Design and development of anisotropic laminate scaffolds of electrospun polycaprolactone for annulus fibrosus tissue engineering applications

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Design and development of anisotropic laminate scaffolds of electrospun polycaprolactone for annulus fibrosus tissue engineering applications

By

Andrea Fotticchia

Doctoral Thesis

Submitted to Loughborough University in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Centre for Biological Engineering

Department of Mechanical and Manufacturing Engineering

Loughborough University

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Abstract

In several cases, current therapies available to treat a large number of musculoskeletal system diseases are unsatisfactory as they provide only temporary or partial restoration of the damaged or degenerated site. In an attempt to maintain a high standard of life quality and minimise the economic losses due to the treatments of these frequently occurring ailments and subsequent lost working days, alternative therapies are being explored. Contrary to the current treatments, tissue engineering aims to regenerate the impaired tissue rather than repair and alleviate the symptoms; thus offering a definitive solution.

The annulus fibrosus (AF) of the intervertebral disc (IVD) is a musculoskeletal system component frequently subjected to degeneration and rupture, characterised by predominance of anisotropically arranged collagen fibres.

In the present thesis, electrospinning technology is used to fabricate polycaprolactone (PCL) scaffolds intended to replicate the anisotropic structure of the AF. Process parameters were adjusted to fabricate a wide range of random and aligned defect-free micro- and nano-fibrous matrices. Mechanical properties were investigated to demonstrate their dependence on the combination of fibre orientation and environmental conditions.

Human mesenchymal stem cells (hMSCs) cultured on mono-, double- and multi-layers demonstrated a strong influence of laminate scaffold features and configuration on cell morphology and viability. Cells showed good integration with 3-dimensionally angle-ply arranged electrospun (ES) PCL layers, and migration capability. Cell viability was found to be dependent on matrix thickness and, to a less extent, fibre diameter. Therefore, the same parameters were studied further in an acellular model; thereby supporting the hypothesis of scaffold morphological features influence on mass transfer and, subsequently, cell viability.
A reliable methodology to mount cellularised double-layers around agarose cores, to resemble the IVD configuration, was developed and deemed adequate when subjected to compressive mechanical stimulation in a bioreactor.

Moreover, a novel magnesium nanoparticles (Mg nucleus pulposus (NPs)) loaded matrix was successfully manufactured and characterised. The assessment of Mg NPs loaded matrix cytocompatibility allowed identification of a new research area with high potential for designing a broad range of vascularised tissue engineered devices. Eventually, preliminary studies were conducted to explore the potential of ES PCL matrix for tendon tissue engineering.
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<tbody>
<tr>
<td>AA</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>AB</td>
<td>Alamar blue</td>
</tr>
<tr>
<td>CF</td>
<td>Chloroform</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>GF</td>
<td>Growth factor</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IPA</td>
<td>Isopropanol</td>
</tr>
<tr>
<td>L-Gln</td>
<td>Glutamine</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>Mg</td>
<td>Magnesium</td>
</tr>
<tr>
<td>NE-AA</td>
<td>Non-essential amino acids</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TGF-βx</td>
<td>Transforming growth factor x (x=1, 2 or 3)</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
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#### Misc.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AF</td>
<td>Annulus fibrosus</td>
</tr>
<tr>
<td>BSC</td>
<td>Biological safety cabinet</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DI</td>
<td>Deionised</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy dispersive x-ray</td>
</tr>
<tr>
<td>ES PCL</td>
<td>Electrospun polycaprolactone</td>
</tr>
<tr>
<td>ESC</td>
<td>Environmental stress cracking</td>
</tr>
<tr>
<td>FDM</td>
<td>Fused deposition modelling</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
</tr>
<tr>
<td>IVD</td>
<td>Intervertebral disc</td>
</tr>
<tr>
<td>MD</td>
<td>Machine direction</td>
</tr>
<tr>
<td>Mn</td>
<td>Number average molar mass</td>
</tr>
<tr>
<td>Mw</td>
<td>Mass average molar mass</td>
</tr>
<tr>
<td>MW</td>
<td>Multiwell</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleus pulposus</td>
</tr>
<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>ODF</td>
<td>Orientation distribution function</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>OM</td>
<td>Optical microscope</td>
</tr>
<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
</tr>
<tr>
<td>PD</td>
<td>Polydispersity</td>
</tr>
<tr>
<td>PGTMC</td>
<td>poly(glycolide-co-trimethylene carbonate)</td>
</tr>
<tr>
<td>PLCL</td>
<td>Poly(lactic-co-caprolactone)</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactide-co-glycolide) acid</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet-rich plasma</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Rt-PCR</td>
<td>Real time polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>T&lt;sub&gt;b&lt;/sub&gt;</td>
<td>Boiling point</td>
</tr>
<tr>
<td>T&lt;sub&gt;g&lt;/sub&gt;</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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**Genes**

- Acan: Aggrecan
- Coll I: Collagen type I
- Coll III: Collagen type III
- Scx: Scleraxis
- SP7: Osterix
- Tnc: Tenasin
- Tnmd: Tenomodulin

**Mechanical properties**

- E<sub>high</sub>: Modulus at high strain
- E<sub>low</sub>: Modulus at low strain
- UTS: Ultimate tensile strength
- ε: Stress
- σ: Strain
1. Introduction

1.1 Problem statement

The human intervertebral disc (IVD) undergoes degeneration with ageing or due to exposure to altered spinal biomechanics as a consequence of injury; conditions that negatively impact on the quality of life (van Tulder et al. 2002). The IVD has a complex composite structure and the aetiology of its degeneration is still not completely understood (Adams & Roughley 2006). Conversely, it is evident that the IVD deterioration process cannot be reversed with the knowledge and technologies currently available. Today’s conservative therapies are able only to partially alleviate the symptoms, whilst surgical treatments are known not to provide a definitive solution (Guterl et al. 2013). Interventions to the IVD may temporarily improve patient’s life quality; however complete healing is rarely achieved especially in the long-term (Raj 2008). Furthermore, these treatments alter the biomechanics of the spine; thereby increasing the risk of degeneration in the adjacent discs (Ekman et al. 2009). Mechanical prostheses provide an alternative solution; however, they suffer from limited biocompatibility and, like the aforementioned therapies, do not restore the original tissue functionality (van den Eerenbeemt et al. 2010).

It is also noteworthy that the average age and life expectancy of the population are constantly increasing in the world. This is paralleled by a growing demand for medical treatments and, therefore, an increasing economic expenditure on healthcare systems (Martin et al. 2008). Today’s governments are, consequently, under great pressure to both reduce the need for and the economic cost of medical interventions.

Tissue engineering is within the regenerative medicine field that aims to restore the physiological structure and functionality of impaired tissues through applying the principles of engineering and biology (Langer 2000). This interdisciplinary approach is believed to provide a better alternative
to the current medical treatments as, by restoring physiological tissue functionality, it not only promises to deliver to patients a much better quality of life, but also reduces healthcare costs by eliminating the need for long-term treatments and follow-up surgeries.

Tissue engineering strategies are, in essence, based on the development of a scaffold (a matrix made of biomaterials) able to deliver cells to the degenerated site; thus triggering healing processes that restore the physiological tissue structure and function (Longo, Lamberti, et al. 2012). Tissue engineering encompasses a variety of approaches that depend strongly on the characteristics of the degenerated site; consequently, the scaffolds for cell delivery are often designed to resemble the structure and mechanical properties of the target tissue.

Electrospinning is a well-established technology that enables polymers to be processed into fibres with a size comparable to those of extracellular matrix components (Liu et al. 2012). Furthermore, it allows the deposition of these fibres in an anisotropic (aligned) manner. This feature is of particular interest for IVD regenerative medicine as it allows the creation of structures that mimic the architecture of the oriented collagen fibres. Furthermore, the use of such anisotropic fibre substrates not only provides cells with opportune topographical cues, but also allows the orientation-dependent mechanical properties of physiological AF tissue to be matched.

The potential of the electrospinning technology is high, as demonstrated by the large number of studies focusing on controlling the process itself (Jeun et al. 2005; Gholipour Kanani & Bahrami 2011), and its application in tissue engineering (Barnes et al. 2007). Electrospun (ES) fibres have been revealed to be able to support cell culture, allowing the manufacturing of simple tissue engineered devices (Liu et al. 2012). In addition, the said scaffold, according to an increasingly popular approach, can be stimulated mechanically to investigate cell response to physical stresses and to enhance tissue development in-vitro prior to implantation (Naing & Williams 2011).

Nevertheless, no tissue engineered artificial replacement has reached the market so far. The steps made in this direction are limited to feasibility studies and demonstration at laboratory
scale. There are several issues that need to be addressed and many questions to be answered before the successful clinical translation of this technology can be achieved.

For example, the effect of the electrospinning parameters on the produced fibres needs to be entirely elucidated. Furthermore, the physical properties of ES PCL matrices, such as wettability and degradation, remain a matter of debate. This framework becomes more complicated when investigating the biocompatibility properties of ES PCL layers. The chemical and topological features that govern cell fate, in terms of morphology and phenotype, are being intensely investigated (Nisbet et al. 2009; Beachley & Wen 2010). However, many controversial results have been obtained in this regard. In particular, there is a lack of knowledge on cellular response to ES PCL matrices arranged in 3-dimensions. There is evidence that cell metabolism is affected by nutrients transport across such scaffolds (Song et al. 2014; Curcio et al. 2014). Nonetheless, the role of morphological scaffold features and size, which are fundamental for the success of the artificial replacement, on cell behaviour is still unclear.

Connected to this, encouraging blood vessel growth into the ES PCL layers or scaffolds is a strategy currently pursued to allow the more efficient delivery of metabolites across the scaffold, even though, inevitably, it also increases the complexity of the scaffold design (Kaully et al. 2009). Moreover, arranging the cellularised scaffold in the desired shape and conveying the opportune mechanical stimuli by means of bioreactor have proven to be challenging (Hudson et al. 2015; Tsai et al. 2014).

To date, several researches have focused on the replication of the AF (See et al. 2011; Park et al. 2012; Hudson et al. 2015) and, to this aim, some have exploited the electrospinning technology to achieve promising results (Koepsell, Remund, et al. 2011; Nerurkar, Sen, et al. 2010). Nevertheless, the approaches pursued so far provide only a partial answer to the AF tissue regeneration challenge. For example, in such studies, primary cells were cultured on anisotropic electrospun mono-layers (Koepsell, Remund, et al. 2011); primary cells were cultured in static conditions in a multilamellar angle-ply (Nerurkar, Sen, et al. 2010) or isotropic (Park et al. 2012)
scaffold; rabbit mesenchymal stem cells were mechanically stimulated in an IVD-like model lacking the AF angle-ply organisation (See et al. 2011; Hudson et al. 2015). Hence, the mechanical stimulation of hMSCs loaded in an artificial replacement, characterised by a stack of anisotropic lamellae and mimicking the organisation of the natural AF, would represent an additional step towards the development of a functional AF artificial replacement.
1.2 Aim of the project

The major aim of this project is to contribute and increase the current knowledge related to the influence of ES PCL matrix with different characteristics and arrangements to human mesenchymal stem cells for application in AF tissue engineering. This aim is pursued by addressing the following specific goals.

The fabrication of a suitable scaffold is carried out by processing polycaprolactone by means of the electrospinning technology to obtain a range of nano- and micro-fibrous matrices with different fibre diameters and patterns (random, semi-aligned and aligned fibres). Extensive morphological characterisation facilitates optimisation of the fabrication process and manufacturing flawless fibrous substrates. The achievement of a reliable fabrication process to obtain good-quality fibres is a key step for the subsequent evaluation of the matrix biocompatibility properties and from the perspective of future industrial scale-up. Moreover, the measurement of the physical properties enables accomplishing a comprehensive understanding of the ES PCL matrices characteristics, which are relevant for tissue engineering applications such as degradation, hydrophilicity and mechanical properties.

The potential of the application of the matrices for tissue engineering purposes is first explored by studying the adhesion and viability of hMSCs seeded on the scaffold surface; in particular, by comparing different fibre patterns. Furthermore, the effect of ES PCL layers arranged in fashion similar to the AF 3-dimensional structure is studied with the aim of evaluating the suitability of such complex scaffold for AF tissue engineering. Adequate investigations methodologies need to be developed to appropriately characterise the novel scaffolds arranged to replicate the AF structure.

Further to the study in static conditions, the scaffolds seeded with cells are mechanically conditioned in a bioreactor, providing the biomechanical stimuli that are anticipated to simulate
the *in-vivo* biomechanical environment. To properly convey the mechanical compression stimulation to the scaffolds, an IVD-like structure must be developed by wrapping the ES PCL layers, loaded with cells, around a hydrogel core.

Two side projects were also conducted parallel to the AF artificial replacement development. Firstly, magnesium nanoparticles were loaded into ES PCL matrix, with potential employment for scaffold vascularisation, and the response of hMSCs seeded on it evaluated. Secondly, the response of tenocytes to ES PCL matrix was investigated in the perspective of tendon tissue engineering as tendon, likewise the AF lamella, is formed of aligned collagen fibres.

### 1.3 Thesis structure

The current study aims to fabricate an artificial replacement of the AF of the human spine using electrospinning and polycaprolactone, as producing technology and material respectively, and applying tissue engineering principles. The project can be divided into two main parts: synthesis and characterisation of the artificial scaffold and cellular response to the scaffold.

Chapter 2 includes the background information needed to have a complete picture of the current AF regeneration issues. The chapter begins by illustrating the IVD anatomy and physiology, paying special attention to the processes that occur during IVD degeneration. Then the most relevant results currently available on AF mechanical properties are reported. Following this section, the issues related to IVD and AF tissue engineering are discussed with a description of the recent advances in the field. In this framework, a detailed description of the electrospinning technology is reported.

In Chapter 3, section 3.1 focuses on how PCL, the material of choice for this project, was processed into nanofibres by means of electrospinning. The outcomes of the fabrication process
are reported in section 5.1 and discussed further in section 7.1, where an explanation of the relationship between fibre morphology and process parameters is provided.

Following the manufacturing process methodology description, the characterisation of the matrices is reported. In this section (3.2), the methodologies presented are: morphology analysis, wettability, degradation and uniaxial mechanical properties. Related results can be found respectively in sections 5.2 and 5.5, and the discussion in 7.1. These sections provide an exhaustive description of the properties of the PCL substrate, with special focus on the mechanical properties and how these properties can be compared with the natural AF tissue. The results of the characterisation lay the foundations for the comprehension of the test described in Chapter 4, where the response of cells is studied in relationship to the morphological features of the PCL layers.

Chapter 4 focuses on the methodologies applied to study the behaviour of hMSCs cultured on the substrates arranged in single and multi-layers configuration (4.1 to 4.5). The major findings pertaining to the response of cells to the ES PCL scaffolds are presented in the results sections (6.1 to 6.7). Sections 4.6 and 4.7 describe the procedures undertaken for studying mass transport through ES layers and the response of hMSCs to lack of oxygen. The relative results can be found in sections 6.8 and 6.9. All the results of the experiments described in the above mentioned sections are discussed in an integrated manner in sections 7.2 and 7.3.

The interaction between hMSCs and the electrospun substrate was also studied in dynamic conditions, simulating the natural IVD anatomy, by fabricating an IVD-like construct. The fabrication and characterisation procedures of the IVD-like construct are detailed in section 4.8. In the same section, the bioreactor used for the mechanical stimulation is described along with the IVD-like manufacturing procedures developed in the current study. The said apparatus was used to demonstrate that cellularised IVD-like constructs are able to bear mechanical stimulation. The outcome of the IVD-like construct fabrication and mechanical stimulation can be found in
section 6.10. The response of preliminary cellularised constructs stimulation tests is extensively commented in section 7.4.

As final remarks, the conclusions and the future perspectives related to the experimental work carried out in this thesis are summarised in sections 8.1 and 8.2.

The appendices include two additional parts which topic is related to the main one. In the first of the two appendices, fabrication of ES matrices of PCL with Mg NPs embedded is presented. Mg has been demonstrated to have beneficial effects on endothelial cells and to play an important role in the cardiovascular system (section 9.1). Such a matrix, intended to be applied as outer layer of the IVD-like construct, was designed to enhance vascularisation with positive effect on nutrients transfer. In sections 9.2 and 9.3, the fabrication process is described alongside the characterisation techniques adopted. The outcome in terms of biological and physical properties is illustrated in section 9.4 and its potential for, but not limited to, AF tissue engineering is discussed in section 9.5.

The second appendix describes the investigation of ES PCL layers for potential employment in tendon tissue engineering. The anisotropy of ES PCL matrices was judged to be a key feature that could be adopted also for tissue engineering of the tendon (10.1 and 10.2). Section 10.3 delineates the methodologies for primary rat tenocytes culture on ES PCL layers. The results of the imaging, viability and gene expression assessment are illustrated in section 10.4. The positive outcome of the experiments, in the perspective of tendon tissue engineering application, is discussed further in section 10.5.
2. Literature review

2.1 Low back pain epidemiology

Back pain, specifically low back pain, in its acute and chronic form, is a relevant concern for national health systems and economies. Although it is widely recognised that back pain is presently a major impairment of people’s life quality, there is no consensus about its definition and specific features (Adams & Roughley 2006). It is noteworthy that a study in USA revealed that 90% of low back pain cases can be attributed to non-specific pain. In other words, a specific cause of the pain could be identified for only a small portion of patients (van Tulder et al. 2002). Back pain might be also classified according to its severity and duration and, despite a large number of people may completely recover after an acute back pain episode, it causes the largest number of inactivity events in under 45 years old people (Andersson 1999). In addition, in the USA it was estimated that back pain is the third cause of surgical operations, with 1 billion USD expenditure only for spinal surgery (Weinstein et al. 2006), whilst studies in Sweden and UK demonstrated that 11-19% and 12% of work absenteeism respectively is due to low back pain cases (Andersson 1999) with related costs, for the UK, estimated in about 4.3 billion GBP (Maniadakis & Gray 2000). Alongside these data, economic analysis further highlighted that the national costs for back pain in UK, Sweden and Netherlands were 3.3, 2.5 and 5.0 billion USD respectively (van Tulder et al. 2002). These expenditures are expected to rise as a research in the US covering nine years highlighted a steady increase of expenditures related to low back pain (Martin et al. 2008). It is also noteworthy that half of the working days loss and social cost is attributed to a small group of more severely affected people (15%)(van Tulder et al. 2002).

The statistics reported above remark the importance of finding a solution for back pain disease; in particular, when the most severe events provoke disability that significantly affects life quality.
2.2 IVD

2.2.1 Structure and function

The intervertebral disc (IVD) is a collagen-rich structure located between the vertebrae of mammalian spine. Its role is to sustain the body weight, adsorb shocks and give the spine a certain degree of flexibility. The IVD structure is composed of three components: the end plates (EPs), the AF and the NP as revealed in Figure 2.1.

The EPs are cartilage layers which lie at the interface between the vertebrae and the other IVD structures. The EPs play a role in mass transfer, as they are, along with the periphery of the AF to a lesser extent, the only regions slightly innervated and vascularised, thus providing the rest of the IVD with nutrients (Kobayashi et al. 2010; Urban et al. 2004). The region comprised between the EPs is filled with two concentric components. The NP in the centre is a rounded hydrogel-like portion mainly made of water, retained by a matrix essentially formed of collagen type II and proteoglycans. The liquid part constitutes up to about 80% of the NP whole weight. The series of plies wrapped around the NP form the AF, mainly made of collagen type I rather than II. In radial direction the AF is not uniform as characterised by a gradual change of ratio of ECM components; the outer AF layers have a higher collagen type I to

Figure 2.1: A) schematic representation of the IVD; B) MRI of a spine motion segment. AAF=anterior annulus fibrosus, PAF=posterior annulus fibrosus, NP=nucleus pulposus, VB=vertebral body. Reproduced with permission from BMJ Publishing Group Ltd. (Nerurkar, Elliott, et al. 2010).
type II ratio that leads to a lower water content as collagen II is more hydrophilic than I (Bron et al. 2009; Clouet et al. 2009). In Table 2.1 the AF and NP main components and relative amounts are reported.

Table 2.1: Average values of the main components measured in non-degenerated IVDs.
Percentage may change according to the location (i.e. inner or outer AF) and age. Adapted from (Le Maitre et al. 2007; Antoniou et al. 1996; Eyre & Muir 1976). IAF=inner AF; OAF=outer AF.

<table>
<thead>
<tr>
<th>Component (% of dry weight)</th>
<th>AF</th>
<th>NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>65-75%</td>
<td>75-85%</td>
</tr>
<tr>
<td>Collagen</td>
<td>50-60%</td>
<td>15-20%</td>
</tr>
<tr>
<td>of which:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>IAF</td>
<td>OAF</td>
</tr>
<tr>
<td>32%</td>
<td>84%</td>
<td>0%</td>
</tr>
<tr>
<td>Type II</td>
<td>64%</td>
<td>16%</td>
</tr>
<tr>
<td>Proteoglycans</td>
<td>15-20%</td>
<td>65%</td>
</tr>
<tr>
<td>Other proteins and elastin</td>
<td>5-25%</td>
<td>20-25%</td>
</tr>
</tbody>
</table>

Both NP and AF play a fundamental role in withstanding the loads to which the spine is subjected. The loads are the result of the complex combination of uniaxial compression due to the body weight and bending and torsional forces caused by back and torso movements. Uniaxial compression, which possesses a static or cyclic nature due to the gait, is withstood by the NP, thanks to the osmotic pressure provided by the hydrophilic matrix. In particular, aggrecan, the main NP proteoglycan, has highly polar side chains; thereby playing a major role in attracting water (Watanabe et al. 1998). In addition, loss of water from NP is also prevented by the presence of the AF layers, which is less hydrophilic and is formed of a densely packed collagen matrix (Nerurkar, Han, et al. 2011). However, during compression water tends to exude, as demonstrated by the IVD height loss during the day and recovery during the night (Grunhagen et al. 2006; Lewis & Fowler 2009). Under a bending motion, the nucleus moves within the IVD in the opposite direction of the movement. This induces stress to the fibres of the AF, which bulge out.
opposite to the compressed side (Jensen 1980; Adams 2004). The AF is subject to both negative and positive deformations in axial and radial direction respectively (O’Connell et al. 2011). The AF is formed of a stack of layers, composed largely of aligned collagen type I fibres that form an average 30° angle with the horizontal plane. Each layer has an alternation of about ±30° of the fibre angle compared with the adjacent layers, proceeding from the outside to the inside in radial direction (Figure 2.2) (Bron et al. 2009). Actually, the said angle is not constant and varies from 28° to 44° towards the inner portion (Bron et al. 2009). The AF structure is not even symmetric along the circumference as the number of plies varies between 15 to 22 corresponding to the posterior and anterior AF respectively (Jackson et al. 2012).

![Schematic of the fibres angle within the AF multilamellar structure. α indicates the angle between the AF lamella collagen fibres and the horizontal plane. Reproduced with permission from BMJ Publishing Group Ltd. (Adams & Roughley 2006).](image)

2.2.2 Vascularisation and nutrition

A remarkable IVD characteristic is its avascular nature. The IVD is the largest avascular structure of the human body with an average size of 15 mm in thickness and 30-50 in diameter (Huang & Gu 2008; Ishihara & Urban 1999). In the whole body, oxygen and nutrients supply and wastes removal are normally accomplished by the blood vessels that actively convey molecules with the perfusion of blood. Molecules diffuse from vessels to the surrounding tissues covering a maximum distance of 200 µm, except for cartilage which is up to 1 mm (Kaully et al. 2009).
Conversely, the closest blood vessels lie on the IVD periphery, namely the outer AF and the EPs (Urban et al. 2004). The AF structural anisotropy has an effect on nutrients supply which has been demonstrated to be more efficient along the axial pathway compared with radial one (Jackson et al. 2012), meaning a more relevant role of the EPs over the AF. The decreased permeability of the EPs, associated to calcification is considered one of the primary causes of molecules diffusion impairment and, subsequently, disc degeneration (Bibby & Urban 2004; Grunhagen et al. 2006; Rajasekaran et al. 2004). Conversely, oxygen and lactate diffusion rate, characterised by opposite concentration trends in radial direction, demonstrated not to be affected by the degeneration degree of the disc (Bartels et al. 1998). The same authors also pointed out a high variability between individuals with a minimum oxygen level equal to 0.7% (Bartels et al. 1998). In a later study, oxygen concentration was revealed to vary from around 8-10% at the IVD periphery to 0.3-0.5% in the centre (Ishihara & Urban 1999). IVD cells cultured at the same low oxygen concentration found in IVD centre exhibited a decrease in proteins and proteoglycans synthesis, thereby proving the difficulties in maintaining the homeostasis in such harsh conditions (Ishihara & Urban 1999). Further studies on IVD cells have revealed that glucose deprivation and low pH negatively affect cell viability thus providing another explanation of disc degeneration (Bibby & Urban 2004).

So far, all the mechanisms involved in IVD degeneration have not been completely elucidated; however, there are a number of evidences indicating that the impairment of an environment favourable for cell function is tightly related to disc degeneration. Moreover, the equilibrium of gases, nutrients, wastes and macromolecules relies on an efficient diffusion mechanism and proper blood supply at the IVD periphery.

### 2.2.3 Degeneration aetiology and progression

Disc degeneration is the result of a combination of causes (impaired nutrients supply, structural defects, genetic inheritance, ageing, traumas) that interact in a way that is not yet well
understood (Adams & Roughley 2006; Beattie 2008; Shankar et al. 2009). A study on 600 IVDs has pointed out that onsets of degeneration appear in the second and third life decade for males and females respectively (White & Panjabi 1990). Furthermore, there is evidence that by age of 50, 97% of the discs are degenerated to some extent; in particular in the lumbar region (Kalson et al. 2008). Once the IVD is damaged, the subsequent cascade of degenerating reactions will not stop because NP and AF tissues are not able to self-repair due to their avascular nature (Adams & Roughley 2006). The processes involved lead to a loss of proteoglycans, a change in the proportion of collagen and a variation of types of collagen (Adams & Roughley 2006). It has been found that fragmentation of proteoglycans normally begins in young people, leading to a decrease from 70% to 20% of the IVD dry weight by 60-80 years old (Kalson et al. 2008). In the inner AF, collagen type II is replaced by type I, along with a decrease in ECM synthesis turnover and cell number (Adams & Roughley 2006; Liebscher et al. 2011; Ding et al. 2013). The diminished amount of hydrophilic macromolecules provokes a loss of water and a subsequent increase of IVD stiffness (Raj 2008; Sen et al. 2012).

Impaired quality of life arises from back pain, a consequence of IVD degeneration that leads IVD assuming unnatural positions and conformations. At this stage, a range of phenomena occurs. IVD might undergo to herniation when nuclear matrix bulges outward or collapses inside the adjacent bones. Alteration of motion segment mobility exerts non-physiological loads on the adjacent vertebrae thus possibly accelerating degeneration of other IVDs (Ekman et al. 2009). These changes, which may ultimately lead to AF rupture and NP leakage, provoke stimulation to pain nerves (Raj 2008; van den Eerenbeemt et al. 2010). Nerves were also found growing inside degenerated IVDs (Freemont et al. 1997).
2.2.4 Current treatments

Treatments of symptomatic IVD degeneration can be divided into three categories: conservative and surgical treatments, and total disc replacement. Conservative treatments aim to manage pain and prevent it to become chronic or to have a remarkable negative impact on life quality, by means of a variety of techniques such as physiotherapy, cognitive therapies and drugs administration (van Tulder et al. 1997). Surgical treatments encompass a range of methodologies that vary according to the conditions of the IVD to be restored. Total disc replacement, conversely, consists of complete IVD removal and the insertion of a mechanical device (Kleuver et al. 2013). However, all these techniques do not offer a permanent solution to the underlying problem of the diseases (Raj 2008; van den Eerenbeemt et al. 2010; Pirvu et al. 2015).

Disc replacement and spinal fusion alter the spine structure, limiting the mobility, and modify the spinal biomechanics and the distribution of tissue stresses, provoking non-physiological loads on adjacent discs (Ekman et al. 2009). Reviews of randomised trials pointed out no significant difference between clinical outcomes provided by disc replacement or spinal fusion; however, there remain concerns for the long-term performance, which tends to be poorer compared with the short-term (Kleuver et al. 2013; Berg et al. 2009). However, even for successful surgery, not all the patients treated consider the outcome satisfactory (Asch et al. 2002).

Furthermore, it must be stated that, the existing disc replacement surgery cases all remain at prosthesis level (van den Eerenbeemt et al. 2010). This means that these artificial replacements have no active role inside the body rather than a simple restoration of the mechanical properties. The re-operation rate is also slightly higher than surgical interventions and there are some concerns regarding the complications consequence of disc replacement treatment (van den Eerenbeemt et al. 2010).
2.2.5 **AF mechanical properties**

In this section, the most relevant findings concerning the assessment of AF mechanical properties are reported focusing on uniaxial tests of single and multilamellar AF samples. In Table 2.2 the major findings concerning tensile tests on explanted AF samples are summarized. Mechanical properties have been evaluated by dissecting the AF in two different ways: isolating single lamellae or cutting thick multilamellar portions of the AF. These approaches return different information and are complementary to each other. Moreover, for both strategies, different mechanical test designs were adopted as well as modulus calculation methodologies.

**Multilamellar mechanical tests**

Multilamellar tests can be performed by adopting a cylindrical coordinate system; thereby stretching tissue samples in axial, radial and circumferential directions.

Circumferential stress has been found to be in the range 1.70-4.71 MPa relative to the toe region with the highest values provided by Isaacs et al. who did not specify the region of the AF investigated (Isaacs et al. 2014; Guerin & Elliott 2006; Elliott & Setton 2001). Ebara et al. have measured a considerably higher modulus; nevertheless, the distinction between toe and linear modulus has not been done, as in the previously reported experiments (Ebara et al. 1996). The outer AF modulus in the linear region has been found to be higher than that of the inner AF (Elliott & Setton 2001). This was ascribed to the higher collagen type I/type II ratio of the outer AF layer in respect to the inner AF (Chan & Gantenbein-Ritter 2012).

The toe modulus always exhibited lower values compared with the linear modulus at higher strain. This is explained by the low energy required at small strain to straighten the collagen fibres (Elliott & Setton 2001). Moreover, the same authors noticed the absence of dependence on the location, for all the stretching directions tested, demonstrating the weak relationship of toe modulus with AF composition.
Conversely, radial modulus has been found significantly lower than circumferential modulus with good agreement between the experiments of Isaacs et al. and Elliott et al. (Elliott & Setton 2001; Isaacs et al. 2014). The low modulus assessed in this configuration has been attributed to the absence of collagen fibres oriented in radial direction contrary to axial and circumferential direction (Elliott & Setton 2001).

With regard to axial directions very high variability was found across few different experiments. Elliot et al. found similar values to those measured in radial direction in the range 0.27-0.96 MPa (Elliott & Setton 2001). A stiffer behaviour (1.5-16 MPa) was detected by Kasra et al. in axial direction, compared with that reported by Elliott et al. (Kasra et al. 2004). The discrepancy could be due to the presence of the upper and bottom bones with AF explants, which, conversely, were removed in the test conducted by Elliott et al. The interruption of the collagen structure integrity as the consequence of sample preparation may also be the cause of the different performance (Kasra et al. 2004). The different source of AF chosen (sheep Vs human) may have been an additional contributing factor to the discrepancy.

**Single lamella mechanical tests**

Holzapfel et al. found the tensile modulus of AF single lamella, measured at the highest strain regime, to be higher for the outer AF (5.96-8.01 MPa compared with 3.79-3.80 MPa of the posterior AF, toe modulus) (Holzapfel et al. 2005). The same trend was confirmed in a further two experiments though measuring higher moduli (Zhu et al. 2008; Skaggs et al. 1994). In addition, these two experiments highlighted a dependence of the modulus from the circumferential position (higher in the anterior region) that, conversely, resulted not significant in Holzapfel et al.’s test (Zhu et al. 2008; Skaggs et al. 1994; Holzapfel et al. 2005). The modulus values obtained by Pezowicz et al. lie within the range of other single lamella studies (Zhu et al. 2008; Holzapfel et al. 2005) even though direct comparison is difficult as the source of the disc and the sample location within the disc are not specified (Pezowicz 2010). Isaacs et al. measured values
remarkably lower than those found in the previously mentioned studies; however, the authors did not provide an explanation for the discrepancy with earlier studies (Isaacs et al. 2014).

**Summary**

Due to the complex structure of the AF, the possible combinations of testing configurations are several; therefore, even a range of AF tensile modulus is difficult to provide. However, the experiments on both multilamellar and single lamella have revealed a trend of the modulus that increases when shifting from the posterior to the anterior and from inner to outer AF, with the exception of Green et al.’s test (Green et al. 1993). Furthermore, concerning multi-lamellar samples, higher values were assessed when tests were carried out in circumferential direction compared with radial and axial, even though some exceptions again emerged (Green et al. 1993; Żak & Pezowicz 2013).

Single lamellae have been revealed to have higher tensile modulus compared with thick AF portions. The difference is related to the structure of the single lamella whose collagen fibres were oriented in the same stretching direction whereas in multilamellar samples collagen fibres have varying angles, depending on the lamella where they reside. Therefore the collagen configuration of the single lamella is more favourable to exert higher tensile strength. This is further confirmed by the higher modulus assessed on single lamella stretched in longitudinal direction compared with transversal (Isaacs et al. 2014).
Table 2.2: Summary of the most relevant findings of the mechanical tests performed on natural AF tissue. ($E_0$=toe region; $E_1$=linear region; $E_1$ or $E_{low}$, $E_2$ or $E_{medium}$ and $E_3$ or $E_{high}$ are three regions of stress-strain curve according to Zhu et al. or Holzapfel et al. respectively (Zhu et al. 2008; Holzapfel et al. 2005); SD is reported only when the precise value is provided by the authors).

<table>
<thead>
<tr>
<th>Direction of stretching</th>
<th>Source</th>
<th>Parameter</th>
<th>Region analysed and average modulus value [MPa]</th>
<th>Reference</th>
</tr>
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<td>Circumferential</td>
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<td>$E_0$</td>
<td>Outer AF</td>
<td>2.52±2.27</td>
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<td></td>
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<td></td>
<td>Inner AF</td>
<td>1.70±1.21</td>
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<td></td>
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<td>$E_1$</td>
<td>Outer AF</td>
<td>17.45±14.29</td>
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<td></td>
<td></td>
<td>$E_0$</td>
<td>Outer AF</td>
<td>4.71±0.77</td>
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<td></td>
<td>$E_1$</td>
<td>Outer AF</td>
<td>11.04±2.24</td>
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<tr>
<td>Radial</td>
<td>Dynamic E</td>
<td>1-3.23</td>
<td>Outer AF*</td>
<td>Inner AF*</td>
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<td>E</td>
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<td>Posterior</td>
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<td>=20</td>
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<td>E</td>
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<td>Anterior</td>
<td>Anterior</td>
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<td>=49</td>
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<td>=10</td>
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<td></td>
<td>Outer AF</td>
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<td></td>
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<tr>
<td></td>
<td>E0</td>
<td></td>
<td>0.19±0.04</td>
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<tr>
<td></td>
<td>E</td>
<td></td>
<td>0.45±0.25</td>
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<td></td>
<td>E0</td>
<td></td>
<td>0.113±0.03</td>
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<td>E</td>
<td></td>
<td>0.212±0.04</td>
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<td>Outer AF~</td>
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<td></td>
<td>E0</td>
<td></td>
<td>≈1.5-2.8</td>
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<td></td>
<td>E</td>
<td></td>
<td>≈11-16</td>
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<td>Pig</td>
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<td>Cervical</td>
<td>Thoracic</td>
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<td>Outer AF~</td>
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<td></td>
<td>E0</td>
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<td></td>
<td>E</td>
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<td></td>
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</tr>
<tr>
<td>Human</td>
<td>Anterior</td>
<td>25.11±12.40</td>
<td>Anterior</td>
<td>20.05±4.58</td>
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<tr>
<td></td>
<td>Outer AF</td>
<td></td>
<td>Inner AF</td>
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</tr>
<tr>
<td></td>
<td>$E_0$</td>
<td>0.27±0.28</td>
<td>0.34±0.21</td>
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<tr>
<td></td>
<td>$E$</td>
<td>0.82±0.71</td>
<td>0.96±1.17</td>
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- **(Elliott & Setton 2001)**

<table>
<thead>
<tr>
<th>Single layer</th>
<th>Human</th>
<th>Anterior</th>
<th>Posterior</th>
<th>27.2±10.2</th>
<th>Anterior</th>
<th>7.2±3.1</th>
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<tr>
<td>$E_{1}$</td>
<td></td>
<td>66.42±34.91</td>
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<td>$E_{2}$</td>
<td></td>
<td>118.60±23.42</td>
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<td>$E_{3}$</td>
<td></td>
<td>118.71±25.17</td>
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</table>

- **(Green and Adams, 1993)**

<table>
<thead>
<tr>
<th>Human</th>
<th>$E_{low}$</th>
<th>Outer AF</th>
<th>Inner AF</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Posterior</td>
<td>Posterior</td>
<td>3.80±5.02</td>
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<td></td>
<td></td>
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<td>Anterior</td>
<td>3.79±2.61</td>
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<tr>
<td></td>
<td></td>
<td>5.96±3.05</td>
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<tr>
<td></td>
<td></td>
<td>8.01±6.50</td>
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</table>

- **(Holzapfel et al. 2005)**

*Numbers represent average values ± standard deviation.*
<table>
<thead>
<tr>
<th></th>
<th>Posterior</th>
<th>Posterior</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>$E_{medium}$</td>
<td>24.1±12.3</td>
<td>14.0±8.63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32.5±12.1</td>
<td>13.9±8.13</td>
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<tr>
<td>$E_{high}$</td>
<td>64.9±48.6</td>
<td>31.2±19.8</td>
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<tr>
<td></td>
<td>77.6±20.0</td>
<td>27.5±12.8</td>
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<table>
<thead>
<tr>
<th></th>
<th>Longitudinal</th>
<th>Transversal</th>
<th>(Isaacs et al. 2014)</th>
</tr>
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<tbody>
<tr>
<td>$E_0$</td>
<td>9.67±1.82</td>
<td>1.38±0.11</td>
<td></td>
</tr>
<tr>
<td>$E$</td>
<td>16.15±1.52</td>
<td>1.85±0.46</td>
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<table>
<thead>
<tr>
<th></th>
<th>Outer AF</th>
<th>Inner AF</th>
<th>(Skaggs et al. 1994)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E$</td>
<td>Postero-lateral</td>
<td>82±43</td>
<td>59±41</td>
</tr>
<tr>
<td></td>
<td>Anterior</td>
<td>136±50</td>
<td>76±50</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>$E_{low}$</th>
<th>37.2-39.3</th>
<th>(Pezowicz 2010)</th>
</tr>
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<tbody>
<tr>
<td>$E_{medium}$</td>
<td>45.7-46.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_{high}$</td>
<td>31.1-35.5</td>
<td></td>
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</tbody>
</table>

*Exact values not provided. The figures reported are extracted from the authors’ charts. Anterior, lateral and posterior at slow, medium and fast stretching rate results were pooled because no significant difference was found.*
* Exact values not provided. The figures reported are extracted from the authors’ charts.

