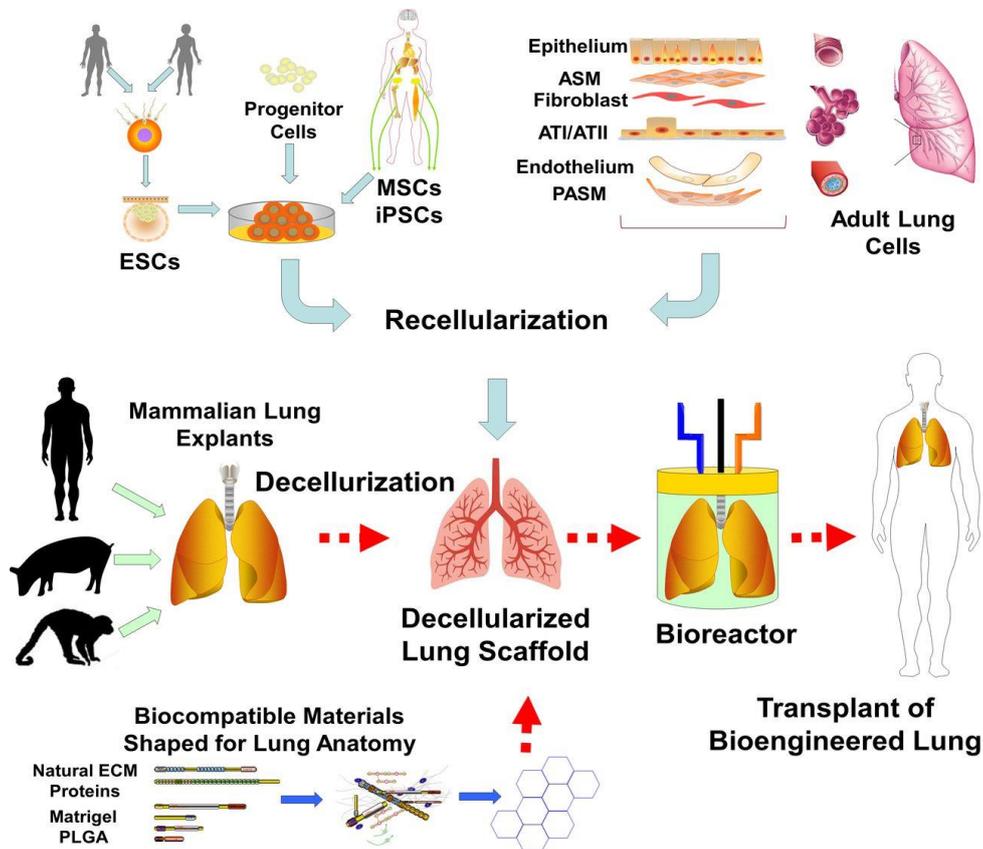
The last category includes acellular tissue matrices achieved by decellularization of organs combined with the preservation of the structure of the blood vessels. Cells can be reintroduced by perfusion through the remaining vascular skeleton to reanimate the organ tissue prior implantation, as in the case of submucosa from bladder and small-intestine. <sup>26,32</sup>

Both naturally derived materials and acellular tissue matrices have the potential to be biologically recognized by the organism into which they would be transplanted. <sup>32</sup> Nonetheless synthetic polymers present the advantage of easier

fabrication and higher level of reproducibility on larger scale once properties such as strength, degradation rate, and microstructure are managed under control.<sup>9</sup>

Currently, regenerative medicine studies focus preferably on the employment of acellular matrices and synthetic materials.<sup>33</sup>

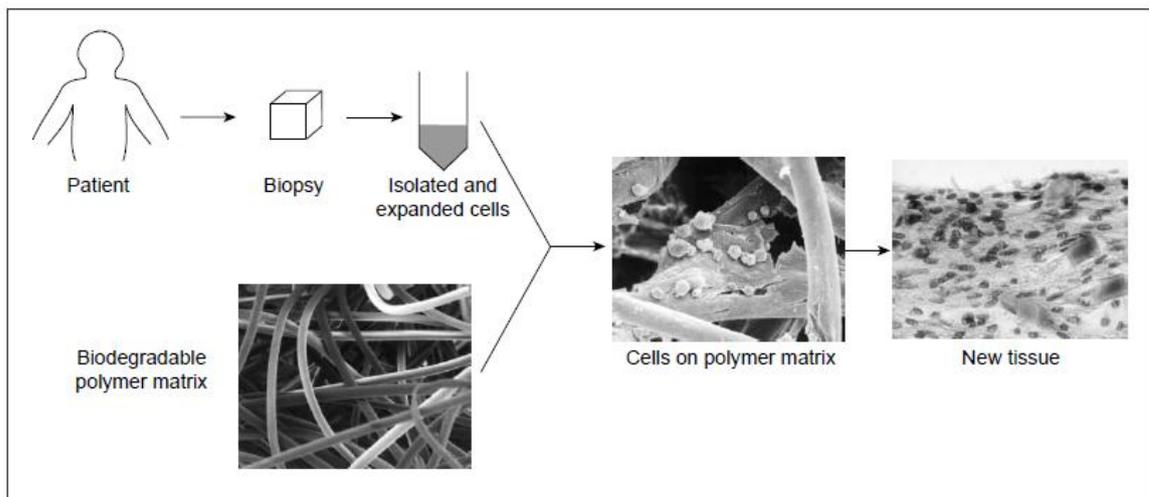
Acellular tissue matrices have had a major impact in scaffolding. They support cell ingrowth and regeneration of genitourinary tissues with no signs of immunogenic rejection. These matrices, usually collagen-rich, are prepared by mechanical and chemical manipulation of a segment of tissue and after implantation they will slowly degraded and replaced by proteins from the extracellular matrix. Living cells can be associated with the construct before transplantation or migrate into the matrix afterwards, once it is already in the human body. Fig. 1.10 illustrates the de/recellularization process.<sup>6,9</sup>



**Figure 1.10.** Example of decellurization performance to bioengineer lungs.<sup>26</sup>

Additionally, fabrication of synthetic scaffolds that possess specific aspects of the material properties and structure of target tissue is currently investigated. Polyesters of  $\alpha$ -hydroxy acids, as the above mentioned PGA, PLA, and PLGA, have been approved for human use in a broad range of applications. One of the fields where their employment is widely spreading is regenerative medicine.<sup>9</sup>

Polymers can be engineered to be biodegradable, enabling gradual replacement of the scaffold by the cells seeded in the graft and by host cells, as shown in Fig. 1.11. They produce nontoxic degradable metabolites that will be eventually eliminated from the organism in the form of carbon dioxide and water. Besides, their thermoplasticity favour the construction of 3D scaffolds with a specific desired structure, shape, and dimension by a number of techniques. Electrospinning has been used to quickly create highly porous scaffolds in various conformations.<sup>2,8,9</sup>



**Figure 1.11.** Tissue engineering approach by seeding cells onto biodegradable polymer ECM, that results in completely natural new tissue after its elimination.

Biomaterials that encompass a large spectrum of mechanical properties are being studied. Not only is bulk mechanical support sought, but also provision of instructive cues to adherent cells, depending on the features of the polymer selected. For instance, soft fibrin–collagen hydrogels have been studied as lymph node mimics, whereas rapidly degrading alginate seeks to repair or regenerate bone defects.<sup>8</sup>

However, one drawback of the synthetic polymers is lack of biologic recognition, although a number of groups are attempting to design synthetic scaffolds which incorporate proteins or other molecules to assist in recognition.<sup>1,3</sup>

## 1.5 BIOFABRICATION TECHNIQUES

In vivo cells encounter a complex physical and chemical environment that differs greatly from common culturing conditions. 3D cell culture techniques are designed to stimulate the environment in a similar fashion.

Biofabrication is a facet of tissue engineering where 3D tissue-like structures comprised of biomaterials and cells are generated to achieve the highest resemblance to the native conditions and architecture.<sup>6</sup>

Traditionally, formation of 3D constructs is accomplished by seeding cells onto pre-shaped scaffolds of porous polymers or by casting a cell-seeded hydrogel into a mold. The product consequent of these approaches achieve the geometrical complexity but lack a defined cell distribution.<sup>11,34</sup>

### **1.5.1 Seeding onto scaffold**

Seeding of cells onto solid scaffolds entails the problem of difficult distribution at specific locations. Moreover, static cell seeding of constructs is inefficient and usually leads to a heterogeneous cell distribution.<sup>34</sup>

### **1.5.2 Hydrogels**

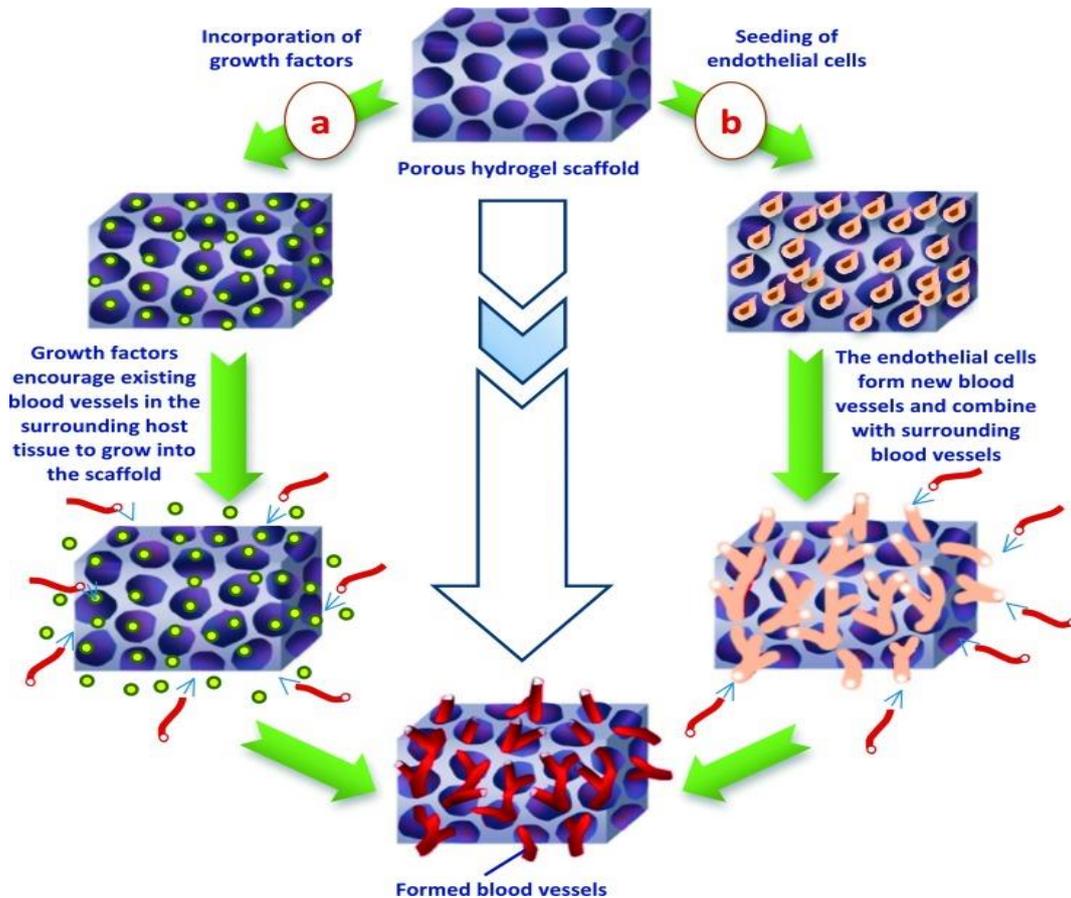
Cell-laden hydrogels are 3D networks composed of hydrophilic polymers crosslinked either through covalent bonds or held together via physical intramolecular and intermolecular attractions. Said crosslinking enables them to swell while retaining their 3D structure without dissolving, which is the principle feature that differentiates them from gels.<sup>28</sup>

Biologically speaking, they are very attractive candidates for the incorporation of cells or other bioactive compounds in biofabrication. The reason why is the provision of an instructive, highly hydrated 3D environment, which recapitulates several features of the natural extracellular matrix, as much as a desirable framework for living cells.<sup>6,34,35</sup>

Hydrogels used in regenerative applications are predominantly based on naturally derived polymers. Alginate, gelatine, collagen, chitosan, fibrin and hyaluronic acid are often utilized for such goal.<sup>28,30</sup>

The abundance of biologically-relevant chemical and physical signals present in these materials are beneficial for cell viability and proliferation. Such signals lead to the development of specific neo-tissues. Furthermore, they favour an efficient and homogeneous cell seeding and allow for various shapes and biomechanical characteristics.<sup>35</sup>

There are two main methodologies by which hydrogels can be employ as scaffold. Fig. 1.12 shows both approaches applied to the bioengineering of blood vessels, (a) by incorporating growth factors or (b) via seeding of endothelial cells into the porous hydrogel.<sup>28</sup>



**Figure 1.12.** Schematic illustration of blood vessels formation encouraged by either (a) incorporating of regulatory growth factors or (b) via seeding of endothelial cells into the porous hydrogel scaffold. <sup>28</sup>

Although progress in hydrogels for enhanced bioactivation, cell survival and tissue formation is raising up, the lack of control over the physio-chemical demands for biofabrication results in a major hurdle for this field. <sup>30,35</sup> Therefore, the successful creation of a printable hydrogel capable to support cell adhesion, migration, and differentiation will significantly advance this promising approach for regenerative medicine. <sup>11</sup> Nevertheless, recent advances have enabled novel strategies for biofabrication, such as 3D printing and electrospinning. <sup>3</sup>

### 1.5.3 3D bioprinting

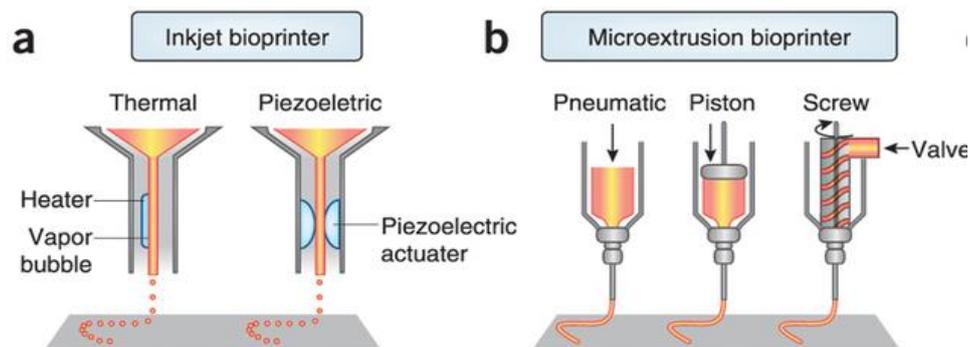
Bioprinting of cells, along with supporting and biocompatible materials into complex 3D functional living tissues, is being applied to tissue engineering to address the high demand of suitable organs for transplantation. <sup>34</sup>

3D bioprinting involves additional complications if compared to non-biological printing. Among them, selection of biocompatible materials, cell types, growth and differentiation factors, and sensitivities of living cells to their microenvironment. <sup>8,36</sup>

This exciting tool displays high resolution control over material and cell placement and has been proved useful for the creation of several tissues to be transplanted. In addition, mimicking the environment of the cells, printed grafts may be utilized to study cell–cell and cell–matrix interactions in development studies as well as models for research, drug discovery and toxicology.<sup>34,36</sup>

Two of the most typical bioprinting strategies are inkjet and microextrusion, whose processing variations are illustrated in Fig.1.13.<sup>36</sup>