** Due to the complexity of the experiment the values of all the disc regions studied were not provided by the author.
2.3 AF tissue engineering

2.3.1 Electrospinning: a technique suitable for AF tissue engineered scaffold

Electrospinning is a process known since the beginning of the last century; however, the inventor may be considered Formhals who published several patents detailing the first electrospinning experimental set-up (Bhardwaj & Kundu 2010). Hence, the technology was disregarded for decades until the 1990s when it began to draw the attention of scientists due to the increasing interest in nanotechnology and related applications (Subbiah et al. 2005). Considering the nanometre- and micrometre-size fibres that electrospinning technology allows easy fabrication, which is comparable to that of cells (tens micrometres) and many biological molecules (for example, a collagen fibril has tens to hundreds nanometres diameter), regenerative medicine is one of the most promising fields for this technology (Braghirolli et al. 2014; Huang 2003). In the following sections, after illustrating the main electrospinning features, current applications of ES materials in tissue engineering are reviewed.

**Description of the electrospinning technique**

A typical electrospinning apparatus is rather simple and, in its basic configuration, is formed by three fundamental components: the pump, the collector and the power supply. The starting material is a polymer dissolved in a suitable conductive solvent. A reservoir, typically a syringe, is filled with the solution and connected to the pump. The pump pushes the solution through a metal needle which is connected to the electrode of the power supply. The counter electrode, usually grounded, is connected to the metal collector. The collector might assume different shapes; in the simplest case it is a metal flat surface. The collector is placed perpendicular to the needle axis, separated by an air gap measuring up to tens centimetres.
There are a number of parameters and variables that influence the outcome of the process and can be divided into environmental, solution and process parameters (Bhardwaj & Kundu 2010). Essentially, the environmental parameters are temperature and relative humidity. They affect the evaporation rate of the solvent and solution viscosity. Indeed, humidity and temperature are difficult to control accurately, as within GMP infrastructures, which is out of the scope for academic-led research activities. Solution parameters depend on the polymer and the corresponding solvent system used. Their combination can lead to various viscosities, conductivities, surface tensions and rates of solvent evaporation, which relates inversely to solvent boiling point (Gholipour Kanani & Bahrami 2011).

**Polymer concentration (and viscosity)**

Concentration is undeniably the most important parameter because it determines the viscosity of the solution, which subsequently governs the fibre diameter. Higher viscosity, proportional to concentration, allows fabricating bigger fibres (Zhang et al. 2005). Viscosity contrasts the repulsive electrical forces, thus hampering fibre stretching. A minimum critical concentration (peculiar for each polymer-solvent system) is required to ensure polymer chains entanglement. Viscosity lower than the critical concentration leads to beads formation (Bosworth et al. 2008) and to a larger deposition area on the collector (Supaphol & Chuangchote 2008). It must be pointed out that viscosity is also affected by other factors, such as: temperature, presence of surfactants and polymer molecular weight (Veleirinho et al. 2007). Further to concentration, molecular weight constitutes an additional parameter for viscosity control. Contrary to the other more controversially interpreted parameters of the electrospinning process, there is a general consensus concerning the positive relationship between fibre diameter and concentration (Gholipour Kanani & Bahrami 2011; Guarino et al. 2011; Pham et al. 2006; Heikkila & Harlin 2008; Deitzel 2001; Cui et al. 2006).
**Flow rate**

Flow rate is the only process parameter that can be changed by the operator, completely independently from the others, contrary to the other parameters that influence each other in a rather complex interdependent manner. Along with polymer concentration, it is the only parameter on which scientists agree regarding its role played in the process. It has been demonstrated that by increasing flow rate, bigger fibre is obtained (Wang & Kumar 2006). This relationship might simply be explained with the increase in mass ejected per time unit whilst the speed of fibres travelling towards the collector remains unchanged because it is governed by the electric tension between spinneret and collector. It has been revealed that, to keep the jet constant, a higher voltage is required to draw away the higher polymer mass per time unit due to the increased flow rate (De Vrieze et al. 2010). Nevertheless, flow rate is not deemed one of the most relevant parameters to modify fibre morphology; instead, it is often used to keep jet development constant. Troubles can rise from too low or high flow rates that would provoke needle clogging or dripping.

**Tension (and electric field)**

The potential difference between the spinneret and the collector is fundamental as the driving force of the electrospinning process. Theoretically, an increase in the electric power causes the raise of the charge density on the jet surface. Consequently, the higher repulsive forces would lead to a more intense stretching and reduction of fibre diameter. Some authors found evidence of agreement with this mechanism (Ding et al. 2002; Y. Li et al. 2006; Wang et al. 2009); nevertheless, other studies highlighted the existence of a positive relationship between voltage and fibre diameter (Fallahi et al. 2008; Zhang et al. 2005; Supaphol et al. 2005). In the latter case the model suggested implies that the rise in tension with increase of charge density makes the drawn jet approach the collector faster, delaying the instability; thereby leaving less time for the polymeric solution to become thin. It is possible that these two opposite effects coexist and one
overcomes the other depending on the combination of the other parameters adopted. This is demonstrated by the concave voltage-diameter curve experienced by Heikkila et al. (Heikkila & Harlin 2008). The authors demonstrated that, upon voltage increase, fibre diameter is subject to a decrease followed by an increase. The monotonic relationship more often reported elsewhere can be explained with the limited voltage range analysed in each study.

The efficacy of the tension applied depends on the solution conductivity, which can be increased by adding ionic compounds, such as salts (Jacobs et al. 2010; Supaphol et al. 2005).

In some cases, the driving force of the process has been addressed as electric field (Gupta & Wilkes 2003; Guerrini et al. 2009; De Vrieze et al. 2010). Dealing with one parameter that includes both voltage and spinneret-collector gap (electric field=voltage/distance) appears as a way to make parameters management easier; however, keeping the electric field constant by modifying voltage and spinneret-collector distance in opposite ways may not lead to constant fibre morphology.

**Spinneret to collector distance**

As mentioned in the previous paragraph, changing the gap between the nozzle and the collector, whilst keeping the voltage consistent, modifies the electric field, the driving force of the process. Higher distance has been found to cause the fibre to get thinner. This occurs because a larger gap allows the fibre having a longer time to be stretched by the twisting and bending movements (Ojha et al. 2008; Chang & Lin 2009). At short distances, the competing effects between the strong stretching forces imposed by the electric field and the duration allowed for the fibre to decrease its size, makes it difficult to foresee the output, as demonstrated by the absence of evident effect reported (Zhang et al. 2005). Furthermore, it has been hypothesised that, if the gap is kept large, the previously described thinning effect of the instabilities may be compromised by the evaporation of the solvent. The faster the fibre dries, the less is its capability to decrease in size (Jalili et al. 2005). Eventually, another consequence is the fibre distribution on the collector.
since it has been proved that the increased nozzle-collector gap leads to wider deposition area (Supaphol & Chuangchote 2008).

**ES matrix features influence on cell behaviour**

In the previous section the versatility of the electrospinning technology and how the process parameters can be adjusted to modify the outcome have been revealed.

The ES matrix has been defined as a “hierarchical structure consisting of both micro and nano levels of scale” (Shalumon et al. 2010), able to mimic the ECM organisation (Lim & Mao 2009). The ECM is a complex matrix that provides cells with the optimal environment from the biochemical and topographical point of view. In the first instance, ES matrix allows cells to adhere on the fibres forming a mono-layer. Nevertheless, there is an extensive literature that focused on individuating the optimal ES matrix features that foster cell growth. This section reviews the response of cells to fibre diameter and arrangement, the most important parameters that can be adjusted as shown previously.

*Fibre diameter*

ES fibre diameter may range from tens nanometres (Greiner & Wendorff 2007) (the size of some biological macromolecules (Moeller et al. 1995)) to several micrometres (Lowery et al. 2010) therefore, with the cell size around few tens micrometres, response of cells attached to the ES matrix depends on fibre size. Intuitively nanofibres provide a huge number of attachment sites for cells whilst big fibres force cells bridging from one fibre to another (Kwon et al. 2005).

Due to the massive number of publications on this topic only the most relevant have been considered, with particular focus on PCL matrix to make the comparison more consistent. The studies reviewed are reported in Table 2.3.
<table>
<thead>
<tr>
<th>Material</th>
<th>Cell type</th>
<th>Fibre diameters obtained</th>
<th>Fibres arrangement</th>
<th>Main outcome</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL</td>
<td>NIH 3T3 mouse fibroblasts</td>
<td>117-1647 nm</td>
<td>Random</td>
<td>More cells on 500 nm fibres rather than on beaded or bigger ones, up to 1 µm. Slightly better on 1.65 µm compared with 1 µm.</td>
<td>(Chen et al. 2007)</td>
</tr>
<tr>
<td>PCL</td>
<td>HUVECs</td>
<td>800 and 3600 nm</td>
<td>Random</td>
<td>Slightly better cell performances with 3600 nm fibres compared with 800 nm.</td>
<td>(Del Gaudio et al. 2009)</td>
</tr>
<tr>
<td>PCL</td>
<td>HMSCs</td>
<td>500-3000 nm</td>
<td>Aligned</td>
<td>No significant difference in cell proliferation. Only more upregulation of collagen II and aggrecan genes on 500 nm fibre.</td>
<td>(Wise et al. 2009)</td>
</tr>
<tr>
<td>PCL</td>
<td>HUVECs</td>
<td>400-2600 nm</td>
<td>Random</td>
<td>Higher proliferation on microfibrous scaffolds.</td>
<td>(Soliman et al. 2011)</td>
</tr>
<tr>
<td>PCL</td>
<td>MG63 osteoblasts</td>
<td>248-6200 nm</td>
<td>Aligned</td>
<td>Higher viability on 248 nm fibre.</td>
<td>(Shalumon et al. 2010)</td>
</tr>
<tr>
<td>PCL</td>
<td>NIH 3T3 mouse fibroblasts</td>
<td>400-1600 nm</td>
<td>Random</td>
<td>400 nm fibres enhanced viability to a greater extent.</td>
<td>(Hadjizadeh &amp; Doillon 2010)</td>
</tr>
<tr>
<td>PEG-PLA</td>
<td>MC3T3-E1 osteoprogenitor cells</td>
<td>140-2100 nm</td>
<td>Random</td>
<td>Better cell density and aspect ratio on 2100 nm fibres.</td>
<td>(Badami et al. 2006)</td>
</tr>
<tr>
<td>PHBV</td>
<td>Rat bone marrow MSCs</td>
<td>383-5200 nm</td>
<td>Random and aligned</td>
<td>Higher cell density on 600-700 nm fibre diameter scaffolds.</td>
<td>(Lü et al. 2012)</td>
</tr>
<tr>
<td>Material</td>
<td>Cell Type</td>
<td>Wavelength (nm)</td>
<td>Orientation</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>----------</td>
<td>-----------</td>
<td>----------------</td>
<td>-------------</td>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PLCL</td>
<td>HUVECs</td>
<td>320-7000</td>
<td>Random</td>
<td>Quite the same cell performances on 320 and 1100 nm fibres. On 7000 nm cells were rounded and sparsely distributed.</td>
<td>(Kwon et al. 2005)</td>
</tr>
<tr>
<td>PU</td>
<td>HMSCs</td>
<td>200-1600</td>
<td>Random</td>
<td>Not significant higher proliferation for cells seeded on 1400-1600 nm fibre.</td>
<td>(Kuo et al. 2014)</td>
</tr>
<tr>
<td>Silk fibroin</td>
<td>HDFs</td>
<td>256-1214</td>
<td>Random</td>
<td>Higher proliferation on 256 nm fibre.</td>
<td>(Hodgkinson et al. 2014)</td>
</tr>
</tbody>
</table>
In most cases, the results reveal that cell viability and proliferation were fostered by both nano- and micro-fibres, in two different size ranges. Positive outcomes were achieved with fibres characterised by a dimension within 250 and 700 nm (Soliman et al. 2011; Badami et al. 2006; Del Gaudio et al. 2009; Chen et al. 2007; Hodgkinson et al. 2014; Hadjizadeh & Doillon 2010) and above 1650 μm (Shalumon et al. 2010; Lü et al. 2012; Chen et al. 2007; Wise et al. 2009). Exception is represented in one case where better cell adhesion was found on micron sized structures (Kwon et al. 2005) or no relevant difference was detected (Kuo et al. 2014). Narrowing the outcomes reported to the ones obtained with MSCs, only two researches can be considered, though the cell source is not the same. Lu et al. and Wise et al. both reported that MSCs performed better on scaffolds formed of fibres with size in the submicron range (Lü et al. 2012; Wise et al. 2009). A study on uniformity of fibres pointed out that beaded nanofibres impair cell attachment compared with other normal fibres of all sizes. This was attributed to the lower surface area to volume ratio of beaded fibres and that the uneven surface of beads do not offer proper attachment sites to cells (Chen et al. 2007).

Fibre arrangement

Further to fibre diameter, fibre arrangement is probably the most frequently modified matrix feature. Fibre pattern can be tuned by changing the electrospinning collector type. Aligned fibres are the most common alternative to random fibres, manufactured using a static collector. The shift towards aligned fibres can be ascribed to two factors. The electrospinning apparatus can be fairly easily modified to orient the fibres along a specific direction (Yang et al. 2005; Pokorny et al. 2010); another reason is that for tissue engineering purposes, aligned fibres are supposed to better mimic specific anisotropic tissues structure (Gu et al. 2011; Raj 2008; Kannus 2000; Longo, Loppini, et al. 2012), compared with random fibres (Courtney et al. 2006; Baker et al. 2011). As can be seen in the relevant works listed in Table 2.4, most of the cell types investigated are those that in mammals populate tissues with a high orientation degree (i.e. nerves, ligaments,
tendons and AF). Indeed, the reviewed studies reveal that cells demonstrate higher proliferation or viability on aligned fibres (Jahani et al. 2012; Gupta et al. 2009; Lü et al. 2012).

Conversely, contrary to expectations, no effect of fibre arrangement has been assessed on AF (Koepsell, Zhang, et al. 2011) and ligament (Lee et al. 2005) cells, with the exception of Wismer et al.’s experiment (Wismer et al. 2014), thereby suggesting that other parameters, rather than the presence of topographical cue itself, may govern cell response. With this regard surface chemistry (Roach et al. 2007) and fibre diameter are likely to play a role.
Table 2.4: Main outcomes of the studies that compared aligned and random fibre patterns.

<table>
<thead>
<tr>
<th>Fibre diameter</th>
<th>Material</th>
<th>Cell type</th>
<th>Cell culture duration</th>
<th>Main outcome</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>383 nm</td>
<td>PHBV</td>
<td>Rat bone marrow MSCs</td>
<td>7 days</td>
<td>Significant higher number of cells detected on random fibres.</td>
<td>(Lü et al. 2012)</td>
</tr>
<tr>
<td>187 nm</td>
<td>PLGA</td>
<td>Rat Schwann cells</td>
<td>7 days</td>
<td>Higher viability on aligned fibres.</td>
<td>(Subramanian et al. 2012)</td>
</tr>
<tr>
<td>400-1500 nm</td>
<td>PCL</td>
<td>Rat MSCs</td>
<td>5 days</td>
<td>Higher viability on random fibres.</td>
<td>(Jahani et al. 2012)</td>
</tr>
<tr>
<td>1.03 nm</td>
<td>PCL</td>
<td>Human Schwann cells</td>
<td>7 days</td>
<td>Slightly higher maturation level (1 gene out of 5) for cells seeded on aligned fibres compared with random.</td>
<td>(Chew et al. 2008)</td>
</tr>
<tr>
<td>200 nm</td>
<td>PCL</td>
<td>Rat Schwann cells</td>
<td>9 days</td>
<td>No difference between the two patterns.</td>
<td>(Gupta et al. 2009)</td>
</tr>
<tr>
<td>160 nm</td>
<td>PCL/gelatine</td>
<td>Rat Schwann cells</td>
<td>9 days</td>
<td>Enhanced viability for cells cultured on random fibres.</td>
<td>(Gupta et al. 2009)</td>
</tr>
<tr>
<td>430 nm</td>
<td>PLLA</td>
<td>Human tendon stem cells</td>
<td>14 days (proliferation) + 7 days (Rt-PCR)</td>
<td>No proliferation difference. More evident upregulation and downregulation of tendon and bone genes expression respectively on aligned fibres.</td>
<td>(Yin et al. 2010)</td>
</tr>
<tr>
<td>187 nm</td>
<td>PLGA</td>
<td>Rat Schwann cells</td>
<td>7 days</td>
<td>Aligned fibres enhanced cell viability in respect</td>
<td>(Subramanian et al. 2012)</td>
</tr>
<tr>
<td>Diameter</td>
<td>Material</td>
<td>Species</td>
<td>Cell Type</td>
<td>Days</td>
<td>Observations</td>
</tr>
<tr>
<td>---------</td>
<td>----------</td>
<td>---------</td>
<td>-----------</td>
<td>------</td>
<td>--------------</td>
</tr>
<tr>
<td>2.52 µm</td>
<td>PCL</td>
<td>Porcine AF cells</td>
<td>14 days</td>
<td>No significant DNA content difference between the two patterns.</td>
<td>(Koepsell, Zhang, et al. 2011)</td>
</tr>
<tr>
<td>657 nm</td>
<td>N/A</td>
<td>PU</td>
<td>Human ligament fibroblasts</td>
<td>7 days</td>
<td>Both the fibres enhance proliferation, achieving the maximum at day 3.</td>
</tr>
<tr>
<td>1 µm</td>
<td>PLLA</td>
<td>HMSCs</td>
<td>5 days</td>
<td>No proliferation difference detected.</td>
<td>(Lee et al. 2013)</td>
</tr>
<tr>
<td>N/A</td>
<td>PLGA</td>
<td>P19 embryonic carcinoma cell</td>
<td>3 days</td>
<td>Aligned fibres allowed enhancing proliferation and viability compared with random ones.</td>
<td>(Irani et al. 2014)</td>
</tr>
<tr>
<td>0.759 µm</td>
<td>PLGA/collagen (50:50)</td>
<td>Human foreskin fibroblasts</td>
<td>14 days</td>
<td>Improved proliferation on aligned fibres regardless fibre composition.</td>
<td>(Full et al. 2015)</td>
</tr>
<tr>
<td>0.712 µm</td>
<td>PLGA/collagen (85:15)</td>
<td>Human foreskin fibroblasts</td>
<td>14 days</td>
<td>Improved proliferation on aligned fibres regardless fibre composition.</td>
<td>(Full et al. 2015)</td>
</tr>
<tr>
<td>1 µm</td>
<td>PCL</td>
<td>HMSCs</td>
<td>5 days</td>
<td>Higher cell proliferation and differentiation into osteogenic lineage for cells cultured on aligned fibres.</td>
<td>(Chang et al. 2013)</td>
</tr>
<tr>
<td>391 nm</td>
<td>Silk/P(LLA-CL)</td>
<td>Schwann cells</td>
<td>7 days</td>
<td>Aligned fibres provided higher viability.</td>
<td>(Zhang et al. 2013)</td>
</tr>
<tr>
<td>2.17 µm</td>
<td>PCL</td>
<td>Porcine AF cells</td>
<td>21 days</td>
<td>Aligned fibres allowed higher cell proliferation as well as PU compared with PCL matrix. Random fibres enhanced GAGs synthesis.</td>
<td>(Wismer et al. 2014)</td>
</tr>
</tbody>
</table>
**Cell penetration in ES layers**

Electrospinning is currently one of the most promising and versatile technologies to efficiently manufacture nano- and micro-fibrous matrices; nevertheless, it is well-known that, in the tissue engineering field, the main drawback of such substrates is the high fibre packing density that might hamper cell penetration capability (Nisbet et al. 2009). In the perspective of using cellularised scaffolds for treatments, achieving cell penetration is fundamental to recreate uniform 3-dimensional tissues. In Table 2.5 the studies reporting the outcomes of cell penetration in ES layers are listed.
Table 2.5: Experimental parameters and results regarding cell penetration evaluation studies.

<table>
<thead>
<tr>
<th>Fibre diameter</th>
<th>Material</th>
<th>Fibres arrangement</th>
<th>Layer thickness</th>
<th>Cell type</th>
<th>Cell culture duration</th>
<th>Main outcome</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/A</td>
<td>PCL + agarose</td>
<td>Aligned</td>
<td>250 μm</td>
<td>Bovine primary AF outer cells</td>
<td>1 week</td>
<td>Cell penetration limited to each layer boundary.</td>
<td>(Nerurkar, Sen, et al. 2010)</td>
</tr>
<tr>
<td>N/A</td>
<td>PCL</td>
<td>Aligned</td>
<td>600 μm</td>
<td>Calf MSCs</td>
<td>12 weeks</td>
<td>Collagen and GAGs present throughout the thickness but more intense staining is detected at the top of each seeded side.</td>
<td>(Driscoll et al. 2011)</td>
</tr>
<tr>
<td>N/A</td>
<td>PLLA</td>
<td>Random</td>
<td>1 mm</td>
<td>Human bone marrow MSCs</td>
<td>4 weeks</td>
<td>Cell distribution similar to the physiological one. Rather uniform accumulation of ECM.</td>
<td>(Nesti et al. 2008)</td>
</tr>
<tr>
<td>N/A</td>
<td>PCL</td>
<td>Aligned</td>
<td>250 μm</td>
<td>Bovine primary AF cells</td>
<td>8 weeks</td>
<td>Cells did not penetrate in the inner third of the construct.</td>
<td>(Nerurkar et al. 2008)</td>
</tr>
<tr>
<td>≈ 4 μm</td>
<td>PCL/poly(glycerol sebacate)</td>
<td>Random</td>
<td>100-150 μm</td>
<td>Human bone marrow MSCs</td>
<td>3 days</td>
<td>Cells migrated up to 50 μm in depth.</td>
<td>(Tong et al. 2011)</td>
</tr>
<tr>
<td>N/A</td>
<td>PCL</td>
<td>Aligned</td>
<td>= 1 mm</td>
<td>Human primary meniscus fibrochondrocytes</td>
<td>9 weeks</td>
<td>Uniform cells presence up to the middle portion of the scaffold (9 weeks).</td>
<td>(Baker et al. 2010)</td>
</tr>
<tr>
<td>Fiber Diameter</td>
<td>Material</td>
<td>Orientation</td>
<td>Fiber Diameter</td>
<td>Cell Type</td>
<td>Culture Duration</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>---------------</td>
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<td>----------------</td>
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<td>----------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>117-1647 nm</td>
<td>PCL</td>
<td>Random</td>
<td>50 µm</td>
<td>NIH-3T3 fibroblasts</td>
<td>5 days</td>
<td>Moderate cell presence only down to the middle of the scaffold Better performance on 428 nm fibres. (Chen et al. 2007)</td>
<td></td>
</tr>
<tr>
<td>800 and 3600 nm</td>
<td>PCL</td>
<td>Random</td>
<td>430 and 680 µm</td>
<td>HUVECs</td>
<td>7 days</td>
<td>Cell infiltration occurred throughout the entire scaffolds, though more homogenously in the microfibres ones. (Del Gaudio et al. 2009)</td>
<td></td>
</tr>
<tr>
<td>3.4-12.1 µm</td>
<td>PCL</td>
<td>Random</td>
<td>1 mm</td>
<td>Human primary venous myofibroblasts</td>
<td>3 days</td>
<td>Homogenous distribution only in scaffolds made of fibres thicker than approximately 6.7 µm. (Balguid et al. 2009)</td>
<td></td>
</tr>
<tr>
<td>248-6200 nm</td>
<td>PCL</td>
<td>Random</td>
<td>50-60 µm</td>
<td>HUVECs</td>
<td>7 days</td>
<td>Cells did not migrate beyond half depth of the high packing density scaffold. Better with low density scaffold but no difference depending on fibre diameter. (Soliman et al. 2011)</td>
<td></td>
</tr>
<tr>
<td>Thickness</td>
<td>Material</td>
<td>Orientation</td>
<td>Depth</td>
<td>Cell Type</td>
<td>Days</td>
<td>Notes</td>
<td></td>
</tr>
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<td></td>
</tr>
<tr>
<td>130-9100 nm</td>
<td>PCL</td>
<td>Aligned</td>
<td>1 mm</td>
<td>PC12 cells</td>
<td>5</td>
<td>Cells reached 867 μm penetration depth on the matrix with the biggest fibres and pores of approximately cell body size. (Zander et al. 2013)</td>
<td></td>
</tr>
<tr>
<td>3.10 μm</td>
<td>PCL</td>
<td>Random</td>
<td>314.9 μm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.17 μm</td>
<td>PCL</td>
<td>Aligned</td>
<td>374.5 μm</td>
<td>Bovine primary AF cells</td>
<td>21</td>
<td>Cells were barely able to migrate inside. More penetration inside PCL layers down to approximately 50-70 μm. (Wismer et al. 2014)</td>
<td></td>
</tr>
<tr>
<td>0.92 μm</td>
<td>PU</td>
<td>Random</td>
<td>257.9 μm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.84 μm</td>
<td>Aligned</td>
<td>224.5 μm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In most of the studies, cell penetration was judged as unsatisfactory. Cells have been found to adhere only to the surface (Nerurkar, Sen, et al. 2010) or have been able to only partially infiltrate, leaving the remote part from the surface empty (Soliman et al. 2011; Chen et al. 2007; Tong et al. 2011; Driscoll et al. 2011; Wismer et al. 2014; Nerurkar et al. 2008). Conversely, few studies claimed the achievement of a complete and homogeneous cell colonisation (Balguid et al. 2009; Del Gaudio et al. 2009; Nesti et al. 2008; Baker et al. 2010). Nevertheless, regardless of the different study outcomes, a relationship emerges between fibre diameter and cell penetration. The studies listed in Table 2.5 comparing different fibre diameter effects, except results from Chen et al. (Chen et al. 2007), highlight poorer infiltration capabilities for cells seeded on small-sized fibres (Balguid et al. 2009; Del Gaudio et al. 2009; Zander et al. 2013).

To date, the electrospinning technology has revealed itself as not fully reliable in terms of providing acceptable cell penetration and nutrient transit, which are mandatory characteristics for the fabrication of uniform tissue engineered scaffold.

### 2.4 State of art of AF artificial replacement: scaffold design

Currently, a number of attempts have been made to accurately replicate the AF natural tissue. Such scaffolds can be theoretically grouped according to their features; the structure complexity, the cell type, the usage of a NP-like core, the adoption of a synthetic or natural material are just some of the possible criteria. For the review that follows, it was decided to apply a classification based on the AF artificial replacement structure, making a distinction according to the complexity degree. Some of the studies replicated the AF as a single unit, a ring-shaped structure, which is anticipated to enclose an NP-like core, whilst in others the AF multilamellar arrangement was simulated. The studies that focused on the fabrication of an entire IVD-like construct device were as well included in this section.
2.4.1 Single unit artificial replacements

The simplest strategy for AF regeneration is designing a scaffold able to support AF or stem cells and maintain the tissue mechanical properties and to prevent leakage of the NP, or any other artificial core.

Sato et al. proposed a porous atelocollagen scaffold that supported AF cell survival when implanted into an in-vivo IVD defect model (Sato et al. 2003). However, this attempt did not intend to replicate the AF structure since it was used largely as a carrier for cells. A single unit degradable AF scaffold was also attempted with poly(1,8-octanediol malate), showing to foster aggrecan and collagen type II genes expression of AF cells (Wan et al. 2007). The same research group proposed an alternative AF artificial replacement using a natural biomaterial. The salt leaching method and demineralisation of bone matrix gelatine allowed to produce a chondrocytes-seeded ring able to act as a containment for a poly(polycaprolactone-triol-malate) inner core (Wan et al. 2008). The introduction of anisotropic pores in a PLA/bioglass composite scaffold was found to affect human AF cells orientation and penetration. Proliferation and synthesis of the main AF ECM components revealed an increase over a culture period of four weeks, especially in presence of higher bioglass content (Helen & Gough 2008). However, although certain scaffolds appear able to support AF cell growth, there are still some concerns about the applicability of such material for AF tissue engineering as the authors did not shape the PLA/bioglass into sheet or ring that could be used as patch for, or whole replacement of, the AF (Helen & Gough 2008).

The shape issue was successfully addressed by Mizuno et al. who used PLA/PGA composite to fabricate a cellularised AF replacement in combination with an NP-like core. The scaffold was implanted in rat ectopic location showing increasing ECM components synthesis and cell number after 12 weeks (Mizuno et al. 2004). Nevertheless histological analysis demonstrated no appearance of the typical AF structure which was hypothesised to be due to the absence of scaffold lamellar arrangement and lack of proper mechanical stimulation.
An improved AF-like structure was proposed by Bowles et al., who designed a ring-shaped collagen structure by means of gel contraction technique. The methodology allowed to achieve collagen fibres orientation in circumferential direction inducing contact guidance phenomenon on primary cells (Bowles et al. 2010). Despite the lack of the characteristic multilamellar AF structure, ring-shaped collagen fibres were wrapped around an alginate core and tested in bioreactor (Hudson et al. 2015) or implanted in rabbit spine showing similar native IVD mechanical properties and disc height maintenance (Bowles et al. 2011). In a later article, using the same technique, the authors were able to replicate accurately the shape of a rat IVD, with the aid of μCT (Bowles et al. 2012). μCT has been as well adopted to prepare customised rat IVD replicates by 3D printing PCL (van Uden et al. 2015). However, many challenges must be addressed prior to translation to humans: further study on big animal models instead of rabbit and the implantation of stem cells instead of primary cells of xenogeneic origin. Indeed, AF scaffold formed by collagen gel contraction technique undergoes considerable shrinkage over time (Bowles et al. 2010), which may raise concerns on performances shift with the duration of in-vitro and in-vivo study.

The strategy of circumferential fibres has been followed by Lazebnik et al. by means of a rather different methodology (Lazebnik et al. 2011). The authors exploited ES PCL, surrounding an agarose core, which contributed to the increase of the whole construct stiffness. As stated by Bowles et al., the circumferential orientation of the AF-like portion (Lazebnik et al. 2011; Bowles et al. 2011), whilst being able to well withstand the stress along fibres direction, is probably not the optimal arrangement considering the biomechanics of the spine which is subject to a complex combination of loads (Jensen 1980).

Another approach, mirroring that of the last two studies mentioned, allowed to fabricate from silk a single unit AF artificial replacement that lacks the fibrous structure but possesses lamellar arrangement. The method did not allow obtaining a circumferential lamellae orientation, as in the AF, and exhibited a lower Young’s modulus and ultimate tensile strength (UTS) compared with the porous version of the same material (Park et al. 2012). Conversely, lamellae showed better biological
performance in terms of supporting collagen and GAGs synthesis and proliferation of porcine AF cells. Single piece AF scaffolds were obtained by decellularising porcine AFs, preserving the internal lamellar structure (Wu et al. 2014). Despite no cytotoxicity being assessed, there are concerns about using xenogenic tissues, as pathogens may be conveyed.

2.4.2 Composite artificial replacements

All the aforementioned studies proposed AF artificial replacements with a relatively low degree of structural complexity. Some investigations have also been carried out to replicate the AF anisotropic multilamellar arrangement.

A scaffold was developed by combining three strips of knitted silk showing to improve the expression of key genes (collagen, aggrecan and decorin) and collagen type II synthesis of MSCs in an IVD model (See et al. 2011). Compared with other materials of natural origin, silk is an exception, due to its considerable tensile strength; therefore, it is a good candidate for regenerating tissues that are subject to high tensile loading (Cheung et al. 2007; Rising et al. 2011).

Koepsell et al. demonstrated the beneficial effect of ES aligned fibres on orientation of porcine primary AF cells, and the synthesis of collagen and GAGs, which are often regarded as important markers with the perspective of IVD and AF regeneration (Koepsell, Remund, et al. 2011). Another successful application of electrospinning technology is evidenced by the development of new collagen and GAGs when calf MSCs are cultured in a 3-dimensional PCL fibrous environment. The presence of multi-layer construct also demonstrated improved mechanical properties, another important indicator for the success of an artificial replacement (Nerurkar, Sen, et al. 2010). Nevertheless, the complexity of the experiment caused reduced cell survival inside the core, possibly due to the impaired nutrients transit across the whole scaffold. Martin et al. demonstrated that ES layers within a tissue engineered replicate can be implanted in a rat caudal spine showing good histological outcome though displacement problems have been highlighted (Martin et al. 2014).
Recently, matrices fabricated with electrospinning technology were integrated to fused deposition modelling (FDM) characterised by sub-millimetre fibres. The authors claimed that the addition of aligned ES fibres improved hMSC viability and overall mechanical properties compared with the FDM layers alone (Kang et al. 2013). Likewise, an example of lack of lamellar orientation has been provided by Chik et al., who adopted three collagen layers to wrap a hydrogel core (Chik et al. 2014). The isotropic morphology of collagen allowed to achieve cells, but not collagen, orientation by application of torsional stimulus. Positive histological results were claimed; however, the procedure adopted to fabricate the triple-layer requires to let each collagen layer contract for three days before the addition of the next layer. This strategy is potentially difficult to implement with a larger number of layers.

These experiments highlight the importance of having a scaffold structure that resembles closely the natural tissue features: the nano- and micro-scale pattern provides topographical cues to cells and contributes to tune the performance of the whole construct.
2.4.3 Artificial AF constructs

Cellularised constructs in static conditions

This section presents the results pertaining to the behaviour of cells cultured in static conditions within scaffolds for IVD regeneration purposes (Table 2.6). The effects of the environmental conditions on cells are shown by analysing morphology, cell penetration, orientation and matrix synthesis. Influence of the addition of GFs has been stated, in addition to matrices with random or oriented fibres.

Studies regarding AF regeneration found contrasting results concerning cell proliferation and matrix synthesis. Koepsell et al. discovered a drop in DNA content at the end of the cell culture experiment, along with a decrease in matrix production (Koepsell, Zhang, et al. 2011). The same research group, in a similar experiment, demonstrated a larger amount of matrix on aligned fibres scaffolds (Koepsell, Remund, et al. 2011). It has been proposed that the decrease in cell number indicates the intense synthesis activity of each cell (Koepsell, Remund, et al. 2011). Wismer et al. observed an increase in cell number (bovine AF cells) and collagen type I gene upregulation on aligned ES PCL fibres whilst downregulation of aggrecan and collagen type II (Wismer et al. 2014). The authors ascribed the lack of phenotype maintenance to the insufficient cell penetration. Heo et al. managed to induce matrix synthesis despite the number of living cells kept constant on oriented ES scaffolds (Heo et al. 2011). Difficulties in maintaining an increase in cell number until the end of the experiment have also been addressed by other research groups. Tong et al. attributed the high matrix content, measured alongside a low cell density after three weeks, to the differentiation state of fibroblasts which ceased proliferating after seven days (Tong et al. 2011). It has been further hypothesised that the absence of MSCs proliferation may be due to the senescent state of cells after prolonged in vitro culture, even though the authors cast doubt regarding this interpretation (Baker et al. 2010). Moreover, the increase of ECM component with culture time was proved by other research groups (Nesti et al. 2008; Wise et al. 2009). Nerurkar
et al. obtained cell proliferation and ECM synthesis increase from cells seeded on aligned ES fibres (Nerurkar & Elliott 2007). An alternative scaffold was designed by Helen et al. who demonstrated the feasibility of inclusion of bioglass in PDLLA scaffold foam to support primary AF cells (Helen & Gough 2008). Another polymer modification was proposed by positively charging the surface of polyamide fibres exhibiting favourable conditions for AF cells deposition of Coll II and GAGs, which are known to carry a negative charge (Gruber et al. 2008).
Table 2.6: Main features and outcomes of the static culture experiments related to the IVD regeneration (comparison between first and last time point).

<table>
<thead>
<tr>
<th>Scaffold type</th>
<th>Cell type</th>
<th>GF</th>
<th>Cell number</th>
<th>GAGs</th>
<th>Collagen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aligned PCL ES fibres</td>
<td>Porcine AF cells</td>
<td>TGF-β1</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>(Koepsell, Zhang, et al. 2011)</td>
</tr>
<tr>
<td>Aligned PCL ES fibres</td>
<td>Bovine AF caudal disc cells</td>
<td>TGF-β3</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>(Nerurkar &amp; Elliott 2007)</td>
</tr>
<tr>
<td>ES poly(glycerol sebacate)/PCL</td>
<td>Human MSCs</td>
<td>-</td>
<td>=</td>
<td>=</td>
<td>↑</td>
<td>(Tong et al. 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTGF</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Aligned PCL ES fibres</td>
<td>Human fibrochondrocytes</td>
<td>TGF-β3</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>(Baker et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>Human MSCs</td>
<td></td>
<td>=</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>Material/Condition</td>
<td>Cell Type</td>
<td>TGF-β1</td>
<td>Gene Expression</td>
<td></td>
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<tr>
<td>Aligned PCL ES fibres</td>
<td>Porcine AF cells</td>
<td>↓</td>
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<tr>
<td>Aligned PCL ES fibres</td>
<td>Human MSCs</td>
<td>=</td>
<td>↑</td>
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<tr>
<td></td>
<td>TGF-β1</td>
<td>=</td>
<td>↑</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
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</tr>
<tr>
<td>ES PLA</td>
<td>Human MSCs</td>
<td>N/A</td>
<td>= (type I)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>↑ (type II)</td>
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</tr>
<tr>
<td>PDLLA/Bioglass (30% wt) foam</td>
<td>Human AF cells</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
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<tr>
<td>ES charged polyamide</td>
<td>Human AF cells</td>
<td>=</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
<td></td>
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<tr>
<td>Lamellar silk scaffold</td>
<td>Porcine AF cells</td>
<td>↑</td>
<td>↑</td>
<td></td>
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<td></td>
<td></td>
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</tbody>
</table>

(Koepsell, Remund, et al. 2011)
(Wise et al. 2009)
(Nesti et al. 2008)
(Helen & Gough 2008)
(Gruber et al. 2008)
(Park et al. 2012)
<table>
<thead>
<tr>
<th>Material</th>
<th>Cell Type</th>
<th>Sign 1</th>
<th>Sign 2</th>
<th>Sign 3</th>
<th>Sign 4</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycarbonate urethane</td>
<td>Bovine AF cells</td>
<td></td>
<td>↑</td>
<td>↓</td>
<td>=</td>
<td>(Kandel et al. 2014)</td>
</tr>
<tr>
<td>poly(1,8-octanediol malate)</td>
<td>Rat AF Cells</td>
<td></td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>(Wan et al. 2007)</td>
</tr>
<tr>
<td>network</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aligned ES PCL nanofibres + FDM</td>
<td>Human MSCs</td>
<td></td>
<td>↑</td>
<td>N/A</td>
<td>N/A</td>
<td>(Kang et al. 2013)</td>
</tr>
<tr>
<td>microfibres</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Aligned ES PCL</td>
<td>Bovine AF cells</td>
<td></td>
<td>↑</td>
<td>↑</td>
<td>↑ (type I)</td>
<td>↓ (type II)</td>
</tr>
</tbody>
</table>
Cellularised constructs in dynamic conditions

As revealed in the previous section, scientists have been attempting to mimic the biological environment through the replication of the ECM structure and by tuning the chemico-physical scaffold features. Recently, another approach has emerged, based on the application of mechanical stimuli to the cellularised construct (Grad et al. 2011; Martin et al. 2004). By providing adequate mechanical stimuli, cells are supposed to behave closer to cells in natural tissues, compared with those cultured in static conditions. Cells are known to be sensitive to mechanical stimuli thanks to trans-membrane proteins that connect the substrate and the ECM to cell cytoskeleton (Guilak et al. 2009). Mechanical forces trigger specific biochemical pathways that affect RNA and protein synthesis (Glossop & Cartmell 2009). The consequence of the process is the regulation of cell activity by the external solicitations. Therefore, extensive research is being carried out with the aim of finding the relationship between cell behaviour in response to mechanical stimuli. Simultaneously, the role of engineers is to design devices, addressed as bioreactors, able to adequately convey the mechanical stimuli and appropriately monitor the stimulation process (Martin et al. 2004).

Mechanical stimulation bioreactors

Bioreactors have been used to study and develop a variety of functional human tissues (Naing & Williams 2011); the IVD is no exception. Considering that the AF and NP are, in-vivo, subject to continuous and intense mechanical loads, the enhancement of scaffold performances by means of bioreactor has been attempted. Akin to the tests in static conditions shown above, both primary and stem cells have been seeded on or in scaffolds and mechanically stimulated. In the literature reviewed, the bioreactors presented are generally custom-made and designed to provide compression or stretching, according to the scaffold and cell type and test objective (see Table 2.7).
Cellular response to stimulation

Table 2.7 presents the most recent experiments that exploited bioreactors for IVD regeneration purposes. Korecki et al. studied the response of AF cells harvested from old and young individuals, and found that less mature cells are more responsive to cyclic compression at 0.1 and 3 Hz in terms of cell proliferation (Korecki & Kuo 2009). Investigation of hydrostatic pressure revealed that AF cells synthesise more ECM when subject to high pressure, possibly because a more intense stimulus triggers mechanisms that facilitate the IVD load bearing capability (Hutton et al. 2001). AF cells stimulated under equibiaxial tensile stress regime also demonstrated different behaviour depending on the intensity of the stress imposed. The equibiaxial tensile stress led to a significant increase in both DNA and ECM production only for strains not smaller than 10% (Hee et al. 2010). In another study, the application of hydrostatic pressure favoured cell infiltration in the scaffold and proliferation of AF cells, with slightly better results for outer AF cells (Reza & Nicoll 2008). Contrary to what expected, outer AF cells behaved more closely to chondrocytes, compared with inner AF cells that are supposed to be similar to cartilage cells. This trend was proved by the increase in collagen type II and the maintenance of GAGs synthesis level of outer AF cells (Reza & Nicoll 2008). These results are consistent with those obtained on human AF cells encapsulated in collagen gel and exposed to low (0.25 MPa) hydrostatic pressure (Neidlinger-Wilke et al. 2005).

Another group of studies focused on MSCs with the aim of finding the proper scaffold and stimulation pattern in the perspective of clinical usage (Rodrigues et al. 2011). Kisiday et al. found that equine MSCs differentiate into chondrocytes under dynamic compression but the increase in matrix production is not significant when compared with unloaded samples. In contrast, unloaded samples, with the addition of TGF-β1 in culture medium, achieved better results. Furthermore, the application of mechanical stimulation, when cells were cultured in GF presence, appeared to hamper cells and matrix development (Kisiday et al. 2009). Dissimilarly, GFs usage within a
spinner flask system demonstrated to be beneficial in terms of increased ECM and enhanced mechanical properties, whilst cell number maintained constant. It is noteworthy that mechanical properties did not reach the values of the benchmark adopted by the authors and that no control test without GFs was performed. Thus, as stated by the author, it has not been possible to separate the contribution of chemical and mechanical stimuli (Janjanin et al. 2008). Recently, by using an ES dynamically compressed engineered AF, Tsai et al. achieved an upregulation of collagen type I whilst unchanged collagen II expression and GAGs synthesis (Tsai et al. 2014). In this experiment, hMSCs were co-cultured and seeded with IVD cells in GFs-enriched medium and as negative control the unstimulated sample was adopted.

By exerting shear stress, discordant results were obtained by Nerurkar et al. who experienced GAGs synthesis drop on MSCs in orbital shaker. It was hypothesised that the proteoglycans may have a higher mobility in respect to collagen (Nerurkar, Sen, et al. 2011). Connelly et al. contributed to the understanding of GAGs synthesis drop by demonstrating a significant GAGs release in medium following a culture period of 21 days’ duration (Connelly et al. 2010).

Thorpe et al. arranged a complex experiment by comparing specimens subject to compression stimuli. The mechanical load was combined with TGF-β3 supplementation obtaining six different patterns. No advantage, in relation to MSCs chondrogenesis, was achieved with continuous stimulation. The highest increase in cartilage matrix synthesis occurred with the continuous addition of the GF, whether or not combining it with the mechanical stimulation after 21 days of culture. The improvement from the mechanical performances point of view was achieved only by the unloaded samples with the addition of TGF-β3. The authors hypothesised that the presence of the GF is a more important element than the mechanical stimulation, to induce MSCs chondrogenesis (Thorpe et al. 2010). A study that shares some features with the aforementioned was conducted to discover the effect of chondrogenic pre-differentiation and mechanical compression on hMSCs (Budde et al. 2010). Nevertheless, in this experiment, no significant increase in either DNA, GAGs content or mechanical properties was detected using any of the
combinations between biochemical and mechanical stimulation. Before the stimulations, cells were cultured in Fibroblast Growth Factor II (FGF-II) enriched medium to enhance differentiation. It was inferred that the lack of cell development was due, not to a decrease in cell number, but rather an “unspecific differentiation” that the tests performed were unable to detect (Budde et al. 2010). Absence of proliferation was also found by Baker et al. on MSCs in GF supplemented medium, though exerting a different stimulation pattern. In this case, cell number drop was ascribed to the achievement of senescence which inhibited proliferation and differentiation (Baker et al. 2011).

The experiment from See et al. is particularly relevant because it is an example of tissue engineered device that mimics the entire IVD. The assembly, seeded with mammalian stem cells, was subject to a rehabilitative regime of increasing compression stimulus with time. At the end of the test collagen II was found to be increased compared with the static control, contrary to collagen I. Cell viability essay demonstrated a diminished activity, which was attributed to cell differentiation or death (See et al. 2011).

Overall, more positive outcomes were reported when cells were cultured and/or stimulated in presence of GFs (Kisiday et al. 2009; Janjanin et al. 2008; Thorpe et al. 2010; Chik et al. 2014); however, to make translation to clinical level easier, it is desirable that the expensive and short-living GFs are abandoned in favour of mechanical stimulation, whereby the approval of beneficial effects of mechanical stimulation and the identification of optimal pattern remain a significant research challenge.
Table 2.7: Main features and outcomes of the dynamic culture experiments (results are presented as comparison with the static control, unless otherwise stated).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Stimulation type</th>
<th>GF</th>
<th>Scaffold</th>
<th>Cell number</th>
<th>GAGs</th>
<th>Collagen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human AF cells</td>
<td>Sinusoidal equibiaxial stretching: Pre-compression (0.4 MPa, 1 Hz, 2 h/0.5d, 7 days) + 5% strain, 1 Hz, 2 h/0.5d, 7 days</td>
<td>-</td>
<td>Flexercell® membrane</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>(Hee et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>Sinusoidal equibiaxial stretching: Pre-compression (0.4 MPa, 1 Hz, 2 h/0.5d, 7 days) + 10% strain, 1 Hz, 2 h/0.5d, 7 days</td>
<td></td>
<td></td>
<td>=</td>
<td>↑</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Porcine bone</td>
<td>Free swelling‡, 42 days</td>
<td></td>
<td></td>
<td>=</td>
<td>↑</td>
<td>↑</td>
<td>(Thorpe et al. 2010)</td>
</tr>
<tr>
<td>marrow MSCs</td>
<td>Delayed cyclic compression‡‡: 10% strain, 1 Hz, 1 h/d, 5 d/wk, 42 days</td>
<td></td>
<td>TGF-β3 Agarose hydrogel</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Continuous cyclic compression‡‡: 10% strain, 1 Hz, 1 h/d, 5 d/wk, 42 days</td>
<td></td>
<td></td>
<td>=</td>
<td>↓</td>
<td>↓</td>
<td></td>
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<tr>
<td>Human bone marrow MSCs</td>
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<tr>
<td>Cyclic compression: 10%, 0.5 Hz, 28 days</td>
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<tr>
<td>Unloaded, 28 days</td>
<td>TGF-β 2+IGF-I</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Acellularised bovine cancellous bone disc</td>
<td>=</td>
<td>=</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Budde et al. 2010)

<table>
<thead>
<tr>
<th>TGF-β 3</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Free swelling †, 42 days</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TGF-β 3</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Delayed cyclic compression ‡: 10% strain, 1 Hz, 1 h/d, 5 d/wk, 42 days</td>
<td>↓</td>
<td>=</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>TGF-β 3</th>
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<tbody>
<tr>
<td>Continuous cyclic compression ‡‡: 10% strain, 1 Hz, 1 h/d, 5 d/wk, 42 days</td>
<td>=</td>
<td>↓</td>
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<table>
<thead>
<tr>
<th>TGF-β 3</th>
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<tbody>
<tr>
<td>Hz, 1 h/d, 5 d/wk, 42 days</td>
<td></td>
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<tr>
<td>Source of stem cells</td>
<td>Cyclic compression: 0.25 Hz, 15 min/d, 5-11-18 and then 25% strain in 4 weeks</td>
<td>Cyclic uniaxial strain: 3h (10%) + 3 h/d (0%), 1 Hz, 21 days</td>
</tr>
<tr>
<td>---------------------</td>
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<tr>
<td>Rabbit bone marrow MSCs</td>
<td></td>
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<td>Calf MSCs</td>
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<tr>
<td>Bovine bone marrow</td>
<td></td>
<td></td>
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<tr>
<td>MSCs</td>
<td>Uniaxial strain: 7 days free swelling + static strain, 21 days</td>
<td>=</td>
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<td>------</td>
<td>-------------------------------------------------------------</td>
<td>---</td>
</tr>
<tr>
<td>Calf bone marrow MSCs</td>
<td>Cyclic uniaxial strain: 6% strain, 1 Hz, 3 h/d, 4 weeks. 6 weeks static preculture</td>
<td>TGF-β3</td>
</tr>
<tr>
<td>Human bone marrow MSCs (co-cultured with IVD cells)</td>
<td>Dynamic compression: 10% strain, 1 Hz, 1 h/d, 21 days</td>
<td>TGF-β1</td>
</tr>
<tr>
<td>Sheep AF cells</td>
<td>Dynamic compression: 1% strain, 1 Hz, 1-1-1 h on-off-on each day, 6 h/wk, 2 weeks</td>
<td>-</td>
</tr>
<tr>
<td>Time Course</td>
<td>Strain</td>
<td>Frequency</td>
</tr>
<tr>
<td>-------------</td>
<td>--------</td>
<td>-----------</td>
</tr>
<tr>
<td>1h on-off-on each day, 6 h/wk, 2 weeks</td>
<td>5%</td>
<td>1 Hz</td>
</tr>
<tr>
<td>Dynamic compression: 5% strain, 1 Hz, 1-1-1 h on-off-on each day, 6 h/wk, 2 weeks</td>
<td>-</td>
<td>↑↑</td>
</tr>
<tr>
<td>10%</td>
<td>1-1-1</td>
<td></td>
</tr>
<tr>
<td>Dynamic compression: 10% strain, 1 Hz, 1-1-1 h on-off-on each day, 6 h/wk, 2 weeks</td>
<td>-</td>
<td>↑↑</td>
</tr>
</tbody>
</table>

* Comparison with free swelling, same TGF-β 3.

*↑↑ Comparison with day 0.
3. Materials and methods: scaffold fabrication and characterisation

3.1 Scaffold fabrication

3.1.1 Electrospinning apparatus description

The electrospinning apparatus (Ske s.r.l, Milano) exploited within the present project does not vary significantly from the typical machine described in the introduction (section 2.3.1).

The apparatus (Figure 3.1) and supporting system used were composed of a syringe pump with a vertical support for accommodation of the nozzle, a collector cylinder, a power supply and an emergency button. The syringe pump could be selected from two types (Nexus 6000, Chemix; NE-1010, New Era Pump Systems) and loaded with differently sized syringes (Terumo, Japan). Once connected to the tube, the nozzle (or spinneret) was placed inside the hole of the vertical support to keep it in a stable position throughout the fabrication process. The support had the possibility to move along a track, parallel to the long axis of the drum collector, allowing keeping the spinneret always perpendicular to the collector axis. The horizontal speed of the spinneret could be adjusted from 0 to 30 mm/s. At a distance varied from 8 to 22 cm, with 1 cm steps, the collector cylinder, 36 cm long with 8.4 cm diameter, was placed. The cylinder is driven by an electric motor that enabled to achieve a rotational speed tuneable between 0 to 3000 rpm. At the maximum frequency the corresponding linear velocity at the surface was 13.2 m/s. The power supply (SL150, Spellman) had its poles connected to the steel drum and the spinneret to keep the desired voltage, adjustable from 0 to 30 kV. In particular the collector was connected to the ground whilst the spinneret was connected to the positive electrode.

The whole apparatus was located within a fume cupboard to allow the solvent to evaporate properly during the process. The chemical hood was located inside a clean room to keep the
environment free from the presence of micro and nano particles that could contaminate the ES layers.

3.1.2 Process parameters and procedure

Polymer and solvents

The chosen polymer was polycaprolactone (PCL, 70-90 kDa), which was purchased from Sigma-Aldrich.
To achieve a broad range of fibre diameters, PCL dissolution in different solvents was planned. Attempts to produce fibres were made by exploiting the following chemicals as polymer dissolution solvents (in brackets the supplier):

- Trifluoroacetic acid, TFA (Carlo Erba)
- Acetic acid, AA (Sigma-Aldrich)
- Formic acid, FA (Sigma-Aldrich)
- Chloroform, CF (VWR)
- Methanol, MeOH (Sigma-Aldrich)
- Dimethylformamide, DMF (Sigma-Aldrich)
- Tetrahydrofuran, THF (Carlo Erba)
- Ethanol, EtOH (Sigma-Aldrich)

**Solution preparation**

The various concentrations of polymer solutions were prepared by measuring the weight of PCL and solvent required using a high resolution balance (Mettler Toledo). The concentrations are thus expressed in weight/weight percentage and were calculated with the following equation:

\[
\frac{W_p}{W_p + W_s} \times 100
\]

With \(W_p\) and \(W_s\) represent polymer and solvent weights respectively.

Immediately after preparation, the vials were weighed and the total weight recorded. The mixtures of polymers and solvents were prepared in disposable 50 ml polypropylene plastic vials (Corning) and stored under a chemical hood until the solution was homogeneous. To speed up the process, especially when using very high concentrations, the solutions were mixed by stirring whilst immersed in hot water bath, with the temperature kept under PCL melting point and solvent boiling point.

Prior to begin each electrospinning session, the vial with the solution was weighed and any weight loss due to solvent evaporation was compensated by adding the equivalent weight of missing solvent. The added solvent was then allowed to mix with the solution to prevent the
solution to be inhomogeneous. Tilting the vial and inspecting it visually to verify the absence of two phases identified 30’ as a more than sufficient time to achieve a homogeneous solution.

**Strategy for process establishment**

To aid the establishment of process parameters, an extensive literature review was conducted to identify the range of machine settings required and select the most suitable polymer concentration for each solvent system. The collector speed was kept as low as possible (60 rpm) to allow the fibres to deposit randomly. An aluminium foil was attached to the collector prior to each session to facilitate matrices removal and handling. The velocity of the drum, being an additional process parameter, was increased in a step-wise manner to ensure only good-quality fibres were produced. The experimental strategy was implemented as three phases.

At the beginning of the first phase, the initial experiments were performed with parameters found in the literature review. Starting from the said process conditions, using one batch of solution a large number of different apparatus settings could be tested by maintaining the spinneret steady so that two or more nanofibre “stripes” on the same aluminium foil could be electrospun by just changing the apparatus settings. This way, direct comparison of different machine settings could be done using the same polymer solution batch; thereby reducing the batch-to-batch variability. During each test only one process parameter was modified compared with the previous test in order to clearly isolate its effects.

The first phase explored a large number of settings and provided information to switch to the second experimental phase.

In the second phase, the experiments were performed by changing the parameters on the basis of the experience gained in the first phase. In this case, key variables identified in the first phase were further fine-tuned allowing to improve the quality of the fibres observed during the first stage.
In the third phase, a quite novel method was applied with the aim of further improving the matrix morphology, in particular to decrease the chance of defects such as spheres and beads that inevitably form if the long-lasting electrospinning process is unstable. This approach, strongly recommended by Van der Schueren et al., consisted of finding combinational effects of parameters, which allow reaching the so-called steady state (Van der Schueren et al. 2011). The steady state implies that during fibres spinning not only a stable Taylor cone is kept, but also a continuous and constant mass of polymer solution during time of injection.

Once a satisfying outcome was achieved during the test, the same parameters were used to electrospin a large batch of polymer solution to produce a thick layer.

Following the establishment of the process parameters, two batch production processes were performed to verify the repeatability and reproducibility of the established methodologies. Such processes were further adopted for widening the specimen collection by adding layers with new features (see appendix I).

**ES PCL matrix collection and storage**

When each aluminium foil was totally covered with ES PCL fibres the apparatus was turned off. The rotating collector was covered with a seamless layer of fibres and the matrix layer attached to the foil had to be cut off with a surgical blade. The lateral matrix portions were removed to avoid the presence of electric field boundary effects on the morphology consistency. The fabricated matrices were then left under gentle air flux for at least one hour to promote the evaporation of residual solvents. Then, all matrices were kept in plastic envelopes sealed to preserve the material from particle contamination.

**3.2 Characterisation of ES PCL scaffolds**

Following the procedures previously illustrated, in the present section the characterisation techniques of the ES layers are described. The electrospinning process parameters were adjusted
to fabricate three types of matrix with as many different arrangements: random, semi-aligned and aligned fibres. This was achieved using 60, 2000 and 3000 rpm collector speed. The former and latter values are the minimum and the maximum speeds available for the electrospinning apparatus exploited herein.

The purpose of the methodologies described in the following sections is the characterisation of the matrices in the perspective of usage as tissue engineered devices. In particular, comparisons between the different matrices were performed to find the most suitable fibre type for the aforementioned application.

### 3.2.1 Morphology evaluation

**Scanning electron microscope (SEM) analysis**

Specimens of less than 1 x 1 cm² surface area were cut with care and placed on metal pin stubs, upon which sticky carbon tabs were glued.

ES PCL matrices were gold sputtered (Polaron 5100) and placed in the SEM (Sigma, Zeiss) chamber without further treatment. The SEM, equipped with two different detectors (namely InLens and SE2), was set to 5 or 10 kV acceleration voltage and the observation was carried out by detecting the emitted secondary electrons.

**Image processing: fibre diameter and orientation distribution function (ODF)**

After acquisition, the SEM images were processed with the aim of measuring quantitative parameters. Despite fibre morphology and number of defects (if any), which define the quality of the layer, two parameters were quantified. Quantification would allow the comparison between samples more reliable. The first parameter is fibre diameter whilst the second is the alignment degree. Diameters of 30 fibres were measured on three different images for each specimen using Image-j (National Institute of Health, USA) software.
Fibre alignment degree was measured through a computer-aided processing tool developed at Loughborough University (Demirci et al. 2011). The software was capable of recognising fibres and analysing their relative position in SEM images (Figure 3.2). The output of the software is the orientation distribution function (ODF) representing the percentage of fibres with an orientation within each angle interval of 10°, across the entire 180° range. Flawless or almost flawless images were selected with magnification of 2000, 5000 or 10000 X. For alignment analysis, the number of fibres included in two symmetrical intervals around 90°, identified as the machine direction (MD), were considered. Alignment degree was evaluated on the basis of the number of fibres that were included in the 80-100° and 70-110° intervals; therefore, the angle was sufficiently close to 90°.

*Figure 3.2: Fibre directions, in red superimposed to the fibres, individuated in the sub-domains of a representative image.*
Matrix thickness measurement

ES PCL matrix thickness was evaluated as it is an essential parameter for calculating the mechanical properties described in section 3.2.4.

ES PCL matrices thickness was measured by optical profilometry. For each sample type, three strips approximately 1 x 2 cm² were cut and placed on the bottom of a Petri dish. To avoid matrix lifting, which could impair the measurement, strips were wetted with 70% EtOH solution which, once evaporated, left the layers in close contact with the bottom surface of the Petri dish.

Subsequently, the Petri dishes containing the samples were placed under the light source of the profilometer (Talysurf CLI 2000, Taylor Hobson). The instrument was set with the following parameter values:

- gauge = CLA-3000
- sampling spacing = 4 μm
- sampling rate = 500 μm/s
- gauge frequency = 500 Hz

A high value of gauge frequency was selected to increase the sensitivity to the background of the Petri dish clear surface. Eventually, for every kind of layer a total of nine scans was performed. A post-processing protocol was established to standardise the acquired results. The sample height was evaluated as the mean height of the sample excluding the first 1-2 millimetres of the two edges due to the frequent presence of artefacts.

3.2.2 Wettability

Interaction between the matrices, the object of the present study, and water was investigated through the wettability test. Water affinity for biomaterials is a relevant indicator to predict the biocompatibility (Vogler 1998). For this project, this material feature was correlated to the mechanical properties assessed in wet conditions (section 3.2.4) and to cell response (section 4.2). Biocompatible materials promote cell attachment and spreading, requirements for a successful
tissue engineered device. However cell attachment is mediated by proteins, which are the first biological components that bind to the material surface (Roach et al. 2007). The mechanism by which proteins adsorb to the surface is governed by chemico-physical interactions (Wilson et al. 2005); thus, an opportune surface design in terms of chemistry and topography becomes fundamental to obtain the desired cell response.

The contact angle apparatus (FTA 1000, First Ten Angstrom) was set to measure the angle of a 5 µl deionised water drop on the specimen. The angle measured was the average between 200 frames acquired immediately after the drop was deposited on the specimen surface. Wettability of ES PCL samples with same fibre size, arranged in three different manners, was compared with that of solvent cast PCL. For solvent cast PCL, disc of 5 mm diameter were prepared using PTFE moulds. PCL 17% wt. was dissolved in chloroform with the same procedure as prior to an electrospinning session.

### 3.2.3 Degradability

Degradation of ES PCL matrix was studied through a test of mass loss and molecular weight reduction over 6 and 12 months respectively. A total of 24 ES PCL specimens (random arrangement, fibre diameter=1.17±0.57 µm, matrix thickness=93.41±4.44 µm) of comparable mass were cut from the same matrix and divided into two groups. The first remained untreated, while the second was exposed to UV light (1h each side) to simulate the sterilisation process prior to cell culture. The specimens were soaked in PBS and kept at 37 °C and 5% CO₂. At defined time points, specimens were treated as described in the following paragraph to perform the measurement. The consequences of ES PCL degradation over time and upon UV sterilisation will be discussed in section 7.1.4.
**Mass loss**

The evaluation of mass loss occurred on a weekly basis by weighing the samples three times with a balance (Adventurer Pro AV114, Ohaus) at $10^{-4}$ g accuracy. Before mass measurement samples were soaked thoroughly in deionised water to prevent salt deposition, which may affect the evaluation and allow it to dry. The average weight loss was calculated by comparing the measured value to those obtained at the beginning of the experiment.

Following the last measurement, two fragments were cut from two specimens per group. The four samples were then mounted on an equal number of pin stubs, sputter coated (EMITECH SC7640 Au/Pd) and observed with the SEM (Sigma, Zeiss). As negative controls the as fabricated matrix and one sample soaked in deionised water for one week were used. The latter enabled evaluation of the short-term effect of the wet environment on the morphology.

**Molecular weight loss**

Aside from those used in mass measurement, the remaining specimens were further divided into three groups, corresponding to each time point: 0, 3 and 6 months. Along with the ES PCL layers, raw PCL, of the same batch used to electrospin the matrices fabricated herein, was also analysed. Measurement was performed by means of gel permeation chromatography (GPC) using an Infinity 1260 apparatus (Agilent Technologies). The calculation of the molecular weight is based on the solution of Mark–Houwink equation that relates intrinsic viscosity to molecular weight as follows:

$$[\eta] = K M^\alpha$$

Where $[\eta]$ is the intrinsic viscosity, $M$ is the molecular weight and $K$ and $\alpha$ are the Mark–Houwink parameters characteristic for the specific polymer-solvent system.

For PCL-THF system Mark–Houwink parameters were retrieved from literature (Schindler et al. 1982) as follow:
3.2.4 **Tensile mechanical tests**

Tensile mechanical performance of ES PCL layers was assessed within a range of experimental set-ups. As discussed in sections 7.1.5 and 7.1.6 the results were analysed in terms of the influence of different experimental conditions and compared with the performances of the native AF.

**Multidirectional mechanical tests**

ES PCL layers were tested by changing the stretching direction compared with the main fibre direction for semi-aligned and aligned matrices in order to capture the contribution of anisotropic mechanical properties of the matrices. The apparatus chosen (MicroTester 5848, Instron) was equipped with a 50 N load cell.

Specimens were cut into 10 x 50 mm² keeping the longest axis at an angle of 0, 30, 60 or 90° relative to the principal fibre direction (Figure 3.3, left). Duct tape square 1 cm x 1 cm was attached to each specimen edge to improve the hold of the grips. The gauge length of each sample was thus 3 cm. Once mounted in the grips the layers were preloaded with 0.1 N prior to being stretched at 5 mm/min rate until breakage. The instrument was connected to a PC where force and grip displacement outputs were recorded.
Data analysis was conducted using the obtained force and displacement data and the thickness values (see section 3.2.1) necessary to calculate samples cross-section; hence the stress and the tensile modulus. The cross-section area was assumed to be constant throughout the whole test. The experiment was conducted in triplicate and the average and standard deviation were calculated for each of the said region. Tensile modulus \( E \) was calculated as the ratio between stress \( (\sigma) \) and deformation \( (\varepsilon) \) in a specific region.

\[
E = \frac{\Delta \sigma}{\Delta \varepsilon}
\]

Where \( \sigma \) and \( \varepsilon \) are defined as follow:

\[
\sigma = \frac{F}{A}; \quad \varepsilon = \frac{\Delta L}{l_0}
\]

With \( F=\)force; \( A=\)cross-section area; \( \Delta L=\)elongation; \( l_0=\)initial length.

For linear behaviour, \( \sigma \) and \( \varepsilon \) should change constantly so every curve region is theoretically suitable for modulus calculation. However, in the present study, two regions could be identified on the stress-strain curve. Thus, two different linear moduli were computed for each sample; one

---

**Figure 3.3:** Left: schematic representation of the applied stress on an ES PCL matrix. Right: ES PCL specimen during the stretching mechanical test.
for each region approximate to a linear portion (Figure 3.4). The moduli were named \( E_{\text{low}} \) and \( E_{\text{high}} \) corresponding to the low and high strain regions respectively. Specimen thickness was measured according to the procedures described in section 3.2.1 and is reported in Table 3.1.

![Figure 3.4: Example of the method adopted to calculate the tensile modulus. The two linear regions in the red circles are used to calculate the two tensile moduli at low and high strain.](image)

<table>
<thead>
<tr>
<th>Sample (fibre arrangement-fibre diameter)</th>
<th>Fibre arrangement</th>
<th>Thickness [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-0.40</td>
<td>Random</td>
<td>37.80±4.19</td>
</tr>
<tr>
<td>SA-0.40</td>
<td>Semi-aligned</td>
<td>25.77±3.49</td>
</tr>
<tr>
<td>A-0.60</td>
<td>Aligned</td>
<td>20.68±3.97</td>
</tr>
</tbody>
</table>
**Mechanical tests in physiological-like condition**

The aim of the mechanical tests described in this section was to further explore ES PCL matrices mechanical properties studied in an environment closer to the physiological one (i.e. PBS at 37 °C). Under these conditions, samples of random, aligned and semi-aligned patterns were tested by conducting stretching at various angles relative to the main fibre directions. In addition to the first assessment carried out in dry conditions, random fibre samples behaviour was investigated by comparing two different stretching directions (0 and 90°), considering that fibre alignment study, carried out after the first mechanical test, revealed the presence of a small orientation degree even on random fibre samples (see section 5.1.4).

**Samples preparation**

Rectangular ES PCL specimens (n=3, 10 x 40 mm²) were prepared with the aid of a custom device (Figure 3.5), which improved the reproducibility of their size and shape. In particular, the device, made of wood with plastic covering, ensured application of the duct tape perpendicular to the specimen’s longest side with high accuracy. Furthermore, the 20 mm gauge length was maintained across all the samples with high accuracy. Specimens (see Table 3.2) were cut out from the layers with known thickness, as previously described. The fibre diameter difference of samples was assessed as statistically insignificant (p<0.05).
Compared with the tests reported in section “Multidirectional mechanical tests”, different specimens, characterised by a higher fibre diameter, were chosen because of experimental constraints. Prior to testing, half of the specimens were preconditioned by soaking in PBS at 37 °C for 24h.

Table 3.2: Thickness of the samples used for the mechanical test in wet and dry conditions.

<table>
<thead>
<tr>
<th>Sample (fibre arrangement - fibre diameter)</th>
<th>Fibre arrangement</th>
<th>Thickness [µm]</th>
<th>Angles cut</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-0.91</td>
<td>Random</td>
<td>28.52 ± 3.37</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>SA-0.67</td>
<td>Semi-aligned</td>
<td>71.23 ± 13.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>A-0.73</td>
<td>Aligned</td>
<td>132.57 ± 39.17</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>90</td>
</tr>
</tbody>
</table>
Testing protocol: test to failure

The testing protocol was as follows:

- Preload: 0.5 N
- Extension rate: 10 mm/min
- Data acquisition rate: 10 Hz
- Test automatically stops when the force drops to 40% of the maximum value

Wet specimens were tested similarly, but the Biopulse attachment (Figure 3.6) was used. This device consists of a chamber that can be filled with PBS, and is equipped with a heating system that ensures the maintenance of a constant temperature set at 37 °C.

Additional mechanical tests on commercial plastic

In light of the behaviour detected on ES PCL samples stretched in wet conditions (see section 5.5 in the results), for which a jagged pattern was evident in the stress-strain curves, the decision was made to conduct further investigations.

Sheets of two different commercial plastics (referred to as "plastic 1” and “plastic 2” from here onwards) were cut and prepared following the procedure described above for ES PCL matrix. The test protocol remained unchanged as well as the environmental testing conditions, with the exception of an additional test performed under PBS at room temperature. The room temperature test was designed to isolate any contribution of 37 °C temperature.
4. Materials and methods: study on the applicability of ES PCL for AF regeneration purposes

This chapter outlines the experiments dealing with the investigation of hMSCs response to ES PCL layers. The tests were designed on the basis of the morphological data obtained from the assessments described in section 3.2.1.

4.1 Biological experiments. Techniques

This section presents the techniques relative to the biological experiments performed in the present thesis. The procedure described had been developed and then applied to the tests described in sections 4.2 to 4.8.

4.1.1 hMSCs standard culture procedures

Standard cell culture procedures include all the techniques applied to expand cells to a suitable number. These procedures were adopted in every cell seeding experiment in the same way, unless otherwise stated.

hMSCs were defrosted and seeded at a density of 5000-6000 cells/cm² in standard tissue culture flasks using culture medium as specified in Table 4.1. Expansion was performed by trypsinisation every time cells reached 80-90% confluency.
For this project, two batches of hMSCs passage 4 (obtained at the beginning and approximately at mid-term respectively) were expanded and used for all the subsequent experiments.

**Cell Crown aided experiment**

Initial cell culture experiments on ES PCL layers were performed by placing the specimens on the bottom of multiwell plates and cell suspension was added on their surface drop-by-drop. This method, despite returning positive outcomes, has some drawbacks. With a punch, specimens with not satisfying standardisation level and wavy surface could be obtained. In addition, cells could drop off the matrix upon seeding.

Therefore, Cell Crown devices (Scaffdex) were adopted. These devices allow to fix a 2-dimensional layer, such as ES PCL, between a ring and a cylinder that fit in standard multiwell plates.

**Viability assessment**

Alamar Blue (AB) is a well-established test based on the capability of the cells to convert the chemical blue dye resazurin into the pink resorufin (Rampersad 2012). This assay was extensively adopted for this project to assess cell viability. The assay was performed according to the manufacturer guidelines and reading the fluorescence signal using a plate reader (FLUOstar...
Omega, BMG Labtech) at 544/590 ex/em wavelength. The positive control is prepared by autoclaving 10% AB solution in order to obtain a 100% reduced resazurin solution.

**Staining procedures**

Fluorescence microscopy is one of the techniques chosen to observe cells on the surface of the ES PCL matrices.

The first staining experiments were performed to ensure the presence of cells on ES PCL specimen surface. Therefore, two dyes that were immediately available were used on hMSCs: Hoechst 33342® (Life technologies) (Quent et al. 2010; Ko et al. 2014; Chen et al. 2007; Liu & Williams 2010; Fotticchia et al. 2013) and Cell Tracker® (Life technologies) (Weir et al. 2008; Flasza et al. 2007). The former is a molecule that, once bound to DNA in cell nucleus and irradiated with UV light, emits in the blue light region (ex/em 346/497 nm). The latter, based on a chloromethylbenzamido compound, once penetrated in the cell is modified into a membrane impermeant compound; thus, its red light emission (ex/em 553/570 nm) is useful in identifying cell shape. Cell Tracker staining was performed on living cells; subsequently, fixed with formalin (Sigma-Aldrich) and further stained with Hoechst 33342 following the manufacturer guidelines.

To better visualise cell shape, as Cell Tracker returned not enough satisfying results, staining of actin filaments was carried out with the aim of evaluating cell elongation and orientation. Phalloidin Alexa Fluor®-555 (ex/em 555/565 nm) was selected according to its compatibility with the microscope filter and to avoid light absorption/emission spectra overlapping with those of Hoechst 33342.

To enhance the understanding of cell adhesion on different substrates, it was decided to study focal adhesions, which are protein assemblies on cell membranes, responsible of cell spreading, attachment and mechanical force transmission (Chen et al. 2003). The primary antibody (Ab)
selected was a polyclonal anti-human vinculin from rabbit whilst the secondary Ab was polyclonal anti-rabbit IgG from goat (Abcam) conjugated with Alexa Fluor-488 dye (ex/em 495/519 nm).

With the aim of evaluating the survival rate of cells cultured within multi-layers live and dead assay (Life Technologies) was selected. The assay is based on two dyes, namely ethidium homodimer-1 (EthD-1, ex/em 528/617 nm) and calcein AM (AM, ex/em 494/517 nm) (Y. Zhu et al. 2010; Park et al. 2012; Yang et al. 2009; Yeganegi et al. 2010). The first is cell membrane impermeable and binds only to DNA of dead cells. Therefore, dead cells are identified because they have impaired membranes. Conversely, AM is able to stain the cytoplasm of living cells as it undergoes enzymatic conversion into fluorescent calcein (Decherchi et al. 1997).

**Fluorescence images processing**

The analysis and processing of the fluorescence microscope images was performed to extract information quantitatively. The purpose was to establish a procedure capable of analysing pictures with as little operator intervention as possible.