Inkjet bioprinting creates brief waves, either electrical heating (thermal) or acoustic (piezoelectric) by pressure pulses, releasing droplets of cell containing ink at the nozzle. On the other hand, microextrusion bioprinting uses pneumatic or mechanical (piston or screw) dispensing systems to extrude continuous stream of material and cells onto a stage. Both are being actively used to fabricate a wide range of tissues.<sup>11</sup>



**Figure 1.13.** 3D bioprinting techniques. (a) Thermal inkjet printers electrically heat the tip to force droplets from the nozzle by air pressure, whereas acoustic printers use pulses formed by piezoelectric or ultrasound pressure. (b) Microextrusion printers use pneumatic or mechanical (piston or screw) dispensing systems to extrude continuous beads of material and/or cells.<sup>11</sup>

Despite inkjet printers have been proved to create cell-laden fibres and structures for scaffolding, their size reaches the few hundreds of micrometers as minimum, if not larger. Moreover, the assessment of cell viability needs yet to be further investigated as populations from a molecular level upwards, to clear doubts regarding the cell's health post-treatment. It has been postulated about this method that could lead to the manifestation of shearing or genetic alterations on cells due to the stress entailed by the physical throughout the procedure, thereby increasing cell mortality rates.<sup>3,8,36</sup>

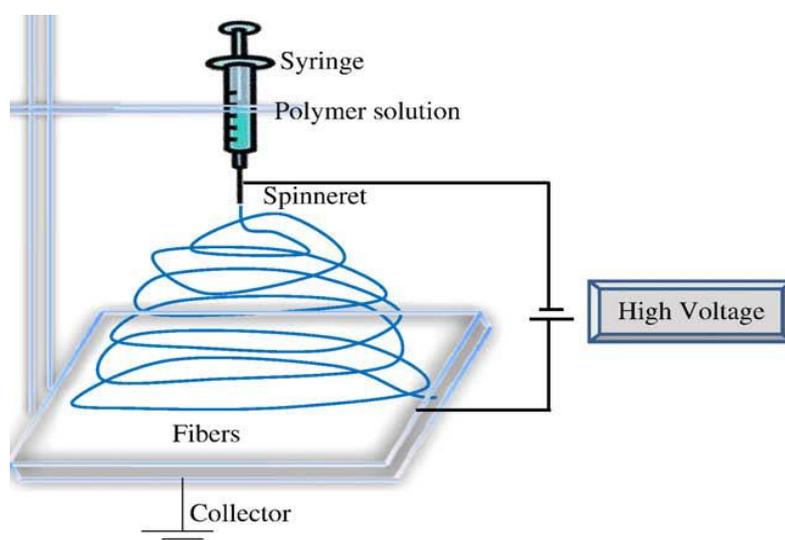
Although extremely promising, bioprinting strategies suffer trade-offs in terms of feature and printing resolution as well as cell viability. The development of technologies that excel in these three aspects would signify a remarkable progress in this field.<sup>34,36</sup>

### 1.5.4 Electrospinning

Electrostatic spinning is a simple technique which utilizes high electrostatic forces to produce polymeric fibres with diameters on the nano and micrometer scale. Its origin can be traced back to half century ago and its currently applied numerous areas, such as, nanocatalysis, protective clothing, filtration, optical, healthcare, environmental, and the one of interest to this study, tissue engineering.<sup>37,38</sup>

There are basically three essential components to fulfil electrospinning: a high voltage supplier, a capillary tube with a needle of small diameter, and a metal grounded conductor acting as collecting screen. Besides, a syringe containing the polymer and a pump to push the solution are required for the process.<sup>39</sup>

A typical electrospinning set-up is illustrated in Fig. 1.14.



**Figure 1.14.** *Left: schematic diagram of electrospinning process and basic components involved. Right: picture of set up of single cell electrospinning machine.*<sup>37</sup>

Overall, this is a relatively robust and straight-forward technique resulting from the combination of electrospray and spinning. In this system, a high voltage is applied to the droplet of a fluid creating an electrically charged jet of polymer solution or melt out of the tip of a needle, which acts as one of the electrodes. This leads to the deformation of the droplet and the final ejection of a charged jet from the tip of the cone, which accelerates towards the counter electrode as the solution jet is either evaporated or solidified. Consequently, continuous nanofibers are generated and then collected as an interconnected web.<sup>38 39</sup>

One electrode is placed into the polymer solution and the other attached to the collector. The electric field is subjected along the capillary tube that holds the fluid by its surface tension, thus a charge will build up on the surface of the liquid.<sup>40</sup>

The electrostatic repulsion and the external electric field cause a force directly opposite to the surface tension of the fluid. As the intensity of the electric field is boosted, the hemispherical surface of the fluid at the tip of the needle elongates to create a conical shape, known as the Taylor cone, shown in Fig. When both forces grow stronger by raising the electric field they overcome the surface tension of the solution and a charged jet of liquid is ejected from the tip of the Taylor cone.<sup>37,39</sup>

As the newly formed jet travels towards the collector, it becomes unstable and elongated by a whipping motion provoked the interaction between its surface charges and the external electric field. This process allows the jet to stretch further and become very long and thin, while the solvent evaporates, giving a solid polymeric fibre as result. Such fibres are randomly deposited onto the collector due to the whipping process aforementioned.<sup>3,39</sup>

Among the several advantages that electrospun nanofibers offer are the extremely high surface-to-volume ratio, porous control, malleability to different sizes and shapes and the ability to control the nanofiber composition to achieve the desired results from its properties and functionality.<sup>3,37</sup>

## **1.6 COAXIAL ELECTROSPINNING**

One of the promising techniques that electrospinning offers is the use of a coaxial tube spinneret with two different solutions, the use of which yields a core shell fibre architecture.<sup>1</sup>

For this purpose, a coaxial nozzle can be employed, with an inner and out nozzle, where each feed their own solution from a pump system. The inner nozzle will be the core and the outer nozzle the shell, which Fig. 1.15 illustrates. Such arrangement allows the fabrication of core-shell structures. The inner solution is sustained at the edge of the outer compound nozzles to undergo the creation of the Taylor cone. Then, in a similar manner as in the single electrospinning process. The jet will be pulled by the electric current and stretched by the bending instability from the droplet. During that time, the solvent evaporated and the compound jet solidifies, yielding the already mentioned core-shell fibres.<sup>41</sup>

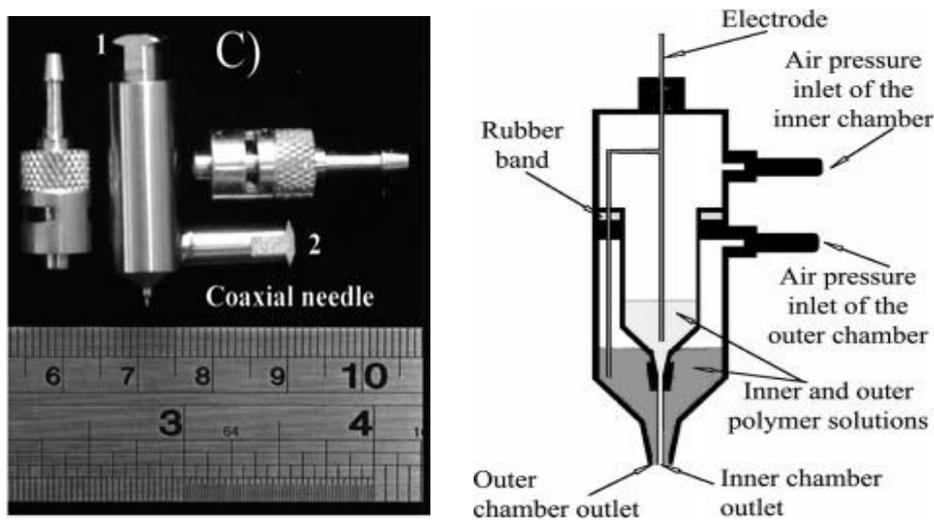
The co-electrospinning procedure should be fast enough to prevent any sort of mixture between the core and shell polymers, as much as any other material embedded on them. To yield a successful fibrous structure, a minimum viscosity is required to maintain the solution in a sufficient entangled regime.

This technique is particularly attractive for the employment of not spinnable materials, either for the shell or core. By means of this strategy, fibres with a liquid core or even a hollow tube fibre could be fabricated. Moreover, core-shell fibres from solutions of miscible polymers are also possible to electrospun, since typically the diffusion is significantly longer than the time it takes the solvent

evaporation. Fibres resulting from this approach usually present a distinct boundary between the core and shell <sup>42,43</sup>

The ratios between the diameter of the core and shell can be modified by adjusting the flow rate, which in turn will change the diameter of the core whereas the size of the shell remains relatively invariable. <sup>37,38</sup>

In contrast with single-component products, core-shell fibres provide a feasible route for a better control of the release of chemicals embedded, which would mean another application in drug delivery. This feature could be achieved by adjusting the fibre microstructure as well as the fibre diameters. <sup>40</sup>



**Figure 1.15.** Left: Basic components of coaxial electrospinner needle. <sup>4</sup> Right: nozzle arrangement for co-electrospinning of core-shell fibres. <sup>41</sup>

### 1.6.1 Cell electrospinning

The electrospinning of cells was first described in 2005 where post-treated cells required to fully assess the cells well-being in a genetic, genomic and physiological level. After several well-established cell viability checks from both single cells and entire populations, survival was demonstrated. Cell suspensions inside the fibre core suffered almost no cellular damage during the electrospinning process and remained active. Such findings led to the idea of whether is feasible to apply this approach to the direct generation of cell-bearing fibres and scaffolds. <sup>43</sup>

Though the focus of this report relies on coaxial electrospinning, other needle configurations are also possible, allowing even different cells types to be compartmentalized within three immiscible media. <sup>3</sup>

The ground electrode in cell electrospinning has significant differences from those which belong to standard set-ups. The collection of the generated fibres

takes place on conducting grounded or oppositely charged mesh, that allows the deposition of the mat in an aqueous medium followed by a later immersion in cell culture media. The primary reason behind such variation is because the newly formed fibres that harbour the cells must not dehydrate as this will negatively influence their viability. <sup>3</sup>

Cells must be maintained at all times within a friendly environment that is capable to accommodate the demanding metabolism of cells. <sup>4</sup>

Regarding its application to tissue engineering, this approach provides a mechanism to fabricate a biomaterial scaffold with controllable distribution of cells throughout the scaffold. Furthermore, the control of fibre shell porosity can be optimized according to the cell needs, and produce an immuno-barrier for implantation. Current research in this field seeks to address the suitability of such constructs for in vivo implantation and release studies of bioactive molecules. <sup>1</sup>

### **1.6.2 Electrospinning parameters**

The influential factors that can effectively alter the outcome of the electrospinning process, regarding the final disposition and morphology of the fibres can be grouped into the three following categories.

#### **Set-up Parameters**

The external conditions at which the electrospinning process is taking place can have certain impact on the fibre morphology.

Voltage is a crucial parameter to promote the surmounting of the surface tension and initiate a Taylor cone. If the voltage applied is too high, the polymer solution that is being fed will not be sufficient for such prolongation thereby provoking the recession of the Taylor cone back into the needle. On the other hand, the effect on morphology induced by the voltage is yet to be defined. Generally, stronger stretching of the jet reduces fibres diameter but the actual result depends mostly on the polymer parameters and set-up. The application of higher voltage tends to cause the formation of beads, however, when reaching certain values, it will generate additional jets which ultimately will yield fibres of a heterogonous distribution of diameters. <sup>4,37,44</sup>

Variations on the spinneret–collector distance also has a clear influence on the electric with similar consequences to the alteration of the voltage. Furthermore, it has an additional effect on the solvent evaporation. There is an optimal time that allows the sufficient evaporation for the achievement of smooth fibres. Nonetheless, not only can too short distances present evidence of bead formation

but large distances as well, likely due to less stretching resulting from a less intense smaller electric field. <sup>4</sup>

Thirdly, seeing as the flow rate determines how much material is ejected, an increment in this rate involves a larger fibre diameter and bead size. In the majority of cases a low flow rate is preferred as this permits the solvent having enough time to evaporate. In the opposite situation, the jet cannot evaporate in time prior deposition on the collector, leading to bead formation. <sup>4,38</sup>

Concerning the collector, electrically grounded conductive ones are often utilized. If the conductivity of the collector is not high enough, charge will be able to accumulate thus the repulsion of like-charges will be increased, resulting in lower packing density of the fibres. <sup>38</sup>

Lastly, the diameter of the internal needle can also affect the process. A smaller orifice decreases the fibre diameter, clogging and bead formation. The plausible explanation for such phenomenon would be the less exposure of the solution to the atmosphere, reducing the amount of dried solution at the tip of the needle. Moreover, since the amount of solution at the tip will be diminished, the surface tension will increase, hence a greater voltage is needed to initiate the jet. <sup>4,38</sup>

### **Solution parameters**

The relevance of polymer solution properties is great in order to provide a spinnable fluid and they have a big influence on the morphology of the fibres and mat structure.

Surface tension of the polymer solution is a critical factor since the initiation of the jet requires to overcome it. This tension largely depends on the solvent composition and it can be adjusted by addition of new miscible components or surfactants which reduce it. Lower surface tension permits the formation of the jet with an electric field of lower intensity, contributing to less beads and more smooth fibres. <sup>37,39</sup>

Another solution parameter is viscosity, affected by the concentration and molecular weight of the polymer. If a jet is drawn from a solution with too low viscosity the jet will break as it is stretched and no continuous fibres will be deposited on the wafer. On the contrary, if too high, the ejection of a jet will be challenging. <sup>43</sup>

Besides, low concentration and molecular weight will lead to bead formation. The molecular weight entails the molecular entanglement which holds the jet together through the stretching process. When both features are incremented, such beads will elongate from spheres to spindle like structures. Furthermore,

higher viscosity increases the diameter of the fibres meanwhile decreasing the deposition area, which results in higher uniformity in the fibre diameter.<sup>38,42</sup>

The charge of the jet depends on its conductivity which in turn depends on the polymeric material, the solvent and their ionizability. The conductivity can be increased by modifying the pH or ion concentration hence decreasing the diameter of electrospun fibres. A higher conductivity raises the electrostatic repulsion thus enhancing the stretching which will produce thinner fibres. Whereas too low conductivity will not permit sufficient whipping of the jet, leading to thicker fibres and bead formation.<sup>4,37,44</sup>

### **Ambient Parameters**

Electrospinning could be affected by the environmental conditions as well. For instance, high temperatures may diminish the solution viscosity which in turn would yield thinner fibres. Besides, it would raise the evaporation rate of the solvent.<sup>45</sup>

Humidity, on the other hand, should be high enough so volatile solvents do not dry too quickly, leading to the clogging of the tip of the needle. A more humid environment would induce the condensation of water on the surface of fibres, inducing pore formation and a larger diameter.<sup>4,38,</sup>

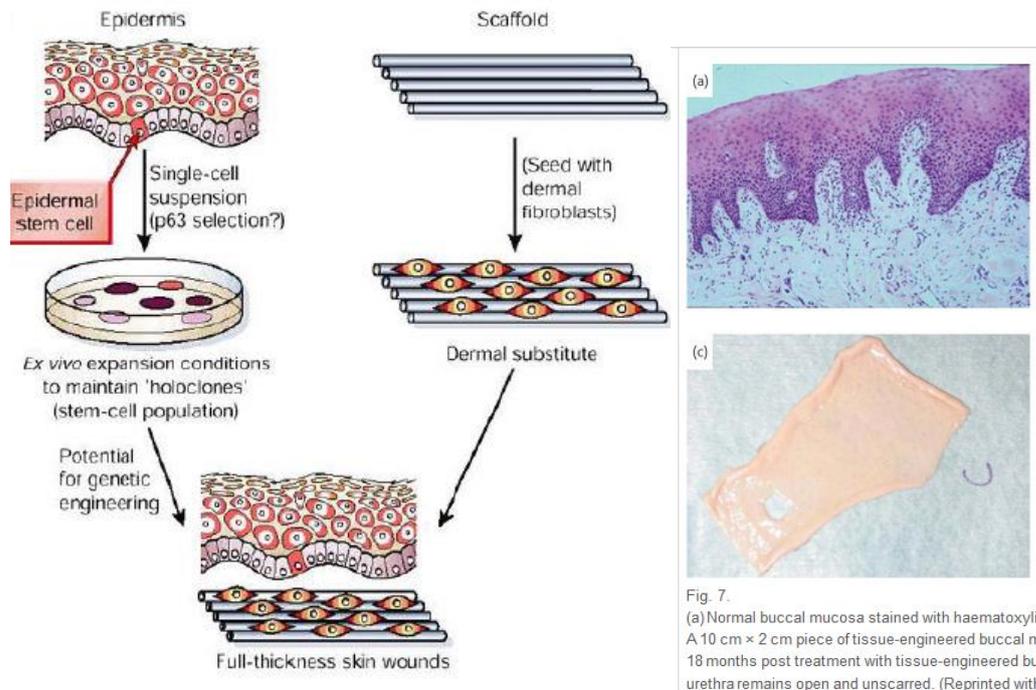
In conclusion, playing with all these factors it is possible to direct the process towards a desired direction, for example, pore dimensions can be optimized by increasing flow rate and polymer concentration, leading to higher viscosity and larger fibre diameter which in turn entails larger pore space.<sup>46 4</sup>

## **1.7 ACHIEVEMENTS AND CHALLENGES**

The science of tissue engineering has been explored for barely two decades. To date, most of the advances in the field are associated to the development of model systems. The study of such constructs has enlightened basic principles of cell biology disciplines related to regenerative medicine. The interaction of different scientific areas together could be key to elucidate missing information.<sup>6,33</sup>

Scaffolds can be molded to fit the particular anatomy of the tissue receptor proving a substantial control over the spatial distribution of cells. New approaches are improving the integration of grafts within the host vascular and nervous systems through the release of growth factors and cell seeding in a controllable manner, enhancing healing response of the body even by immune system modulation.<sup>7,8</sup>

Organs such as skin and cartilage has been successfully replaced by bioartificial substitutes. In the case of skin, replacements, which are shown in Fig.1.16, are relatively simple. They serve to the purpose of covering the body but the lack of immune cells prevents a higher degree of protection, among other physiological functions.<sup>33</sup>



**Figure 1.16. Left:** methodology of engineered skin substitutes. **Right:** graft images for the replacement buccal mucosa.<sup>47</sup>

Furthermore, tissue-engineered vascular grafts are entering clinical trials, for treating congenital heart defects in human patients. A variation on this strategy regards the fabrication of blood vessels in vitro and their subsequent decellularization before placement in patients requiring kidney dialysis.<sup>8</sup>

Another decellularized tissues, without posterior re-seeding, have also reached the market as medical devices and have been used as substitutes for some muscle defects.<sup>13,48,49</sup>

In some cases, the polymer's mechanical properties alone are believed to produce a therapeutic effect. For example, injection of alginate hydrogels to the left ventricle reduced the progression of heart failure in models of dilated cardiomyopathy and is currently undergoing clinical trials.<sup>24,36</sup>

Despite the significance of such progress, several challenges remain unsolved for the majority of organs.

Mechanical properties of tissues might be affected by the decellularization process, removing several types of ECM signalling molecules.<sup>5,50</sup>

Moreover, the transplanted tissue could degenerate gradually over time if there is not a progressive replacement by host cells. Hence, the procedures used to strip cells and other donor immunogenic components and recellularize the tissue before implantation are being actively optimized.<sup>8</sup>

Other obstacles involve the limited supply of immunologically compatible cells with the receptor body. Identifying renewable sources and obtaining sufficient numbers of therapeutic cells is often a challenge. Besides, biomaterials with desired mechanical, chemical, and biological properties are insufficient; and they are not able to generate large vascularized tissues of easy integration into the patient's circulatory system.<sup>8,9,33</sup>

In summarize, a number of issues will be important for the advancement of regenerative medicine as a field. First, stem cells, whether isolated from adult tissue or induced, will often require tight control over their behaviour to increase their safety profile and efficacy after transplantation.<sup>5</sup>

The creation of microenvironments, often modelled on various stem cell niches that provide specific cues, including morphogens and physical properties, or have the capacity to genetically manipulate target cells, will likely be key to promoting optimal regenerative responses from therapeutic cells.<sup>7,9,49</sup>

Second, the creation of large engineered replacement tissues will require technologies that enable fully vascularized grafts to be anastomosed with host vessels at the time of transplant, allowing for graft survival.<sup>26,36,51</sup>

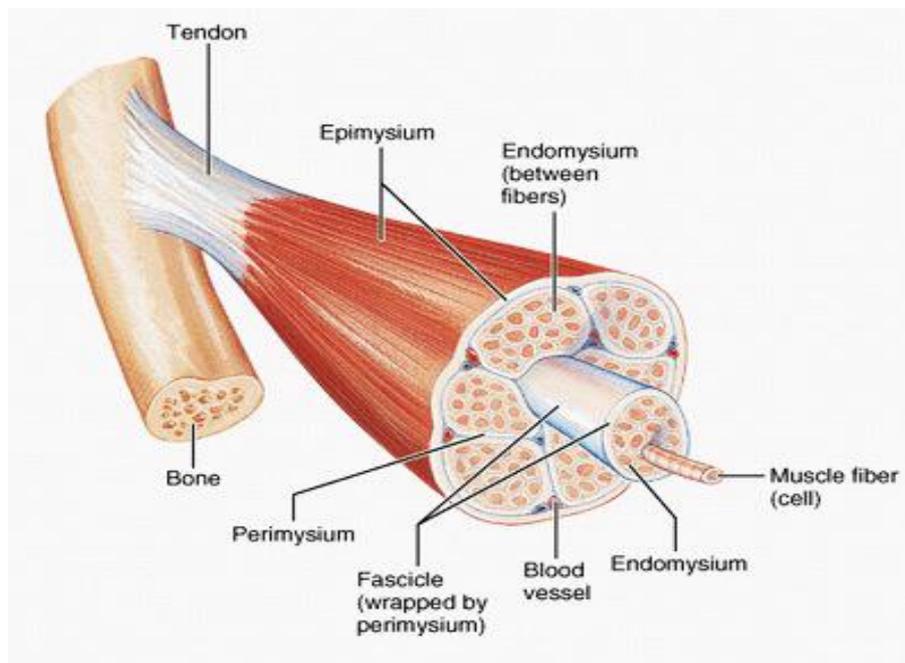
Thirdly, creating a pro-regeneration environment within the patient may dramatically improve outcomes of regenerative medicine strategies in general. An improved understanding of the immune system's role in regeneration may aid this goal, as would technologies that promote a desirable immune response. A better understanding of how age, disease state, and the microbiome of the patient affect regeneration will likely also be important for advancing the field in many situations.<sup>5,6,12</sup>

Finally, 3D human tissue culture models of disease may allow testing of regenerative medicine approaches in human biology, as contrasted to the animal models currently used in preclinical studies. Increased accuracy of disease models may improve the efficacy of regenerative medicine strategies and enhance the translation to the clinic of promising approaches.<sup>8,36</sup>

## 1.8 PRINCIPLES OF SKELETAL MUSCLE

The human body harbours two sorts of muscle tissue, smooth and skeletal muscle, being the later the focus of this research. The smooth muscle is generally present in a mechanically dynamic environment, such as cardiovascular, gastrointestinal, and urinary tissues.<sup>13</sup> The skeletal muscle on the other hand, plays an essential role in voluntary movement as well as in the stabilization of the skeleton and viscera protection. Moreover, it actively collaborates in aid respiration process, metabolic homeostasis, hormone and cytokines production and thermoregulation of the body.<sup>52</sup>

Skeletal muscle represents the body's largest tissue, accounting to more than 40% of the human body weight. It is comprised of bundles of elongated multinucleated cells, known as muscle fibres. These membrane-bound fibres display bundles of myofibrils in a striated pattern of repeating units named sarcomeres, which comprise the fundamental contractile structure of the skeletal muscle tissue. Such structure is depicted in Fig. 1.17. Given a certain neuronal or electrical stimuli said myofibrils respond by setting off a mechanical contraction to apply the necessary force that is required by a particular skeletal muscle to start its motion.<sup>52,53</sup>



**Figure 1.17.** Anatomy of skeletal muscle tissue and components.<sup>49</sup>

In addition, skeletal muscle also contains several populations of stem cells, whose function is the maintenance of the tissue's integrity as well as reparation mediators of any potential damage to the muscle. The endogenous progenitor cells of skeletal muscle, namely satellite cells are in charge of postnatal muscle regeneration, representing the predominant muscle stem cell population.<sup>52,53</sup>

### 1.8.1 Skeletal Muscle Regeneration

In embryogenesis skeletal muscle is developed from the mesoderm. Mesenchymal stem cells (MSCs) differentiate to form the individual musculoskeletal tissues, which include muscle, cartilage, bone, tendons and ligaments.<sup>54,35</sup>

During myogenesis MSCs differentiate to myoblasts, that will further grow and fuse into multinucleated myotubes. Once these myotubes mature, they create bundles of myofibres in an embryo. These myotubes mature into myofibres which bundle together to form the skeletal muscle which will be retained over the life span of the organism.<sup>53,55</sup>

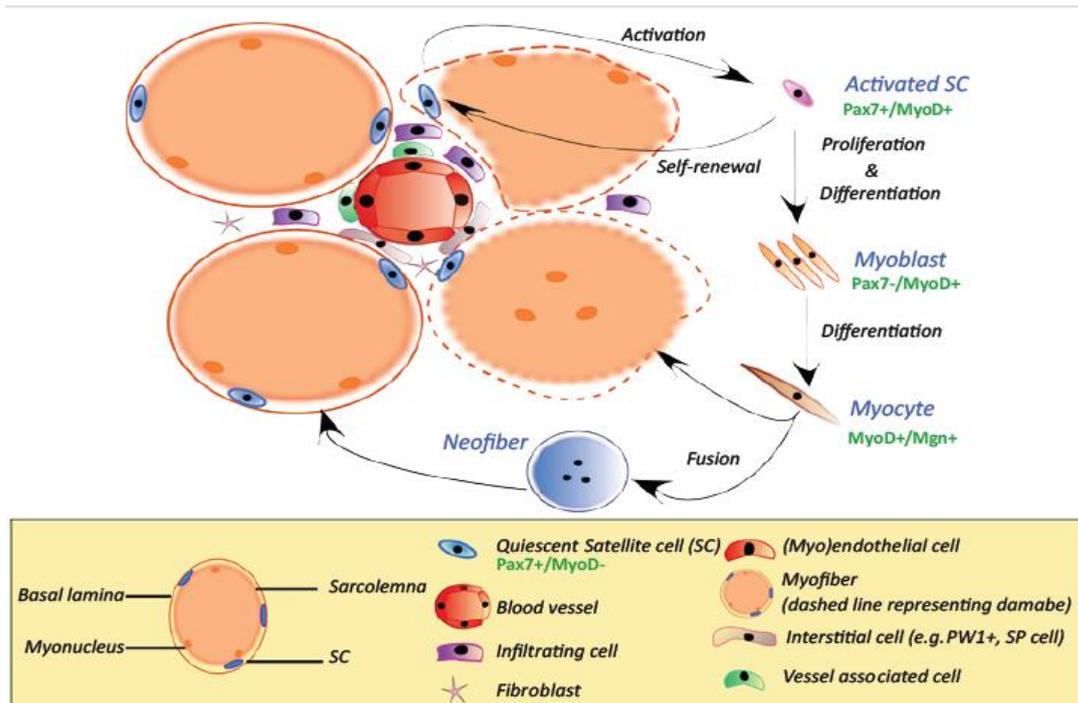
In spite of the exceptional regeneration capacity that adult muscle tissue possesses, situations such as aging, skeletal muscle disorders and traumatic lesions provoking large muscle losses make unfeasible the full restoration of the tissue to a healthy state.<sup>52,54</sup> Therefore, a clinical solution is needed to fulfil such skeletal muscle deficiencies. These may range from congenital myopathies, dystrophies and aging, to cancer, surgical procedures, accidents or neuromotor atrophies, resulting in the impediment of locomotion and low life quality.<sup>52,56</sup>

Nowadays, the only viable therapeutic approach is tissue transplantation, which involves all the drawbacks previously exposed. *Ex vivo* engineered muscles, consisting of scaffolds containing differentiated muscle progenitor cells, may represent a viable alternative to replace or regenerate the damaged tissue.<sup>55,57</sup> Although significant advances have been achieved in recent years, several practical challenges still remain. One major hurdle consists in procuring the appropriate amount of muscle progenitor cells.<sup>48,53,58</sup>

Another important requirement is to establish the optimal conditions for cell proliferation, maturation, and assembly of the skeletal muscle fibres. Finally, clinically relevant amounts of tissue will require means to provide for vascularization and innervation.<sup>53,56</sup>

The first step towards skeletal muscle regeneration is the proper disposition and differentiation of the muscle fibres.<sup>52,54</sup>

The variations that the skeletal muscle may undergo throughout life, either by growth or exercise will be accommodated by recruiting a population of mononucleated stem cells known as satellite cells. In their quiescent state, these resident cells lie dormant within the myofibre, immediately under the basal lamina.<sup>54</sup>



**Figure 1.18.** Skeletal muscle regeneration process by muscle resident stem cells. <sup>52</sup>

In essence, skeletal muscle regeneration in adults relies on satellite cells to be ready to function in response to muscle damage. When such event takes place, they will undergo asymmetric divisions, thus not only will the satellite cell compartment be again replenished but also new fully functional myofibres will be generated. This scheme can be seen in Fig. 1.18. <sup>53</sup>

The degree of understanding in skeletal muscle regeneration achieved relies on the fidelity of *in vitro* models engineered to study the role of microenvironmental cues in myogenic cell proliferation and differentiation, which require high control and definition. Isolated satellite cells have been employed as well for that purpose but primary myogenic cultures encounter certain limitations, among them, low replicative capacity and cellular heterogeneity. <sup>57,59</sup>

In consequence, the preferred cell line employed for *in vitro* studies is the immortalized mouse myoblast cell line C2C12, as in the case of this work. The encapsulation of skeletal myoblasts has shown evidence of viability and differentiation to myotubules in collagen gel. <sup>55 35</sup>

Despite the recent progress and the successful culturing and differentiation *in vitro* of a number of skeletal muscle progenitors, challenges still remain concerning the organization in the correct disposition to form functional arrays of myotubes. <sup>53</sup>

The scope of this project aims to address such issues by the employment of C2C12 cell-embedded electrospun fibres.