Cell nuclei shape was exploited as nucleus assumes an elongated profile when the entire cell is extended in a particular direction, and change from rounded shape into a more elliptical one (Dalby et al. 2003; Raghunathan et al. 2013). The protocol, based on Image-j software, comprised several steps that allowed identification of nuclei in ellipses by the software itself. As a geometrical entity, described mathematically, two parameters were calculated to exploit the measurement of the angle and length of the ellipsis axes. The detailed description of the procedure is described hereafter.

1) The multichannel pictures are imported into Image-j and the channels are split to isolate the blue component only, which corresponds to Hoechst 33342 dye signal, thus to the nuclei (Figure 4.1).
2) In case of specimens with a certain fibre orientation the pictures are rotated to align fibres to the horizontal axis, which corresponds to 0°, assumed as the MD. The contrast of the signal can also be enhanced to have very bright blue nuclei. The picture is then transformed into a binary image where nuclei are black spots on a white background (Figure 4.2).

3) Odd shapes that would be clearly interpreted in the wrong way by the software could be manually deleted. Software detection options are set to allow performing the
transformation of the spots into ellipses at best.

4) One of the smallest nuclei is individuated and its area is measured using the opportune tool. The value is used to set the lower threshold for particle analysis to exclude isolated pixels or very small spots. Subsequently, the analysis is performed leading to the replacement of the black shapes with black-contoured ellipses (Figure 4.3). Along with the processed pictures, a set of numerical data is generated.

5) The parameters of interest are angle and circularity. The former refers to the angle formed between the major ellipsis axis and the horizontal axis of the image, which is aligned to the MD for aligned fibre samples. This gives a quantification of the nuclei alignment. In contrast, circularity parameter (C) is calculated as follows:

\[
C = 4\pi \times \frac{\text{Area}}{(\text{Perimeter})^2}
\]

C value could span between 0 and 1. If C is equal to one, the nucleus analysed is perfectly rounded, whilst if it is close to zero the shape tends to be infinitely elongated.

6) The output of the software is presented in such a way that the parameters of each ellipsis can be associated to the correspondent ellipsis on the image; therefore, permitting a
visual checking of the analysis consistency. Data aggregation and subsequent calculations were performed with MS excel®.

The above described method was applied to over 1100 nuclei.

*Verification of images analysis protocol effectiveness*

The protocol described in the previous section was verified through the analysis of images of cells with known characteristics.

*Cell orientation*

A set of nine ellipses of different size was drawn by changing the angle of the major axis, from 90 to 0°, with 10° steps (Figure 4.4). The same set was copied and pasted twice to have three copies for each angle. All the ellipses of this array were then modified manually by randomly introducing shape irregularities (Figure 4.5). This step was undertaken to verify the effectiveness of the image processing method when it was used to recognise shapes similar to cell nuclei, which can be not perfectly elliptical (see the example in Figure 4.6).

All the images with the tilted, original and deformed ellipses were analysed following the protocol described in the previous section. For each angle, the average of three deformed ellipses was calculated and compared with the original known value.
A similar procedure was carried out to demonstrate the circularity of cell nuclei. A set of ellipses with the ratio of their axis varying from 1 to 0.17 was drawn (Figure 4.7). The set was replicated twice and randomly manually modified to introduce flaws in the contour (Figure 4.8). The analysis was performed following the steps described in the previous section and the results were compared with the data obtained from the original image.

**Cell nuclei circularity**

A similar procedure was carried out to demonstrate the circularity of cell nuclei. A set of ellipses with the ratio of their axis varying from 1 to 0.17 was drawn (Figure 4.7). The set was replicated twice and randomly manually modified to introduce flaws in the contour (Figure 4.8). The analysis was performed following the steps described in the previous section and the results were compared with the data obtained from the original image.
Demonstration of cell alignment analysis method

The values obtained on the ellipses with varying angles are shown in Table 4.2. The angles measured with the procedure are all very close to the values of the regular shapes as prepared, with the difference that exceeds 1° in only one case (ellipse number 9 at 10°). To be noticed that in the same case the ellipse was the smallest of the set suggesting that, as expected, the reliability is lower when the software handles tiny objects.

Table 4.2: Comparison between the angle values of the ad hoc prepared ellipses and relative measurements following the protocol.

<table>
<thead>
<tr>
<th>Ellipse number</th>
<th>Original angle [°]</th>
<th>Measured angle [°]</th>
<th>Difference between original and measured values [°]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90.00</td>
<td>90.19</td>
<td>0.19</td>
</tr>
<tr>
<td>2</td>
<td>80.00</td>
<td>79.74</td>
<td>-0.26</td>
</tr>
<tr>
<td>3</td>
<td>70.00</td>
<td>69.90</td>
<td>-0.10</td>
</tr>
<tr>
<td>4</td>
<td>60.00</td>
<td>59.91</td>
<td>-0.09</td>
</tr>
<tr>
<td>5</td>
<td>50.00</td>
<td>50.19</td>
<td>0.19</td>
</tr>
<tr>
<td>6</td>
<td>40.00</td>
<td>39.99</td>
<td>-0.01</td>
</tr>
<tr>
<td>7</td>
<td>30.00</td>
<td>29.75</td>
<td>-0.25</td>
</tr>
<tr>
<td>8</td>
<td>20.00</td>
<td>20.64</td>
<td>0.64</td>
</tr>
<tr>
<td>9</td>
<td>10.00</td>
<td>11.14</td>
<td>1.14</td>
</tr>
<tr>
<td>10</td>
<td>0.00</td>
<td>0.04</td>
<td>0.04</td>
</tr>
</tbody>
</table>

The values measured on the ellipses with the flaws randomly introduced in their contour are reported in Table 4.3. As anticipated, the difference with the original values is higher than that reported in Table 4.2; thereby proving that the deformation in the ellipses contours increases the variability and, consequently, the error. Yet, the difference is of the order of magnitude of few degrees (mean difference =1.79±2.34°).
Table 4.3: Comparison between the angle values of the ad hoc prepared ellipses and those measured on the manually modified ellipses.

<table>
<thead>
<tr>
<th>Ellipsis number</th>
<th>Original angle [°]</th>
<th>Measured angle average [°]</th>
<th>Measured angle SD [°]</th>
<th>Difference between original and measured values [°]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90.00</td>
<td>95.37</td>
<td>1.81</td>
<td>5.37</td>
</tr>
<tr>
<td>2</td>
<td>80.00</td>
<td>82.26</td>
<td>1.23</td>
<td>2.26</td>
</tr>
<tr>
<td>3</td>
<td>70.00</td>
<td>68.72</td>
<td>2.51</td>
<td>-1.28</td>
</tr>
<tr>
<td>4</td>
<td>60.00</td>
<td>63.55</td>
<td>1.40</td>
<td>3.55</td>
</tr>
<tr>
<td>5</td>
<td>50.00</td>
<td>53.25</td>
<td>1.72</td>
<td>3.25</td>
</tr>
<tr>
<td>6</td>
<td>40.00</td>
<td>37.44</td>
<td>2.04</td>
<td>-2.56</td>
</tr>
<tr>
<td>7</td>
<td>30.00</td>
<td>31.25</td>
<td>1.71</td>
<td>1.25</td>
</tr>
<tr>
<td>8</td>
<td>20.00</td>
<td>22.19</td>
<td>0.36</td>
<td>2.19</td>
</tr>
<tr>
<td>9</td>
<td>10.00</td>
<td>10.89</td>
<td>4.89</td>
<td>0.89</td>
</tr>
<tr>
<td>10</td>
<td>0.00</td>
<td>2.92</td>
<td>0.74</td>
<td>2.92</td>
</tr>
</tbody>
</table>

Concerning the circularity, the outcome of the comparison between the values of the as prepared ellipses and the ones measured with the procedure is revealed in Table 4.4.
Table 4.4: Comparison between the circularity values of the ad hoc prepared ellipses and relative measurements following the protocol.

<table>
<thead>
<tr>
<th>Ellipsis number</th>
<th>Original circularity</th>
<th>Measured circularity</th>
<th>Difference between original and measured values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.00</td>
<td>0.92</td>
<td>-0.08</td>
</tr>
<tr>
<td>2</td>
<td>0.98</td>
<td>0.91</td>
<td>-0.07</td>
</tr>
<tr>
<td>3</td>
<td>0.92</td>
<td>0.87</td>
<td>-0.05</td>
</tr>
<tr>
<td>4</td>
<td>0.80</td>
<td>0.78</td>
<td>-0.02</td>
</tr>
<tr>
<td>5</td>
<td>0.60</td>
<td>0.59</td>
<td>-0.01</td>
</tr>
<tr>
<td>6</td>
<td>0.32</td>
<td>0.36</td>
<td>0.04</td>
</tr>
</tbody>
</table>

When the deformation was introduced the difference increased slightly (Table 4.5) but it always remained equal or lower than 10%. It was also noticed a decreasing trend of the error the more the ellipses analysed are elongated. This indicates that ellipses with circularity equal, or close, to 1 (circles) have their circularity more affected by deformations. The same trend could be observed to a lesser extent in Table 4.4.
Table 4.5: Comparison between the circularity values of the ad hoc prepared ellipses and of those measured on the manually modified ellipses.

<table>
<thead>
<tr>
<th>Ellipsis number</th>
<th>Original circularity</th>
<th>Measured circularity average</th>
<th>Measured circularity SD</th>
<th>Difference between original and measured values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.00</td>
<td>0.90</td>
<td>0.01</td>
<td>-0.10</td>
</tr>
<tr>
<td>2</td>
<td>0.98</td>
<td>0.90</td>
<td>0.01</td>
<td>-0.08</td>
</tr>
<tr>
<td>3</td>
<td>0.92</td>
<td>0.85</td>
<td>0.03</td>
<td>-0.08</td>
</tr>
<tr>
<td>4</td>
<td>0.80</td>
<td>0.75</td>
<td>0.12</td>
<td>-0.05</td>
</tr>
<tr>
<td>5</td>
<td>0.60</td>
<td>0.62</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>6</td>
<td>0.32</td>
<td>0.30</td>
<td>0.11</td>
<td>-0.03</td>
</tr>
</tbody>
</table>

**SEM imaging**

Electron microscopy allowed correlating cell shape obtained with fluorescence techniques with fibre orientation. All the chemicals for this procedure were purchased from Sigma-Aldrich. Samples preparation was performed by fixation with glutaraldehyde followed by soaking in Osmium tetroxide 1% in sodium cacodylate 0.1 M solution. Dehydration was achieved by means of 5’ rinsing passages in increasing EtOH concentration solutions. Subsequently, samples were soaked in three hexamethyldisilazane solutions with increasing concentration for 30’ each; after which they were let dry overnight. Fixed dried samples were then mounted on pin stubs and silver paste was applied to increase the conductivity. Subsequently, the specimens were sputter coated (EMITECH SC7640 Au/Pd), loaded into the chamber of the SEM (Sigma, Zeiss) and analysed with 5 kV acceleration voltage.
4.2 Investigation of the response of hMSCs cultured mono-layers

In this section, ES PCL layers with different fibre orientation, characterised as reported in section 3.2.1, are used as substrate for culture hMSCs which response was investigated from the viability and morphology perspective (see section 4.1.1 for cell culture methods).

4.2.1 Samples preparation

From the original ES PCL sheets, a suitable number of pieces per type was cut out, and sterilised by UV exposure for 1h per side. At the end of the sterilisation procedures, the ES PCL layers were mounted onto the Cell Crown devices as described by the manufacturer (Figure 4.9). The devices, mounted with matrices, were inserted into the wells of a 12 multiwell plate. Cell Crown inserts were not pressed in close contact with the bottom of the wells, but allowing the presence of medium between the bottom of the well and bottom of the matrices. 2 ml of culture medium for hMSCs was added in each well in order to precondition the matrices for 24h.

Figure 4.9: Detail of an ES PCL layer mounted on a Cell Crown device.
The medium used to precondition the samples was removed and 30 µl of cell suspension (5 x 10^4 cells) were pipetted onto each ES PCL sample mounted on Cell Crowns. The plates were then moved inside the incubator for approximately 2h to allow the cells to adhere to the fibres. Afterwards, 2 ml of culture medium was topped up for each sample and the plates incubated again for 24h. The medium was replaced every 2-3 days and viability was monitored at regular intervals by means of AB assay (see section 4.1.1).

### 4.2.2 Investigation of hMSCs morphology and orientation

**Fluorescence optical imaging**

To assess cell shape and adhesion, specimens of five different fibre types were prepared, as described above. Three samples with different fibre arrangement and same fibre diameter (range 0.40-0.60 µm) were selected to study fibre alignment effect on hMSCs. Furthermore, two random fibre samples with two- and three-fold bigger fibre diameter respectively were chosen. HMSCs were cultured for 20 days according to standard procedure and then double stained with Hoechst 33342 and Alexa Fluor-555 dyes (section 4.1.1). Prior to staining all the fixed layers were removed from the Cell Crowns and the portions outside the ring cut out with a scalpel to obtain disc-like shape specimens, which are perfectly flat.

Once cut, the specimens were maintained at all times soaked in PBS with the seeded side upwards, and they were turned over only when they had to be observed under the inverted microscope. The same layers were also analysed to assess cell penetration. The discs were divided in two parts along the diameter and a narrow strip parallel to the diameter was cut out with a scalpel. The strip was tilted by 90° to expose the cross-section to the objective of the microscope.

**SEM imaging**

SEM was employed to evaluate the morphology and orientation of cells compared with the topographical features of the substrates. Three samples with different fibre arrangements and
comparable fibre diameter were selected. At the end of the culture period, samples were fixed and appropriately treated for SEM imaging (section 4.1.1). Prior to gluing the specimens on the pin stubs and before performing the sputter coating, all the specimens were cut in two parts, one of which was turned over.

**Focal adhesion investigation**

Immunocytochemistry technique was used to further identify focal adhesion of hMSCs on ES PCL fibres. Specimens, of three different fibre types, were mounted on Cell Crowns and hMSCs seeded and cultured for seven days. Vinculin staining was performed (see section 4.1.1) followed by counterstaining with Hoechst 33342 and phalloidin Alexa Fluor-555 conjugated that was performed according to the standard procedure (section 4.1.1).

### 4.3 Investigation of the response of hMSCs cultured in a double-layer model

The investigation of hMSCs response to ES PCL mono-layers described in section 4.2 laid the foundation for the design of the tests presented in this section. Particular focus was given to the influence of ES PCL fibre diameter and matrix thickness to cell viability and orientation (see section 3.2.1).

#### 4.3.1 Samples preparation

Sample preparation protocol described hereafter was adapted from the procedure carried out for mono-layers, presented in section 4.2.1. The ES PCL layers were cut into squares, after which two corners on the same side were cut out to facilitate the identification of the main fibre direction (Figure 4.10), and sterilised. Three single layers per type were mounted individually on Cell Crowns devices, inserted in 12 multiwell plate and soaked in culture medium for 24h prior to cell seeding.
On day 0, 30 µl of hMSCs suspension was seeded at a density of $5 \times 10^4$ cells/sample on the matrices, fixed to Cell Crown inserts. Cells were let adhere to ES PCL for 2h and then 2 ml medium per well were added. After 24h cells viability was evaluated through AB as per standard protocol (see section 4.1.1). On day 1, the ES PCL matrices were arranged into double-layers. Cell Crown inserts were disassembled and the unseeded layers overlapped the seeded ones with the attention of the main fibre orientation. A schematic representation is shown in Figure 4.11.

**Figure 4.10:** Schematic of the layout of the ES PCL matrices for the first seeded (left) and the second unseeded (right) layer respectively. The corners were cut out to help overlaying of the reciprocal layers with 60° fibre orientation.
The constructs were allowed to stabilise for one day, after which another viability assessment was performed. Medium was changed every 2-3 days and viability regularly monitored by means of AB assay (see section 4.1.1).

### 4.3.2 Viability study

Specimens cut from two different matrices with aligned fibres of different diameters (0.60±0.22 and 1.72±0.50 µm) were prepared, as described previously. As a control, two layers of the same types of those used to build the double-layers were maintained as mono-layer throughout the experiment’s duration. AB assay was performed as per standard procedure (section 4.1.1) to monitor cell viability up to 25 days.

*Figure 4.11: Overlapping strategy adopted to build an ES PCL double-layer. The top layer was superimposed on the cellularised bottom layer.*
4.3.3 Investigation of hMSCs morphology and orientation

Fluorescence OM imaging

The double-layers seeded with hMSCs studied in the previous section were further investigated. Cell morphology was evaluated by means of Alexa Fluor-555 and Hoechst 33342 dyes (see section 4.1.1). Thin cross-sections were cut with a scalpel, along the diameter, and placed into multiwell plates to observe cells presence between the juxtaposed matrices (see schematics in Figure 4.12 and Figure 4.13).

![Figure 4.12: Schematic representing how each layer was cut into thin strips prior to optical microscopy analysis.](image1)

![Figure 4.13: Schematics of the surfaces observed under the microscope.](image2)
Furthermore, narrow strips were cut out from the samples and each layer separated. The downwards side of the bottom matrix and the upward side of the top one were placed in a well plate facing the microscope objective and observed. The strips were placed parallel under the microscope objective thus the angle between the two layers imposed during double-layer assembling was maintained.

**SEM imaging**

HMSCs were cultured in a double-layer random fibre model (0.46±0.14 µm fibre diameter, 72.52±1.63 µm single layer thickness) according to the procedure previously described (4.3). The specimen was treated according to the fixation protocol for SEM analysis (see section 4.1.1) and narrow strips were cut from the double-layers as shown in Figure 4.12 and Figure 4.13. Hence the strips were mounted on special holders with vertical wall so that the cross-section faced the SEM detector. Prior to sputtering and loading, one layer of the cross-section was partially lifted to expose both the interface profile and the inner surface.

**Toluidine staining**

Toluidine blue was used to stain double ES PCL layers (random fibres, 1.27±0.49 µm fibre diameter, 59.63±10.43 µm thickness) prepared accordingly to section 4.3.1 and provide further evidence of cell presence and distribution (see previous paragraphs, section 4.3.3). The dye was chosen because, further to cells body, it has been applied for proteoglycans and GAGs histology (Shepard & Mitchell 1976) and quantification (Terry et al. 2000). Proteoglycans, abundant in cartilages and intervertebral disc, are protein cores with attached polysaccharides (GAGs) chains which, like chondroitin, heparin and keratin sulphate, are rich in SO₃⁻ groups (Beaty & Mello 1987). The high electron density allows complexes formation with toluidine blue (Sridharan & Shankar 2012; Terry et al. 2000).
HMSCs were cultured as for standard procedures (see section 4.1.1) and seeded ($5 \times 10^4$ cells/sample) on ES PCL mono-layers. The seeding steps were performed in accordance with preliminary tests that demonstrated enhanced uniformity of cells attached to top and bottom layers. Immediately after seeding, a second matrix of the same type was overlaid for 1h. Hence the double-layer was turned over and let stabilise for 1h, after which samples were mounted on Cell Crown devices. A control without cells was kept in medium for the same time span as seeded scaffolds. AB test (see section 4.1.1) was performed since the day after seeding and on regular basis till the end of cell culture on day 14. On the last day, samples were stained with toluidine blue (Sigma-Aldrich), following the manufacturer guidelines.

Stained specimens were observed with an optical microscope for surface analysis (Optihot, Nikon). Some specimens were analysed by separating bottom and top layers with tweezers and observing the inner sides; other samples were mounted between two glass microscope slides and protruding edges bent outward to expose the interface. The slides were then placed vertically under the optical microscope objective for observation.

### 4.4 Effects of matrix thickness and fibre diameter on hMSCs viability in ES PCL double-layers

The experiments reported in this section are the logical consequence of the test described in section 4.3, whereby cells were cultured in different ES PCL double-layer constructs. Indeed, the outcome suggests that viability could be affected by both layer thickness and fibre diameter (see section 6.3). Nevertheless, in the same test, the contribution of each individual variable could not be isolated, also considering that the experiment had not been designed for this purpose. Thus, the investigations reported herein aim to examine the individual contribution of layer thickness and fibre diameter on cell viability.
Preparation of samples of the two studies was conducted simultaneously exploiting Cell Crown devices and taking advantage of the same cell batch. Samples were prepared and cells cultured for 21 days according to the procedure described in section 4.1.1 and 0.

### 4.4.1 Fibre diameter

For this experiment, the comparison was made between two double-layers characterised by approximately three-fold fibre diameter difference: 0.46±0.14 and 1.72±0.50 μm respectively. As a control, hMSCs were cultured on 24 multiwell plates keeping the same cell density as on ES PCL matrices. The test was conducted in triplicate.

### 4.4.2 Layer thickness

Two different structures were exploited using one matrix type (77.97±10.28 μm thick, aligned fibres with 0.70±0.38 μm diameter) to study the effect of layer thickness on cell viability. The scaffold concept was the same as in the experiments above (4.4.1). In one case both the bottom and the top layer were made of simple mono-layers whilst in the other the bottom and top layers were formed of two matrices respectively. A schematic is illustrated in Figure 4.14.

![Figure 4.14: Schematic of the thin (left) and thick (right) double-layers named D and DD-layer respectively.](image-url)
Fibre orientation was kept at 60° between the layers above and underneath the cells; within each double-layer. Samples were named D-layer or DD-layer depending on the arrangement in one or two double-layer fashion.

4.5 HMSCs culture within ES PCL multi-layer

This section presents the methodologies related to the fabrication of a cellularised multi-layer made of overlapped ES PCL layers. The procedures developed here take advantages of those established in sections 4.2 and 4.3 where hMSCs were seeded on mono-layers and in double-layers.

4.5.1 Multi-layer assembling and seeding

Eight layers (random fibres, 1.27±0.49 µm fibre diameter and 59.63±10.43 µm layer thickness) were prepared as per standard procedure (see section 4.2.1). HMSCs were cultured according to the procedure described in section 4.1.1 and 30 µl solution drop containing 5 x 10^4 cells was pipetted on each ES PCL specimen and incubated at 37 °C for 24h (see schematic in Figure 4.15, top). Cell viability at day 1 culture was assessed through AB test on mono-layers (see section 4.1.1). On the same day, after 24h from seeding, Cell Crown devices were disassembled and the seeded layers were overlapped to build two multi-layers, each one made of four layers with the seeded side facing upward (see schematic in Figure 4.15, bottom). Each multi-layer scaffold was mounted in a single Cell Crown and soaked in culture medium. The other Cell Crowns were placed in the same plate as the scaffolds and soaked in medium till further usage. The first AB test on multi-layers was performed the day after assembling (day 2). Cell culture in multi-layers continued for 7 days when viability was evaluated. Afterwards multi-layers were removed from the Cell Crowns, mounted individually on eight Cell Crowns and viability assessed on each mono-layer the following day (day 9) to let cell stabilise. Layers were named from “A” (bottom layer) to “D” (top layer) according to their location depth within the multi-layer.
Figure 4.15: Schematic showing the steps carried out to build each multi-layer. Top: 4 layers, to be mounted on Cell Crowns on day 0, seeded individually with an equal number of cells; bottom: the four layers are overlapped obtaining a seeded multi-layer on day 1.
4.5.2 Live and dead essay

The day after the AB test, the layers were split and stained with a live/dead assay (Life Technologies) to assess the number of living and dead cells (see section 4.1.10). At least eight pictures per layer, taken on both samples, were analysed by applying a grid on the images using Image-j software and manually counting the cells. The percentage of living and dead cells was calculated and the data statistically analysed.

4.6 Investigation of ES PCL matrix permeability

On the basis of the results of the tests that investigated the viability of hMSCs seeded in double-layers with different morphological features (see section 6.3), ad hoc experiments were designed for acellular ES PCL matrices. It was hypothesised that the different viability performances were due to different nutrients diffused through the substrates. Thus, the present section investigates the diffusion of AB dye through ES PCL layers fixed to Cell Crowns. Two experiments were performed in parallel to the cellularised double-layer experiments described in section 4.4, to examine independently the effect of matrix thickness and fibre diameters.

Concerning ES PCL thickness experiment, from a random fibre matrix (1.17±0.57 µm fibre diameter and 93.41±4.44 µm layer thickness) square specimens were cut, sterilised by UV light exposure, and soaked in PBS for 24h. Hence, they were arranged into mono-, double- and triple-layer configurations and mounted on sterile Cell Crown devices. For fibre diameter, investigation matrices with fibres of 0.40±0.15 and 1.59±0.49 µm were selected (151.20±16.76 and 156.00±28.30 µm thickness respectively). Cell Crowns were subsequently inserted in standard 12 multiwell plates so that, in each well, two compartments, separated by the ES PCL layer, were created (Figure 4.16, left). At the same time 10% AB standard solution (blue) and 100% reduced AB solution (pink) were prepared as described in section 4.1.1. PBS was then completely aspirated from the wells containing the Cell Crowns and replaced with 2.7 ml of AB solution. Then 300 µl of
100% reduced AB were slowly pipetted in-between the well and Cell Crown walls. Both the solutions were brought to RT prior to start and at each time point (30’ and 1, 2, 4h) 50 µl of solution was taken from the region inside the Cell Crown and pipetted in a 96 multiwell plate. The layers’ diffusion capability was evaluated as the amount of 100% reduced AB solution that diffused through the layers into the Cell Crowns (Figure 4.16, right).

For each experiment, the highest permeability obtained was adopted as the reference (100%). All the other values were then computed as ratios of such reference value.

Diffusion was modelled using Fick’s second law. Prism (Graphpad) was used to find the best fit of the experimental values to the negative exponential equation (1) derived from Fick’s second law.

\[ y = y_0 + (p - y_0) \cdot \left(1 - e^{-\frac{t}{\tau}}\right) \]  

(1)

The characteristic time \( \tau \) of the negative exponential curve was then utilised to calculate the diffusion constant \( D \) from equation (2) (Boss et al. 2012; N. Wang et al. 2013).

\[ D = \frac{d}{S} \cdot \frac{V_A V_B}{V_A + V_B} \]  

(2)

Where \( d \) is the thickness of the ES PCL layer, \( S \) the surface area of the layer and \( V_A \) and \( V_B \) the volumes of the two compartments.
4.7 Effect of low oxygen concentration on hMSCs

hMSCs response to hypoxic condition was studied to clarify the role of a lack of oxygen; a condition found physiologically in the IVD (see section 2.2.2). The results of the test described herein aided interpretation of the outcome of cell viability tests in double- and multi-layer models (sections 6.3, 6.6.7 and 6.7), as discussed in section 7.3.3.

Six wells of two standard 24 multiwell plates were seeded with hMSCs. Cells were expanded, trypsinised as for standard procedure (see section 4.1.1) and plated at a density of $1 \times 10^4$ cells/well. After seeding the two plates were kept in incubator under standard condition (5% CO$_2$) for 24h, after which viability was assessed to evaluate the cell initial state. Immediately after the test one plate was moved into the hypoxic chamber (Invivo2 400, Ruskinn), where the AB solution

---

Figure 4.16: Schematic of the top and side view of a Cell Crown insert inside a well. On the right the diffusion mechanism of 100% reduced AB test is illustrated.
left in the wells was replaced with fresh medium that had been preconditioned in hypoxic environment (2% O₂, 5% CO₂) for several hours. Simultaneously, AB solution of cells culture in standard condition was replaced with normal medium. HMSCs were left growing for 7 days and the last viability evaluation was performed at the end of the culture. Every two days, preconditioned and normal medium were respectively changed in each plate. The viability values obtained for each culture condition were averaged between all the six wells. For cells maintained in hypoxic environment both medium and AB solution were preconditioned in the hypoxic chamber prior to every medium change and AB assay.

4.8 IVD-like construct assembly and mechanical stimulation through bioreactor

The BOSE bioreactor available at Loughborough University permits maintaining live cells in-vitro whilst exerting mechanical stimulation. Nevertheless, the bioreactor, which is equipped with compression platens, does not allow the stimulation of 2-dimensional seeded samples, such as the ES PCL matrices of the present work. Thus, considering the radial stress experienced by AF under IVD compression, the physiologically-like mechanical stimulation of such samples could be effectively achieved through the fabrication of an IVD-like construct, resembling the structure and material mechanics of the natural IVD, which could be stimulated by imposing compression forces. Furthermore, it is deemed that stimulation of cellularised ES PCL sheets could be more beneficial when the matrices are arranged in a configuration similar to the natural one. Under these premises, it was planned to couple the ES PCL matrices, acting as the AF, to a core, acting as the NP. The double-layer model presented in section 4.3 was established in preparation of the development of the IVD-like model described hereafter.
4.8.1 **NP-like disc preparation**

Agarose (Sigma-Aldrich) was selected for the fabrication of the NP-like core and a synthesis protocol was established in accordance with the manufacturer guidelines.

Once ready, the hydrogel is removed from the beaker and cut into two thinner discs with a scalpel. An 8 mm diameter puncher is used to cut as many cylinders as possible from each big disc. Hence, to make the thickness of the 8 mm diameter discs uniform, a custom-made device formed of two parallel scalpels glued together (Figure 4.17) is adopted to slice each 8 mm cylinder. The final thickness of each 8 mm disc was assumed to be equal to the distance between the two blades (3.7 mm). Agarose discs were stored at RT in PBS. Prior to being used to build cellularised IVD –like assemblies, the discs were exposed to 1h UV light for sterilisation.

*Figure 4.17: Double surgical blade used to cut 8 mm diameter agarose discs of constant thickness.*
4.8.2 Cellularised IVD-like constructs preparation

A number of aligned or semi-aligned ES PCL matrices was selected to be seeded with hMSCs and arranged into AF-like structures. Rectangular shaped samples (1.4 x 3.5 cm²) were cut out from the ES PCL sheets with two opposite fibre directions, + and −30°, similarly to what has been done in section 4.3. Before seeding, medium was removed in order to keep the layers flat and adherent to the Petri dish bottom. Cells, cultured in standard flasks, were harvested according to standard procedures (section 4.1.1) and concentrated at a density of 5 x 10⁴ cells/10 µl. On each rectangular shape, belonging to +30° fibre orientation group, cells were seeded by pipetting 10 µl cell solution drop on six different locations as shown in Figure 4.18. This seeding strategy was planned to ensure the most even distribution possible in the middle portion of the rectangular samples, and minimise the solution dropping out of the specimen, on the Petri dish bottom.

Figure 4.18: Top view of the seeding and cutting strategy adopted to fabricate AF-like strips.
The portions on the left and right side, as revealed in Figure 4.18, were removed before wrapping the layers around the agarose discs. Immediately after cell seeding +30° samples were overlaid on -30° rectangular specimens and let them stabilise for 1h in incubator. After this time span, the
double-layer samples were turned over, transferred to six multiwell plates and again incubated for 1h before topping medium up.

After 24h incubation double-layers were cut as represented in Figure 4.18 thereby obtaining two double-layer strips of approximately 4 x 35 mm² each (dashed red lines). The exceeding material at the top and bottom, as shown in Figure 4.18, will be excluded at a later stage. The cutting operation was undertaken under the BSC using a disposable surgical blade and without the aid of rulers, using the agarose discs height as reference for the width. Each double-layer strip was laid on an open Petri dish and an agarose disc was placed on top of it on its curved side. With tweezers, the strip was wrapped around the agarose cylinder and sealed with commercial waterproof polyurethane resin (Wilkinson) keeping the AF-like strip tightly in contact with the agarose disc. It must be noted that glue was not applied on the contact points between agarose and ES PCL but between the two edges of the ES PCL strips. This operation left the edges of the strip glued together and protruding out of the IVD-like strip (Figure 4.19). The glue was let dry for one minute and then the construct was soaked in warm medium. Figure 4.20 is a representation of the final structure of the construct. An opportune cytocompatibility test on the commercial glue used was performed to evaluate any adverse effect on cells.

**Preliminary viability test in static conditions**

Three ES PCL strips were seeded with hMSCs as described above, wrapped around agarose discs and sealed with glue. An equal number of ES PCL strips was kept as control. The objective of this test was to assess whether and how the assembly procedure affects cell viability, compared with the seeded ES PCL strips.
In addition, each seeded ES PCL double strip, after being split into two smaller double strips, was used to prepare two specimens: one was used as control sample and the other one was used to make an IVD-like construct (Figure 4.21). Hence a direct comparison between identically seeded samples could be done and thus any effects from sample handling and processing could be captured more accurately.

**4.8.3 Bioreactor mechanical stimulation**

**System description**

The BOSE 5900 BioDynamic® bioreactor is a complex system that allows *in-vitro* mechanical stimulation of cellularised scaffolds for tissue engineering. The apparatus is contained in an incubator, which ensures the maintenance of 37 °C and 5% CO₂ at all times. Inside the incubator there are three main components: the pumping system, the electrical motor, with a frame supporting a chamber containing samples and cell culture liquid, and the valve system. All the stated elements, with the exception of the incubator, are controlled through a PC equipped with the WinTest® software provided by the supplier.

**Figure 4.21: Samples preparation strategy.** A, B and C are the parent cellularised double-layers, which, after day 1 AB assessment, are cut to make AF-like strips and AF-like strips used to build IVD-like constructs.
The top of the vertical electrical motor (Figure 4.22) is connected to the chamber (Figure 4.23) that has four platens allowing individual samples to be mounted. The chamber is fixed to a metal structure (Figure 4.25) and the connection to the motor occurs through four metal shafts (Figure 4.24). Force sensors are located on top of each shaft (Figure 4.26). A peristaltic pump and a valve allow to tune the medium flow rate and pressure inside the chamber.
Mechanical tests were performed to evaluate the role of the double-layer strips within IVD-like assemblies played during confined and unconfined compression. Four IVD-like assemblies were prepared as described in section 4.8.2 without seeding cells on them. The mechanical behaviour of these unseeded IVD-like constructs was compared with that of four agarose discs, without the outer ES PCL layer. The mechanical stimulation apparatus integrated in the BOSE bioreactor was used to exert a compression at a constant rate of 0.1 mm/s till failure. Samples were preconditioned in PBS for 24h and preload was applied manually in the range 0.3-0.1 N.
compression modulus was calculated on the linear region that followed the initial toe region, after removing the initial preload value which was not constant across the specimens.

**Mechanical stimulation of IVD-like constructs**

The mechanical stimulation of IVD-like constructs is to understand the biological response to the radial stress simulating the biomechanics experienced by AF laminates during cyclic compression loading. Two main experiments of IVD-like construct mechanical stimulation were carried out by changing the displacement values. The following section describes the experimental procedure and characterisation techniques applied.

**Samples loading**

Loading samples in the chamber is a crucial step for every mechanical stimulation experiment. In particular, the bioreactor has been designed in such a way that, whatever loading strategy was used, the cellularised scaffolds and the chamber were exposed briefly to a non-sterile environment. To minimise bacteria contamination, which occurred twice during experimental trials, the antibiotic and antimycotic concentration used in standard cell culture medium (see section 4.1.1) was raised to 200 µg/ml and 0.50 mM respectively. Before commencing any test all the chamber components were autoclaved and, after mounting, perfused with EtOH and sterile PBS. Four IVD-like specimens, prepared as described in section 4.8.2 and maintained in the incubator, were treated to fit the cylindrical rubber membranes (Figure 4.27). To this purpose, the edges of the ES PCL outer layers, resulting from the fabrication process, were cut out. The specimens, held in the membranes, were loaded inside the chamber on the upper flat surface of each bottom platen. A manual preload of about 1 N was applied. The outer clear rubber membranes were arranged to fit the shaft edges. The role of the membranes is to keep the IVD-like constructs in the correct position as it had been noticed that the slimy nature of agarose makes the specimens slip out. A simple preliminary test was performed to ensure the membranes,
which are not tightly sealed to the shafts, allow the medium in the chamber to reach the samples. Hence pre-warmed medium was circulated in the chamber to keep cells alive. After filling the chamber, the load sensors were zeroed again because when the shafts were immersed in liquid environment the force detected by the sensor was subject to a variable shift.

![Diagram of membrane mounted on shafts](image)

Figure 4.27: Schematic representation of the membrane mounted on the shafts. Left: the membrane is mounted on the shaft edges before applying the preload on the sample. Right: After application of the preload and upon mechanical stimulation the membrane is able to deform.

Subsequently WinTest software was set with the experiment parameters. The testing parameters (Table 4.6) were decided on the basis of the published literature though there is no consensus about what is the best stimulation pattern to stimulate cellularised intervertebral disc artificial replacements (Baker et al. 2010; Thorpe et al. 2010; Hee et al. 2010; See et al. 2011; Ching et al. 2003; Korecki & Kuo 2009; Reza & Nicoll 2008; Hutton et al. 2001; Neidlinger-Wilke et al. 2006; Kisiday et al. 2009; Thorpe et al. 2008).
At the end of each experiment, the data collected by the sensors were analysed with the aim of gaining information related to the stimulation. Every 100”, for a 2” time span, temperature and force values recorded at 100 Hz sampling rate.

**Statistical analysis**

The output of all the experiments returning quantitative data was further analysed by statistical analysis tools. Depending on the licence available, SPSS® (IBM) or Prism® (Graphpad) softwares were adopted.

For comparison between two samples paired or unpaired, Student’s t-test was applied and Levene’s test was used to verify the equality of variance. Multiple comparison analysis was performed by means of one-way or two-way ANOVA and post hoc tests. In the results section, the output of the statistical analysis is indicated as p value threshold.
5. Results: ES PCL layers fabrication

Approximately 300 different electrospinning configurations were explored and the results obtained are presented in this section.

To obtain more stable production process with precisely controlled electrospinning apparatus, it is important to monitor the transition process from polymer solution to fibre. In particular, the Taylor cone must be observed to reach a steady state condition as explained in materials and methods (section 3.1). The Taylor cone shape is the result of the interaction of surface tension, electrostatic force and gravity force; hence, it depends strongly on the solvent system and concentration used. With CF based solvent systems the Taylor cone was very elongated (few centimetres beyond the spinneret tip) before the fibres became too thin to be seen to the naked eye. The classical conical shape was a feature of THF and TFA based solvent systems but with a length within millimetres. With DMF, CF+MeOH and with FA+AA solvent systems the Taylor cone is rather invisible and a spray-like jet came out from the spinneret tip. The type of jet observed could give cues about whether the concentration needs to be increased or decreased. Moreover, the presence of drops or clogs that compromises the formation of a stable cone was indication of the necessity to adjust the balance between the flow rate and the electric field.