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## II. Materials and Methods

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### 2.1 Materials

- Sterile phosphatebuffered saline solution (sPBS) from Gibco, Cat# 15140
- MiliQ-water
- *E.coli* expressing GFP supplied by Peter Fojan
- *S. cerevisiae* from Malteserkors tørgær
- Mouse myogenic C2C12 cells from AmericanTissue Type Culture Collection (LGC Standards, Boras, Sweden).
- Culture flasks (75 cm<sup>2</sup>) from BD Biosciences
- Trypsin/EDTA (Life Technologies, Cat#15546/ Sigma Aldrich, Cat# E808)
- DMEM + Glutamax culture medium from Life Technologies
- Resazurim Sodium Salt from Sigma Aldrich
- Penicillin/Streptomycin antibiotics from Invitrogen (Pen/Strep)
- Fetal calf serum from Helena Bioscience.
- Heat-inactivated horse serum from Invitrogen
- Trypan blue from Sigma Aldrich Cat#884217
- JC-1 from Invitrogen Cat#86774
- Hoechst 33342 from Invitrogen Cat#63259
- Calcein AM from Invitrogen Cat#3099, 1 ml.
- Propidium Iodide from Sigma P-4170 Cat#12543653
- 0.1% Triton X-100 from Sigma

#### **Polymer solutions**

PCL (70–90 kDa) from Sigma-Aldrich, is comprised of a 12w/w% PCL solution in an organic solvent mixture of Chloroform, from Sigma Aldrich, and Dimethylformamide (DMF) from Iris Biotech GMBH. (60%:40% w/w).

Polyethylene glycol (PEG) from Sigma-Aldrich, used as foaming agent.

PEO(900 kDa) from Sigma-Aldrich, is dissolved to 2% in deionized water and left overnight at 80°C for proper mixing with a bar magnet.

NaA from Sigma-Aldrich, is alternatively added to solutions containing different proportions of PEO, always reaching a 4% final mixture of both together.

## **Yeast Medium**

The liquid medium provided to grow *S. cerevisiae* is prepared by mixing 10 g/L yeast extract, 20 g/L peptone and the necessary volume of MilliQ-water to reach the desired solution. Besides, a stock solution of 10x dextrose is dissolved to 20% in MilliQ-water as well. Both solutions are autoclaved at 121 °C for 30 min. When the temperature is about 60°C, the appropriate volume of said dextrose is added to the mixture to get the desired growth medium.

## **Luria-Bertani Medium (LB)**

*E. Coli* bacteria grown in LB medium prepared by dissolving 10 g/L tryptone, 10 g/L NaCl and 5 g/L yeast extract into a volume of MilliQ-water according to what is needed. The resulting solution is properly mixed with a magnet and autoclaved at 121 °C for 30 min. For a later disposal, the medium is stored in the fridge.

## **Proliferation medium for C2C12**

Minimum essential medium alpha with GlutaMAX (DMEM; Gibco) is supplemented with 10% fetal calcium serum (FCS, Helena Bioscience) and 1% penicillin/streptomycin (Pen/Strep; Pen: 10,000 U/mL, Strep: 10 mg/mL).

## **Differentiation medium**

Differentiation medium consists of DMEM medium supplemented with 2% heat-inactivated horse serum (Invitrogen) and 0.5% penicillin/streptomycin.

## **Agar Plates**

Agar plates are prepared to seed either yeast or bacteria colonies when required. For this purpose, an amount of 15 g/L or 20 g/L agar is added to certain volume of the yeast or LB medium respectively. After autoclaving and mixing with a magnet, a desired number of petri dishes are filled with medium at temperatures not lower than 60°C to avoid early solidification. Once the medium on the petri dishes is no longer liquid, plates are stored inverted in the fridge. In the case of LB plates, ampicillin can be added to a final concentration of 200 mg/L, in order to prevent the growing of undesired bacteria.

## 2.2 Methods

### **Turbidimetry**

1ml of cells is placed into a disposable cuvette and the optical density (OD) is measured at 600 nm in a spectrophotometer from Pharmacia Biotech. Sample is always compared to a negative control, which contains only media with no cells. For OD<sub>600</sub> 0.05-1.0, the reading is considered proportional to cell number. If measurement of the culture gives a higher OD value, sample is diluted 1:10 and re-measured.

### **Subcultivation of C2C12**

After checking cell appearance under the microscope, medium is removed from T75 flask. Cells are washed gently twice with 10 mL s-PBS. 3 mL trypsin/EDTA are added to the flask and distributed over the cells by tilting the flask. After cells are detached (approximately 3 min of incubation), 10 ml culture media are used to flush and resuspend the cells by pipetting up and down. Centrifuge the cell suspension in a 15 ml centrifuge tube for 5 min at 125 g at 20°C. Supernatant is discarded and pellet resuspended in 2 ml medium by pipetting again. When required, seed in a new flask 2,500 cells/cm<sup>2</sup>, according to the counting calculations. Medium must be added according to the volume of the flask, and changed every two days.

### **Differentiation of C2C12**

The induction of cell differentiation is performed on seeded cells, when confluency is reached, by substituting proliferation media by differentiation media (day 0). Differentiation media was replaced every 2 days.

### **Cell concentration estimation**

10 µl of cell suspension are transferred to the counting chamber of a hemocytometer. The microscope (Olympus CKX41) is set to 10x and the cells are counted to calculate the cell concentration per ml by the following formula:  
*average cell count* · 10<sup>4</sup>/mL = cells/mL

### **Cell counting**

After visualization with fluorescent microscope (AxioCam MRc5, Zeiss) the calculation of the number of C2C12 cells present on the samples is carried out digitally by ZenPRO analysis and Image J software.

## Coaxial electrospinning of cells

Cells are centrifuged prior utilization and the cell pellets are resuspended in the core solution. In the case of C2C12 and additional FBS solution is added up to 50% volume. Both shell and cell/core solutions are loaded into 10mL syringes (B.D) and placed in the Yflow electrospinner machine (Nanotechnology Solutions 2.2D -500). The conditions selected for an optimum electrospinning are 1.5 kV/cm at room temperature and the flow rates of the shell and core solutions were maintained at a ratio of 6:1 as suggested in previous reports.<sup>60</sup> The fibres are electrospun directly into a MilliQ- water bath containing salts following the procedure already described in other studies.<sup>61</sup> The core-shell fibres are then collected on the surface of the water bath and left for 15 min prior removal, followed by immersion of the sample in proliferation medium when electrospinning C2C12 cells,

## Yeast viability assays

- AlamarBlue® assay (Life technologies): yeast cells harboured inside the electrospun fibres are checked for survival with an Resazurim Sodium Salt as described by the manufacturer. Fibres are placed in a small culture plate and incubated with 1mL of 10% AlamarBlue® solution at 37°C shield from light for four hours. Characterization of viability is assessed by colour change.

-Proliferation assay: agar plates are used to control the capability of the yeast cells to produce new colonies by incubating the sample of electrospun fibres at 30°C overnight.

-Catabolization activity: the sucrose degradation to CO<sub>2</sub> through glycolysis is tested to assess the metabolic capability of *S. cerevisiae* cells. The later appearance of bubbles on the petri dish lid would indicate such catabolization.

## C2C12 viability assays

-Trypan Blue: 0.5% trypan blue solution is utilized one day after recollection of the fibres. The dye is incubated for 1 hour and the viability is characterized by colour change, dead cells would correspond to dark staining and viable to bright.

- Fluorescent Staining: the following description applies for all the viability assays performed with dyes that required fluorescent microscopy.

After the culture period, cell-bearing scaffolds are transferred to a six-well plate and washed twice with sPBS. 200 µl of the dye selected for the experiment is added to the samples and incubated at 37°C for one hour. At last, samples are rinsed twice with sPBS and kept in sPBS at 4°C until the observation time.

A later fixation was performed using 4% formaldehyde for 15 min.

Prior staining, positive control simple is permeabilized by incubation for 15 minutes with 2% Triton X-100 in PBS.

The above procedure is followed for the use of DAPI/PI, Hoechst/JC-1 and Calcein/PI.

### **Seeding over scaffold**

Empty electrospun fibres containing PCL and PEO are divided in pieces and transferred to a six-well plate. After addition of the proliferation medium, C2C12 are added to the samples, with a density of approximately 5000 cells/cm<sup>2</sup>.

### **Statistical analysis**

Student-t-test, Microsoft Excel and GraphPad Prism are used for statistical analysis of the data. P values lower than 0.05 are considered significant. In the figures asterisks indicate P value as follows: \* < 0.05; \*\* < 0.01; \*\*\* < 0.005 or no symbol if the results are not significant.



### III. Results and Discussion

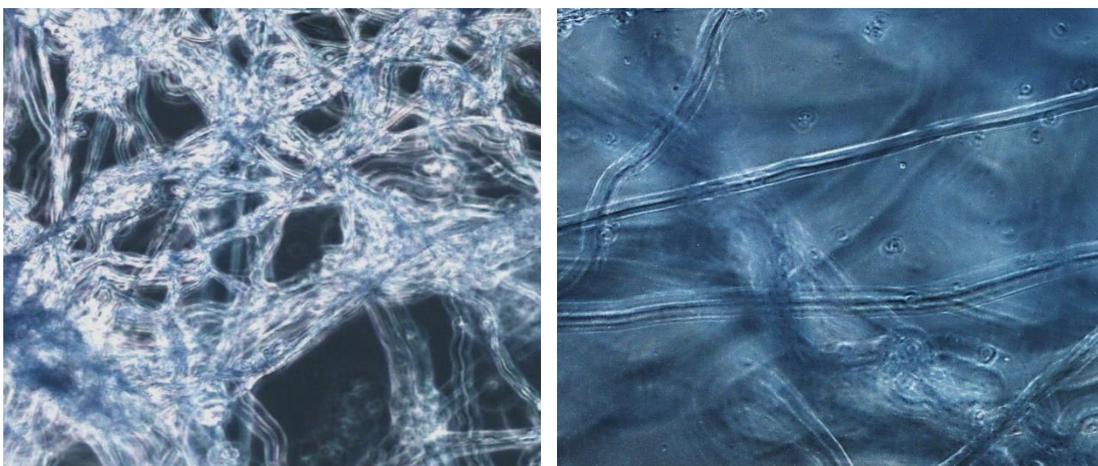
#### 3.1 CHARACTERIZATION OF FIBRES – SCAFFOLD

##### PCL/PEO electrospun fibres

The successful electrospinning of PCL and PEO solutions separately indicated that the polymer solutions were spinnable and the parameters were suitable for fibre production. When employing PEO and PCL together through coaxial electrospinning, there was no detectable miscibility in the emerging jet at the needle nor evidence of mixing between them in the resulting fibres.

As observable in Fig. 3.1 the fibrous mat collected presented a remarkable randomness in the distribution of the fibres and the superposition of the majority of them made the characterization rather difficult. Moreover, seeing as the scaffolding for skeletal muscle engineering is the final purpose of this work, the disposition of the fibres should be aligned and entail certain order to direct the proliferation of the cells in that direction, thus favouring the creation of myotubes.

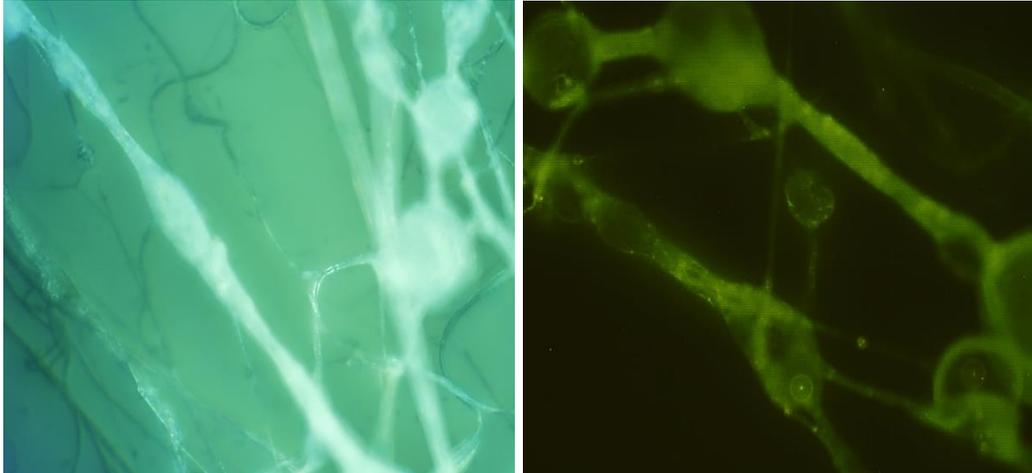
Following such premise, the electrospinning process was optimized by applying a program to set a dynamic pattern which the electrospinner can register to move the needle in consequence. Fig. 3.1 shows the distribution of PCL/PEO fibres generated under dynamic conditions programmed to create a horizontal pattern of fibres. Although the result achieved was not entirely homogeneous, the distribution of the fibres was considerably corrected, allowing a better visualization of the sample too. The presence of fibres that are still deposited in alternative directions could be explained by the time that the jet spends travelling from the needle to the collector, which could favour freedom of fibres distribution.



**Figure 3.1.** *Left: PCL/PEO fibres statically electrospun. Right: PCL/PEO fibres dynamically electrospun for a better alignment.*

### Entrapment of PEO/*E. coli* cells

A strain of *E. coli* expressing the green fluorescent protein (GFP) was utilized to ensure the visualization of the cells within the fibres. *E. coli* cells were suspended in the PEO/FBS solution followed by the electrospinning into the core of PCL/PEO fibres. Standard microscopic images revealed a high degree of alignment on the fibrous mat, as evident from Fig. 3.2.



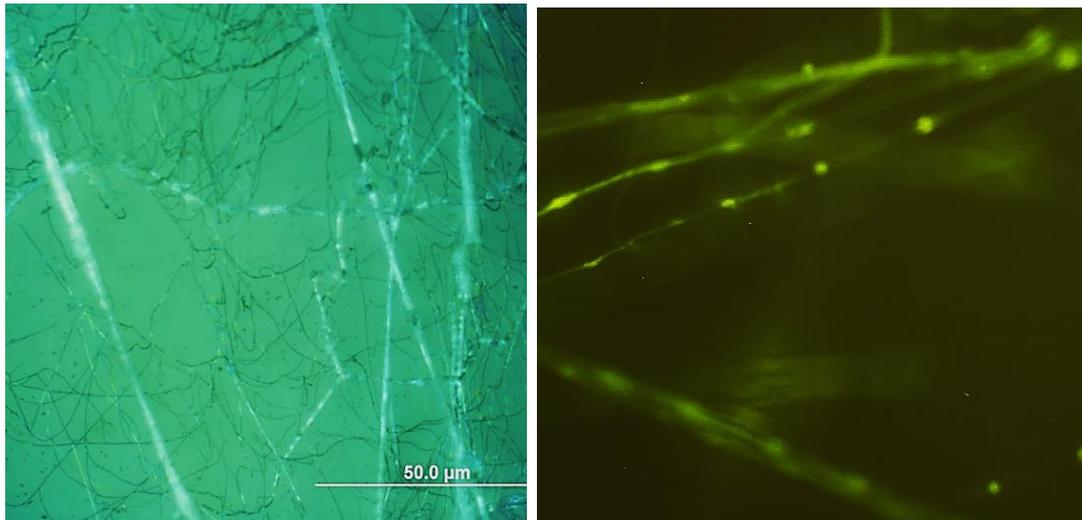
**Figure 3.2.** PCL-shell and PEO/GFP-*E. coli* cells–core electrospun fibres. Left image corresponds to standard microscope visualization. Right image corresponds to 500 nm fluorescent excitation filter. Scale bar = 30  $\mu\text{m}$

Electrospun fibres also presented spherical and oval formations along their structure. The imaging with fluorescent microscope uncovered the presence of grouped cell populations within the fibre in said spots and also in the rest of the core area, proving the entrapment of *E. coli* cells within the fibres core.

Such deformations commented above seem to point to a quite flexible shell material, which would represent a positive feature for the engineering of a potential tissue because it would allow the growth and expansion of the cells inside the fibre while keeping the desired alignment for muscle regeneration.