Concerning the final matrices, after removal from the collector it was noticed that a rather smooth and defect-free surface at the macro-scale was a mandatory condition to have good quality fibres at the micro- and nano-scale (whilst the opposite was not always true).
5.1 Effect of polymer solution formulation and process parameters on matrix morphology

5.1.1 Effects of solvents used

The first electrospinning experiments were carried out using the simplest solvent systems, composed of only one chemical. In fact the addition of a second solvent would introduce another variable to the system, namely the solvents ratio. The first electrospinning attempts were carried out using TFA and CF. These solvents were selected because they were compatible with silicon Tygon tube, initially available. Fibres electrospun in CF showed to be quite big and rough, far beyond the size expected based on the literature review on the effects of process parameters on fibre morphology (Figure 5.1).

PCL dissolved in TFA, conversely, led to fine fibres, albeit with the presence of defects (Figure 5.2). The two order of magnitude difference in size detected (approximately 10 µm and 100 nm), despite the difference in the process parameters, is affected significantly by the electrical permittivity and boiling point, two important intrinsic properties of the chemicals used as solvent. In fact, TFA had higher electrical permittivity and slightly lower evaporation rate.

Figure 5.1: Example of ES layer obtained from PCL dissolved in CF. Scale bar 10 µm.

Figure 5.2: Example of ES layer obtained from PCL dissolved in TFA. Scale bar 10 µm.
Further experiments with CF revealed that it was not feasible to fabricate fibres with diameter below 10 μm. For this reason CF was mixed with other solvents to modify the properties of the solvent system. Tests with FA alone demonstrated that the polymeric solution was not electrospinnable; thus, AA was added to it as suggested by Van der Schueren et al. (Van der Schueren et al. 2011). Trials with also CF+MeOH solvent systems led to quite large and rough fibres (Figure 5.3); whereas FA+AA allowed producing ultrafine fibres without defects (Figure 5.4).

The alteration to the ratio between CF and MeOH from 7:1 to 5:1 enabled a slight improvement in the quality. Even by replacing MeOH with EtOH, no major improvement was noticed.

Further development of FA+AA polymer solutions led to production of two different defect-free layers with increased fibre size. Despite the different diameter as shown in Figure 5.5 and Figure 5.6, a completely different arrangement can be seen. In one case (Figure 5.5) fibres are almost straight whereas in the other (Figure 5.6) they assume a curling shape. This outcome might be explained with the higher concentration (30% wt against 21% wt) and the lower electric field (0.9 against 0.65 kV/cm) of the sample in Figure 5.6 compared with the one in Figure 5.5.
Afterwards two additional solvent systems were studied: DMF+CF 1:1 and THF+DMF 1:1 (Croisier et al. 2012; Del Gaudio et al. 2009; Pham et al. 2006; Jeun et al. 2005). The first experiments using these two solvent systems indicated their potential to produce high-quality fibres (Figure 5.7 and Figure 5.8).
5.1.2 **Control of fibre alignment by adjusting the collector rotation speed**

Tests with increased collector speed were conducted as well. In Figure 5.9 and Figure 5.10, the difference between semi-aligned and aligned fibres can be seen. They were electrospun in a row using the same solution batch. The fibre alignment evaluation results are detailed in section 5.1.4.

![Figure 5.9: Example of ES layer obtained from PCL dissolved in THF+DMF 1:1 and 2000 rpm collector speed. Scale bar 10 μm.](image1)

![Figure 5.10: Example of ES layer obtained from PCL dissolved in THF+DMF 1:1 and 3000 rpm collector speed. Scale bar 10 μm.](image2)

After demonstrating the feasibility of using DMF+CF 1:1 and THF+DMF 1:1 as solvent systems to produce good-quality aligned fibres it was decided to produce thick samples, suitable for cell culture experiments, mainly with established solvent systems.

5.1.3 **Standardisation and mass production of layers used for cell culture**

After establishing the fabrication process, a small-scale mass production of ES layers was carried out. The fabricated layers were characterised quantitatively to facilitate further biological experiments.
It must be emphasised that, between the two periods, the electrospinning apparatus was used by other users but was subject to no relevant change in any of its components. Materials, polymer and the solvents used were all supplied by the same companies as those in establishment of process parameters. Hence, it was deemed reasonable to replicate layers with same features as those observed during process establishment stage by only setting up the machine with the same process parameters. Contrary to this assumption, during the first experiments, with all the same apparatus set-up to the one used for reference samples, different outcomes were noticed. What is shown in Figure 5.11 is an example of the situation encountered when fibres, obtained with standardised process parameters, were observed. The inconsistency of the fibre morphology would be most likely attributed to the only variables that are not under the operator control: the environmental parameters. In the laboratory, these parameters, namely temperature and humidity, followed the external weather condition; albeit somewhat altered by the building air conditioning system.
Thus, in most cases, all the recipes and process parameters had to be re-adjusted (see table in Figure 5.12) to obtain flawless fibres with specified features. Figure 5.12 provides an example of two samples whereby fibre diameter difference is not statistically significant but had to be produced with a somewhat relevant parameters modification.

The effect of solvent system physico-chemical features on fibre morphology will be discussed in details in section 7.1.
5.1.4 Image processing: ODF

The ODF could be calculated with an opportune software allowing the generation of a graph representing the percentage of fibres characterised by a specific orientation relative to the software reference axis. The 0-180° range was split into 18 quantiles and the fibres grouped within them as shown in Figure 5.13 and Figure 5.14. The quantiles around 90° were further compared as shown in Table 5.1.

Figure 5.12: Comparison between a sample of the first batch (left) and its replicate (right) with the same fibre arrangement and diameter. Scale bar 2 µm. Below the images the parameters subjected to amendments are reported.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>First batch sample</th>
<th>Second batch sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 Concentration [% wt]</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>16 Tip to collector distance [cm]</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>3 Flow rate [ml/h]</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>18 Voltage [kV]</td>
<td>18</td>
<td>20</td>
</tr>
</tbody>
</table>

5.1.4 Image processing: ODF

The ODF could be calculated with an opportune software allowing the generation of a graph representing the percentage of fibres characterised by a specific orientation relative to the software reference axis. The 0-180° range was split into 18 quantiles and the fibres grouped within them as shown in Figure 5.13 and Figure 5.14. The quantiles around 90° were further compared as shown in Table 5.1.
The examination of the percentages clearly demonstrated a significant difference between random fibres and the other two patterns (p<0.01). A large number of fibres collected at high collector speed (2000 and 3000 rpm) acquired a direction consistent with the cylinder rotational direction, perpendicular to its long axis.

ODF relative to random specimens (Figure 5.13) indicates that there is a contribution, though not as relevant as on aligned and semi-aligned mats (Figure 5.14), of the slow collector rotational speed. A constant percentage of fibres per each angle interval was in fact expected whilst the distribution demonstrated to be not uniform as demonstrated by the lack of fibres perpendicular to the collector rotation direction (0-20 and 160-180° in Figure 5.13).

![Random fibres](image)

*Figure 5.13: Representative ODF of a random fibre matrix. Lack of fibres perpendicular to the collector rotational direction is evident.*

Regarding the matrices fabricated at high collector speed, in the narrower range considered the frequency values were 33.79±9.56 and 34.26±9.56%, for semi-aligned and aligned fibres respectively whilst 53.96±12.14 and 55.68±12.00% when studying the wider interval (70-110°,
Table 5.1). As confirmed by the statistical analysis, there is no significant difference between the two types of matrices.

Figure 5.14: ODF of two representative samples with semi-aligned and aligned fibres. The higher percentage of fibres parallel to the collector rotational direction is clear.
The assessment of fibre orientation allowed correlating cell response of cells cultured on ES PCL mono- and double-layers with different morphologies and arrangements (see sections 6.2, 6.4 and 10.4).

### 5.2 Wettability

Contact angle was applied to quantify hydrophilicity and three ES PCL samples were compared with solvent cast PCL. Fibre diameters of the said matrices are considered equal since no significant difference between them was identified. The results of the investigation are shown in Figure 5.15. Solvent cast PCL clearly behaved in completely different way compared with any of the ES PCL samples. Contact angle passed from a somewhat hydrophilic value to a highly hydrophobic. The ANOVA test showed a significant difference (p<0.05) only between random and aligned fibres. All the ES samples differed significantly from the solvent cast one (p<0.01).
5.3 ES PCL matrix degradation

5.3.1 Mass loss

Untreated and UV light exposed PCL samples were immersed in PBS and their mass loss measured over 12 months. At the end of the experiment mass variation turned out to be not statistically significant, being -0.16 ± 2.55% for untreated specimens and +1.25 ± 2.55% for those that had been subjected to sterilisation process.

At the end of 12 months two specimens per type were observed with the SEM (Figure 5.17, A-D and Figure 5.17, E-J). The images, reported below, confirmed the absence of difference between untreated and UV treated samples (Figure 5.16, A-B and C-D).
However, the comparison with a control sample of the same matrix type demonstrated that fibres surface, originally smooth (Figure 5.17, F), was characterised by a slight roughness (Figure 5.16, B and D). In addition, at low magnification, fibres soaked for 12 months exhibited a more irregular arrangement, with curlier fibres (Figure 5.16, A and C). This is compared with the control sample (Figure 5.17, E and F), which is characterised by more straight fibres. A similar change was noticed on the matrix soaked in water for 7 days immediately after the synthesis (Figure 5.17, G), therefore indicating that contact with water might affect fibres though independently from time. A third feature detected on degraded samples is the presence of some particles of micro and submicrometric size, on the fibres surface (Figure 5.16, B and D). The nature of these particles is unknown, but it was hypothesised that they could originate from PBS salts, which were not completely washed away by the rinsing steps performed with deionised water. The same particles were not found on the as fabricated sample or on that soaked for seven days in deionised water (Figure 5.17, H).
Figure 5.16: A) and B) micrographs of untreated ES specimens soaked in PBS for 12 months; scale bars 20 and 2 μm. C) and D) UV treated ES specimens soaked in PBS for 12 months; scale bars 20 and 2 μm.
5.3.2 **Molecular weight loss**

ES PCL specimens of the same type of those used for the mass loss test and treated in the same way were analysed by means of GPC to evaluate the change, if any, in molecular weight over six months. The charts in Figure 5.18 illustrate the Mn trend of PCL specimens.

*Figure 5.17: E) and F) images of untreated ES specimen; scale bars 100 and 2 µm. G) and H) nanofibres soaked in water for 7 days; scale bars 100 and 2 µm.*
All the specimens clearly exhibited a decrease of Mn over six months. It must be noted that, due to machine availability and budget constraints, the experiment could not be performed in triplicate. However, considering each time point the highest value always belonged to ES PCL whilst the lowest to UV treated ES PCL. This evidence provides greater consistency to the result; thereby indicating that UV treatment affected ES PCL.

The implication of the ES PCL layers will be discussed thoroughly in section 7.1.4.

5.4 Investigation of mechanical properties under different testing conditions

The outcomes reported in this section will be further analysed in terms focusing on the reliability of the results depending on the experimental set-up and compared with the performances of the natural AF tissue in the perspective of ES PCL usage in a clinical setting (see section 7.1.5 and 7.1.6).
5.4.1 **Stretching direction effect on ES PCL mechanical properties**

ES PCL layers were tested by changing the angle between stress and fibre directions. Load and displacement values were then used to calculate the stress and the tensile modulus in two stress-strain curve linear regions at low and high strain.

Performance analysis was conducted by comparing the values between different specimen types and, within each one, taking into account the different stretching directions. In Figure 5.19, the tensile moduli of all the specimens are compared. The values span between two orders of magnitude; from the highest to the lowest.

The highest tensile modulus is achieved by the aligned samples when stretched in direction parallel to the fibres ($E_{\text{low}0^\circ}=53.64\pm12.26$ MPa, $E_{\text{high}0^\circ}=21.42\pm7.17$ MPa). By varying the angle from 0 to 30, 60 and 90° the same property decreased to the following values: $E_{\text{low}90^\circ}=2.37\pm0.47$ MPa, $E_{\text{high}90^\circ}=0.49\pm0.06$ MPa. In this last case the tensile moduli of either region were the lowest among all. Semi-aligned specimens behaved the same way but compared with those with aligned fibres had a lower tensile modulus at 0° ($E_{\text{low}0^\circ}=27.94\pm11.63$ MPa, $E_{\text{high}0^\circ}=11.92\pm3.81$ MPa) and at 90° the two moduli were fairly close to each other ($E_{\text{low}90^\circ}=1.39\pm0.15$ MPa, $E_{\text{high}90^\circ}=0.71\pm0.11$ MPa). Random samples demonstrated intermediate values: $E_{\text{low}}=2.77\pm0.28$ MPa and $E_{\text{high}}=1.25\pm0.09$ MPa.
The analysis of the stress-strain curve shape (see Figure 5.20) highlights two important differences between the specimens behaviour. Along with a substantial decrease in the tensile modulus, when changing the stretching angle from 0 to 90°, examples in Figure 5.20 clearly show a reduction in the UTS value and a rise in the stretching ability. By comparing the curves obtained for each sample type and stretching direction combination, the highest UTS obtained was 35.58 MPa for aligned samples at 0°. As a comparison, for the semi-aligned specimens in the same condition UTS was 15.52 MPa. Decreasing the stimulation angle for aligned layers the UTS was as follows: 14.72 (30°), 4.96 (60°) and 2.38 MPa (90°).

Figure 5.19: Tensile moduli of layers with random, semi-aligned and aligned PCL fibres. Logarithmic scale was used for the vertical axis because of the wide range covered. Bars represent significant difference (p<0.05).
Concerning the extension, as already said above, it achieves the maximum value when the sample is stretched in perpendicular direction compared with the fibres. Semi-aligned samples showed an increase of extension from about 0.3 (0°) to more than 1 (90°). The range of aligned matrix
extension is even higher, spanning from approximately 0.2 to 1.6, when subject to parallel and perpendicular load respectively.

5.5 Testing environment influence on ES PCL matrices mechanical properties

The mechanical test results described in this section were performed to provide insight into ES PCL properties under conditions closer to the physiological environment.

The shape of the curves obtained in wet conditions is very similar to those in dry conditions although more elongated on the strain axis. Furthermore, for many samples stretched in PBS at 37 °C the diagrams demonstrated a jagged pattern, not observed on dry specimens.

5.5.1 Wet environment effect on ES PCL tensile modulus

Figure 5.21 illustrates the comparison between the tensile moduli at low strain (E_{low}) measured in dry and wet conditions showing a decrease in modulus with the increase of the testing angle. For all the specimen types, while not significant for the semi-aligned pattern, the modulus is lower when the test is administered in wet conditions. This was also true for the tensile modulus evaluated at high strain (E_{high}) as the charts in Figure 5.22 report. E_{high} of aligned fibres at 0 and 30° stretching could not be calculated as the stress-strain curves did not exhibit the two typical linear regions; moreover, in those cases, it was decided not to replace E_{high} with E_{low} due to the overall small strain displayed. Similarly to E_{low}, E_{high} of semi-aligned fibres did not show any significant difference between dry and wet conditions.
Figure 5.21: $E_{\text{low}}$ modulus comparison for all the tests performed in wet and dry conditions.

* indicates significant difference ($p<0.05$) between dry and wet conditions modulus within the same stretching angle.
Figure 5.22: $E_{\text{high}}$ modulus comparison for all the tests performed in wet and dry conditions. Some data is missing because of the impossibility to individuate a proper linear region in the correspondent stress-strain charts. * indicates significant difference ($p<0.05$) between dry and wet conditions modulus within the same stretching angle.
The results above show that both orientation and environmental conditions individually exert a relevant role on the tensile modulus. The combination of the stretching orientation and environmental condition was studied further by means of two-way ANOVA statistical test. The significance of the results of $E_{\text{low}}$ and $E_{\text{high}}$ modulus is reported in Table 5.2. This analysis provides an additional insight; thus demonstrating that the effect of wet conditions is different depending on the stretching direction applied. For both aligned and random fibres, soaking samples in PBS at 37 °C exerted a more relevant effect on larger moduli: 0 and 90° for aligned and random fibres respectively.

Coherent with the charts in Figure 5.21 and Figure 5.22, $E_{\text{high}}$ of aligned fibres and both the moduli of semi-aligned fibres demonstrated a lower significance. In these cases, the effect of wet environment is not as relevant as on random fibres and on aligned fibres modulus at low strain. However it is likely that the low significance calculated on $E_{\text{high}}$ of aligned fibres is affected by the missing data at 0 and 30°, which makes the detection of a trend in dry conditions difficult, over the entire 0-90° angle range.

**Table 5.2: Significance level of the combined effect of stretching orientation angle and environmental conditions for all the measured moduli.**

<table>
<thead>
<tr>
<th>Fibre arrangement</th>
<th>Tensile modulus</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aligned</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_{\text{low}}$</td>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td>$E_{\text{high}}$</td>
<td></td>
<td>0.037</td>
</tr>
<tr>
<td><strong>Semi-aligned</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_{\text{low}}$</td>
<td></td>
<td>0.028</td>
</tr>
<tr>
<td>$E_{\text{high}}$</td>
<td></td>
<td>0.099</td>
</tr>
<tr>
<td><strong>Random</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_{\text{low}}$</td>
<td></td>
<td>0.004</td>
</tr>
<tr>
<td>$E_{\text{high}}$</td>
<td></td>
<td>0.002</td>
</tr>
</tbody>
</table>
5.5.2 **Wet environment effect on ES PCL strain at UTS**

Strain at UTS demonstrated to be strongly influenced by the environmental conditions, in particular on aligned and random fibres (Figure 5.23). Wet conditions fostered an increase in strain at UTS compared with dry conditions and an increasing trend was noticed when testing samples from 0 to 90°. Moreover under wet conditions strain at UTS exhibited a small drop between 60 and 90° in both aligned and semi-aligned fibres.
Figure 5.23: Strain at UTS comparison for all the tests performed in wet and dry conditions.

* indicates significant difference (p<0.05) between dry and wet conditions strain at UTS within the same stretching angle.
The combined effect of wet conditions and stretching angle on strain at UTS was evaluated by two-way ANOVA analysis as reported in Table 5.3. Whilst aligned fibres demonstrated a high influence of both the testing variables on strain at UTS, on semi-aligned and random samples the combination of stretching angle and wet environment was not significant.

Table 5.3: Significance level of the combined effect of stretching orientation angle and environmental conditions for all the measured strains at UTS.

<table>
<thead>
<tr>
<th>Fibre arrangement</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aligned</td>
<td>0.000</td>
</tr>
<tr>
<td>Semi-aligned</td>
<td>0.372</td>
</tr>
<tr>
<td>Random</td>
<td>0.452</td>
</tr>
</tbody>
</table>

5.5.3 Wet environment effect on ES PCL UTS

Wet environment showed the exertion of a small influence on UTS of ES PCL layers. The only exception is represented by semi-aligned and aligned fibres at 30 and 60° respectively (Figure 5.24). The trend from 0 to 90° stretching orientation is similar to that of tensile modulus; thus revealing higher values at 0° for aligned and semi-aligned fibres and at 90° for random fibres.
Figure 5.24: UTS comparison for all the tests performed in wet and dry conditions. * indicates significant difference (p<0.05) between dry and wet conditions UTS within the same stretching angle.
Similarly to strain at UTS, the combinatory effect of orientation and environmental conditions demonstrated to be significant only for aligned fibres.

**Table 5.4: Significance level of the combined effect of stretching orientation angle and environmental conditions for all the measured UTS.**

<table>
<thead>
<tr>
<th>Fibre arrangement</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aligned</td>
<td>0.004</td>
</tr>
<tr>
<td>Semi-aligned</td>
<td>0.670</td>
</tr>
<tr>
<td>Random</td>
<td>0.248</td>
</tr>
</tbody>
</table>

5.5.4 **Jagged pattern evidence on ES PCL layers in wet conditions**

Considering the results obtained on specimens tested in PBS at 37 °C, which demonstrated a jagged pattern; particularly on random and, to a lesser extent, semi-aligned fibres towards 90° orientation (Figure 5.25). Therefore, it was decided to perform the same experiment using alternative materials. Two commercial plastic sheets (plastic 1 and 2) were tested by combining different parameters. The outcome of the test carried out to replace the duct tape with the sandpaper, as described in the relative materials and methods section (3.2.4), is not revealed as in wet environment the sandpaper dissolved and softened; thus rendering it useless.
In Figure 5.26 the curves relative to representative specimens of the tests performed are revealed. Despite the differences in curve shapes, which are not intended to be studied here, no distinctive relationship between line patterns and experimental conditions emerged.
5.5.5 **Unexpected response of semi-aligned fibres to different environments**

Random and aligned fibres showed a noticeable different behaviour when stretched in wet and dry environment. In general, samples in dry conditions exhibited a higher tensile modulus and lower strain at UTS in dry conditions (Figure 5.21 to Figure 5.23). Essentially, semi-aligned fibres were anticipated to behave similarly to aligned fibres; however, the stated trend was not

![Stress-strain charts relative to representative samples of plastic 1 (top) and plastic 2 (bottom).](image)

*Figure 5.26: Stress-strain charts relative to representative samples of plastic 1 (top) and plastic 2 (bottom).*
identified. Further comparison between aligned and semi-aligned fibres in dry conditions showed a quite different curve shape, especially towards 0° stretching orientation. Figure 5.27, which reports two representative curves, shows that semi-aligned fibres display higher strain at the same load level as aligned fibres and a considerably more pronounced toe region. It was hypothesised that the different trend might be due to the samples fibre morphology. Samples were selected because of their equivalent fibre diameter; however the SEM images show, at low magnification, that semi-aligned sample has a larger number of curling and crimped fibres compared with aligned fibre sample (Figure 5.28). The correlation between fibre morphology and response to mechanical stretching will be examined in more details in the discussion chapter.
Figure 5.27: Shape comparison between representative samples of aligned and semi-aligned fibres stretched at 0° orientation. The zoom reported in the bottom chart clearly indicates the larger toe region exhibited by the semi-aligned fibres.
5.5.6 **Unexpected response of random fibres to different stretching angles**

The mechanical test was performed on random fibres, adopting 0 and 90° stretching angles because the software aided morphology analysis, reported in section 5.1.4, revealed the presence of a slight anisotropy. The mechanical test results obtained are in contrast with what expected on the basis of the trend assessed on aligned and semi-aligned fibres. The difference between $E_{low}$
tensile moduli, at 90 and 0° orientation (Figure 5.21 and Figure 5.22), was found to be significant in both dry and wet conditions (p<0.05).

6. Results: biological experiments on ES PCL scaffolds

This chapter illustrates the findings relative to the interaction between hMSCs and ES PCL layers. Section 6.1 demonstrates the penetration issues of cells into ES mono-layers while section 6.2 shows the behaviour of cells cultured on the surface of ES mono-layers. Subsequently the tests related to culturing cells within ES layers arranged in 3-dimensional fashion are presented in sections 6.3 to 6.7. The results associated with the issues relative to the lack of oxygen, for hMSCs survival, and mass transport through ES layers are presented in sections 6.8 and 6.9. The above mentioned sections are analysed in details in the discussion (sections 7.2 to 7.3). The last section of this chapter (6.10) deals with the outcome of the characterisation of the IVD-like construct, described in section 4.8, and its mechanical stimulation after cellularisation. An exhaustive interpretation of these results is provided in section 7.4.

6.1 HMSCs penetration assessment on mono-layer cross-section

Cross sections were observed to assess the presence of cells between the fibres underneath the surface. Figure 6.1 illustrates that the cells within the matrix are extremely rare; thereby proving the penetration difficulties of hMSCs.
In a similar experiment, the combination of two dyes (Hoechst 33342 and Cell Tracker) was exploited with the aim of enhancing the reliability of cells identification by fluorescent staining, in case nucleus staining alone was not sufficient. Again, this approach confirmed the low infiltration capabilities of hMSCs inside ES PCL matrices (Figure 6.2).

Figure 6.1: Cross sections of semi-aligned and aligned layers stained with Hoechst 33342 dye, visualised under the fluorescence microscope. Scale bar 100 µm.
The lack of cell infiltration assessed herein triggered the investigation of double- and multi-layer structures; whereby fabrication procedure and outcomes are reported in sections 4.3 and 6.3 respectively.

6.2 **HMSCs culture on matrices with various fibre arrangement**

This section presents the experiment evaluating the influence of different matrix types, characterised in section 5.1.4, to cell morphology. Viability of hMSCs for all the samples, whose features are reported in Table 6.1, was positive at the end of the 20 days culture prior to fixation and staining.
Table 6.1: List of the specimens tested and relative main features.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Fibre arrangement</th>
<th>Fibre diameter [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-0.40</td>
<td>Random</td>
<td>0.40±0.15</td>
</tr>
<tr>
<td>SA-0.40</td>
<td>Semi-aligned</td>
<td>0.40±0.14</td>
</tr>
<tr>
<td>A-0.60</td>
<td>Aligned</td>
<td>0.60±0.22</td>
</tr>
<tr>
<td>R-0.93</td>
<td>Random</td>
<td>0.93±0.38</td>
</tr>
<tr>
<td>R-1.67</td>
<td>Random</td>
<td>1.67±0.48</td>
</tr>
</tbody>
</table>

6.2.1 Fibre alignment effect on cell morphology

**Fluorescence imaging**

Almost flat ES matrices could be removed from the Cell Crown devices after staining with Hoechst 33342 and Alexa Fluor-555 phalloidin dyes, which labelled cell nuclei and cytoskeleton actin respectively (Figure 6.3). Especially at higher magnification on semi-aligned and aligned fibres, more cells are oriented towards the fibres. Cells on these samples mostly have a bipolar shape, as evidenced by the directional-arrangement of actin filament, whilst those on random fibres mostly have more than two elongated regions; thus assuming a triangular-like or polygonal shape as revealed by the isotropic arrangement of actin filaments.
SEM imaging

The SEM confirmed the results obtained with the optical microscope and directly correlate cell morphology and distribution with the microscopic features of the ES fibre on the same images. Three samples with different fibre arrangement were studied (Figure 6.4, A-H). Cells seeded on random fibres are shown in images A-D. In the areas where the cell density is higher, cells touch each other through protrusions (A and B). HMSCs sometimes assume an elongated shape with no specific orientation and in some cases, in the centre of the cell, the nucleus can be identified as a slight circular bulging at higher magnification (see arrows in image B).

Semi-aligned fibre specimen demonstrated a lower density of cells compared with the random one. However, every cell assumed a bipolar shape and, furthermore, their elongation is oriented...
clearly towards the MD of fibres (E-H). Also on this kind of specimen cells look well integrated with the matrix, with protrusions developing along the fibres, as can be seen in image H. Concerning aligned fibres, the SEM results were not positive as no cell could be observed and since the presence of cells was confirmed using optical fluorescent imaging, it is anticipated that the sample drying process might cause detachment from the matrix.
6.2.2 Focal adhesion assessment through vinculin staining

HMSCs seeded on standard ES layers and the control (polystyrene wells) were stained for vinculin, nucleus and actin filaments observation.
Representative three channels images are shown in Figure 6.5 (A-L). HMSCs seeded in control wells grew in mono-layer with a higher density in the middle where the cell suspension drop was pipetted. Control cells had no specific orientation in the centre, though towards the outer portion of the mono-layer cells assumed a radial arrangement (J-L). Sub-cellular structure, stained with fluorescent dyes, can be clearly distinguished. Actin filaments, in red, are indicative of the moderately elongated shape of the cells (J). Vinculin protein resulted to be abundant and localised in small spots, identified by bright green dots or dashes (K, red circles). Actin filaments and vinculin patterns are almost consistently co-localised with each other and they can be both used as indicators of cell shape. On ES PCL samples, images are more blurred as, despite the precautions taken to keep the samples flat, not all the cells lie on the same focus plane (A-I). The different fibre diameter of the two random specimens, R-0.53 and R-1.59, had no noticeable influence over vinculin arrangement or pattern and cells adopted the polygonal shape (A-F) previously observed (section 6.2.1). Furthermore, vinculin shows the same dotted pattern observed on cells seeded in control wells. Conversely, on aligned fibres the bright vinculin dots appeared undistinguishable and the signal rather strong (H).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fibre arrangement</th>
<th>Fibre diameter [um]</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-0.53</td>
<td>Random</td>
<td>0.53±0.36</td>
</tr>
<tr>
<td>A-0.47</td>
<td>Aligned</td>
<td>0.47±0.24</td>
</tr>
<tr>
<td>R-1.59</td>
<td>Random</td>
<td>1.59±0.49</td>
</tr>
<tr>
<td></td>
<td>Actin filaments and nuclei</td>
<td>Vinculin and nuclei</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>R-0.53</td>
<td><img src="image1.png" alt="Image A" /></td>
<td><img src="image2.png" alt="Image B" /></td>
</tr>
<tr>
<td>R-1.59</td>
<td><img src="image4.png" alt="Image D" /></td>
<td><img src="image5.png" alt="Image E" /></td>
</tr>
</tbody>
</table>
Figure 6.5: Fluorescence images of hMSCs seeded on ES PCL layers with different fibre diameter and arrangement. Sample names are indicated as “arrangement-fibre diameter”. Cells are stained in red, correspondent to actin filaments, and green, correspondent to vinculin protein; nuclei are counterstained in blue. In left and middle column the following cell components are represented: actin+nuclei, vinculin+nuclei; in the third column all the channels are merged. Scale bar 50 µm.
6.3 Viability tests on ES PCL mono- and double-layers

The ultimate aim of the present project is to engineer an artificial replacement for the AF. AF peculiar multilamellar structure can be replicated using ES PCL aligned fibre layers overlapped in an angle-ply manner. After obtaining positive results of cell growth on ES PCL mono-layer, an experiment where cells were cultured between two matrices was designed.

HMSCs were cultured on ES PCL double- and mono-layers up to 28 days. The matrices were chosen on the basis of their different fibre diameter, not considering the thickness (Table 6.3). Viability results plot in Figure 6.6 show a similar trend for all the samples. Cells on both mono and double-layer experienced a drop in viability after 3 days of culture. Hence, all the other checkpoints, with the exception between day 10 and 15, demonstrated an increase in viability. Despite A-0.60_Double followed the same trend as the other samples, the final fluorescent value was significantly lower (p<0.05). Furthermore, the difference between the first and the last day (51663±26397 and 78413±2932 AU respectively) was not significant after the evaluation with the Student’s t-test, assuming the variances not equal, according to the Levene’s test. This contrasts with the significant difference between day 1 and 28 related to the other three sample types.

Table 6.3: List of the specimens used to culture cells in double-layer constructs.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Type of substrate</th>
<th>Thickness of the whole construct [µm]</th>
<th>Fibre diameter [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-0.60_Mono Mono-layer</td>
<td>233.22±26.35</td>
<td>0.60±0.22</td>
<td></td>
</tr>
<tr>
<td>A-0.60_Double Double-layer</td>
<td>466.44±52.70</td>
<td>0.60±0.22</td>
<td></td>
</tr>
<tr>
<td>SA-1.72_Mono Mono-layer</td>
<td>88.67±22.55</td>
<td>1.72±0.50</td>
<td></td>
</tr>
<tr>
<td>SA-1.72_Double Double-layer</td>
<td>177.34±45.10</td>
<td>1.72±0.50</td>
<td></td>
</tr>
</tbody>
</table>
6.4 Oriented double-layer influence on hMSCs morphology and adhesion

6.4.1 Fluorescence imaging

Handling double-layers during disassembling process of Cell Crown inserts was not problematic as the overlaid upper layer did not delaminate from the bottom one and, together, they formed a rather solid construct with both layers integrated. After fixation cells nuclei and actin filaments were stained using Hoechst 33342 and phalloidin Alexa Fluor-555 dyes. Narrow strips were cut out from the double-layer and the cross-section was observed as well as the internal surfaces (see schematics in Figure 4.12 and Figure 4.13).

Figure 6.6: Viability of hMSCs cultured on ES double-layers. The first two time points refer to the first assessment after cells were seeded 1 day on mono-layers and 1 day after overlapping the second layer respectively. Bars on the right represent significant difference between samples at day 25 (p<0.05). * indicates significant difference between the first and the last time point (p<0.05).
The quality of cross-section images was compromised by the wavy nature of the specimens. An example of each specimen is shown in the top row of Figure 6.7. Observation resulted in rather blurred images where only a localised area with relative strong fluorescent signal could be identified thanks to fluorescent signal provided by the cells. Bright circles, corresponding to cells, were aligned along a specific line that was thus identified as the interface between the layers.
With regard to the surfaces, the observation methodology, outlined in Figure 6.8, which kept the ES PCL strips parallel under the objective, allowed to assess the presence of a specific angle between the direction of cells attached to the bottom layer, compared with the top one (Figure 6.9). The green arrows in Figure 6.9 indicate the predominant fibre directions of top and bottom layer respectively. Cell bodies, stained in red with actin binding dye, are oriented in the direction of the fibres. This demonstrates that hMSCs are able to respond actively to the new topographical configuration encountered upon migration. On the top layer, fewer cells were detected, possibly due to the difficulty for cell to migrate from bottom to top layer. The arrows

Figure 6.7: Cross sections observed with fluorescence microscope. Top: A-0.60_Double; bottom: SA-1.72_Double. Dotted lines mark the interface between the two layers. Cells were stained for actin (red) and nuclei (blue). Scale bar 100 µm.
overlaid to the main fibre direction show that the angle between the layers fibre angle, measured with Image-j on the representative samples in Figure 6.9, is approximately $76^\circ$ and $55^\circ$. These values differ slightly from the $60^\circ$ angle adopted during sample preparation. This slight mismatch is possibly due to the impossibility of accurately control the fibre direction during layer cutting and overlapping after cell seeding. Moreover, the green arrows were added manually without the opportunity to estimate the fibre angle with the software, as outlined in section 5.1.4 using SEM images.

![Figure 6.8: Schematic representing how the layers were cut for cell alignment comparison.](image)

**Two ES strips were cut and placed parallel under the microscope objective.**

![Figure 6.9: Representative image of the combination bottom and top layers, showing the orientation of the cells on each layer and the angle between them. Left: SA-1.72_Double; right: A-0.60. Scale bar 100 µm.](image)
6.5  **HMSCs adhesion at the inner surface of double-layers**

6.5.1  **SEM imaging**

Double-layer strips were mounted on special pin stubs and studied by means of SEM. In Figure 6.10 (A and B) the structure of the double-layers is clearly visible. The plies have been manually separated before samples mounting in order to expose the inner sides to the SEM detector. Some areas (arrows in Figure 6.10 B, C, and D) are almost completely covered by cells, similarly to what has been observed on mono-layers (section 6.2.1), and possibly ECM. In Figure 6.10 E the detail of a cell aggregate can be observed whilst in Figure 6.10 F the cell mono-layer does not allow to distinguish between single cells probably due to the dense matrix deposited by cells. The difference in cell number might be ascribed to the inhomogeneity of cell distribution and to the small surface area available with the cross-section configuration adopted for this analysis.
Figure 6.10: SEM images of double-layers with hMSCs presence at the inner surface. Red arrows show cells attached to the matrix in a low (C) and high (B and D) cell density area respectively. B and D show the migration of cells from one ply to the other. Scale bar 20 µm (A), 10 µm (B, C, D), and 2 µm (E, F).
6.5.2 OM imaging

To provide further evidence (see sections 6.4 and 6.5.1), hMSCs presence and distribution between the two ES PCL matrices was assessed with toluidine blue after a culture period of 14 days. Observation at the unaided eye clearly showed a blue hue on the whole surface whilst the control did not retain the stain (Figure 6.11).

![Image](image_url)

*Figure 6.11: Three double-layers (left) and control sample (right) stained with toluidine blue.*

After splitting the layers, both top and bottom matrices revealed the same blue colour, slightly stronger on some areas than on others (Figure 6.12).

![Image](image_url)

*Figure 6.12: Comparison between bottom and top layers which both show a clear blue stain.*
Figure 6.13 shows the inner sides of the specimens, observed with an optical reflection microscope. No significant difference was noticed between top and bottom layers with localised dark blue spots within a diffused blue tone. Intensity differences are deemed to be due to local cell density inhomogeneity. The regions that are more intensely stained are supposed to be populated by a higher number of cells which likely synthesised more ECM. The qualitative evaluation, performed by unaided eye and microscope observation, revealed an overall darker staining on the top layers.

<table>
<thead>
<tr>
<th>Bottom</th>
<th>Top</th>
</tr>
</thead>
</table>

*Figure 6.13: Optical microscope images of top and bottom cellularised ES layers stained with toluidine blue. Cell bodies are clearly visible. Scale bar 50 µm.*
In Figure 6.14, the details of the double-layer interface are shown. In Figure 6.15, control samples are shown. Unseeded specimens, after being subject to the staining procedure, did not present any blue colour nor darker areas.

**Figure 6.14:** Cross-section images of ES cellularised double-layers stained with toluidine blue showing the blue hue at the interface, surrounded by cells. Scale bar 100 µm.

**Figure 6.15:** Control samples, acellular ES layers, stained with toluidine blue. Scale bar 50 µm.

6.6 Double-layer thickness and fibre diameter influence on hMSCs viability

This section reports the results regarding the tests designed to investigate the viability of cells cultured between different double-layer types are reported. The systematic investigation of the
scaffold morphological features influence on cells was done following the results presented in section 6.3 where fibre diameter and scaffold thickness effects could not be decoupled. The parameters studied were fibre diameter and layer thickness. In one experiment, fibre diameter was kept constant and cells were seeded between matrices with different thickness. In the other test, a comparison was made between two different fibre diameters.

6.6.1 Layer thickness effect

In this experiment hMSCs were seeded between two double-layers, one of which having thickness two-fold than the other; therefore the samples are indicated as D- and DD-layer from here on. Cells cultured on all the double-layers, as well as on the control, experienced an increase in viability (Figure 6.16). 24h after double-layers assembly, there was a decrease in viability, relative to the control samples (cells in multiwell). From this point onwards, viability increased faster for cells on the ES PCL samples compared with the control. Fluorescence intensity at day 21 was significantly higher for D-layer, compared with both DD-layer and the control.
6.6.2 Fibre diameter effect

Two samples of SA-0.46 and SA-1.72 characterised by 0.46 and 1.72 µm fibre diameters with the matrix thickness controlled were studied. Final results demonstrated the same viability trend observed in the previously reported experiment which studied the influence of layer thickness. Higher fluorescence intensity was found on control wells on the day after double-layer mounting, compared with the samples under examination (Figure 6.17). At the following time points, hMSCs metabolic activity inside the double-layers is even higher than that of the control and such higher viability becomes more dramatic and significant by day 21. SA-0.46 increase was steeper than SA-1.72 and, on day 21, viability turned out to be higher; albeit statistically insignificant.

Figure 6.16: Viability of cells seeded on double-layers of different thickness. The black bar indicates significant difference (p<0.05).
Verification of the effect of fibre diameter decoupled from double-layer effect

The experiment was designed to clarify the role of fibre diameter on cell viability for slightly longer culture duration; therefore helping interpret the above reported results obtained on double-layers.

Cell viability on ES PCL matrices with different fibre diameter was assessed seven times over 35 days, the longest cell culture experiment on ES PCL layers conducted within this project. AB assay results are shown in Figure 6.18. Cells demonstrated a maximum viability value on day 21 with a not significant increase compared with day 14. The last two time points at 28 and 35 days demonstrated a decreasing trend (not significant) in cell metabolic activity. Cell viability was not significantly different between the sample types studied at any time point.

Figure 6.17: Viability of cells seeded on double-layers characterised by different fibre diameters. Black bars indicate significant difference (p<0.05).
In light of the results obtained on cell viability within double-layers with different thicknesses (see section 6.6.1), the experiment was designed to evaluate how the viability of hMSCs seeded in an ES PCL multi-layered scaffold is affected by seeding depth.

### 6.7 HMSCs viability within ES PCL multi-layered constructs

In light of the results obtained on cell viability within double-layers with different thicknesses (see section 6.6.1), the experiment was designed to evaluate how the viability of hMSCs seeded in an ES PCL multi-layered scaffold is affected by seeding depth.

#### 6.7.1 Metabolic activity assay

At the end of cell culture, viability analysis could be performed of cells grown on mono- and multi-layers. Viability of two multi-layered constructs was assessed on day 2 and 8 after seeding, showing a significant increase on both the samples (S1 and S2), as reported in the chart in Figure 6.19.
Conversely, cells on each layer from the multi-layer constructs, evaluated on day 1 and 9, before (day 1) and after (day 9) multi-layer assembling and disassembling, demonstrated a decrease (Figure 6.20).

![Viability result relative to each of the two assembled multi-layers.](Figure 6.19)

![Viability of each layer, average of sample 1 and 2.](Figure 6.20)

*Figure 6.19: Viability result relative to each of the two assembled multi-layers.*

*Figure 6.20: Viability of each layer, average of sample 1 and 2. From the bottom to the top: A=first layer, B=second layer, C=third layer, D=fourth layer. Bars represent significant difference (p<0.05).*
6.7.2 **Live and dead assay**

After 10 days culture, each mono-layer was treated with live/dead staining and observed with fluorescence microscope. The qualitative evaluation of every mono-layer surface revealed a smaller total number of cells on the bottom layer (A) compared with the others; however, the difference was more pronounced when compared with those observed on the top layer (D). Representative images of the layers are shown in Figure 6.21 A-D.

![Images of living (green) and dead (red) cells seeded in ES multi-layer. Each letter identifies the location in the multi-layer (A=bottom; D=top).](image)

Cell counting results are reported in Table 6.4 and revealed in the chart of Figure 6.22. An increasing percentage of living cells was observed with the layers proceeding from the bottom to the top, still a statistically significant difference was found only between layer A and all the others.
Observation of the bottom side of each layer individuated a negligible number of cells, compared with the top surface.

*Table 6.4: Percentage of cells detected on each layer. (A=bottom; D=top).*

<table>
<thead>
<tr>
<th>Layer</th>
<th>Living cells [%]</th>
<th>Dead cells [%]</th>
<th>Standard deviation [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>70.62</td>
<td>29.38</td>
<td>8.23</td>
</tr>
<tr>
<td>B</td>
<td>81.14</td>
<td>18.86</td>
<td>6.35</td>
</tr>
<tr>
<td>C</td>
<td>84.71</td>
<td>15.29</td>
<td>5.70</td>
</tr>
<tr>
<td>D</td>
<td>87.96</td>
<td>12.04</td>
<td>6.55</td>
</tr>
</tbody>
</table>
In light of the viability results revealed in section 6.6, a test was performed to study the correlation between morphology and permeability of ES PCL layer. As with the cellularised model, the diffusion of a traceable molecule was analysed in function of thickness and fibre diameter independently. The molecule diffusion through layers with different thickness, over time, expressed as percentage of the maximum values obtained, is reported in Figure 6.23.

**Figure 6.22:** Graph representing the increasing trend in number of living cells, proceeding from the bottom to the top across the multi-layer, and the symmetrical decreasing trend of dead cells. Black lines represent statistically significant difference (p<0.05).

### 6.8 ES PCL matrix features influence on permeability

In light of the viability results revealed in section 6.6, a test was performed to study the correlation between morphology and permeability of ES PCL layer. As with the cellularised model, the diffusion of a traceable molecule was analysed in function of thickness and fibre diameter independently. The molecule diffusion through layers with different thickness, over time, expressed as percentage of the maximum values obtained, is reported in Figure 6.23.
All the samples displayed an increase of resorufin concentration, more noticeable on the mono-layer, following the expected negative exponential trend. At the last time point (4h), considerable higher diffusion of 100% reduced AB solution was observed, compared with that of the double- and triple-layers.

Diffusion constants were computed for each sample as revealed in Figure 6.24. Mono-layer exhibited significant higher diffusion constant compared with both double- and triple-layers.

Figure 6.23: Time-dependent diffusion of resorufin through mono-, double- and triple-layers mounted on Cell Crowns devices.
In Figure 6.25 the results concerning the influence of fibre diameter on diffusion are shown. At the last time point big fibres demonstrated a higher concentration of the module in the second compartment compared with small fibres.
Figure 6.25: Time-dependent diffusion of resorufin through layers characterised by 0.40 (SF) and 1.59 µm (BF) fibre diameters, mounted on Cell Crowns devices.

Figure 6.26 illustrates the diffusion constants determined for layers characterised by small and big fibres. Diffusion constant demonstrated to be higher for the ES PCL layer characterised by small compared with big fibres.
Effect of low oxygen concentration on hMSCs

HMSCs’ behaviour in low oxygen concentration environment was investigated by comparing the viability of cells cultured inside a hypoxic chamber (2% O₂, 5% CO₂) with that of cells kept in standard conditions. Cell metabolism was assessed at day 1 and day 7 and the results are shown in Figure 6.27. The average viability values, calculated on six wells for each plate, are almost identical with very small standard deviations. This outcome indicates that low oxygen concentration does not impair cell metabolism. Thus, the same gas concentration parameters can be used to culture cells under dynamic mechanical stimulation.

This outcome will be further analysed (section 7.3.3) in terms of the relationship with cell viability drop upon seeding depth assessed in section 6.7.
6.10 IVD-like constructs as scaffolds for AF tissue engineering

IVD-like constructs were exposed to confined cyclic compression, using BOSE bioreactor, to understand the cellular and materials response to biomechanical stimulation. ES PCL double-layers with 30° shifting angle were mounted on the basis of the results obtained on flat double-layers cultured in static conditions (section 6.3). The gross appearance inspection showed the repeatability of the agarose disc fabrication process. The presence of the protruding edges (red circle in

![Figure 6.27: Viability values measured on cells cultured under normal and low oxygen concentration respectively.](image)

![Figure 6.28: Agarose cylinder surrounded by an ES PCL strip. The fringe used to safely hold the specimen during the sealant application and for loading in bioreactor is clearly visible in the red circle.](image)
Figure 6.28) facilitated the handling of the samples. When lifting the IVD-like constructs from the edges, with tweezers, no removal or slippage of the core was observed; thereby indicating that the agarose disc core was confined tightly by outer ES PCL layers.

### 6.10.1 Mechanical performances of IVD-like constructs

Agarose discs alone and IVD-like constructs were compared in terms of mechanical behaviour when subject to a ramp of compression till failure. The compression modulus is revealed as significantly higher (p<0.05) for the IVD-like construct (Table 6.5).

**Table 6.5: Compression modulus of agarose discs and IVD-like constructs.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Compression modulus [kPa]</th>
<th>Standard deviation [kPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose disc</td>
<td>96.71</td>
<td>17.81</td>
</tr>
<tr>
<td>IVD-like construct</td>
<td>145.64</td>
<td>35.37</td>
</tr>
</tbody>
</table>

Another noticeable difference was detected at the end of the test when the samples were removed from the grips. Agarose disc shape was no more recognisable and only squeezed fragments of the hydrogel were retrieved. In contrast, IVD constructs could be removed with almost intact configuration by using the tweezers.

### 6.10.2 Viability of hMSCs seeded in the IVD-like constructs in static conditions

The effect of the IVD-like construct mounting procedure on cells was evaluated through the comparison of cell viability before and after the assembly. Viability results are reported in Figure 6.29 over a 14-day culture period. In the first assessment after preparation, AF-like strips and IVD-like assembly demonstrated reduced viability compared with the parent strip. This is ascribed to the manipulation steps undertaken to prepare both the AF-like strips and IVD-like
assembly which led to dimension reduction. Cells in IVD-like constructs demonstrated an average lower viability compared with their counterpart (AF-like strip), albeit statistically insignificant. Both sample types revealed a positive increasing trend over time.

![Graph showing fluorescence levels over time for different samples.](image)

*Figure 6.29: Viability of cells seeded on ES PCL layers. The first column on day 1 refers to the average of three matrices before being split into 3 AF-like strips and three strips used to build as many IVD-like assemblies.*
6.10.3 Mechanical stimulation effect on IVD-like constructs

Effect on hMSCs viability

Viability of three days mechanically stimulated IVD-like constructs was compared with that of the control samples, cultured in static conditions in the same incubator that contained the bioreactor. Results of samples subject to 10 and 5% displacement, are shown in Figure 6.30 A and B respectively. Viability of cells seeded in specimens stimulated with 10% dynamic compression demonstrated to have almost no viability. The negative values are likely due to retention of medium in the agarose phase prior the AB test that might induce a shift in fluorescence values. The AB test involved washing the samples with PBS to remove any medium trace; however, due to its hydrogel nature, agarose released gradually the medium absorbed during cell culture, over the course of the assay; thereby inducing a slight shift of fluorescence towards lower values. Conversely, 5% displacement induced only a viability reduction compared with the static sample.
Effect on hMSCs morphology and distribution

Sample observations were performed on one sample per type by means of fluorescence microscope (Figure 6.31), taking advantage of the methodologies developed in section 4.3.3. On samples stimulated with 10% displacement, only few cells were observed, compared with the static samples, and appeared to be not much evenly distributed across the surface. In particular, cells revealed as patches, larger on the outer layer. Specimens subjected to 5% displacement stimulation revealed an overall higher number of cells and a more even distribution compared with those stimulated with 10% strain though static controls still demonstrated to be better in this regard.

Figure 6.30: Viability results of 10 (A) and 5% (B) mechanically stimulated IVD-like constructs. The assay was performed on both static and stimulated samples, at the same time points, before and after applying mechanical stimulation. * and ** indicate p<0.05 and p<0.1 significance respectively.
Figure 6.31: Representative images acquired at the end of mechanical stimulation experiments. Cells were stained for actin (red) and nuclei (blue). Scale bar 100 µm.
7. Discussion

7.1 Matrix preparation and characterisation

7.1.1 Establishment of a stable preparation process

The tissue engineering replacement developed in the present work was fabricated by means of the electrospinning technology. The fabrication method was chosen on the basis of a number of considerations that make this technology one of the most suitable to replicate the AF. Electrospinning is a versatile technique which allows to process a large range of polymers into non-woven fabric and nano- and microfibres matrix. In addition, by opportunistically changing the apparatus configuration (i.e. the collector) fibre arrangement can be modified to obtain random or aligned fibres (Agarwal et al. 2008). The advantage of such fibre arrangement is that it closely resembles the structure of the ECM which, according to its role, might be characterised by a certain extent of anisotropy (Guterl et al. 2013). Among the other benefits, micro-, but especially nano-fibres, offer a huge surface area to volume ratio thus making available for cells a high number of attachment sites (Lim & Mao 2009). Porosity, which is inversely related to fibre diameter, is a more controversial issue because the void to bulk material ratio and the interconnectivity are very high but, conversely, it is recognised that the pore size is small, in many cases too small to let cells penetrate inside the matrix (Liu et al. 2012). Nevertheless, even when cells are only able to grow on the surface, ES matrices are more desirable than flat surfaces because the active role of fibres topographical cue to cell orientation is undeniable (Nisbet et al. 2009). It has been demonstrated that micro- and nano-fibres exert specific effects on cell morphology by inducing cell elongation (Truong et al. 2010), and cell phenotype by guiding cell fate (Guilak et al. 2009).

For this project PCL was selected among the wide range of natural and synthetic materials that can be processed through electrospinning. PCL is in fact recognised as an inexpensive
biocompatible polyester with quite high flexibility ($T_g=-60^\circ C$ and $T_m=60^\circ C$) and long-term degradation properties (Cipitria et al. 2011).

In the current project a prototypal electrospinning machine was used to fabricate PCL fibrous matrices. The first fabrication attempts were performed after extensive literature study in order to establish a knowledge base on the effects of process parameters to the feature of matrices prepared. As it often happens when trying to reproduce the results by following the methods available in literature, the outcome, even from the same experimental set-up, turns out to be rather different. Hence, with such knowledge base used as approximate starting point, it was possible to study the effects of electrospinning parameters on the scaffold morphology, allowing fine-tuning of the parameters of scaffold preparation process.

The first set of experiments was carried out using single solvent systems, namely TFA, FA and CF. To the author’s knowledge, the former has never been used to electrospin PCL. TFA was selected based on its efficacy observed during author’s previous trials with other polymers (e.g. polybutylene terephthalate). In addition it has been demonstrated that trifluoroethanol (TFE), a compound with similar structure and features as TFA, could be successfully used to dissolve and electrospin PCL defect-free matrices (Koepsell, Remund, et al. 2011). Nevertheless the fibres obtained herein were of poor quality, with too many defects like beads, a fairly common defect type often resulting from incorrect process parameters selection (Van der Schueren et al. 2011). In addition, the electrospinning process revealed to be difficult to control in the long-term due to solvent drops sprayed along with the polymer solution, which locally dissolved the matrix, leading to reproducibility issues.

A trial with pure FA was also performed though after few attempts the difficulties in handling such set-up became clear. Similarly to what experienced by Gholipour et al. the low viscosity made the Taylor cone unstable with a lot of solution lost or electrospayed rather than electrospun (Gholipour Kanani & Bahrami 2011).
CF on the other hand demonstrated to be a good solvent for PCL leading to high viscosity solution even at low PCL concentration. The drawback of this system was that the high viscosity and high CF evaporation rate ($T_b=61.2^\circ C$, (Guarino et al. 2011)) made the solution partially solidify on the spinneret therefore this system was deemed to be not reliable enough to be run for large batch production. Furthermore, the fibre diameter obtained demonstrated to be difficult to control: only big fibres with rough morphology were obtained. The difficulty in obtaining submicron fibres is attributed not only to the low boiling point but also to the low relative permittivity of CF (Guarino et al. 2011; Balguid et al. 2009; Van der Schueren et al. 2011).

The failure of single solvents to electrospin PCL led to the trials with compound mixtures. 50% AA was added to FA leading to improved electrospinnability as revealed also by Van der Schueren et al. and Lavielle et al. (Lavielle et al. 2013; Van der Schueren et al. 2011). The better performances of the binary solvent system were probably due to the AA contribution in terms of lower relative permittivity (6.2 against 58) and surface tension (26.9 instead of 37 mN/m) which led to a less stretchable polymeric solution, and more stable Taylor cone under the same electric field (solvents physical properties are reported in Table 7.1). Apparently the relative permittivity contribution was more relevant than that of viscosity since AA viscosity is 1.22 cP, which is slightly lower than FA (1.57 cP). With this solution system several defect-free samples were prepared and one was spun for enough time to be selected for further experiments. However, the sample could not be utilised for biological experiments as, for unknown reason, the removal from the underlining aluminium foil turned out to be impossible, although no report has been found for such behaviour. It was hypothesised that the FA+AA used to electrospin the fibres would not completely evaporate due to the low volatility before the fibres deposited on the collector. Thus, the still partially soft fibres would stick to the collector upon solvent evaporation. However, no evidence of incomplete evaporation was found when analysing the upper matrix side and the observation of the aluminium foil-matrix interface was not feasible due to the impossibility to properly detach the matrix. Since FA+AA permitted to fabricate smaller fibres (192 nm) compared
with the other solvent systems, a procedure to detach the fibres would allow the investigation of fibres of such low size. One possible strategy would be coating the drum collector at the beginning of the electrospinning session with a polymer, with different solubility than PCL, which could be removed at the end of the process by rinsing with an opportune solvent. Moreover, in a recent article PCL molecular weight trend was studied when solubilized in FA+AA mixture. Results demonstrated that the acidic environment led to PCL hydrolysis which can be interestingly used to tune fibre diameter, but also demonstrates that the technique must be used carefully to preserve PCL integrity (Lavielle et al. 2013). Hence, it is also possible that the molecular weight decrease as the consequence of acidic hydrolysis led to reduction of polymer solution viscosity that allowed the fibres to reach the collector before they completely solidify.

With CF the rationale of a second compound addition was the opposite as that for FA. As a matter of fact, the fibres obtained with CF were too big, indicating that the polymeric solution was too viscous and volatile. Thus, similarly to what was found in literature MeOH was mixed with CF (Pham et al. 2006; Soliman et al. 2011; Blakeney et al. 2011; Shalumon et al. 2010) in different ratios (4:1, 5:1 or 7:1 CF:MeOH) or 50% DMF (Pham et al. 2006; Bolgen et al. 2005; Jeun et al. 2005; Lee et al. 2003). Compared with CF, MeOH boiling point is only 3 °C higher but with a much higher dielectric constant (33 instead of 4.8), which makes the electric field more effective. DMF has both a very high boiling point and dielectric constant (153 °C and 38.3 respectively). These parameters balance the high surface tension, equal to 37.1 mN/m, compared with 26.5 mN/m of CF, which is supposed to curb polymeric solution elongation. Both MeOH and DMF addition led to fibre diameter decrease and quality improvement. The effect of DMF was further confirmed in literature by Lee et al. but not by Jeun et al. (Lee et al. 2003; Jeun et al. 2005). Nevertheless, in the latter work no explanation of this inconsistent behaviour was given and, furthermore, it was not specified whether the other process parameters have been kept constant when comparing the effect of CF and CF+DMF. Eventually the quality of the matrices obtained with CF+DMF solvent system was higher (Figure 5.7) than that of CF+MeOH (Figure 5.3), therefore the latter
method was discontinued. As a matter of fact, the fibres fabricated with CF+DMF had smoother surface, absence of beads and the other process parameters could be tuned allowing to modify the fibre diameter without introducing defects.

THF was the last solvent used and, since the first attempt, it was mixed 1:1 with DMF. To the author’s knowledge there is no report on using pure THF to electrospin PCL and, comparing CF and THF features, it turns out that they share similar characteristics (see Table 7.1) (Heo et al. 2011; Croisier et al. 2012; Li et al. 2007). Therefore, it was worth to try THF immediately as an alternative to CF in the binary solvent system. The trials were successful and the process demonstrated to be very stable hence, to fabricate thick matrices, CF and THF respectively mixed with DMF were exploited.

In the present work solvents were chosen only on the basis of the outcome in terms of fibre quality and size; nevertheless, in the perspective of industrial scale up the integration of such chemicals in the product chain may not be trivial. First of all CF, THF and DMF are toxic and, the first two, potential carcinogenic therefore imposing to put in practice strict safety measure to protect the personnel.

Moreover each of the three solvents has specific drawbacks. DMF, because of its low vapour pressure (Sigma-Aldrich 2015a) and therefore low evaporation rate, may pose challenges in terms of solvent retention in the fibres. In the present work no assessment to verify residual solvent presence was carried out. Nonetheless the exposure to air flow after matrix fabrication and rinsing in medium prior to cell culture is anticipated to decrease the probability of residual DMF presence in the scaffolds. In the pharmaceutical world residual solvent assessment is common as several organic solvents are needed to accomplish drug synthesis (Medley et al. 2014) and it is usually performed by means of gas chromatography as it currently is the most widespread technique (Camarasu et al. 2006; B'Hymer 2003). Besides medicines, residual solvent analysis has been demonstrated to be successfully performed on polymeric microparticles (Dixit et al. 2015) and ES PCL fibres (Nam et al. 2013).
THF is characterised by low flash point of -17 °C (Sigma-Aldrich 2015b). The flash point is defined as the lowest temperature at which the vapour of a liquid ignites when exposed to a flame. Thereby, the working environment must be completely free from any source of ignition such as flames or sparks. Sparks, within an electrospinning facility, may be a hazard as the accidental formation of electric arc upon incorrect machine setting or malfunctioning can occur. However good ventilation like that provided by a chemical hood may prevent vapour accumulation near the needle tip. Special storage and handling control measures within the industrial environment must be taken into consideration (BASF Corporation 1998). Formation of peroxides when THF comes in contact with oxygen, especially in absence of opportune inhibitors, and their accumulation may also increase the risks of explosion (BASF Corporation 1998). The nature of THF, therefore, inevitably increases the complexity of the fabrication process due to the necessary exceptional control measures and, consequently, the associated costs.

CF is a chlorinated solvent with low boiling point. The hazards carried by CF are mainly related to its volatility and toxicity (Fang et al. 2008; Lock 1989). Personnel handling CF in the working environment may easily inhale CF vapour if not adequately protected. CF is known to react with oxygen and release phosgene (WHO 2004), a toxic compound (Tri-gas 2008) which is also believed to be responsible of CF toxicity once metabolised by liver and kidneys (Di Consiglio et al. 2001). Further to acute and chronic toxicity it is suspected to be cause of cancer in humans (IARC 1999). Therefore, as control measure, the exposure to CF has been restricted by supranational institutions like the EU and the WHO (EC 2000; WHO 2004). Obviously the health hazards force any company dealing with CF to put in practice handling control measures and monitoring of the air and of the workers.

Concerning the process parameters, within the same solvent system, specific attention was given to concentration as it has been recognised as the most important parameter that governs the electrospinning outcome (Greiner & Wendorff 2007). Although a systematic investigation of each
parameter influence on fibres morphology was beyond the aim of this project, the results
demonstrated that an increase of concentration leads to bigger fibre formation, which is
consistent with those reported by the other researchers (Guarino et al. 2011; Gholipour Kanani &
Bahrami 2011). Therefore, concentration was the main parameter that has been modified to tune
fibre diameter; the others, albeit relevant, were mainly adjusted depending on solution
concentration with the purpose of keeping the process stable. It has been stated that the
maintenance of steady state conditions, in particular a stable Taylor cone and a constant amount
of polymer ejected per time unit, enables to obtain beadless fibres and high reproducibility (Van
der Schueren et al. 2011). The results of the experiments on PCL revealed here further confirmed
that these conditions are crucial to get a neat matrix at the macro-scale level and are necessary,
but not sufficient, to allow flawless fibres to be produced.

Table 7.1: Features of the chemicals used to electrospin PCL (Bhardwaj & Kundu 2010).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Surface tension [mN/m]</th>
<th>Relative permittivity (or dielectric constant)</th>
<th>Boiling point [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trifluoroacetic acid</td>
<td>13.5</td>
<td>8.4</td>
<td>72.4</td>
</tr>
<tr>
<td>Chloroform</td>
<td>26.5</td>
<td>4.8</td>
<td>61.6</td>
</tr>
<tr>
<td>Formic acid</td>
<td>37</td>
<td>58</td>
<td>100</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>26.9</td>
<td>6.2</td>
<td>118.1</td>
</tr>
<tr>
<td>Methanol</td>
<td>22.3</td>
<td>33</td>
<td>64.5</td>
</tr>
<tr>
<td>Dimethylformamide</td>
<td>37.1</td>
<td>38.3</td>
<td>153</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>26.4</td>
<td>4.8</td>
<td>61.6</td>
</tr>
</tbody>
</table>

The reproducibility issue is related not only to the maintenance of a stable process but also to the
environmental factors. As mentioned in the results section, variations were noticed between the
matrices produced at different times. Duling et al. also found a higher variability of fibre
morphology when the polymer was electrospun on different days in respect to sheets prepared on the same day (Duling et al. 2008). Electrospinning processes using the same system and at the same site took place in different seasons, thereby environmental factors, namely temperature (T) and relative humidity (RH), were uncontrolled. These two parameters are considered to be responsible of the outcome change since the electrospinning apparatus was placed under a chemical hood, which is not equipped with T and RH control system. As a matter of fact, layers produced on the same day with the same machine parameters set were found to be subject to no significant variability. In addition, using constant machine set-up, matrices fabricated within the same batch were consistent contrary to those fabricated in different batches. It must also be considered that the modifications applied to the protocols from one day to the following, within the same season, led to similar changes independently of the environmental conditions. For example, the concentration increase adopted expecting to obtain fibre diameter growth would actually lead to fibre diameter growth regardless the environmental conditions. This and other similar experiences allowed to exclude that the changes in the output were due to random noise triggered by intrinsic reproducibility issues of the apparatus.

Contrary, in the comparison between the two batches made in two different seasons as reported in the results section (Figure 5.11), the same process parameters led to different fibre morphologies. A decrease in fibre diameter was observed when T and RH were both lower. According to literature a higher RH leads to thinner fibres as it prevents polymeric solution from drying, therefore the polymeric jet has more time to be stretched and become thinner before solidifying (Van der Schueren et al. 2011; Marsano et al. 2010). On the other hand previous studies demonstrated that T increase causes fibres to become thinner (Tsou et al. 2011; Wang et al. 2009). At higher T the evaporation rate increases whilst viscosity and surface tension decrease (Pakravan et al. 2011). Apparently viscosity and surface tension decrease has predominant effect thus favouring formation of small fibres. Nevertheless it must be noticed that in the quoted studies the experiments were conducted on different polymer/solvent systems and, furthermore,
the morphology behaviour change was investigated in a range of $T$ above 25°C. Instead, in the range investigated here ($\approx$25 °C, 50-60% RH and $\approx$22 °C, 20-30% RH and) seems that the predominant contribution is given by the decrease in temperature that reduced the evaporation rate thus keeping the viscosity high for longer time, eventually leading to fibre diameter decrease.

7.1.2 Computer-aided characterisation of fibres orientation

ES PCL fibres configuration was studied with the aid of a validated software, developed by E. Demirci using Matlab language (Demirci et al. 2011). The software allowed analysing SEM images of ES PCL matrices and grouping the fibres according to their angle relative to a reference axis. Detected fibres were included in one of the 18 categories spanning from 0 to 180°.

The usage of a computer-aided method has several advantages over a manual evaluation, which is often adopted in similar studies (Koepsell, Remund, et al. 2011; Li et al. 2007; Hong & Kim 2013). Firstly, the number of fibres individuated is higher compared with the manual counting thus leading to a wider statistical sampling and higher accuracy. Secondly the method is independent from the operator measurement capability and fibre choice thus ensuring a high reliability and repeatability by reduction of bias due to variations of imaging interpretation from individuals. In the perspective of scaling up the manufacturing process the availability of an automated protocol is a mandatory requirement to obtain a standard output and allow reliable quality controls over time. Examples of anisotropy investigation by means of custom programmes written in Labview and Matlab are provided by Truong et al. and Courtney et al. respectively (Truong et al. 2010; Courtney et al. 2006).

In semi-aligned and aligned samples a higher number of fibres was found to lie near the 90° region (correspondent to the drum collector rotational direction) compared with random samples. This result is expected and the difference spotted is clear on a superficial analysis. Nevertheless, the software also allowed making other interesting considerations. Regarding random specimens, theoretically, due to perfect homogeneous distribution, a percentage of approximately 5.6% for
each quantile was expected. Therefore, the intervals examined should encompass 11.2% and 22.4% for 80-100° and 70-110° respectively. However, as shown in Table 5.1 of the results section, within the said ranges, the percentages are approximately two-fold. This trend is reasonable if examined along with the distribution chart (an example of a representative graph is given in Figure 5.13). Fibres rarely assumed orientations almost perpendicular to the drum collector rotation direction as revealed by the low percentages in the eight quantiles in the range of 0-40 and 150-180°. Random fibres were fabricated by maintaining the collector speed as slow as possible (60 rpm); nevertheless, it was clear that even at the low speed the way the fibres were pulled and deposited on the drum collector was able to affect fibre arrangement.

7.1.3 Effects of surface topographic features on wettability

Hydrophilicity (or hydrophobicity) of a biomaterial is an important property as it affects cell attachment (Roach et al. 2007). The interaction between cells and the material is rather complex because it is mediated by proteins which is affected, for instance, by the surface charge level and type and by the relative concentration of proteins that compete with each other (Wilson et al. 2005).

Among polymers PCL is classified as rather hydrophobic according to Vogler’s definition for which a surface is defined as such if the contact angle is above 65° (Vogler 1998). At the molecular level this is due to the absence of polar chemical groups in PCL chains as shown in Figure 7.1. Since it has been observed that moderate hydrophilic materials hold better cell adhesion performances (Dalton et al. 1998; Takebe et al. 2000; Lee et al. 1998) it was deemed interesting to measure wettability of PCL samples. Solvent cast PCL wettability was also measured and it turned out to be lower of at least 26° than those observed in similar tests (Yeh et al. 2011; Gupta et al. 2012; Bolgen et al. 2005). Even though these published results span over a 15° range,
the difference relative to the result presented here remains rather high. This can be due to the thin film preparation process, presence of impurities and imperfections or molecular weight differences, although the last parameter was revealed to have only a small influence (Yeh et al. 2011).

ES PCL specimens demonstrated a higher hydrophobicity compared with the contact angle value of solvent cast PCL, consistent with other findings (Hong & Kim 2013; Gupta et al. 2012; Bolgen et al. 2005) and very close to 129° reported by Ekaputra et al. (Ekaputra et al. 2009). The super-hydrophobic effect of ES fibres might be explained with the lotus-leaf theory, for which very high contact angle values can be achieved through the combination of a hydrophobic material and the proper nano-topography (Gupta et al. 2012; Sas et al. 2012; Hong & Kim 2013). The alignment degree also influenced its surface wettability as the contact angle increased with the orientation degree. An opposite trend was found by Kim et al. and Truong et al. albeit this result is consistent with Subramanian et al. who suggested that the lower pore size of aligned fibres might have a role in reducing the wettability (Subramanian et al. 2011; Subramanian et al. 2012; Truong et al. 2010; Kim 2008).

7.1.4 Effects of environment and UV sterilisation on in-vitro degradation

As other polyesters, PCL undergoes degradation by means of hydrolysis in presence of water (Singh & Sharma 2008). In aggressive environment like the human body oxidative degradation is deemed to play a role too (Lyu & Untereker 2009). The combination of liquid medium degradation and mechanical stress accelerates the drop in mechanical properties due to what is called environmental stress cracking (ESC) (Saharudin et al. 2016). ESC leads polymers to faster failure compared with polymers exposed to chemical degradation or mechanical stress only (Kuipers et al. 2004). Propagation of cracks through the polymer structure is the mechanism of failure of bulk polymers. However, for fibrous materials, another mechanism that does not
involve rupture of the bulk polymer (i.e. rupture of the intermolecular bonds of polymer chains) is present. This mechanism relies on rearrangement and separation of fibres that leads to propagation of cracks at macroscopic level (Koh & Oyen 2015). Changes at molecular level, which affect the physical properties, can be monitored by means of a range of techniques such as FTIR (Motiwalla et al. 2013), spectroscopy techniques (Rizzarelli & Carroccio 2014) and GPC (Lam et al. 2007). In the present work GPC was used to evaluate the changes of ES PCL molecular weight over time and after sterilisation.

It is well-known that PCL has a slow degrading kinetic compared with other similar polyesters (Cheung et al. 2007; Cipitria et al. 2011; Puppi et al. 2010; Dong et al. 2009) and complete degradation occurs in approximately 1-4 years depending on molecular weight and environmental conditions (Lam et al. 2007; Cipitria et al. 2011). It has been shown that there actually are differences between in-vivo and in-vitro degradation with the latter requiring longer time. The in-vitro mass loss obtained herein is consistent with those published in literature. After one year, in fact, the ES PCL sheets demonstrated to be subject to no mass loss. This is consistent with what observed on PCL scaffolds immersed in PBS for several months (Lam et al. 2008; Coombes et al. 2004). The only tangible sign of degradation obtained in the present work was the slight increase in roughness detected on fibres surface by means of qualitative SEM evaluation. This could be the initial step of a much evident change in morphology as observed by Zeng et al. on PCL soaked in enzymatic solution, which is more aggressive than PBS (Zeng et al. 2004). PCL immersed in medium in presence of lytic enzymes or chemicals, which catalyse the hydrolytic reaction, has exhibited a higher degradation rate (Lam et al. 2008; Cottam et al. 2009). In addition, despite autocatalysis is deemed to accelerate degradation (Dong et al. 2009; Lam et al. 2008), such mechanism has been actually demonstrated not to be involved (Siparsky et al. 1998). The influence of morphology has also been proved as ES PCL is degraded faster than bulk PCL (Dong et al. 2009). High surface erosion was attributed to the larger surface area to volume ratio of the nanofibres compared with the bulk. As surface erosion depends on the surface area available, PCL

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nanofibres offer a larger surface area compared with bulk PCL (Dong et al. 2009; Lam et al. 2007). Nevertheless both bulk and surface erosion mechanisms (Göpferich 1996) had been assessed under different environmental conditions and sample types (Z.-Y. Wang et al. 2013; Yeo et al. 2008; Lam et al. 2008). For example accelerated degradation had been found to induce surface erosion in the short term whilst bulk erosion was the predominant mechanism in the long-term (Lam et al. 2008). Despite surface erosion was anticipated to be the main degradation route, considering the features of the sample studied in the present work, the experimental data concerning molecular weight loss supports that bulk erosion was the main degradation mechanism, consistently with Coombes’ et al. results (Coombes et al. 2004). However, the different experimental conditions adopted in other works do not allow direct comparisons, thus leaving speculations over the main degradation mechanism at theoretical level.

Light may be another cause for chemical and physical changes in polymers. The extent of changes is higher with high energy light (i.e. short wavelength), therefore in the UV spectrum range. The UV-A, UV-B and UV-C are subclasses of UV ray at 400-315, 315-280 and 280-100 nm wavelength ranges (Kowalski 2009). The radiation-induced chemical changes depend on the spectra of the light and on the material chemistry which determine the amount of energy reflected, transmitted and absorbed by the material. The higher the absorbed fraction is, the higher is the interaction between photons and the material molecules. For polymers it is believed that covalent bonds cleavage is not due to direct photolysis but rather the energy brings the molecule to a higher excitation state that eventually leads to bonds breakage as consequence of free radical formation or accumulation of heat (Feller 1994). Polymers in general have small absorption depth, tens µm (Kowalski 2009), thus photodegradation occurs mainly on the surface; however it has been demonstrated that UV irradiated PCL develops degradation products even at hundreds µm depth (Christensen et al. 2008). In the same study it was revealed that the absorption spectra of PCL has a main peak at 250-200 nm and a smaller one at 320-260 nm (Christensen et al. 2008). The
penetration depth of UV light, considering both the sterilisation and photodegradation potential, may differ for fibrous PCL layers as light may be reflected and scattered multiple times thus having different consequences compared with bulk PCL.

As previously mentioned for the chemical changes induced by the environmental conditions, the alteration in the material physical properties upon high energy irradiation must be carefully evaluated. For example, on PCL, it has been found that gamma irradiation decreased the degradation rate inducing a decline in Mn but an increase in Mw and PD (Cottam et al. 2009). This phenomenon could be attributed to the fact that radiation energy broke and crosslinked polymer chains at the same time. Herein the results obtained with GPC demonstrated a decrease of both Mw and Mn along with an increase in PD therefore UV light had the only effect of generating smaller PCL chains. This is in agreement with the results obtained on polyesters which underwent Mn (Mw not measured) (Yixiang et al. 2008) or both Mn and Mw (Yeh et al. 2011) decrease proportional to the exposure time. Possibly the lower energy of UV compared with gamma ray does not induce the crosslinking effect observed by Cottam et al. (Cottam et al. 2009).

Sterilisation is obviously an essential issue in tissue engineering and the choice of a proper method depends on the scaffold characteristics. In the present study UV light sterilisation was proved to be a reliable sterilisation method as contamination never occurred during cell culture test in static conditions. Nevertheless there is a trade-off between the energy conveyed by UV light which should be kept at the lowest level possible to cause negligible changes at molecular level but must be sufficient to kill the bacterial burden. Bacteria have been demonstrated to be sensitive to both UV energy (inversely proportional to the wavelength) and time of exposure (Giese & Darby 2000; King et al. 2011). In the perspective of the device introduction on the market, the simultaneous impact of UV sterilisation on the bacterial burden and physico-chemical changes. For an AF artificial replacement the evaluation of the mechanical properties change is particularly important as undesired alterations may compromise the expected load bearing capabilities of the artificial replacement.
7.1.5 Stretching direction affects mechanical properties of ES PCL layers

The purpose of the mechanical tests conducted herein was to assess the performances of the ES PCL layers compared with those of the native AF. In the AF the collagen fibres of the lamellae are arranged approximately at ±30° angle relative to the spine horizontal plane (Raj 2008). Taking also into account the wide range of forces, which the AF is subjected to, it was worth to mechanically characterise the ES PCL layers in a directional-dependent manner (Guterl et al. 2013). ES PCL matrices cut from random, aligned and semi-aligned sheets were thus stretched by changing the predominant fibres direction in respect to the pulling direction, till failure.