### Entrapment of NaA/*E. coli* cells

NaA was tested as well as an alternative solution to constitute the fibre core, with the aim to provide a comparison of the outcomes to then select the most optimal material for the embedding of the cells. As visible from Fig. 3.3, electrospun fibres with *E. coli* cells and NaA as core solution showed a considerably reduced size compared to the one presented with PEO. This deviation in size could be attributed to the different composition of the polymer solution which would lead to a different electrospun product due to the influence of its specific parameters in the process.



**Figure 3.3.** **Left:** PCL-shell and NaA/yeast cells core electrospun fibres. **Right:** PCL-shell and NaA/GFP-*E.coli* cells-core electrospun fibre visualized at 500 nm fluorescent excitation filter. Scale bar = 50 µm.

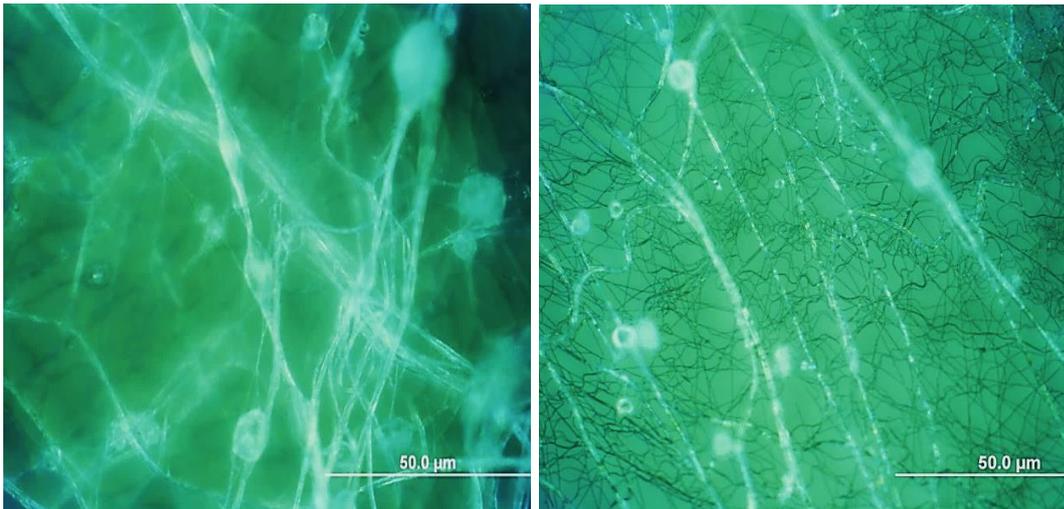
It is worth noting that the entrapment of the cells inside the fibre shell was not as clear by standard microscopy techniques. Nevertheless, fluorescence emission as result of *E. coli* GFP expression did demonstrate the presence of embedded cells inside the fibres by fluorescent microscopy, as it can be seen in Fig 3.3 (left).

Alignment of the fibres appears to be improved compared to the PEO results. Almost all the fibres are deposited in the same direction, and the deviations are barely noticeable. On the other hand, it is relevant to note that difficulties to achieve a stable Taylor cone were encountered during the electrospinning procedure, which could represent a considerable disadvantage on the use of NaA as a core solution.

### Entrapment of *S. cerevisiae*. cells

The coaxial electrospinning of *S. cerevisiae* cells along with PEO yielded a mat of fibres similar to that of *E. coli* cells. As shown in Fig. 3.4 numerous fibres presented rounded deformations along them, suggesting the presence of grouped cells. These features seemed to be correlated by the GFP expression in *E. coli* cells, which leads to think that in this sample that is also the case, despite the lack of fluorescent evidence.

When depositing electrospun *cerevisiae* cells with NaA as core solution and PCL as shell, the result was again a smaller fibre diameter and a better alignment when collected.



**Figure 3.4. Left:** image shows PCL-shell and PEO/ *S. cerevisiae* cells–core electrospun fibres. **Right:** image shows PCL-shell and PEO/ *S. cerevisiae* cells–core electrospun fibres.

Moreover, standard microscopic images as Fig. 3.4 pointed to the plausible presence of cell populations provoking shell deformations. The reason why this was not detectable in the bacterial samples could be associated with concentration differences, where a higher concentration of yeast cells (of larger size as well) could favour the formation of such structures.

However, the instability of the Taylor cone was repeatedly experienced when utilizing NaA as core solution, which favour the employment of PEO as the spinnable material of preference.

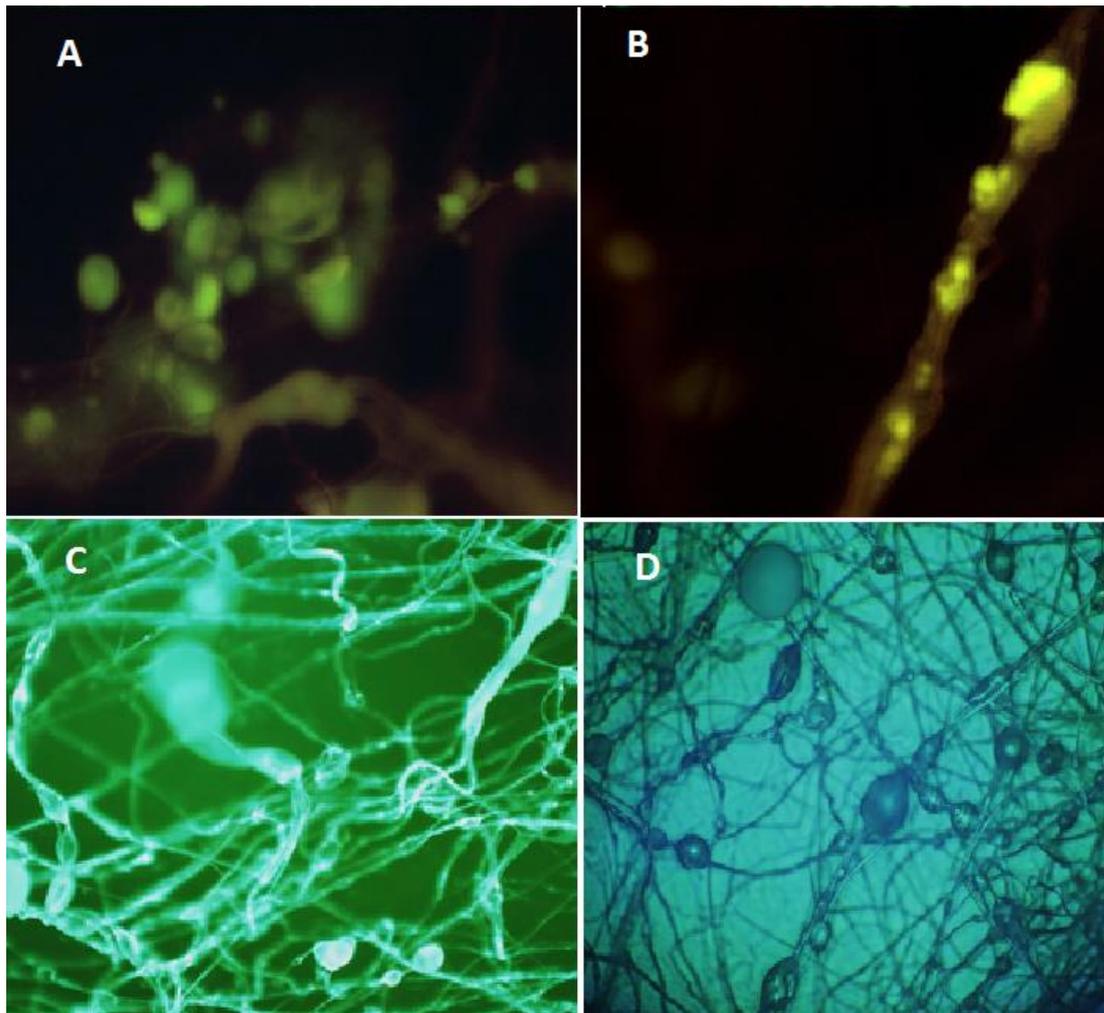
### Entrapment of C2C12 cells

Advancing towards the purpose of this research, the mouse myoblast cell line C2C12 was incorporated to the PEO/FBS core solution and electrospun into PCL shell for the fibre formation.

The resulting cell-bearing scaffold can be seen in Fig. 3.5B where fibres appear with multiple oval deformation that point towards the entrapment of C2C12. The alignment found followed the pattern of previously described core-PEO spinned solutions, though a tendency in the orientation of the fibres is rather obvious.

Furthermore, a C2C12 line expressing GFP was utilized to ensure the confirmation of the entrapment of the cells within the electrospun fibres, Fig.3.5A.

In a first attempt to check the cells condition, a simple viability assay was performed by Trypan Blue staining. Although all necessary precautions were followed, the experiment yielded not conclusive results. As it can be observed in Fig. 3.5C there is no clear distinction between the hotspots that were considered to harbour cells and no trace of cells clearly stained either dark blue or bright as it should be expected from this dye.

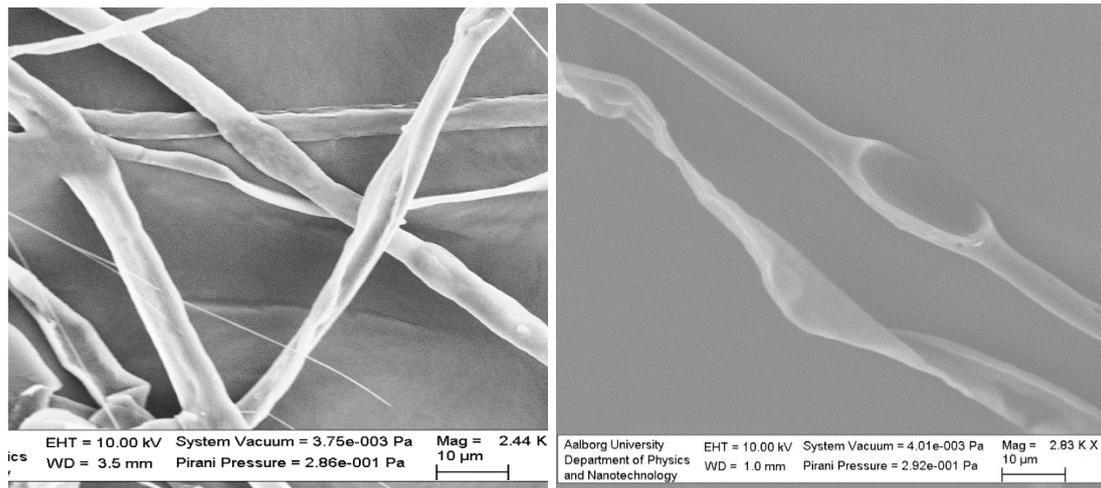


**Figure 3.5.** **A and B:** C2C12 entrapment visualization by GFP fluorescence. **C:** PCL-shell and PEO/C2C12-core electrospun fibres. **Right:** Trypan Blue staining for C2C12 viability within PCL/PEO fibres, shows the impossibility to distinguish any colour changes after incubation. Scale bar = 50  $\mu$ m.

It could stand to reason that the permeability of the membrane was not sufficient to allow the exchange of any compound, and consequently no Trypan Blue molecules could enter the inner fibre. If this was the case, porosity of the PCL shell should be enhanced in order to provide the required communication between cells and medium., which is essential for the cellular proliferation and progress further with the study.

### 3.2 FIBRE SHELL POROSITY

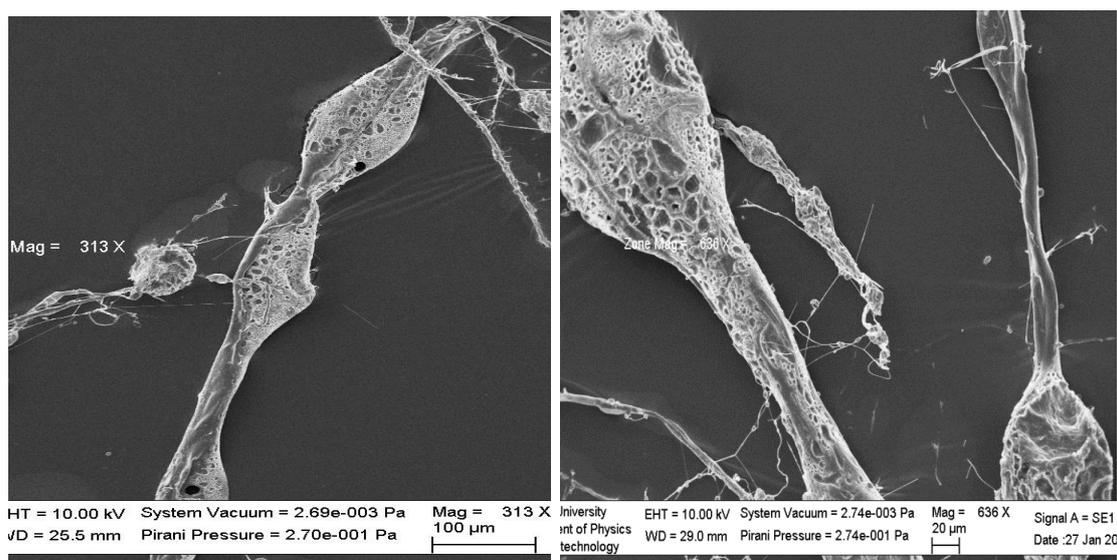
Scanning Electronic Microscope (SEM) imaging of the C2C12-laden fibres allowed the perception of embedded cells, as a thickening in certain areas along the fibre, which can be seen in Fig. 3.6. Nonetheless, it is also observable in this set of imaged the absolute lack of porosity on the shell membrane, pointing out an inadequate configuration that prevents to continue further with the development of skeletal muscle engineering.



**Figure 3.6.** Left: SEM Image of PCL-shell and PEO/C2C12 cells–core electrospun fibres. Right: zoomed image of C2C12 entrapment. Scale bar = 10 µm.

The resources employed by previous researchers, involving the collection of the fibres in a water bath, did not suffice to create pores in this study. Seeing as PEG has been described as a foaming agent, the approach proposed to tackle this issue was the incorporation of PEG to the PCL solution prior electrospinning. In addition to this change, PEO and NaA were combined in the ratio 1:1 in a 4% solution to take advantage of the benefits of both materials for an optimum environment for the cells.

SEM visualization of the electrospun compounds mentioned above along with C2C12 cells revealed the fibrous scaffold structures presented in Fig. 3.7 These images lead to conclude that an adequate level of porosity on the fibre shell was successfully achieved.



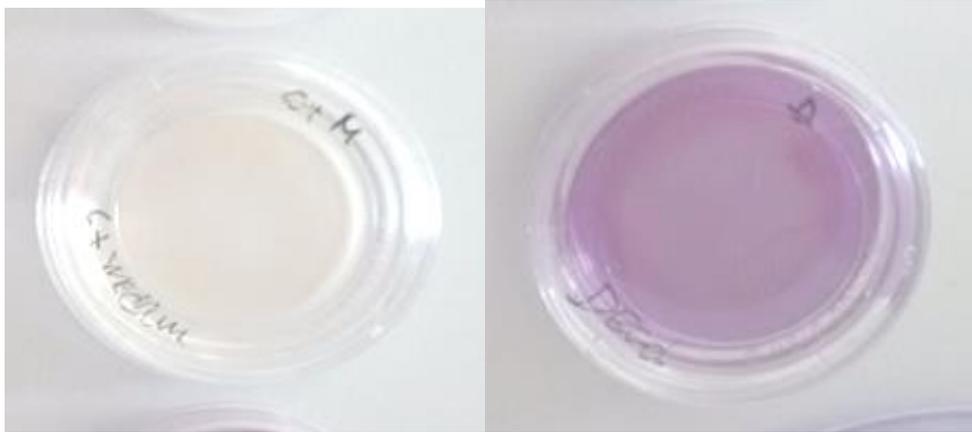
**Figure 3.7.** SEM of PCL-shell and 50% PEO - 50% Alginate / C2C12–core electrospun fibres. Scale bar = 20 µm. The formation of pores on the fibres shell is evident adding PEG.