Results demonstrated that ES PCL matrices stretched parallel to the fibres direction (0°) hold the highest tensile modulus and UTS which decreases when the load angle changes to 90°. Conversely the elongation at break demonstrated an opposite increasing trend with maximum values measured at 90°. A similar behaviour of ES PCL matrices has been assessed by Nerurkar et al. though the modulus values obtained are not comparable possibly because of different experimental conditions (Nerurkar & Elliott 2007). The shape of the stress-strain curves was found comparable across the different orientations with two linear regions clearly detectable and consistent with the studies on similar substrates (Gupta et al. 2012; Hong & Kim 2013; Kim 2008; Kharaziha et al. 2013).

When measurement was carried out at various stretching directions, the difference of the modulus between stretching at 0° and 90° angle for both aligned and semi-aligned fibres is of approximately 20 times. However, when comparing these two fibre patterns, a significant difference of the moduli was not always detected. This is consistent with the low alignment degree difference identified in section 5.1.4. The tensile modulus value measured herein on aligned fibres stretched in parallel direction is comparable to that found by Hong et al. but higher than those reported in other similar studies (Hong & Kim 2013; Kim 2008; Li et al. 2007).
Besides the modulus, the other remarkable difference among the specimens was the strain at UTS which was found to be much higher on 90° stretched samples than 0°. The explanation for this behaviour could be that, the more the fibres are stretched in parallel direction, the more they are subject to breakage (Figure 7.2); contrary, when the load is applied perpendicularly, fibres do not actually split but they rather just disentangle from the adjacent ones (Figure 7.3). As such, the force necessary to break a bundle of fibres along its axis (stretched at 0° angle) is greater than that required to overcome the weak bonds between parallel fibres (stretched at 90° angle). Therefore, parallel fibres are able to separate from each other, increasing the overall specimen length, before the specimen breaks.

*Figure 7.2: Schematic representing fibres behaviour when stretched along the orientation direction (0°).*
At the beginning of this section the results of mechanical properties assessed on ES PCL matrices by changing the stretching angles have been examined. In the following paragraphs the outcome of a similar experiment is discussed. The difference between the two experiments is that, disregarding the stretching direction, in the second one the effect of two environmental conditions (air or PBS at 37 °C) was studied. The effect of PBS at 37 °C will be thoroughly dealt below; nevertheless, in this second experiment the baseline values in dry conditions were essentially measured as in the first test discussed above.

Comparing the two experiments, it can be overall observed that the moduli of dry samples tested are higher than those of wet samples. In addition to the different mechanical testing apparatus adopted, which can be source of systematic error, the discrepancy can be explained by the difference in the samples utilised in the two tests. Due to matrix availability constraint, for the second experiment samples with bigger fibre diameter had to be selected. In dry conditions $E_{\text{low}}$ of aligned fibres passed from 53.64 to 24.80 MPa when the diameter was 0.60 and 0.73 µm whilst for semi-aligned fibres $E_{\text{low}}$ passed from 27.94 to 7.38 MPa for 0.40 and 0.67 µm. $E_{\text{high}}$ and UTS were also found to follow the same trend whilst strain at UTS exhibited an increase when passing from small to big fibres. The modulus and UTS decrease (Chew et al. 2006; Lim et al. 2008; Wong et al. 2008), and strain increase (Chew et al. 2006) assessed here on matrices characterised by

![Figure 7.3: Schematic representing fibres behaviour when stretched perpendicularly to the orientation direction (90°).](image-url)
bigger fibres is consistent with other studies. It has been hypothesised that the polymer crystallinity depends on fibre diameter, therefore affecting polymer molecules interaction and eventually mechanical properties (Wong et al. 2008). Lim et al. investigated single fibre molecular structure demonstrating that, in big fibres, polymer macromolecules are organised in stacked lamellae perpendicular to the fibre axis whereas in small fibres the formation of only small lamellae, which tend to arrange in fibrils parallel to the fibre axis, occurs (Lim et al. 2008). As a consequence, this last configuration is able to enhance fibre tensile modulus.

7.1.6 Mechanical properties depend on the environmental conditions

**Tensile modulus and strain at UTS significantly change in wet conditions**

Specimens for each type of fibre pattern were subjected to stretching at breakage in dry and wet conditions (PBS at 37 °C). The comparison of the curve shapes, between the two experimental settings, showed a good similarity since in most of the cases two linear regions were detectable. The shift of the curves towards lower moduli and higher strains is clear though not relevant for semi-aligned fibres. Considering each stretching direction the tensile modulus demonstrated significant decrease from dry to wet conditions for the majority of the samples. More importantly this experiment revealed the presence of the combined influence of wet conditions and stretching angle on the tensile modulus of aligned and random fibres, for which the effect demonstrated to be more significant.

ES PCL softening in PBS at 37 °C is consistent with what experienced by Duling et al. (Duling et al. 2008). Lower moduli were measured also on synthetic (Yeganegi et al. 2010) and natural (Bonani et al. 2011) ES polymers when studied in wet conditions. Nevertheless, Thomas et al., studying a range of ES polymers, found that aqueous environment affected the tested materials in opposite ways (Thomas et al. 2007). The authors assessed an increase in modulus in wet conditions on one material, which had a higher hydrophilicity compared with the others that exhibited a decrease in modulus. Therefore the greater modulus was attributed to the high hydration of that single
material, namely poly(glycolide-co-trimethylene carbonate) (PGTMC). PCL was not among the materials tested in the aforementioned study and the wettability of PGTMC was not directly evaluated. However another study measured PGTMC film contact angle equal to $70 \pm 1^\circ$ demonstrating PGTMC to be more hydrophobic than PCL used in the present thesis (Ishaug-Riley et al. 1999). According to Thomas et al.’s interpretation the higher hydrophobicity of PGTMC compared with the other materials tested and to PCL, is responsible of the increase of modulus in wet environment (Thomas et al. 2007). Instead, more hydrophilic materials, along with PCL used here, experienced softening when stretched in water. Water sorption effect has also been investigated on a range of polymers revealing that the glass transition temperature ($T_g$) decreases with increased water content (Verhoeven et al. 1989; Batzer & Kreibich 1981) thereby leading to softening (Ito et al. 2005). At the molecular level water is supposed to weaken the bonds between polymer chains thus facilitating their reciprocal movement, acting as a plasticiser (Ito et al. 2005). Along with water plasticising effect temperature may have affected the mechanical properties change. PCL is a polymer characterised by a $T_g$ of -62 °C and a melting temperature ($T_m$) in the range 55-60 °C. Thereby when passing from RT to 37 °C, closer to $T_m$, PCL is subject to softening as it has been detected by dynamic mechanical analysis (Sengupta et al. 2007).

**Combined effect of environmental conditions and stretching angle**

Despite the softening effect at molecular level, the significant combined effect of stretching angle and wet conditions displayed leads speculating over wet conditions interaction with the fibre network. This combined influence, more significant on aligned and random fibres, made the difference between dry and wet conditions more marked where the experimental conditions lead to higher modulus and UTS. For example on aligned fibres at 0° the tensile modulus difference between dry and wet conditions is higher than the difference at 90° and, simultaneously, the overall modulus at 0° is higher than at 90°. At 0° aligned and semi-aligned fibres are already almost all aligned in the stretching direction thus the decrease in modulus is mainly due to water
plasticising effect that induces each fibre lengthening (Figure 7.4). On the other hand at 90° fibres experience disentanglement and sliding between each other (Figure 7.5). This mechanism involves more friction between fibres than at 0° where the reciprocal fibre movement is limited.

Figure 7.4: Schematic representing fibres behaviour when stretched along the orientation direction (0°). By applying the same load the strain obtained in wet conditions is higher than that observed in dry conditions due to water plasticising effect.
A unique configuration of stress-strain curves, characterised by jagged pattern, was noticed in wet environment, in particular when ES PCL samples were stretched at angle close to 90°, with the only exception of random sample at 0° likely due to random pattern with low anisotropy. It was possible that the pattern was provoked by the slippage of the samples. The sequence of stress accumulation with grips displacement followed by small sample portions slipping out the grips might explain this behaviour. However, the sample preparation methodology used was kept consistent across all the specimens, which leads to the exclusion of this possibility. The tests carried out with the alternative (non-fibrous) plastic materials demonstrated no influence of the testing environmental parameters on the emergence of such jagged pattern. This finding implies

90° stretching orientation in wet environment made jagged pattern to emerge

Figure 7.5: Schematic representing fibres behaviour when stretched perpendicularly to the orientation direction (90°). The difference between the strain measured in wet and dry conditions, being the load equal, is less marked than that obtained at 0° due to the reduced role of the plasticising effect.
that the unique combination of material (PCL), structure (nano- and micro-fibres) and testing conditions did enhance the appearance of the jagged pattern. Therefore, the fibre network, consequence of the electrospinning processing, along with the wet environment induced the fibres to progressively rearrange upon stretching. Rearrangement induces fibres sliding on each other, involving frictional forces. The mechanism of energy stored and subsequently released at discrete intervals to overcome friction may explain the jagged pattern. In addition, a higher extent of fibre sliding movement expected on the fibres stretched at 90° compared with 0°, demonstrated by the higher elongation at UTS, is consistent with the jagged pattern found more evident towards 90° stretching angles.

Remarks on the unexpected behaviour of semi-aligned fibres

Semi-aligned fibres did not exhibit a significant behaviour difference, when stretched in dry and wet conditions, contrary to random and aligned samples. The analysis of the stress-strain curves demonstrated a noticeable difference in the trend at low strain, with semi-aligned fibres displaying a more evident toe region, especially at 0° stretching angle compared with aligned fibres. This peculiar trend was attributed to the morphology of the fibres which, as revealed in the results (section 5.5.5), fibres on semi-aligned sample were found to be more crimped. Studies on the influence of fibres crimping on the mechanical properties have revealed that the more fibres are curled, the wider the toe region is (Brown 1955). The toe region is associated with fibre straightening, thus the wide toe region detected on semi-aligned fibres is consistent with the crimped structure shown in the SEM images. Another aspect worth to consider is the relationship between fibres crimping and the matrix permeability, which is subsequently related to density and porosity. It has been demonstrated that permeability is higher for matrices with high porosity (Zhu et al. 2014; Milleret et al. 2011) and crimp level (Lamb & Costanza 1979). Therefore, it is hypothesised that the higher porosity and inter-fibre distance encountered on semi-aligned fibres reduces the role of water played in fibre-to-fibre interaction, in respect to random and aligned
fibres, when fibres are subject to rearrangement upon stretching. This argument could be extended to explain the less significance of the synergic effect of stretching angle and environmental conditions on modulus and strain, compared with aligned and random fibres.

The similar behaviour revealed by semi-aligned fibres in wet and dry conditions is apparently in contrast with the water plasticising effect discussed in the previous paragraph for random and aligned samples. According to what stated above, semi-aligned fibres are expected to be subject to the same plasticising effect as random and aligned arrangement. However, due to semi-aligned fibres peculiar crimped arrangement, the results obtained here for semi-aligned fibres suggest a more relevant role of wet conditions in the alteration of fibre-fibre interaction over water plasticising effect. Further tests are needed to more accurately elucidate the mechanism by which wet conditions affect ES PCL mechanical properties. In particular, tests carried out on bulk PCL are deemed to be useful in this sense in order to eliminate the contribution of the fibres that are responsible for the additional complexity degree from the morphological point of view. Likewise, such experimental conditions can be applied to the evaluation of single ES PCL fibre mechanical properties. An accurate correlation of the electrospinning process output with the mechanical properties would help predict the behaviour of a hypothetical implanted scaffold.

*Remarks on the unexpected behaviour of random fibres*

Tests carried out by stretching random fibres at 0 and 90° revealed an unforeseen higher tensile modulus at 90° stretching angle. To ensure that no experimental errors, i.e. specimens swap, the test was repeated once with specimens cut from the same ES PCL matrix and once with a sample from another batch (results not shown). The trend did not change across the tests therefore the mechanical behaviour observed is genuine and was not caused by the bias related to the experimental procedures and set-up. To the author’s knowledge in no published work a similar test has been attempted nor was any evidence of such mechanical behaviour highlighted. Thus it
is hypothesised that the highest modulus detected at 90° (in either wet and dry conditions) is caused by the specific structure of the ES PCL matrix.

The random ES PCL matrix, according to the software aided morphology analysis, is formed of a network of fibres having a small orientation degree. The results obtained in this project demonstrated that this structure creates inter-fibre interactions that are stronger when the matrix is stretched perpendicularly. For the present work random fibres were fabricated by keeping the electrospinning drum collector speed as low as possible (60 rpm). This set-up was initially not supposed to induce any relevant fibre orientation. Nonetheless it is notable that, following this strategy, a uniform ES PCL sheet could be produced. Conversely, the common method to fabricate random matrices is using a static collector (i.e. a metal plate or foil) that does not induce any fibre orientation (Lee et al. 2005) but does not allow manufacturing a homogenous matrix in terms of thickness.

Further investigation of the peculiar mechanical behaviour encountered on random fibres can be carried out by studying the mechanical properties of matrices fabricated using a range of collector speeds close to the lowest available. This would allow to assess whether this unexpected effect on mechanical properties is displayed using also other collector speeds to detect the threshold speed beyond which the trends typical of semi-aligned and aligned fibres are exhibited. In addition, computational simulation tools might be useful to correlate the mechanical behaviour of the whole matrix to the fibre-to-fibre interaction upon tensile stress.

*Is ES PCL scaffold suitable for AF tissue engineering?*

The experiments performed under wet conditions detected a change in mechanical properties of ES PCL matrices, allowing correlation with the natural tissue properties of AF. As revealed in the previous sections, the ES PCL modulus measured in dry conditions falls within the lowest and highest values assessed on single natural AF lamella (3.79 and 136 MPa respectively (Holzapfel et al. 2005; Skaggs et al. 1994)). Thereby, the decrease of ES PCL tensile modulus measured here
upon wet conditions must be taken into consideration since it brings the modulus closer to the lowest measured on AF lamella. The UTS is not matter of concern as in wet conditions the change is negligible. Studies on single lamella (Table 7.2), except that conducted by Zhu et al., have revealed that the strength at failure is in agreement with the UTS assessed here on ES PCL.

Natural AF portions, multilamellar or single layer either, are characterised by lower ultimate strain at failure compared with ES PCL thus the latter provides a sufficient safety margin (Table 7.2). A study by Kingsley et al. demonstrated that moderate running activity provokes an IVD height and volume loss of 6.3 and 6.9% respectively (Kingsley & D’Silva 2012). This data suggests that the load conveyed to the AF whilst running would unlikely reach the failure values reported in Table 7.2.

<table>
<thead>
<tr>
<th>Strain at failure</th>
<th>UTS [MPa]</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2*</td>
<td>29.20±1.81</td>
<td>(Zhu et al. 2008)</td>
</tr>
<tr>
<td>0.65</td>
<td>0.9-3.8</td>
<td>(Green et al. 1993)</td>
</tr>
<tr>
<td>0.09-0.15</td>
<td>3.6-10.3</td>
<td>(Skaggs et al. 1994)</td>
</tr>
<tr>
<td>0.09-0.18*</td>
<td>1-3.2</td>
<td>(Ebara et al. 1996)</td>
</tr>
</tbody>
</table>

AF axial strain has been found to be subject to strains of similar magnitude in both compression and tension for posterior and anterior AF respectively (O’Connell et al. 2011). The same study identified the radial strain to be maximum in the posterior region, in compression, and equal to 0.039. Heuer et al. compared the effect of different mechanical stimulus combinations on a whole vertebral segment model. They measured the highest AF strain (11.9%) when 500 N axial strain and 7.5 Nm moment were applied simultaneously, compared with the same loads individually applied (Frank Heuer et al. 2008). A similar moment value was found to be responsible of the
onset of damage on an AF section in a fatigue experiment; however the authors estimated that, for an entire IVD within the spine, a higher load, unlikely achievable in the everyday life, is needed to induce AF failure (Green et al. 1993). Despite the wide range of properties values identified, it is implausible that a hypothetical ESPCL replacement would be stretched up to the region identified with $E_{\text{high}}$ modulus (Figure 7.6). Thus, among the data obtained in the present work, $E_{\text{low}}$ is deemed to be a more reliable indicator when confronting the scaffold and the natural tissue performances. Eventually there is the need of novel data concerning the mechanical performances that an artificial IVD replacement would have once implanted in-vivo. Properties such as modulus and strain under compression of each component are difficult to predict as they strictly rely on a number of parameters (i.e. construct size, material of the core, number of the outer layer plies, fibre arrangement).

![Figure 7.6: Example of stress-strain diagram. The black dotted line indicates 100% strain.](image)
7.2 HMSCs shape and orientation on ES PCL mono-layers

7.2.3 ES PCL fibre alignment induces contact guidance phenomenon on hMSCs

Validation of cell morphology evaluation procedure proves high reliability

The tool applied in the present thesis to investigate nuclei morphology deserves to be commented prior to discussing the results in the following section. In a number of published studies a similar choice has been made (Morgan et al. 2012; Nathan et al. 2011; Z.-Y. Wang et al. 2013) and, for such investigation, there is no standardised procedure that can be used as a reference. Therefore, it was considered useful to demonstrate the effectiveness of the protocol established herein by setting up a test using ad hoc prepared shapes and patterns (sham images Figure 4.5 and Figure 4.8) that simulate the images obtained with the fluorescent microscope. The absence of a gold standard did not allow proper validation of the methodology; nevertheless, being aware of how the software behaves when fed with known inputs, permitted to improve the confidence about the reliability of the results obtained on the samples. The demonstration of effectiveness was set up similarly to what Demirci et al. performed on fibres orientation (Demirci et al. 2011) but in the present work some random noise was added to the sham images to make the demonstration more realistic. As anticipated the customised protocol allowed to measure the images prepared before introducing the noise with a higher accuracy compared with the noise-modified images. However, the analysis of the nucleus major axis angles on the images with noise revealed that the average error made was 1.79±2.34°, which is quite low considering that the entire angle span is 90°. Similarly, the error for circularity calculation, being equal or lower than 10%, is deemed to be acceptable.

In conclusion the robustness of the image analysis procedure was successfully demonstrated through an ad hoc test which confirmed the reliability of the results obtained on the fluorescence
micrographs of hMSCs cultured on ES PCL layers. The validation presented herein becomes especially valuable under the light of the variety of methodologies proposed and the absence of a gold standard procedure.

**HMSCs morphology**

Cells contain several different types of sub-cellular components such as organelles, structural proteins and enzymes covering all the functions necessary for cell life. Cell motility and shape depend on sub-cellular structural proteins among which actin is the most common in many eukaryotic cells (Pollard & Borisy 2003). Actin is present in the cytoplasm, playing a major role in maintaining cell shape, but with higher concentration in lamellipodia, the protruding structures that enable cell motility (Pollard & Borisy 2003). These lamellar structures project and retract according to actin polymerization and disassembly in a dynamic equilibrium mechanism called *treadmilling* (Bugyi & Carlier 2010). Contractility allows cells to attach to and move on the substrate by means of the interaction between focal adhesions and actin filaments (Parsons et al. 2010). Considering its involvement in cell movement and adhesion, actin is a relevant indicator of sub-cellular structure and thus overall cell shape. An interesting experiment further supporting actin importance has revealed that, among the cytoplasmic filaments, actin is the more sensitive component for conveying mechanical and topographical stimuli and turn them into shape changes (Nathan et al. 2011).

In the present work actin filaments distribution gave a clear qualitative indication about cell orientation and morphology on ES PCL fibres; however, nuclei shape and orientation were further exploited quantitatively to find a correlation between cell orientation and fibre arrangement. Cell nuclei exhibited an elongated shape on aligned and semi-aligned fibres, compared with random fibres, and were able to re-orient according to the substrate topography features. Nuclei and actin filaments evaluation demonstrated that both these sub-cellular structures presented an elongated morphology and oriented consistently to the aligned and semi-aligned fibre pattern.
Other studies have confirmed, by means of quantitative methods, that in most of the cases there is a high correlation between nucleus and cell body shape (Raghunathan et al. 2013; Dalby et al. 2003). Even though it was also noticed that, on microgrooves, the whole cell body is more aligned than the nucleus (Dalby et al. 2003), which is stiffer and less prone to deformation (Raghunathan et al. 2013), the nucleus was adopted as marker of cell shape in the present work because of its sharp fluorescent signal, conferred by Hoechst 33342 dye, and well-defined boundaries. As a matter of fact, in fluorescence micrographs, nuclei appeared with a well-defined, constant shape, uniform colour and high contrast in respect to the background whilst cytoskeleton was characterised by irregular shape, lower contrast and inhomogeneous intensity.

In the present work, nucleus morphology investigation was performed to evaluate cell alignment and elongation degree. The significant difference between the main direction of cells grown on random, semi-aligned and aligned fibres, confirmed that ES PCL fibres are able to affect cell orientation. Also, the evaluation of the circularity values demonstrated a difference in nuclei elongation when compared with the effects of random fibres, which demonstrated higher circularity, but no difference was found between semi-aligned and aligned fibres. The absence of a significant difference is consistent with the results of fibre alignment analysis, performed with a different, automated, computer-aided tool, which demonstrated only a small not significant difference between aligned and semi-aligned patterns (section 5.1.4). This small difference was not actually expected to be reflected on nucleus elongation since the nucleus is shaped by a cascade of reactions, at molecular level, and different substrate interactions (i.e. ES PCL/cell membrane, cell membrane/cytoskeleton and cytoskeleton-nucleus) which might reduce the extent of influence of the initial triggering cue provided by fibre alignment (Buxboim et al. 2010). In addition, in this thesis, it was proved that cell nucleus orientation is consistent with fibre orientation. However, this was demonstrated in indirect way since two different tools had to be adopted: SEM to correlate cell body to the direction of the fibres underneath and fluorescence...
microscope to assess cell nuclei orientation (section 6.2.1). Thus future work will include the direct evaluation of fibres and nuclei direction. This objective can be achieved by incorporating fluorophores into ES PCL fibres hence making them visible together with stained nuclei.

In recent years research begun focusing deeply on the role of cell nucleus and its interactions with the cytoskeleton and the surrounding environment (Buxboim et al. 2010). It is now well-established that nucleus acts as mechanosensor modulating gene transcription and ultimately cell fate (Mammoto et al. 2012; Thomas et al. 2002; Vergani et al. 2004). The nuclear lamina, the protein network inside the nucleus, regulates chromatin organisation and interacts with the cytoskeleton. It has been shown that the plasticity of the nucleus is modulated by the equilibrium of forces between the lamina and the cytoskeleton (Isermann & Lammerding 2013; Tsimbouri et al. 2014). Interestingly it has been found that the so-called pre-stress, induced by the cytoskeleton fibrillar network, depends on cell differentiation state and affects nucleus shape (Mazumder & Shivashankar 2010; Mashinchian et al. 2014). In addition, it has been revealed that nucleus, along with the cytoskeleton, is strongly affected by shear stress. Yu et al., by studying the endothelium of the vortex vein system, observed different endothelial cells nucleus shapes depending on the locations, characterised by different flow patterns and consequently shear stresses (Yu et al. 2013). In-vitro, sub-nuclear components of HUVEC and HeLa cells have been tracked demonstrating nucleus internal rearrangements upon different shear stress regimes (Booth-Gauthier et al. 2012). Nuclear rearrangement has been hypothesised to modify transcription factors access to specific DNA regions thereby affecting gene expression (Booth-Gauthier et al. 2012). The influence of shear stress is of particular interest because, as it will be discussed in section 7.4.1, this type of mechanical stimulus is deemed to play an important role in determining hMSCs microenvironment within the artificial replacement.

Once the influence of specific external stimuli such as topography and mechanical stimulation on differentiation is fully elucidated, the analysis of cell nucleus morphological features can be
adopted as a quick tool to monitor cell fate. The author of the present thesis believes that imaging of the nucleus may bring advantages over whole cell or fibrillar network analysis, thanks to the nucleus regular shape, neat boundaries and high contrast.

**Focal adhesion distribution on ES PCL matrices**

Focal adhesions are protein assemblies across cell membranes that mediate the connection between the ECM and the cytoskeleton. They are responsible for cell adhesion, migration and force transmission (Chen et al. 2003). Recent studies revealed that vinculin is one of the most important proteins that form focal adhesions, binding to other focal adhesion constituents such as paxillin and talin (Gallant et al. 2005; Carisey & Ballestrem 2011). Furthermore vinculin has been found to bind to actin thereby playing a role in force transmission and cell shape modulation (Humphries et al. 2007; Parsons et al. 2010). Vinculin has been selected in a number of studies as a target for focal adhesions identification to investigate cell adhesion (Whited & Rylander 2014; Van Tam et al. 2012). This strategy was applied herein as an additional tool to study the effects of topographical feature of ES PCL matrix to cell adhesion.

An evident correspondence between cell shapes outlined by vinculin and actin was detected. This was somewhat anticipated as specific binding sites on vinculin have been found to hold strong affinity for actin filaments (Humphries et al. 2007; Janssen et al. 2006). As it was observed on cells grown on treated tissue culture plastic at the bottom of multiwell plates, vinculin is clearly detectable as dots which identify focal adhesion (Figure 6.5 B,E and K) (Carisey & Ballestrem 2011; Gallant et al. 2005; Lavenus et al. 2011). The comparison between the two random ES PCL matrices showed no clear difference in vinculin distribution pattern or fluorescence intensity, consistently with what observed by Whited et al. who found similar vinculin synthesis on matrices with random fibres of different diameters though only in the range 100-300 nm (Whited & Rylander 2014). In contrast, on aligned fibres, vinculin proteins appeared to be arranged in a denser configuration, as revealed by the more homogeneous intensity distribution. Elsewhere
higher vinculin synthesis was found on cells growing on aligned fibres in respect to random ones (Whited & Rylander 2014). Nevertheless, in the present work the protein quantity could not be measured and, in the author’s opinion, vinculin pattern could be possibly ascribed to the smaller overall membrane surface area of the extended cells on aligned fibres which makes vinculin single units closer to and less distinguishable from each other. Vinculin single units were also clearly detectable on cells spread on flat tissue culture plates, with no specific oriented pattern, similarly to what found on isotropic random fibres (Humphries et al. 2007; Van Tam et al. 2012). Aligned fibre arrangement could lead cell membranes to adhere for a higher percentage of surface area to each fibre, as fibres develop in the same direction and have a higher packing density and lower porosity (Doustgani et al. 2012; Moffat et al. 2009). This is less likely to happen on random matrix, with higher porosity, which makes cells extend their protrusions from one fibre to another in a bridge-like fashion.

**HMSCs orientation is consistent with fibre orientation**

SEM is a powerful imaging tool that allowed studying the shape of cells cultured on ES PCL layers. Influence of fibre arrangement on cells, already assessed with fluorescence optical microscopy, was confirmed. Nevertheless, compared with fluorescence microscopy, SEM evaluation added important information that is the consistency between fibre and cell orientation. As a matter of fact, fluorescence images, whilst allowing to detect cell orientation, do not let properly distinguish the fibrous substrate thereby leaving uncertainty whether their direction is in agreement with the orientation of attached cells.

HMSCs seemed to adhere tightly to the fibres thanks to the large number of protrusions (or filopodia) extending from cell bodies. Regardless fibre arrangement, in most of the cases protrusions were found to co-localise with ES PCL fibres and push out in the same direction of the fibres. This is especially evident on semi-aligned fibres where cell bodies, characterised by bipolar shape, are already oriented in the main fibre direction. The predominance of protrusions
extending parallel to the fibres, rather than bridging from one fibre to the other in transverse direction, may explain the adaptation of cells to the substrate topography. The protraction of protrusions is the mechanism by which cells sense the substrate and anticipate the direction of cell motion (Heckman & Plummer 2013). Filopodia are essentially membrane extensions where focal adhesions are located. The interaction of focal adhesions with the substrate triggers a cascade of interactions involving integrin proteins and cytoskeleton. The cytoskeleton, and subsequently the whole cell body, modifies its shape according to the information obtained at the membrane edges (Geiger et al. 2009). According to the description of Heckman et al., during environment exploration, filopodia retract upon repulsive target sensing. Conversely, if the target is attractive, attachment process begins on the protrusion, thus favouring cell spreading towards the target (Heckman & Plummer 2013). This model explains the finding of the present work, where hMSCs were observed extending filopodia parallel to ESPCL fibre directions. Filopodia, upon substrate exploration, encounter more favourable conditions when attaching along the fibres, therefore inducing the cytoskeleton to extend in the same direction. On random fibres the protrusions extend in all the directions as ESPCL fibres directions are almost equally distributed over 360°. Hence cells on random fibres assume polygonal shape with central symmetry and no specific orientation. On semi-aligned fibres the adhesion process essentially develops in the same way; however, with the presence of anisotropic structure to certain extent, cells extend along the predominant fibre direction, eventually assuming bipolar shape.

Filopodia also allow cells to establish physical contact with each other. On random fibres contact between cells occur in all the directions, consistently with their homogeneous distribution and polygonal shape. On semi-aligned fibres cells were arranged in rows; however, filopodia, allowing cell-to-cell contact, were mainly found between cells in the same row rather than between cells in parallel rows. The development of contacts between cells in the same, rather than in adjacent, rows recalls the interaction of cells established in anisotropic tissues like tendons (McNeilly et al. 2016).
1996) and the AF itself (Bruehlmann et al. 2002) which may indicate the propensity of hMSCs to arrange in specific ordered pattern, driven by the topographical cues provided by ES PCL substrate.

Regarding the lack of cells on aligned fibres specimens observed with the SEM it was hypothesised that the small size of the specimens did not allow to capture areas of high cell density, contrary to what observe with the fluorescence microscope that accommodates larger specimens. Since the number of cells was also reduced on semi-aligned fibres compared with random it is possible that the sample treatment for SEM observation was detrimental for cell attachment, in particular on aligned fibres. Aligned specimens demonstrated in fact to be very weak when stretched perpendicularly to the main fibre direction, even weaker than random arranged fibres (section 5.4.1), thus their handling could have led to cell detachment.

7.3 **HMSCs viability and adhesion on ES PCL double-layers**

7.3.1 **ES PCL matrix thickness and fibre diameter influence hMSCs viability**

Several experiments were performed with the aim of investigating the features of ES PCL matrices that affect cell viability, namely fibre diameter and matrix thickness. Tests were performed to compare cell viability on mono-layers, with cells on top, opposed to double-layers, with cells in between. It has been revealed that cells behave differently when cultured as mono-layer (e.g. standard cell culture flasks) or in 3D environment (Kozlowski et al. 2009; Vergani et al. 2004). According to the results illustrated in section 6.4, it can be assumed that a double ES PCL layer acts as a 3-dimensional environment as there is evidence of cell migration from one layer to another. Also, the test was relevant considering the final goal of the project that is building a cellularised model of the AF.
The experiment demonstrated that the sample with lower viability (A-0.60_Double) had fibre diameter about three times smaller and higher thickness (232.22±26.35 µm) compared with its counterpart (88.67±22.55 µm). The combination of a smaller fibre diameter and higher thickness, may have hampered the diffusion of nutrients and waste metabolic products to and from inside the double-layer. Therefore, the lack of nutrients and wastes abundance in the cell rich area could have been an impediment for hMSCs as indicated by the low cell viability observed.

To further elucidate the contribution of fibre diameter and thickness to the survival of cells within double-layers, additional work on thickness contribution was carried out as illustrated in section 6.6. The AB assay demonstrated lower value of cell viability for the thicker sample whilst the fibre diameter is controlled, thereby partially confirming the results of the previous experiment where viability was found to be dependent on both layer thickness and fibre diameter. The hypothesis of reduced viability for cells seeded between thick layers due to impaired nutrients transit was corroborated by the experiment with acellular samples. The experiment demonstrated significantly higher diffusion constant for mono-layer compared with double- and triple-layers. Diffusivity has been revealed to be negatively affected by thickness also on electrospun polyurethane fibres (N. Wang et al. 2013) and non-woven polyester matrix (Zhu et al. 2014). The values of the diffusion constants found here were lower than those computed by Wang et al. who monitored the concentration change of glucose. Glucose, being a molecule smaller than resorufin, may diffuse more slowly as D is inversely proportional to the molecule radius according to Stokes-Einstein equation:

\[
D = \frac{kT}{6\pi\eta R}
\]

where \( k \) is the Boltzmann constant, \( T \) the absolute temperature, \( \eta \) the viscosity of the solution and \( R \) the hydrodynamic radius of the molecule.
In addition, a symmetrical test was performed also to examine the influence of double-layer fibre diameter on cells seeded between them, maintaining constant double-layer thickness. Higher viability for the sample with the smaller fibres was noticed, though the level of significance was lower than 0.05. In this test it was assumed that cell viability is affected by two variables: the mass transfer and the capability of cells to proliferate on a specific substrate morphology. Both these variables depend on fibre diameter. Therefore, to isolate the contribution of mass transfer, the influence of fibre diameter alone was studied by evaluating the viability of cells on monolayers with different fibre diameters. The absence of fibre diameter influence on the response of cells seeded on different mono-layers suggests that the viability difference on double-layers is due to the variation in mass transfer, consequence of the different fibre size.

The diffusivity test repeated with an acellular model demonstrated that small fibres are actually characterised by higher diffusion constant. This is consistent with the results obtained on cellularised scaffolds, corroborating the hypothesis that lower cell viability can be ascribed to impaired nutrients transit across the layers with big fibres. The correlation found between small fibres and higher diffusion constant is apparently in contrast with the evidence provided in literature where permeability was found to increase with fibre diameter (Hussain et al. 2010; Sell et al. 2008) and therefore pore size (Soliman et al. 2011; Milleret et al. 2011; D. Li et al. 2006; Zhu et al. 2014). However, despite claiming that models predict a higher diffusion constant with higher porosity, Wang et al., found conflicting results in diffusivity test, obtaining an unexpected low diffusion constant for membranes with large pores (N. Wang et al. 2013).

Further to the well-known correlation between fibre diameter and porosity (Hussain et al. 2010), fibre density may be a parameter that can affect permeability. It has been stated that different electrospinning set-ups can influence the deposition area, therefore the number of fibres per surface area unit (fibre density). It has been revealed that a different deposition area can be achieved by a changing feed rate (Teo et al. 2007), electrical potential (Supaphol & Chuangchote 2008) and polymer and ions concentration (Ramakrishna et al. 2005; Supaphol & Chuangchote
The small and big fibres used for the permeability test have been fabricated by significantly increasing PCL concentration from 11 to 16% whilst PCL increment for the fabrication of the layers used in the correspondent test with cells was from 12 to 17%. As it has been shown that concentration increase leads to smaller deposition area (Supaphol & Chuangchote 2008; Ramakrishna et al. 2005) it is possible that, further to fibre size increase, an increased fibre density was obtained. Higher fibre density may thereby have contrasted the increase in pore size due to higher fibre diameter, leading to diminished permeability. Nevertheless, the combined contribution of fibre diameter and density to the overall matrix porosity needs further investigation. Nano-computed tomography, once available at the adequate resolution, may be useful for morphology and porosity investigation across the 3-dimensional structure of ES PCL scaffolds (Cipitria et al. 2011).

In summary, besides matrix thickness, fibre diameter demonstrated to be an additional parameter that can affect cell viability by means of nutrients transit impairment. However, the combination of the results on matrix morphology, obtained with and without cells (sections 6.6 and 6.8), suggests a more significant role of thickness over fibre diameter, at least in the ranges investigated in the present work. Repeating the experiments with the aid of sensors able to locally measure gases and compounds dissolved in medium is believed to be necessary to clarify the correlation between matrix morphology and nutrients concentration.

7.3.2 Influence of ES PCL multi-layer assembly on hMSCs viability

The fabrication and characterisation of cellularised double-layers, as described above, permitted to design a multi-layer which was evaluated by means of AB and live/dead assay. The comparison of viability measured on mono-layers, before and after multi-layers assembling and disassembling, demonstrated no recovery, which is unexpected on the basis of the results obtained on double-layers described in the previous section. This might be due to nutrient limitation, experienced by
cells, which did not occur on the top layer (D) in relevant manner. Migration of cells from one layer to another, a factor that could have had an influence on single layer viability, was not massively observed. Extensive migration phenomenon, in fact, could potentially alter the number of cells present on each layer therefore modifying the viability of a single layer over time.

Cells observed with fluorescence microscope revealed a higher percentage of living cells on the top layer, consistent with the higher viability assessed with AB assay prior to microscopy analysis. The outcome of the experiment discussed in this section adds evidence to the matrix thickness issues already highlighted in double-layer cell viability investigations. Furthermore, the presence of a higher percentage of dead cells on the bottom layer can be related to the low viability, obtained for samples with thick double-layers. AB assay measures cell viability but a certain viability value may be the result of low cell number with intense metabolic activity or large cell number with low metabolic activity. It has been revealed that a linear cell number-signal relationship is valid only for specific concentration ranges and metabolic activity does not always correlate with cell number in time-dependent tests (Nakayama et al. 1997; Al-Nasiry et al. 2007; Quent et al. 2010; Corral et al. 2013).

These findings suggest that the low viability measured on the bottom layer is due to the low number of living cells rather than to the reduction of the metabolic activity of each individual cell.