### 3.3 ASSESSMENT OF CELL VIABILITY

#### *S. cerevisiae* embedded cells

The entrapment within fibres should not alter the cells properties nor their viability. For a potential scaffold, cells must retain a functional metabolism and an intact plasma membrane. Therefore, an Alamar Blue assay was performed in order to assess the viability rate of the cells after undergoing the electrospinning process.

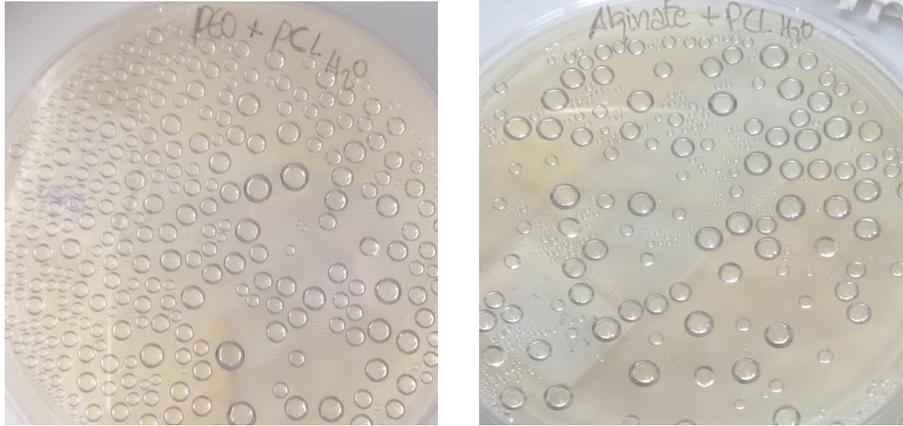
As Fig. 3.8 reveals, *S. cerevisiae* cells proved to survive the procedure by the positive oxidation of the agent, represented by the disappearance of purple colour, due to the presence of cell metabolism.



**Figure 3.8.** Colour difference between positive control (right plate, containing dead cells) and scaffold samples (left plate, containing *cerevisiae* cells embedded).

The viability assessment was further investigated by a sucrose degradation assay of the fibres generated by both core solutions PEO and NaA.

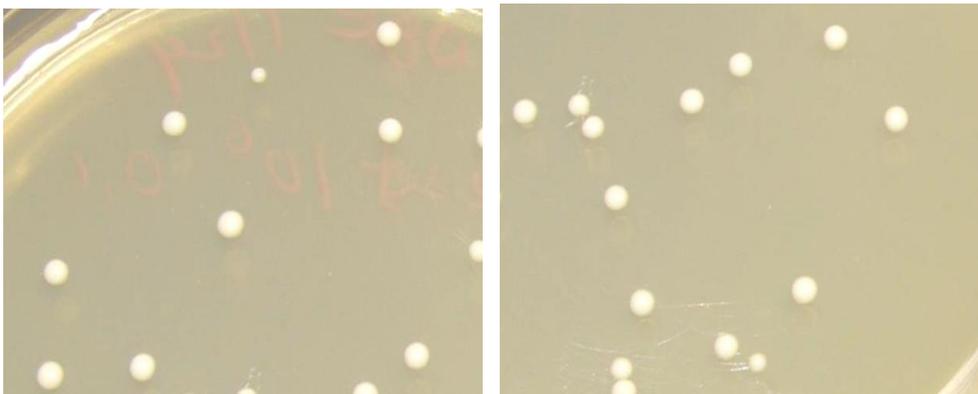
Analogue experiments can be seen in Fig. 3.9 where a high number of bubbles is observable after 24 h in both samples investigated, corresponding to CO<sub>2</sub>. This gas is released as a final step of the catabolization process of the sugar that these yeast cells are capable to perform, leading to the conclusion that not only did the cells survive the fibre generation but also, they remained still alive inside their entrapped environment.



**Figure 3.9.** *S. cerevisiae* sucrose degradation assay after overnight incubation at 30°C. **Right** image shows CO<sub>2</sub> formation of PCL-shell fibres containing PEO as core solution. **Left** image shows CO<sub>2</sub> formation of PCL-shell fibres containing NaA as core solution.

An additional test for the prolonged survival of entrapped *S. cerevisiae* cells was their capacity of proliferation once they are already inside the fibres. For this goal, the electrospun scaffold samples were placed on agar plates after collection and visualization.

As evident from Fig. 3.10, the proliferative power of *S. cerevisiae* cells appears to remain intact, which was demonstrated by the appearance of colonies after overnight incubation at 30°C.

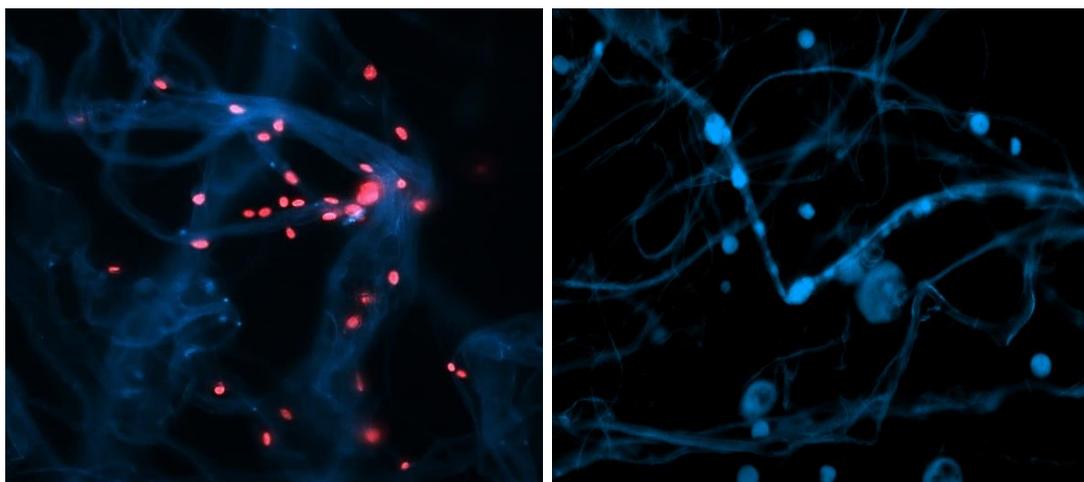


**Figure 3.10.** *S. cerevisiae* colonies appeared after overnight incubation at 30°C. **Left** image shows plate containing PCL-shell fibres containing PEO as core solution. **Right** image shows CO<sub>2</sub> formation of PCL-shell fibres containing NaA as core solution.

### C2C12 embedded cells

Once the control over pore formation was properly established, different methods were tested in order to assess the suitability of the entrapment of C2C12 cells into the biomaterials selected as potential scaffolds for skeletal muscle. If not stated otherwise, assays were performed 24h after the coaxial electrospinning of the fibres, followed by the incubation in proliferation medium at 37°.

The staining with PI and DAPI was chosen as is one of the most straightforward techniques commonly employed to distinguish dead cells.

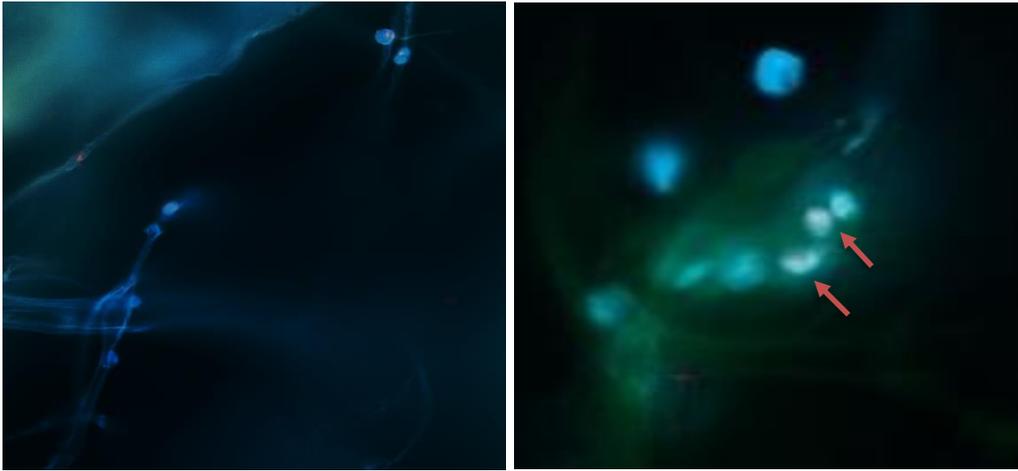


**Figure 3.11.** DAPI/PI viability assay of entrapped C2C12. **Right:** Positive control of dead C2C12 after incubation with Triton 0.2%. **Left:** DAPI/PI staining, shows blue coloured dots.

At a first glance to the Fig. 3.11, the results from this assay suggested a high number of viable cells within the fibres, due to the presence of bright blue spheres. However, after analysing the shape and size of DAPI stained nuclei, doubts were raised about the origin of such spherical spots marked by DAPI. When comparing with the PI stained nuclei from positive control images, differences between both morphologies were evident. Not only did blue spots present high heterogeneity in terms of size and shape, but also the structures highlighted did not seem to coincide with the expected oval shape from C2C12 nuclei.

Furthermore, it was possible to find blue spots of similar intensity in positive control samples as well, which pointed towards low credibility of DAPI marking. Given the core-shell architecture of the fibres and the challenging separation of blue highlighted morphologies from the fibrous structure, it stands to reason that the doubtful outcome of this assay is due to fluorescent reflection of the PCL material comprising the shell of the fibres.

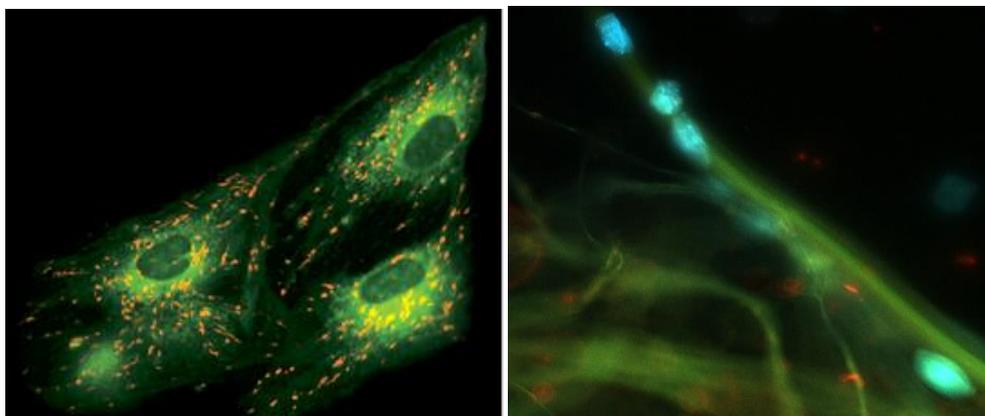
Another strategy to evaluate the viability of fibre-embedded cells was an indicator of mitochondrial membrane potential marker, JC-1, together with Hoechst and DAPI, for the cell cytoplasm and nucleus respectively.



**Figure 3.12.** *JC-1/ Hoechst viability assay of entrapped C2C12. **Left:** positive control of dead C2C12 after incubation with Triton 0.2%. **Right:** presence of red-marked active mitochondria in the cytoplasm of the cells, pointed by the red arrows, indicating cell viability.*

Although this staining allows to clearly distinguish the shape of the cells inside the fibrous scaffold, other issues arose from the visualization of the experiment. Cell cytoplasm does not seem visible in green, as it should be expected from the Hoechst dye. As shown in Fig 3.12, the results indicated the presence of a high number of dead cells, observable by the lack of red spots within the cytoplasm, which would correspond with the membrane potential of living mitochondria. Nonetheless, it is possible to find red dots in within few cells, which would suggest mitochondrial activity and so cell viability.

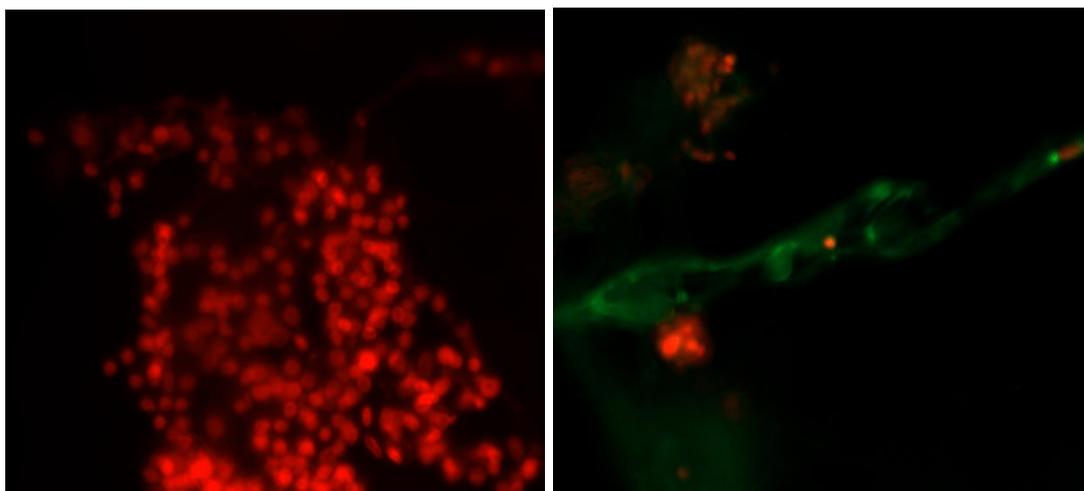
Unfortunately, such red dots can also be found in other places throughout the scaffold where cells are not located. When comparing to results of previous studies which successfully addressed the viability of cells, it is noticeable that all the red dots should correspond to the cytoplasmic area, whereas in the samples from this study presented them randomly distributed around the electrospun material. Such anomaly could be explained by a poor homogenization of the staining solution or the attachment to the scaffold shell.



**Figure 3.13.** *Comparison between a standard result from JC-1 mitochondrial marker assay (left) and the result obtained in this study, where random red dots are spread over the scaffold*

Therefore, the issues above mentioned made unfeasible the utilization of this method as a reliable proof of cell viability.

An alternative method to those previously mentioned is the staining with Calcein/PI, which present the characteristic of being exclusive dyes. This advantage might help to avoid contradictory results and allow a clearer distinction of the state of the cells.



**Figure 3.14.** Calcein/PI viability assay of entrapped C2C12. **Right:** Positive control of dead C2C12 after 15 min. incubation with Triton 0.2%. **Left:** result from Calcein/PI staining, showing viable cells coloured as green and dead as red.

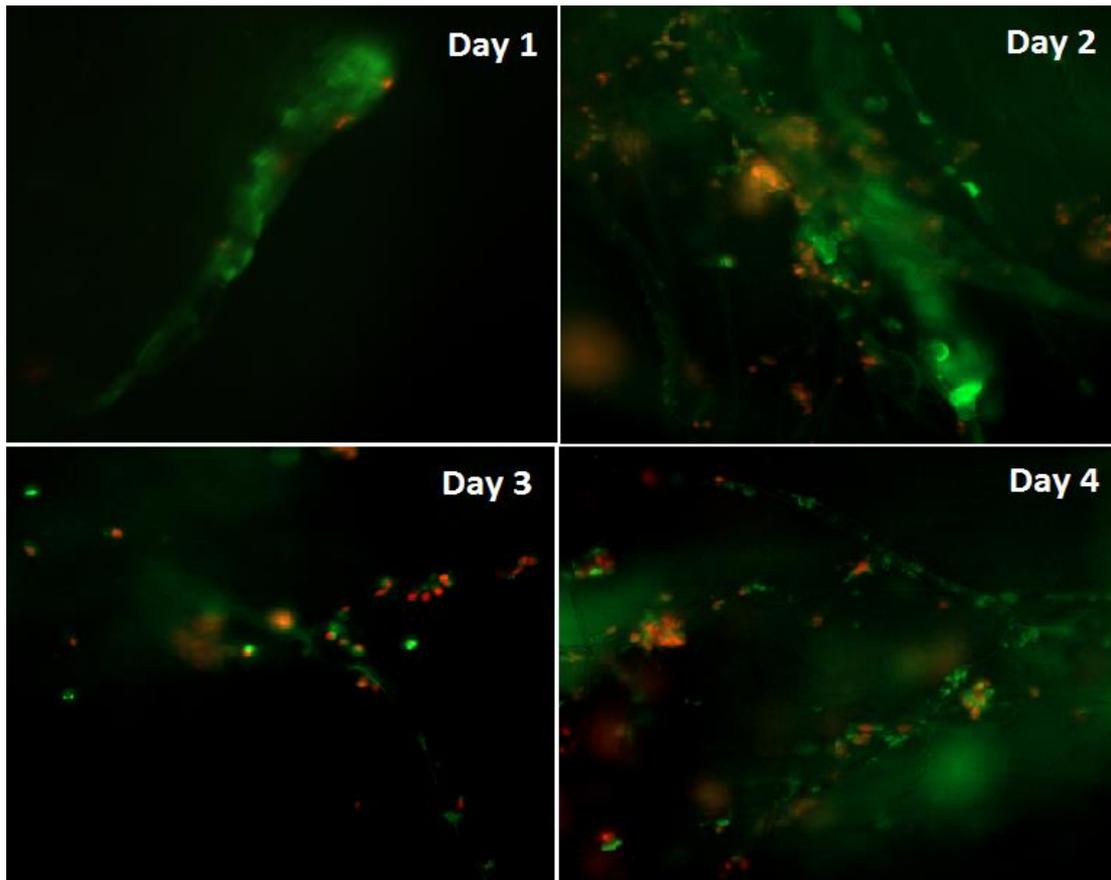
The visualization by fluorescent microscope of the experiment results can be observed in Fig. 3.14. The number of dead cells represented in red are perfectly defined. Regarding the detection of living cells, it was found that even if the fibre shell material did reflect some of the fluorescence along the structure, oval-shape morphologies that would coincide with the presence of cells are still easily distinguishable from the rest of fibrous area.

Such results led to the conclusion that Calcein/PI staining should be employed as a preferred option when analysing the viability of fibre embedded cells electrospun in PCL/PEO-NaA polymer solutions.

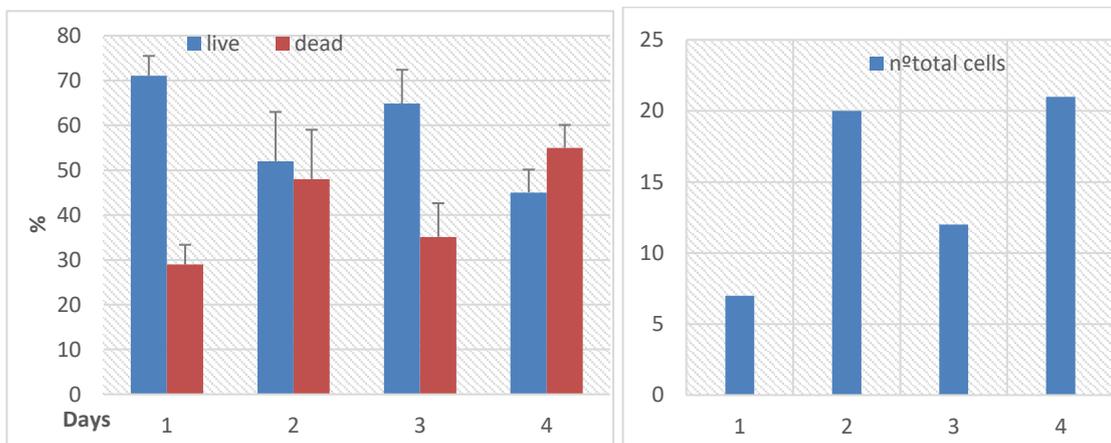
## I.4 PROLIFERATION AND DIFFERENTIATION

### Proliferation of fibre embedded cells

The state of C2C12 cells was followed by the successive repetition of Calcein/PI assays during four consecutive days of incubation in proliferation medium, aiming to study the progression of the cell growth and control if C2C12 cells remain viable for a longer period of time after being subjected to the electrospinning.



**Figure 3.15.** Calcein/PI viability assay of entrapped C2C12 showing viable cells coloured as green and dead as red at one, two three and four days after the electrospinning.



**Figure 3.16.** Statistical analysis of variations in the number of viable and dead C2C12 cells one, two, three and four days after the electrospinning procedure took place.

Fig. 3.15 shows the presence of living cells distributed along the PCL/PEO-NaA fibres. Such populations remained viable even after four days post-electrospinning. However, there was a high number of dead cells in most of the samples. The analysis of the data obtained from the fluorescent microscopy visualization yielded the graphic previously included in Fig. 3.16.

The viability of C2C12 appears to be the most favourable 24 hours after deposition of the electrospun PCL/PEO-NaA cell-laden scaffold. The second day shows almost the same amount of dead and live cells, which could suggest that some of the cells were stressed to some degree during the process and they were not able to survive passed the first day. Nonetheless, there seems to be a relapse on cell viability on the third day, which is proceeded by another fall in the number of viable cells four days after the coaxial electrospinning took place.

The possible explanation for such event, could be deduced from looking at the graphic from Fig.3.15 regarding the average number of cells that were found in the set of images for each day. The first and third point to the presence of a much lower number of C2C12 cells entrapped within the fibres, than the following days, the second and the fourth, respectively. The meaning of such increment could be related to the cellular proliferation inside the scaffold, which would indicate a suitable environment for cell maintenance.

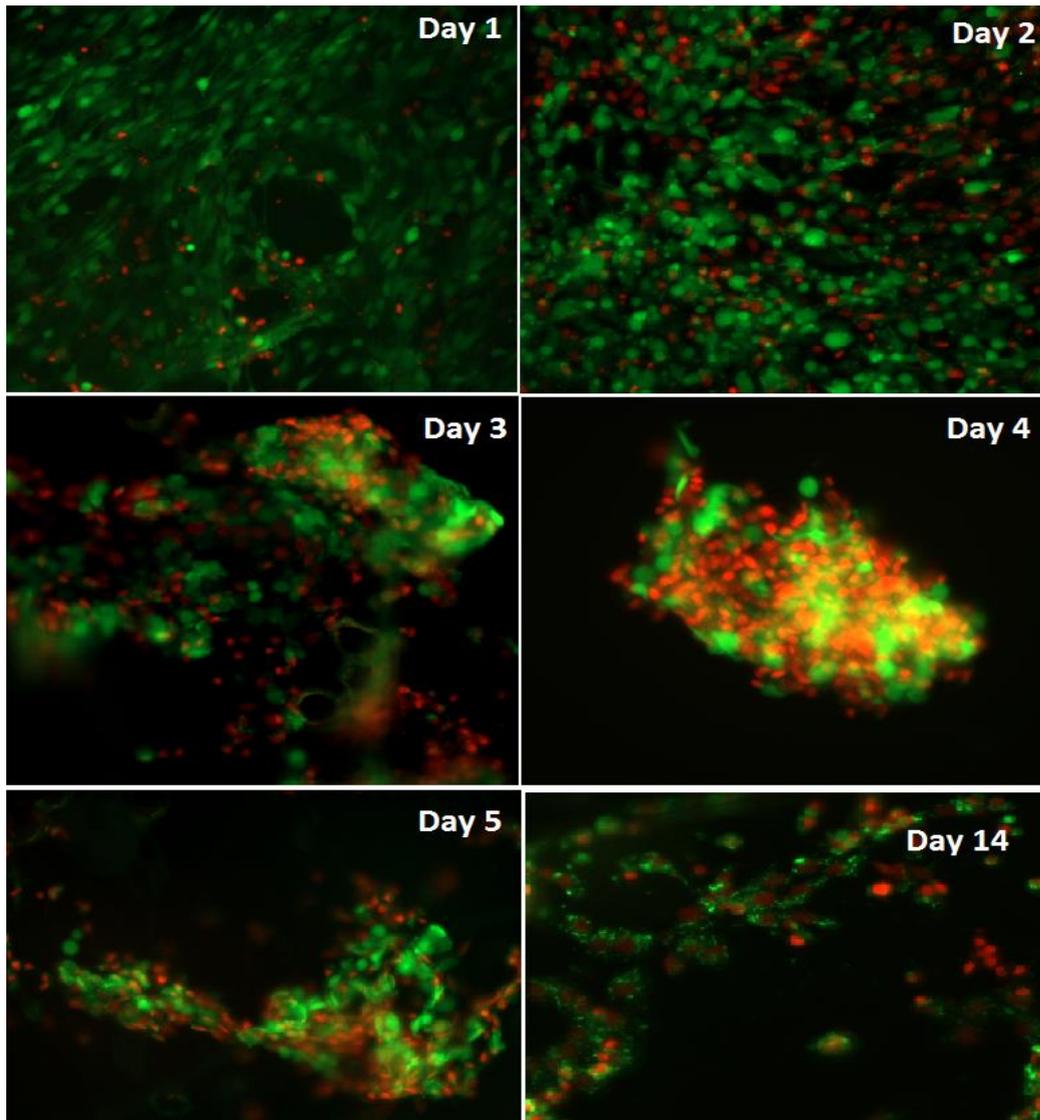
On the other hand, the sudden reduction of the cell population after the second day is most likely the result of changing the growth medium to follow the standard methodology for cell incubation. Since dead cells cannot attach to the plate, a considerably large part of the population will be removed along with the old medium. In this fashion, the quantity of dead cells will be dramatically decreased compared to the actual value. Although viable cells number should not be affected by the change medium, the lesser presence of mortality would raise up the proportion of viable cells, which appears to be the case in this experiment.

### **C2C12 seeding over scaffold**

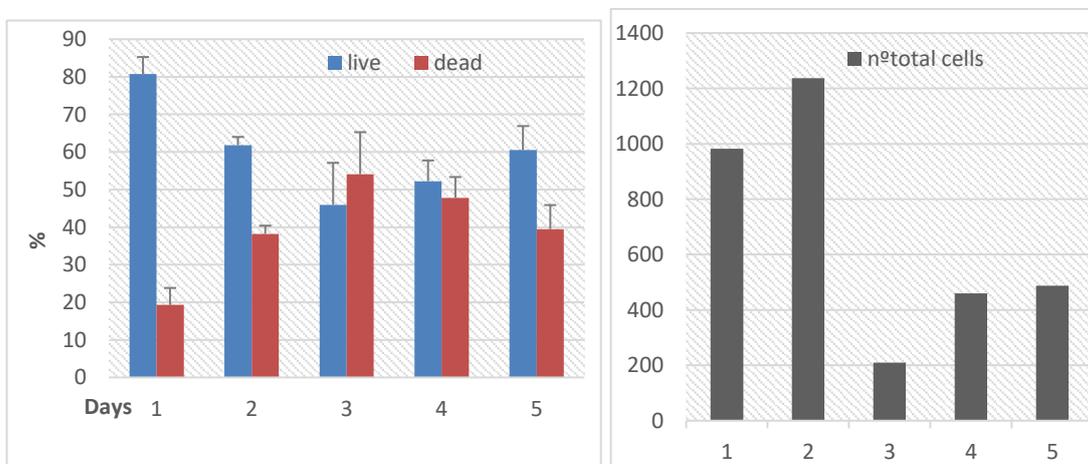
C2C12 proliferation and viability were also investigated for the cell seeding over the PCL/PEO-NaA electrospun scaffold. In this case, experiment was performed after cells reached confluence, followed by incubation in differentiation medium, pursuing to enlighten the potentiality of cell differentiation in this environment. The distinction between live/dead cells was checked with Calcein/PI assays too, repeatedly over 14 days after the seeding of the cells in the fibres.

The results yielded were similar as those previously commented of entrapped C2C12, as is observable in Fig. 3.17. Viable and dead cells are present at all stages, whose proportion is altered as well after 48 hours, when the medium was changed. This would have caused the reduction of the mortality rate on the third day, which was then recovered by cell proliferation the following days (Fig.3.17).

Graphic shown in Fig. 3.18, where the average cell population is represented, supports the hypothesis of removal of loose dead cells due to the loss of their structures of adhesion. Thereby, when old medium was replaced by fresh one, a part of the unviable population of cells was lost, and the percentage of living cells experienced an increment.



**Figure 3.17.** Calcein/PI viability assay of seeded C2C12 over the electrospun PCL/PEO-NaA scaffold for five consecutive days. Green and red correspond to viable and dead cells.



**Figure 3.18.** Statistical analysis of variations in the number of viable and dead cells one, two, three and four days after electrospinning procedure took place. (Day 14 not included in data)

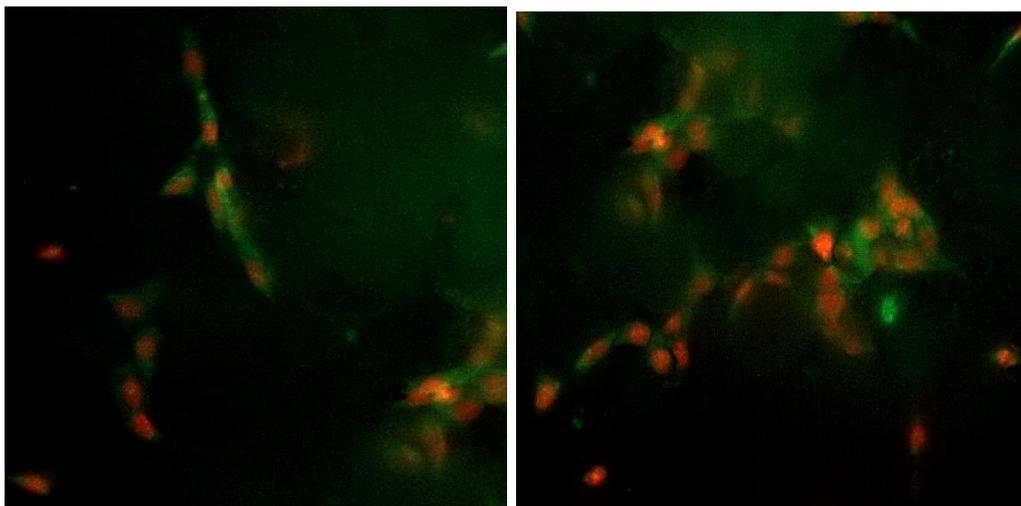
The study of cell viability was prolonged for 14 days to check the long term influence in the cells well-being when seeded onto the scaffold. As it can be deduced from Fig. 3.17, there are cells that remain viable after such period of time, which would indicate that the materials employed to generate the structure, namely PCL and PEO/NaA, as well as the electrospinning process, do not harm the cells in a dangerous level. However, the number of dead cells is indeed increased over time, but the reason for this event needs yet to be addressed, since the utilization of differentiation medium typically provokes an increment on cell death.

### Myotubes formation

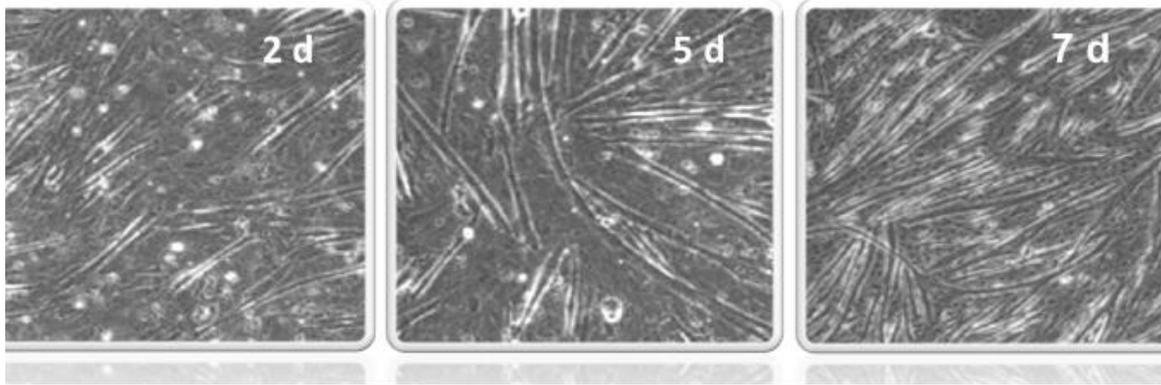
Aiming to provide a better characterization of the differentiation process of C2C12 seeded over the electrospun scaffold, samples were analysed searching for the presence of myotubes, which would indicate the first step in the development of C2C12 cells towards a skeletal muscle tissue regeneration.

However, there was no clear evidence of such structures, and if cell populations were found in a similar morphology, as in the case of Fig. 3.19, the red coloured nucleus pointed their unviability, leaving the conclusion that there is no proof of a differentiation process initiated after 5 days of incubation of C2C12 over PCL/PEO-NaA electrospun scaffold.

When comparing with the standard development of C2C12 in normal medium condition, it was found that cell did start to differentiate after the same period of time, as it can be noted in Fig.3.20.



**Figure 3.19.** Fluorescent microscopy images of myotubes-like morphology, showing sign of death by the red-marked nuclei.



**Figure 3.20:** Muscle precursors after two, five and seven days of incubation in differentiation medium, showing the formation of myotubes already after five days. <sup>53</sup>

These results lead to question the particularities of the ambient surrounding C2C12 cells within the PCL/PEO-NaA fibres, raising doubts about a possible harmful effect that it could be provoking in the cells.

Although it has been demonstrated that cells are indeed capable to survive under such environment, perhaps it has altered the cellular behaviour or response to stimuli.

It could also signify that during the electrospinning process C2C12 undergo high level of shear stress, which in could in turn alter the cell properties and even induce genetic mutations.

In any case, further research should be done in order to assess the nature of these results and to investigate deeper the potentiality of coaxial electrospinning for tissue engineering scaffolds that in future would allow the creation of a skeletal muscle model.

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## IV. Conclusion

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To date, tissue engineering has led to new therapeutic strategies that have been able to tackle certain pathologies. Extensive research has enabled the fabrication of grafts that exploit the properties of biomaterials and cell technologies for regenerative medicine.

In this work, cell embedding fibres have been successfully produced with different organisms, from bacterial to eukaryotic cells, by coaxial electrospinning. The materials of choice have been PCL as fibre shell and different core solutions, aiming to compare the products and so, optimize the composition of the scaffold.

The utilization of PEO yielded a mat of fibres with greater orientation freedom whereas NaA fibre core resulted in electrospun fibres highly aligned, which would favour the right disposition of the growing cells along the fibre, mimicking the myotube structure of native skeletal muscle tissue and possibly facilitating the future engineering process.

However, instability issues in the Taylor cone occurred when electrospinning with NaA and consequently the resulting fibres were thinner than those containing PEO, suggesting the employment of the combination of both as core solution to benefit from their properties.

Regarding the porosity of the fibres, it was found that to reach an adequate number of pores is required the addition of PEG to either the shell solution or to the collecting medium.

Another crucial part of this study, was the evaluation of viability of cells when electrospun into fibres. So far, *S. cerevisiae* cells have demonstrated to be able to survive after being electrospun into the polymeric solutions and retain their metabolic and proliferative capabilities.

Concerning the cell line C2C12, the assessment of viability within electrospun coaxial fibres proved to be rather challenging. Despite the complications, this report presents evidence of viable cell populations embedded in PCL/PEO-NaA fibres. From experience, this report recommends the use of a Calcein/PI assay for such purpose.

Lastly, the development of C2C12 towards skeletal muscle differentiation could not be addressed by the incubation of seeded cells over time onto the electrospun fibrous scaffold, since no clear evidence of myotube formation was found. A future continuation of this research could perhaps identify and solve the problem behind such phenomenon, pursuing the advancement in skeletal muscle regeneration.



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## V. Outlook

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This chapter discusses some strategies by which the work presented could be improved upon.

The majority of the issues encountered along the project were likely due to the fluorescence reflecting capacity of the PCL fibre shell, which complicated greatly the procurement of clear conclusion, especially in terms of viability assessment.

Having had more time, repetition and incorporation of new assays could have been performed to include a further characterization of the cells condition within the scaffold. Alternative materials could also be studied to design the most optimal environment for the thrive of the cells, since only three materials are thoroughly tested in this work.

Moreover, the progression towards skeletal muscle differentiation was not addressed to C2C12 embedded cells, but only to those seeded onto the PCL/PEO-NaA fibres. Future investigation should tackle this to bring light to the potentiality of the construct as a suitable scaffold for skeletal muscle, since the lack of myotubes formation did not allow to conclude the specifics on that matter.

In any case, concerning the viability results from the Calcein/PI assay, data analysis showed not significant results given the large statistical error, resulting from the very random distribution of cells within or onto the scaffold. Apart from overcoming this lack of homogeneity, repetition of all experiments should be performed at least until statically significant values are yielded from the results.

Besides, entrapped cells inside the fibres could meet another medical need as the controlled release of biomolecules or therapeutic drugs to the body, through cell-surface receptor interactions. Additionally, their applicability as disease models could be explored, since the current research has focused mostly on their substrate-like behaviour. In this fashion, they could help treating several pathologies with high prevalence in the worldwide population such as cancer, diabetes mellitus or cardiovascular diseases.<sup>62</sup>

On the other hand, recently multiple-tool biofabrication has emerged as another available methodology for tissue engineering. For instance, hydrogel constructs are reinforced by the deposition of thermoplastic polymer PCL fibres, bringing together inkjet printing and electrospinning techniques. The advantage of such combinations is the lower polymer concentrations to process the hydrogels, while shape and strength of the overall construct will be secured by the polymer network. By this means, more complex shaped tissue grafts could be fabricated, and their application to skeletal muscle engineering could be studied.<sup>11</sup>

Despite electrospinning yields higher resolution of PCL fibres compared to 3D bioprinting, and the resulting mat mimics better the structure of natural ECM, complications to control fibre deposition and extremely small pore size of the resulting fibres become often an obstacle. Nonetheless, melt electrospinning techniques may address both limitations, given that fibres are deposited with high spatial resolution and orientation. Furthermore, it could be advantageous to achieve solutions of interested when dealing with solubility issues, as melt-electrospinning ensures the lack of solvent toxicity problems by the very only use of the polymer melted. <sup>4</sup>

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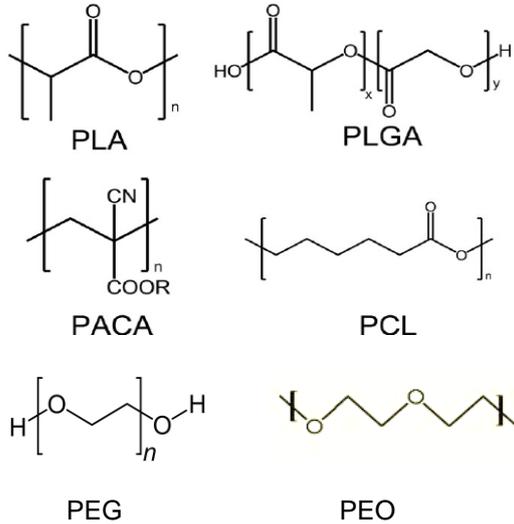
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## Appendix

Synthetic polymers commonly used for biofabrication.



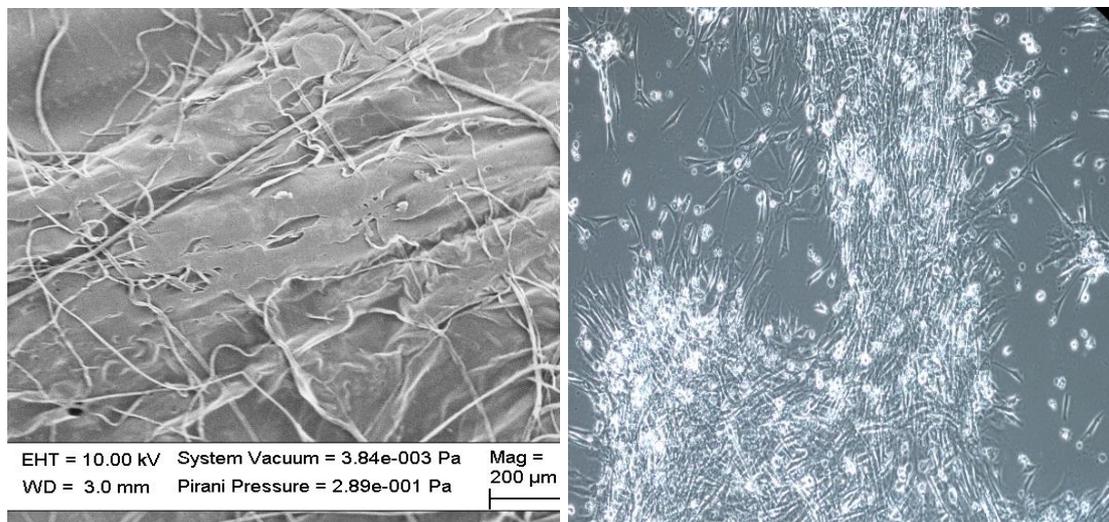
Polyurethanes (PEU)	Artificial hearts and ventricular assist devices Catheters
Poly(tetrafluoroethylene) (PTFE)	Pacemaker leads Heart valves Vascular grafts Facial prostheses Hydrocephalus shunts Membrane oxygenators Catheters and sutures
Polyethylene (PE)	Hip prostheses Catheters
Polysulphone (PSu)	Heart valves Penile prostheses
Poly(ethylene terephthalate) (PET)	Vascular grafts Surgical grafts and sutures
Poly(methyl methacrylate) (pMMA)	Fracture fixation Intraocular lenses Dentures
Poly(2-hydroxyethylmethacrylate) (pHEMA)	Contact lenses Catheters
Polyacrylonitrile (PAN)	Dialysis membranes
Polyamides	Dialysis membranes Sutures
Polypropylene (PP)	Plasmapheresis membranes Sutures
Poly(vinyl chloride) (PVC)	Plasmapheresis membranes Blood bags
Poly(ethylene-co-vinyl acetate)	Drug delivery devices
Poly(L-lactic acid), Poly(glycolic acid), and Poly(lactide-co-glycolide) (PLA, PGA, and PLGA)	Drug delivery devices Sutures
Polystyrene (PS)	Tissue culture flasks
Poly(vinyl pyrrolidone) (PVP)	Blood substitutes
Hydroxyapatite	Bone repair
Collagen	Artificial skin

**Figure 7.1. Top:** chemical formulae of different polymeric compounds typically employed in biofabrication of scaffolds. **Bottom:** table containing a list of polymers and current extend of their applications. <sup>2,29</sup>

### Further Characterization of Scaffold Seeding

For a better description, SEM pictures were taken in order to visualize the aspect of the cell-free scaffold of PCL/PEO-NaA fibres after the electrospinning with no C2C12 cells. As evident in Fig. 7.1 the images revealed the deposition of long and thin fibres with homogeneous sizes, indicating no formation of additional jets on the needle during the electrospinning.

Besides, an additional image is provided in Fig, showing the disposition of C2C12 cells after seeding onto the scaffold. It is noticeable a tendency from C2C12 to group together and cover the scaffold structure until is barely visible anymore.



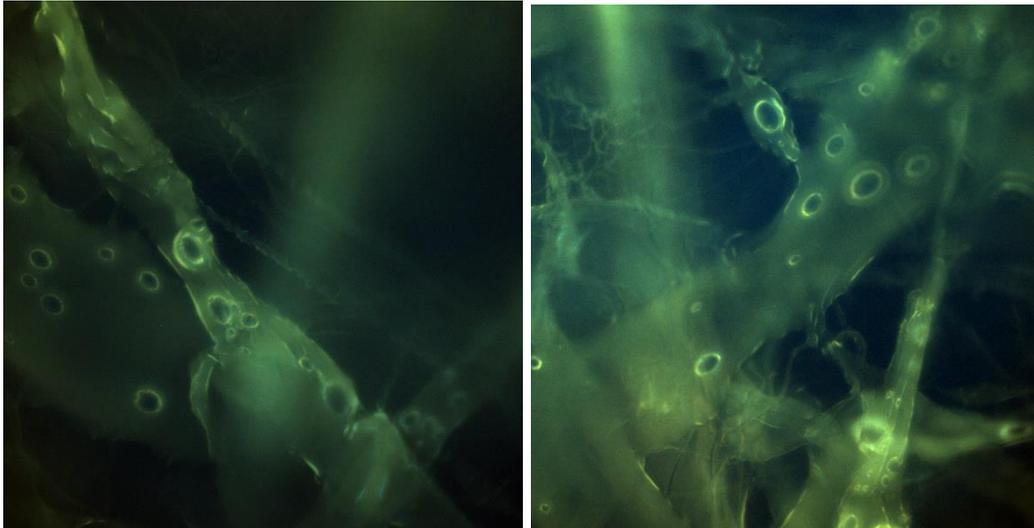
**Figure 7.2. Right:** SEM visualization of the scaffold of PCL/PEO-NaA electrospun fibres without cells. **Left:** standard microscopic image of C2C12 cells seeded over the electrospun scaffold Scale bar = 200 µm

### Characterization of PEU/PEO electrospun fibres

Aiming to assess which polymer is the most suitable for the electrospinning process, polyurethane (PEU) was tested for the fibre-shell generation, solution due to its ability to allow O<sub>2</sub> diffusion through it..

The fibrous scaffold yielded by this material, shown in Fig. 7.3. High level of porosity is presented all over the fibres, with a larger diameter than previously described fibres.

However, when analysing cell viability by staining with Calcein/PI, fluorescent microscopy visualization was not conclusive in the sense that no presence of cells could be found within the fibrous scaffold.



**Figure 7.3.** *Fluorescent microscopy visualization of the scaffold of PEU/PEO-NaA electrospun fibres with C2C12 cells that are not visible by Calcein/PI staining.*

The reason behind the unsuccessful entrapment of cells could be result from a variation in the electrospinning parameters due to the modification of the shell material, from PCL to PEU. Seeing as the polymer solution properties have a great influence in the outcome of the procedure, it stands to reason that this polymer is not as easily spannable as PCL, leading to discard the selection of this material for further investigation.