Sekine et al. studied cell viability by stacking a variable number of cell sheets on the bottom of culture plates, noticing that cell survival is impaired beyond 40 µm depth (Sekine et al. 2011). In the same work cells were cultured by using special inserts, similar to the Cell Crown adopted herein, which allowed to provide nutrients both from the top and the bottom. Viability performance improved but cell concentration at the bottom was found to be lower than those at the top and the maximum thickness of cellularised layers was approximately 100 µm (Sekine et al. 2011). In the experiment reported in the current study a more relevant viability drop (from 81 to 71% of living cells) was noticed at approximately 180 µm (between the two deepest layer A and
B), which might be explained with the different experimental conditions (layers number, seeding density, cell type, type of viability assessment). Using a collagen scaffold Radisic et al. correlated the viability variation across a cellularised matrix to oxygen concentration finding a 60% live cardiomyocytes at 500 µm depth, where oxygen concentration at such depth was assessed to be one order of magnitude lower than in-vivo (Radisic et al. 2006). In the same article the characterisation of a cellularised matrix, suspended in flowing medium revealed that oxygen concentration can be kept at high level both at the top and bottom of the sample, contrary to static conditions (Radisic et al. 2006). The measurement of the gradient of molecules concentration in-vivo is challenging but there is evidence that diffusion is the mechanism that contributes most to mass transfer, compared with convection (Urban et al. 2004; Grunhagen et al. 2006; Jackson et al. 2012). However computer-aided simulations have revealed that disc volume changes, due to mechanical loads, might have an influence on solutes transport (Etienne et al. 2011), in particular in the case of high molecular weight molecules (Ferguson et al. 2004). In addition to these findings, mass transfer can be improved in in-vitro dynamic culture conditions provided by bioreactors, via medium perfusion and hydrostatic pressure (Bueno & Bilgen 2005; Pörtner et al. 2005; Tiğli et al. 2011), showing that diffusion limitation can be overcome by means of enhanced convection mechanism.

As a final remark, the empirical evidence provided by the experiments discussed in this section suggests that using ES PCL matrix may have intrinsic limitation when applied for regenerative medicine purposes. Despite the encouraging performance of ES PCL matrix, care must be taken when adopting such scaffolds to fabricate artificial replacements. To address the mass limitation problems assessed here the versatility of the electrospinning technology may be of help as the adjustment of process parameter would allow fine-tuning of ES PCL matrix morphology. In particular it would be interesting to study more in depth the balance between fibre diameter,
alignment and density and layer thickness, and how these parameters affect cell adhesion, penetration and mass transfer.

The experiment discussed in the present section was conducted by overlapping random fibre matrices therefore, stacking aligned fibres at 60° angle would allow study an arrangement closer to that of the natural AF structure. In addition another crucial parameter is the overall construct thickness that must be increased as the natural AF has a size of the order of magnitude of centimetres. Here a decrease of cell viability was assessed to be 70% at 180 µm depth, which can be considered acceptable, but it is likely to be further reduced within a thicker scaffold.

A consideration that deserves further investigation is the balance between single layer thickness and the total number of layers. A construct with 15 to 25 layers (Jackson et al. 2012), each one with its specific orientation, would have the same number of lamellae as the AF. This number could be increased in order to achieve a more homogeneous cell distribution within each artificial lamella, overcoming the lack of cell penetration of ES PCL layers. By applying this strategy, inside each lamella cells would be seeded between layers with the same fibre angle. Nevertheless such high number of layers certainly poses challenges regarding ES PCL matrix handling. Conversely using a lower, more manageable, number of layers, would not mimic as closely the natural AF and would make processing PCL into ES fibres difficult. Each AF lamella is 0.38-0.76 mm thick (Holzapfel et al. 2005), thus electrospinning a matrix with similar or higher thickness poses challenges from the manufacturing point of view. It is known that long electrospinning sessions to achieve high thicknesses cause the alteration of the electric field over time, due to the presence of a thick dielectric layer represented by the fibres deposited on the collector. The alteration of the electric field may therefore influence the morphology of the fibres, compromising the uniformity of the matrix across its depth (Smyth et al. 2014).

Nevertheless above is presented a borderline case where the number (15 to 25) and thickness (0.38 to 0.76 mm) of lamellae is comparable to those found in the natural AF. A scaffold not
intended for whole AF or IVD replacement but to aid AF repair would probably need fewer and less thick layers.

7.3.3 **HMSCs viability is maintained in hypoxic conditions**

In the present work it was demonstrated that hMSCs are able to survive in 2% O₂ environment, a concentration compatible to that found in the IVD *in-vivo* (Mwale et al. 2011). *In-vitro* studies on AF cells have confirmed that these cells are not negatively affected by hypoxic conditions, as revealed by the maintained capability to live and physiologically synthesise GAGs (Mwale et al. 2011; Feng et al. 2013). Besides IVD cells, which reside in the largest avascular structure of the body, MSCs that dwell in the bone marrow have been found to be subject to a similar environment concerning the O₂ pressure (Hawkins et al. 2013). The role of hypoxic conditions has been further investigated and it has been shown that hMSCs are able to proliferate and maintain a greater differentiation potential compared with hMSCs cultured in normal O₂ conditions (Grayson et al. 2006) and that the differentiation capability is not impaired towards adipogenic and chondrogenic (Jin et al. 2010). In addition it has been revealed that hypoxic conditions are more effective in inducing MSCs differentiation towards NP cells, compared with normoxic conditions (Ni et al. 2014).

The researches mentioned above clearly demonstrate that hMSCs can be successfully cultured in hypoxic conditions and the results revealed in the present thesis further support these findings. No viability difference was noticed when culturing hMSCs in normal and low O₂ conditions. Within the present project this result is relevant for two reasons. The first is that hMSCs confirmed their suitability for the application in AF tissue engineering as their metabolic activity was not impaired by hypoxic conditions. The second reason is that this result would aid the interpretation of the experiment outcomes of cell viability in double- and multi-layers. The unchanged cell viability in hypoxic conditions suggests that the impaired viability of cells cultured within specific ES PCL layer configurations may be not related to lack of O₂ but to lack of other nutrients or
accumulation of waste products. Deschepper et al. demonstrated that MSCs survive to hypoxic conditions given that adequate glucose supply is provided (Deschepper et al. 2011). This suggests that future studies may be profitably directed to primarily investigate glucose concentration and metabolism in ES PCL multi-layer model.

7.3.4 **HMSCs-enabled ES PCL layer integration**

In the present work double- and multi-layers were overlapped in a variety of fashions with the aid of devices (i.e. Cell Crowns) that allowed maintaining a tight contact between all the layers. The efficacy of this strategy was proved by the integration of layers observed during manipulation and the transmigration of cells between the layers assessed by fluorescence imaging techniques. This achievement is relevant in the view of the development of an implantable AF artificial replacement. In the light of the renowned lack of cell penetration in ES PCL individual layer, cell transmigration would allow a homogeneous colonization of the 3-dimensional environment provided by the multi-layer configuration. Upon transmigration cells demonstrated to re-orient according to the topographic features of the layer they moved and attached to. Despite this positive achievement which anticipates that ES PCL multi-layer are suitable to build an angle-ply cellularised structure, a further step is required to improve biological and physical properties of ES PCL multi-layer and make it closer to the natural AF. Bruehlmann et al. demonstrated that in the interlamellar space of AF cells do not have the same elongated shape as cells residing within each lamella but rather have more rounded shape (Bruehlmann et al. 2002). This difference is deemed to be due to the interlamellar matrix which lacks the structure of the lamellae. The interlamellar matrix has been revealed to be mainly composed of proteoglycans with no specific orientation (Scott et al. 1994; Postacchini, F; Bellocci, M; Massobrio 1984). According to what observed, lack of differentiation into AF cells and insufficient culture time in static condition adopted here might have prevented significant deposition of ECM even though direct assessment was not carried out. It has been revealed that ECM synthesis (GAGs and collagen) can be
enhanced by means of mechanical stimulation (Hee et al. 2010) and by growth factors supplementation (Janjanin et al. 2008). As shown in section 6.10.3 the strategy pursued in the present work is the application of mechanical stimulation of hMSCs seeded between ES PCL layers by means of bioreactor.

Toluidine blue further confirmed cell transmigration as it allowed to assess cell presence (Sridharan & Shankar 2012) on hMSCs seeded between double-layers. Preliminary tests on mono-layer had revealed that no blue hue was retained outside the circular seeding area defined by the Cell Crown therefore confirming that the stain was due to cell presence (Sridharan & Shankar 2012; Johnstone et al. 1998; Kisiday et al. 2009). Inspection by the naked eye demonstrated intensity differences across the cellularised surface. This indicates that cell distribution is not perfectly uniform. The study of double-layer cross sections and inner surfaces clearly revealed the presence of dark blue cell bodies at the interface, within a diffused lighter blue, that might be due to some ECM deposition (Balguid et al. 2009; Gruber et al. 2008; Schätti et al. 2011). Further investigation is required to confirm the presence and evaluate the nature of synthesised matrix. Considering the application that the ES PCL matrix is designed for, it would be of particular interest the quantification of GAGs and collagen, which are the major constituents of the AF, and their relative ratios.

Eventually cross sections were analysed by means of SEM following a novel approach. The images confirmed the presence of cells not only adherent to both the inner surfaces but also acting as a bridge between them. This proves that ES PCL double-layers are able to provide a 3-dimensional environment for hMSCs, closer to the natural one in respect to mono-layer.

The contact guidance phenomenon confirmed that hMSCs are able to adopt an elongated morphology along the fibres similar to that of AF cells observed in-vivo (Baer & Setton 2000; Errington et al. 1998). The evidence that substrate and cell shape are factors intimately related to cell differentiation (J. Zhu et al. 2010; Gimble et al. 2008) further supports the adequacy of the
substrate developed herein for AF tissue engineering applications. In addition it has been demonstrated that cell orientation affects ECM synthesis; Wang et al. demonstrated that only cells on oriented substrate synthesise oriented collagen whilst on random fibres only disorganized collagen is produced (Wang et al. 2003). Inducing the synthesis of oriented collagen fibres, similar to the arrangement observed in natural AF lamellae, would be a further step towards the fabrication of an artificial AF replacement. According to the tissue engineering strategy an implanted scaffold, during its degradation, should be replaced by cells and ECM. The decreasing mechanical performance of the scaffold upon degradation is expected to be compensated by the newly synthesised ECM. In the body ECM is synthesised in order to respond in the optimal way to external mechanical stimuli as revealed, for example, by the longitudinal collagen fibres in tendons (Lake et al. 2010) or in bone trabeculae arrangement (Gdyczynski et al. 2014; Frost 1994). However the remodelling process may require a long time, therefore, facilitating cells to synthesise ECM in-vitro, by providing topographical cues, would allow obtaining a somewhat functional artificial replacement ready prior to implantation. This more efficient artificial replacement development would certainly be beneficial for the patient thanks to less time consuming hospitalisation and recovery.

7.4 IVD-like constructs for AF tissue engineering

7.4.1 Construct fabrication process and mechanical properties characterisation

Agarose discs were fabricated with the objective of providing the IVD-like construct with a core able to apply radial stress to the ES PCL matrix, acting as AF equivalents, under confined compression. The outcome of the NP-like core manufacturing was positive as the small clear cylinders obtained were homogenous in terms of morphology and their texture stiff enough to be handled without being damaged. However mechanical tests conducted on IVD-like constructs
built with the agarose discs demonstrated higher variability compared with agarose discs alone: whole IVD-like construct exhibited higher standard deviation of the modulus under compression, due to the complex steps involved to wrap the ES PCL layer around the agarose disc. The mechanical behaviour of agarose discs alone proved to be different than that observed on IVD-like constructs. The higher compression modulus and strain at UTS measured on IVD-like constructs demonstrates the interaction between agarose and ES PCL. It is hypothesised that this connection enables the compression exerted on the IVD-like construct to be transmitted to the ES PCL outer layer, therefore applying tensile and shear stress on the cells attached (Figure 7.7). The test carried out on the IVD-like construct allow anticipating that a similar transmission of mechanical stress would occur also when applying the ES PCL layers to aid natural AF tissue repair. The contribution of the outer layer to the mechanical properties, within an IVD-like construct, was also assessed by Wan et al. and Lazebnik et al.; interestingly the latter adopted different agarose and PCL arrangement but obtained values of the same order of magnitude (Lazebnik et al. 2011; Wan et al. 2008).
Another important difference is the environment where the mechanical stress is conveyed. The IVD is subject to compression by means of the vertebrae bottom and top surfaces which are not flat (Panjabi et al. 1992) therefore leading to not uniform stress distribution in the IVD. Conversely the platens of the bioreactor adopted herein are perfectly flat.

In addition confinement does not originate only from the vertebral bodies but also from the tissues laterally surrounding the IVD. To the author’s knowledge there are no experiments carried out on IVD in confined compression, possibly because of specimen variable size. Conversely portions of NP of definite size have been tested in both unconfined and confined compression revealing a modulus from few kPa (Cloyd et al. 2007; Recuerda et al. 2011) to 0.31 MPa (Périé et al. 2005). Likewise, it is anticipated that the mechanical properties of an artificial IVD-like

![Figure 7.7: Schematic representing the forces involved in IVD-like construct](image)

Axial compression

Radial stress

Circumferential stress

*axial compression.*
construct would change dramatically when tested in both confined conditions and after implantation. The performance discrepancy between testing conditions is due to the physical constraint provided by the environment configuration (i.e. confined testing chamber or surrounding tissues) and by the hydrostatic pressure that the sample exerts in response to compression. Within the IVD-like construct the hydrostatic pressure would be provided by the core of agarose. The capability of a hydrogel to withstand pressure relies on the resistance of water to leave the hydrogel and the breakage of the crosslinks between hydrogel molecules, which, for agarose, are physical crosslinks (Roberts et al. 2011). It has been revealed that, by raising agarose concentration, the modulus at compression (extrapolated from equilibrium moduli) increases (Roberts et al. 2011; Buckley et al. 2009; Gu et al. 2003) along with a decrease in water content and permeability (Gu et al. 2003; Roberts et al. 2011). Thus modification of agarose concentration can be adopted to fine-tune the hydrogel and, consequently, the IVD-like construct physical properties. Considering the whole IVD-like construct, water leaving the agarose core would have to pass through the ES PCL layer in a sort of semi-confined condition. The permeability of ES layer has been found to be $7.87 \times 10^{-12}$ m$^4$/Ns on aligned PLGA fibres (Moffat et al. 2009) thereby it is conceivable that the value for fibres of PCL, a polyester like PLGA, would not vary considerably. Despite more permeable than agarose ($6.61 \times 10^{-13}$ m$^4$/Ns at 2% concentration (Gu et al. 2003)), ES PCL fibres would resist to fluid flow, causing an increase of the whole IVD-like construct hydrostatic pressure thus increasing the compression modulus.

In summary the contribution of the ES PCL layer to the IVD-like construct mechanical compliance originates from the capability of ES PCL layer to act as physical constraint (when subject to radial stretching) for the core and from the efficacy in limiting fluid exudation from the core. It is unknown which is the relative contribution of the two mechanisms to the overall IVD-like construct mechanical properties. Nevertheless, by changing ES PCL matrix features like fibre diameter, arrangement and pore size, which affect permeability, it would be possible to further
fine-tune the mechanical properties of the artificial replacement. More information at this regard can be specifically acquired on ES PCL matrices with different characteristics by measuring, further to tensile properties, their permeability to water and culture medium and the effect on the whole IVD-like construct.

7.4.2 IVD-like constructs are suitable for compressive mechanical stimulation

IVD-like constructs were cellularised by seeding hMSCs between the ES PCL double-layer. The effect of the IVD-like constructs mounting procedure on cells was studied by comparing the viability of cells seeded between AF-like strips and those seeded in AF-like strips used to fabricate IVD-like constructs. The experiment revealed only a small loss of viability on ES PCL strip mounted on agarose core, which is likely due to samples handling procedures and to the addition of glue to seal the strips around the agarose discs.

IVD-like constructs mechanical stimulation induced a decrease in cell viability proportional to the extent of displacement applied. In particular 10% displacement demonstrated to be detrimental for cell survival: after three days no viability sign was detected. Constructs exposed to 5% displacement demonstrated some impaired cell viability. Therefore it seems that the survival of cells between the double-layers depended on the extent of displacement. A number of studies focused on the influence of mechanical stimulation parameters on cells by applying various regimens to vertebral segments in animal models. The parameters under investigation are frequency, duration and magnitude of mechanical stimulation (Ding et al. 2013). In static in-vivo conditions (mouse), it has been demonstrated that from 0 to 1 MPa a logarithmic relationship with disc cell apoptosis exists. Using excised mouse IVDs, 1 MPa mechanical stress was further confirmed to increase apoptosis compared with lower values (Ariga et al. 2003). In addition Maclean et al. found that the same stress level enhances the expression of catabolic genes in AF cells compared with 0.2 MPa (Maclean et al. 2004). These results highlight the importance of
mechanical stimulation magnitude for the maintenance of disc homeostasis. In particular the
mentioned authors emphasized the existence of an upper stress magnitude threshold.
Therefore it is hypothesised that the magnitude applied in the present study might have been the
dause of the reduced cell viability identified in the IVD-like constructs, where different stimulation
magnitudes were used. According to both evidence from literature and the tests carried out here
the magnitude of mechanical compressive stimulation on IVD and IVD-like construct affects cell
behaviour. However it is not clear how compressive stress is transferred to cells. It is believed that
NP cells are mainly subject to hydrostatic pressure due to NP isotropic structure and high water
content (Chan et al. 2011; Wilke et al. 1999). Conversely the structure of the AF, which is a ring
formed of a stack of lamellae arranged with different orientations, makes AF to be subject to
more complex combination of loads that may induce delamination (Adams & Roughley 2006).
Degeneration and rupture caused by delamination have been demonstrated to be the result of
the presence of shear stress between the lamellae and between collagen fibres within the same
lamella (Iatridis & ap Gwynn 2004). It is expected that, upon disc compression, the angle of
collagen fibres of each lamella relative to the horizontal plane decreases and that a similar effect
would be expected in ES PCL layers arranged around the agarose core (Figure 7.8).
As a consequence the angle relative to adjacent lamellae changes too, imposing considerable shear stress on cells at the interface. Using a double-layer model Driscoll et al. demonstrated the higher shear stress induced by adopting the AF lamellar configuration (60° angle shift) on ES PCL double-layer, compared with double-layers with parallel fibres (Driscoll et al. 2013). Shear stress has been shown to be an important stimulus that can modify the expression of genes involved in formation of integrins, implicated in cell attachment to ECM (Glossop & Cartmell 2009), and guide cell differentiation (Schätti et al. 2011). With regard to vinculin it has been found to be synthesised in higher amount upon shear stress, demonstrating the role played by focal adhesion in the regulation of cell adhesion adaptation to external stimuli (Tan et al. 2010; Girard & Nerem 1995). However, for practical reason, shear stress has usually been imposed by applying fluid flow, which may exert different effects compared with the shear stress induced by two ES PCL layers, arranged in an IVD-like construct, sliding against each other.
A better understanding of the role of mechanical stimulation magnitude can be provided by opportune computational simulation. Guerin et al. demonstrated the high non-linearity of AF mechanical response and the relevant contribution of fibres shear stress to whole disc mechanical performances (Guerin & Elliott 2007). Conversely, as stated by the authors, the interactions between adjacent lamellae were not included in the model. On ex-vivo models shear strain and stress were demonstrated to change as function of load applied on the IVD (O’Connell et al. 2011), and both position and load direction (Fujita et al. 2000) respectively. These results show that accurate AF modelling should include the anatomical structural gradients like the change of fibre lamellae angles and increased water content proceeding from outer to inner AF (Holzapfel et al. 2005; Le Maitre et al. 2007). However, due to practical limitation, computational model usually assume uniform anatomical characteristics. Goel et al. designed a complete vertebral segment modelled with finite element model that specifically included inter-lamellar shear stress (Goel et al. 1995). The model demonstrated shear stress to be in the range 135-2250 kPa depending on axial load and region (higher stress in the inner postero-lateral region). Also, the presence of cracks and degeneration were found to amplify the delamination effect of shear stress (Goel et al. 1995). Hollingsworth et al. fitted the solutions of different AF computational models to the experimental dataset published (Hollingsworth & Wagner 2011). In general they found good agreement between models results and experimental data that was used for non-linear regression but further simulation demonstrated limitation in the prediction of shear stress data that was not included in the regression (Hollingsworth & Wagner 2011).

It is believed that the aforementioned computational models should be applied to enhance the understanding of natural AF and eventually to improve the IVD-like construct developed in the present work. Modelling the IVD-like construct would be simpler than modelling entire vertebral segments as the IVD-like construct has a less complex structure: it has central symmetry and its elements have almost uniform properties. The data obtained from the mechanical tests could be
included in the model with the aim of estimating stress distributions across the construct. Of particular interest is the investigation of the distribution of shear stress and strain at the interface of the ES PCL double-layer in order to estimate the force exerted on cells seeded between.

The results presented in this thesis demonstrated diminished viability for cells seeded in IVD-like construct stimulated with 10% compared with 5% and 0% displacement. In addition a lower number of cells and altered cell distribution was detected on mechanically stimulated samples compared with the control. Nevertheless low cell number was hypothesised to be related not only to the magnitude of the mechanical stimulus conveyed but also to the duration, possibly too long compared with other studies using bioreactors (Baker et al. 2011; Thorpe et al. 2010; Hee et al. 2010; See et al. 2011; Korecki & Kuo 2009; Ching et al. 2003; Chan et al. 2011); however, such stimulation pattern was selected to guarantee a sufficient total amount of stimulation time, considering the short duration of the overall experiment.

Besides mechanical stimulation parameters another important difference compared with the characterisation tests of natural IVDs is the usage of different cell types: hMSCs instead of AF cells. Primary IVD cells were found to be reliable in a limited number of in-vivo IVD artificial replacement trials. Mizuno et al. implanted an IVD artificial replacement in rat ectopic region whilst Bowles et al. replaced a natural IVD in rodent spine (Mizuno et al. 2004; Bowles et al. 2011). Despite showing positive biological markers, both devices lack the anisotropic stacking structure that would closely resemble the natural AF. In addition, primary cells are not deemed to be the optimal solution at clinical level because of the low proliferation potential and risk of damaging healthy body sites upon harvesting (Grad et al. 2011; Kuh et al. 2009). However, usage of hMSCs set an even higher challenge as their exploitation involves cell differentiation, an additional step required, by providing the proper biochemical (Steck et al. 2005), topographical (Kishore et al. 2012) and mechanical cues (Bosworth et al. 2014). See et al., stimulating an IVD-like assembly
seeded with hMSCs, reported an increase in viability followed by a drop. Interestingly the stimulation regime was designed to adopt an increasing displacement suggesting a relationship between this parameter and cell viability, consistently with the outcome obtained in the present work (See et al. 2011).

The aim of the preliminary experiment revealed here was to demonstrate that a novel IVD-construct can be mechanically stimulated; with further optimisation, this technique will allow hMSCs proliferation and differentiation towards fibrochondrocytes phenotype for AF regeneration. Furthermore it would be remarkably attractive the accomplishment of such goal without growth factor or other biological molecules supplementation.

7.4.3 Limitations of the mechanical stimulation process using the current bioreactor

The development of a tissue engineered artificial replacement poses formidable challenges, from scaffold design to cell handling. Artificial replacement stimulation by means of bioreactor, used to induce specific cell response, adds further complexity to the process. For this reason IVD-like constructs fabrication and stimulation, object of the present section, have some limitations. The first is represented by the sealing method implemented. The glue adopted was commercially available and not developed for biological purposes. Despite no negligible effect was assessed on cell viability, the consequences on gene expression and protein synthesis are unknown. PCL is already an FDA approved material but the potential risks of any other material of the artificial replacement like the glue must be thoroughly verified according to international standards. BS EN ISO 10993-2009 standard (Biological evaluation of medical devices) has been issued to provide a guideline for the assessment of the safety of medical devices, including permanent and temporary implantable devices (ISO BS EN 2009a). Part 5 of the mentioned standard especially
focuses on the *in-vitro* verification of the cytotoxic effect of a material on cells by means of direct or indirect culture techniques (ISO BS EN 2009b). In the case of tissue engineered devices the regulations are more strict and the safety of the cells that are going to be implanted must be verified too, in compliance with ISO 13022:2012 (Medical products containing viable human cells - Application of risk management and requirements for processing practices) (ISO BS EN 2012). For future work the selection of sealants more suitable than commercial glue, such as cyanoacrylate or fibrin glue (F Heuer et al. 2008; Spotnitz 2010; Schek et al. 2011), unlikely exerting any adverse effect, is desirable. Other sealing strategies have also been taken into consideration. Suturing has been attempted in a number of preliminary tests but found to be not as effective as glue because, firstly, it did not guarantee a tight contact between the disc and the double-layer and, secondly, the sample manipulation process turned out to be too complex and time consuming to be adequately tolerated by cells. Finding the optimal sealing strategy is an issue of clinical relevance in the perspective of using an artificial replacement *in-vivo*. Along with the renowned surgical strategies that might be applied also to the AF artificial replacement (F Heuer et al. 2008; Hill et al. 2012), a number of new developed systems are under investigations such as a barbed closure devices (Bron et al. 2010), a temperature sensitive shape memory polymer (Sharifi et al. 2013) and a poly(trimethylene carbonate) scaffold sealed with a PU membrane (Pirvu et al. 2015). Nevertheless none of these methodologies was judged to be easily applicable to the scaffold fabricated herein in particular because of the scaffold small size.
8. Final remarks

8.1 Conclusions

The ageing population and the high rate of hospitalisation due to musculoskeletal diseases in the western countries is a matter of concern. The absence of decisive therapies and the re-intervention prevalence impair the quality of life of a large percentage of population. Governments must tackle high expenditure due to the direct and indirect costs associated with hospitalisation and working benefits, respectively. Thus, the demand for treatments that provide a definitive solution to musculoskeletal diseases is fairly pressing, especially considering the progressively increased life expectancy.

Tissue engineering is believed to have the potential to regenerate ruptured and deteriorated tissues by restoring the physiological structure and function. This is supposed to be achieved by delivering cells seeded or embedded within a suitable scaffold. However, no such artificial replacement, developed for IVD restoration, has received approval for clinical use to date. Studies that focus on scaffolds for IVD replacement are currently limited to the laboratory scale. Indeed, many unanswered questions must be addressed before reliable products reach the market. The success of an artificial replacement depends on properly directed cell fate, which is achieved by opportunely designing the scaffold that supports cells. Nevertheless, a huge number of variables can be tuned to modify the scaffold features; therefore, the results accomplished in different studies are of difficult comparison and are frequently incomplete or controversial.

The purpose of this thesis was to investigate some of the aspects that still require an answer with regards to scaffold design and cell response in view of aiding AF artificial replacement development.

The experimental work conducted in the first part of the thesis focused on the fabrication and characterisation of ES PCL layers. Electrospinning was the technology of choice to produce
scaffolds that replicate the anisotropic fibrous structure of the AF. Layer fabrication process optimisation demonstrated that two of the solvent systems explored (THF+DMF and CF+DMF, both in 50:50 ratio) are the most versatile and reliable to fabricate fibres that are free from defects. The process optimisation steps led to the conclusion that the fibre diameter is affected significantly by the environmental parameters. This is because the decrease of both T and RH leads to the fabrication of smaller fibres. In addition, excessive drum collector rotational speed induces air turbulence that may influence the process of fibre deposition and, subsequently, fibre pattern.

For polymeric artificial replacements, it is fundamental to be aware of the degradation kinetics in order to predict the post-implantation integrity change and prevent any significant property change to long-term storage. The results demonstrated that, in PBS at 37 °C, the main degradation mechanism is bulk erosion; moreover, UV light exposure is responsible for a further decrease in molecular weight.

The influence of PCL fibrous morphology on wettability is indisputable, as the contact angle value increases significantly, compared with solvent cast PCL. In addition, aligned fibres arrangement leads to a further increase in hydrophobicity due to its lower porosity compared with random fibres.

ES PCL fibres anisotropy is reflected in the mechanical properties with higher tensile modulus and UTS found on aligned fibres stretched parallel to the main fibre direction. From the perspective of the scaffold application inside the human body, the effect of wet environment at 37 °C was investigated to induce a decrease in tensile modulus and increase in strain at UTS. To a greater extent, the effect of wet environment emerged on fibres stretched parallel to the main fibre direction.
The second part of the thesis focused on the response of cells seeded on ES PCL layers and in laminate angle-ply multi-layer scaffolds. Through a validated imaging protocol hMSCs orientation was proved more pronounced on semi-aligned and aligned fibres. Cells seeded in ES PCL double-layers transmigrated from one layer to another; thereby replicating the laminate structure of the AF. The evidences gained from tests on multi-layers with and without cells allowed hypothesise that ES PCL layers limit mass transport and are the cause of impaired cell viability of cells seeded farther in depth.

A double-layer scaffold configuration seeded with hMSCs was adopted to develop an IVD-like model, which successfully withstands cyclic compression in bioreactor. Experiments at different strain magnitudes demonstrated that this parameter negatively affects cell viability in a proportional manner.

Under recent investigation for its supposed benefits on endothelial cells and vascular system, Mg was incorporated successfully in ES PCL fibres, in the form of nanoparticles. Such novel scaffold demonstrated to have no adverse effect on hMSCs adhesion and viability therefore showing potential for the application of Mg NPs loaded matrices in IVD revascularisation.

The adhesion, morphology and phenotype of rat primary tenocytes seeded on ES PCL layers were investigated; thus, proving that the topographical cues provided by aligned fibres permit to better maintain tenocytes extended morphology and, to a lesser extent, phenotype, compared with random fibres.
8.2 Future work

The experimental work conducted in the present project extended over several different research areas from material processing and characterisation to cell response in a variety of conditions in view of AF and tendon tissue engineering. This research increased the knowledge with regard to ES PCL characteristics and hMSCs behaviour towards the development of an AF artificial replacement. Alongside the issues addressed here, several questions arose. The author believes that some of the topics studied here are particularly worthy of further investigation.

The angle-ply structure made of ES PCL aligned fibres provides an adequate environment for hMSCs. The encouraging achievements obtained here permit speculation on the potential of such scaffolds for undertaking further research and, ultimately, the clinical pathway. Two main investigation routes are deemed deserving of special attention.

The first relates to the scaling up of the IVD-like assembly fabrication. The facilities and materials available allowed the manufacture of a small IVD-like assembly of size not comparable with that of the human IVD. Increasing the size presents several challenges; in particular, the development of a reliable method to overlap and seal a large number of angle-ply ES PCL layers, maintaining high cell survival rate. Cell survival depends on the availability of gas and nutrients. The work states clearly that ES PCL in double- and multi-layer configuration impairs mass transfer. The permeability performance of a multi-layer within the IVD-like assembly certainly needs to be investigated as mandatory step to increase the size of the artificial replacement. The size of the current IVD-like assembly may be suitable for in-vivo tests in small animal model. This mandatory passage of the clinical pathway would be useful to evaluate the artificial replacement response to the in-vivo harsh environment especially with regards to the degradation rate which is expected to be faster than that observed here in-vitro.
The second investigation route relates to hMSCs response in terms of phenotype. For the present project, hMSCs were selected because, contrary to AF primary cells, they can be harvested and expanded more easily without damaging the donor site or incurring in senescence. However, the risk of hMSCs lack of or incorrect differentiation is the main drawback for the usage of this type of cells. This study demonstrates that ES PCL angle-ply multi-layer scaffold provides a suitable environment in which cells can thrive and modify the morphology into that typical of AF cells. The next step will be the assessment of the phenotype change. To achieve this aim, it is believed that further work with the bioreactor is essential to convey the proper mechanical stimulus to drive hMSCs fate to AF cell phenotype. Therefore, it is crucial to explore different stimulation patterns as, currently, there is no consensus concerning the optimal mechanical stress configuration.

Similarly, the exploration of mechanical stimuli can be studied for the development of a tendon replacement, which requires a bioreactor able to exert stretching force, contrary to the compression bioreactor adopted for the IVD-like assembly stimulation. The author believes that this strategy may be more effective than the simple static culture to drive cell fate as it provides an environment with a higher complexity degree, closer to the in-vivo conditions.

Concerning the revascularisation of the artificial replacement the strategy of providing chemical cues was pursued. Studies on Mg effects on endothelial cells and vascular system have revealed that this element plays an important role in vascularisation processes. Therefore, the incorporation of Mg NPs in ES PCL matrix, which demonstrated no adverse effect on hMSCs viability, deserves further investigation. Specifically, future research should focus on hMSCs differentiation and its relationship with the amount of Mg. Consequently, the accurate knowledge of Mg release kinetic, which can be fine-tuned by applying opportune modification to the matrix fabrication process, becomes crucial.
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9.1 Introduction

The IVD is the largest avascular tissue in the body (Adams 2004) and the nutrient supply relies on the mass transport from the endplates and the periphery of the AF (Kobayashi et al. 2010; Urban et al. 2004). Damaged or degenerated IVDs are characterised by impaired environment and possibly cell density below the physiological level. Therefore, any attempt at cell injection or implantation of cellularised devices may fail due to the low cell survival rate in such an environment. The development of an IVD artificial replacement capable of enhancing mass transfer would have a higher chance of restoring the natural IVD.

Mg is a fundamental element for mammalian life. The total amount of Mg in humans is about 24 g and it is distributed among bone, muscles and soft tissues; accounting for 53, 27 and 19% of the total amount respectively. 0.3% is found in serum, of which the highest part is in the form of Mg$^{2+}$ ion (Jahnen-Dechent & Ketteler 2012). This metal, which is the fourth most common cation in the human body, participates in hundreds reactions as enzyme co-factor (Elin 1987). Proper Mg concentration is needed for key chemical reactions that lead to synthesis of biological components, and Mg is involved in energy metabolism (Jahnen-Dechent & Ketteler 2012). In addition, Mg has been demonstrated to directly affect cell activity; particularly within the cardio-vascular system. Maier et al. have revealed that a concentration up to 10 mM Mg is able to enhance endothelial cells proliferation and release of NO, an antithrombotic compound (Maier et al. 2004). More recently, a similar value has been demonstrated as the lower threshold, above which endothelial cell viability and migration is impaired (Zhao & Zhu 2015). The importance of Mg role in the extracellular environment and, consequently, in the vascular system development and disease, has also been highlighted in other studies (Shechter 2010; Shechter et al. 2000; Maier 2012; Maier et al. 1998;
The purpose of the fabrication of Mg embedded matrices within the present work arises from the consideration that the outer IVD and tendon are slightly vascularised; therefore, an artificial replacement with a higher neo-vascularization potential could improve the success of the implant as it would facilitate nutrients diffusion and consequently the healing process.

This section develops a novel type of ES PCL matrix with embedded Mg nanoparticles (NPs). The ultimate goal is to incorporate this matrix in the outer part of an IVD-like construct in order to promote neovascularisation at the periphery.

### 9.2 ES PCL fibres embedded with Mg NPs

Along with normal ES PCL matrices, layers with embedded Mg NPs were also fabricated. Two different Mg NPs types were selected: magnesium carbonate (US-nano) and magnesium oxide (Sigma-Aldrich), with MgCO₃ and MgO as the chemical formulae respectively. For the former, the supplier claimed 10 nm as the average diameter while the latter had nanoparticles smaller than 50 nm. Preliminary experiments were performed to gain information about their behaviour; in particular, when added to organic solvents. The main concern with NPs is that NPs tend to form aggregates due to their extremely high surface to volume ratio. This behaviour also depends heavily on the environmental condition and treatment (Hotze et al. 2010).

The usual preparation of the electrospinning solution (section 3.1.2) was adjusted as follows. NPs of either type were weighed and put in vials filled with THF+DMF 1:1 solution. Uniform NPs dispersion was achieved through heating and vigorous magnetic stirring for 24h, until uniform dispersion was obtained. Hence, PCL was added and the stirring speed lowered because of the higher viscosity of the solution. When the solution was sufficiently homogeneous in terms of viscosity and turbidity, it was ready to be electrospun. The parameters were selected according to those used to successfully fabricate fibres with not extreme diameter values and without defects. The first attempt with NPs was done by using a concentration of 0.05% wt between the NPs and the whole solution, thus
corresponding to approximately 0.33% wt between NPs and PCL mass. Subsequently, the concentration was raised to 0.5% (3.3% NPs/PCL ratio). All the experiments described in this chapter were performed in the same way for either kind of NPs, unless otherwise stated.

9.2.1 Dynamic light scattering

To investigate NPs aggregation behaviour the dynamic light scattering (DLS) technique was exploited. DLS measurements (Zetasizer Nano ZS90, Malvern) were performed on different NPs loaded solutions or solution systems. NPs concentration varied between 0.01 and 0.5% wt. Initially NPs dispersion was prepared only by stirring and heating at 50 °C the solution; however, subsequently, other strategies followed, such as sonication, addition of surfactants and their combination. Sonication was first applied for few minutes but was extended to 2h for all the dispersions. Polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) were adopted as biocompatible surfactants. Each sample was evaluated three times for a variable number of measurements, in accordance with the automatic settings of the instrument.

9.2.2 Wettability

Contact angle measurements were conducted on Mg NPs loaded specimens with the aim of checking the NPs contribution to ES PCL hydrophilicity. A comparison was made between normal and Mg NPs loaded ES PCL by following the procedure described in section 3.2.2.

9.2.3 SEM imaging

SEM study was conducted in the same way as for normal ES PCL matrices, concerning specimen preparation and machine setting (see section 3.2.1).
9.2.4 **Energy dispersive X-ray spectroscopy (EDX)**

The assessment of Mg NPs presence was carried out through energy dispersive X-ray spectroscopy (EDX), a method that allows distinguishing the occurrence of almost all the periodic table chemical elements. The elemental investigation of the present study was performed by using a 127 eV detector (XFlash 5030, Bruker) connected to the SEM apparatus already used for morphological characterisation. Data analysis was carried out with the aid of Quantax 400 software, which exploited the PB-ZAF quantification algorithm.

An ES PCL matrix with Mg NPs (MgCO$_3$) embedded was scanned allowing the association of the elements identified to the morphology features. The EDX test was launched and left running for two days.

9.3 **Biological experiments on Mg NPs loaded ES PCL matrices**

9.3.1 **Cytotoxicity test on Mg NPs embedded ES PCL**

To assess any adverse effect of ES PCL layers with embedded Mg NPs on hMSCs, a cytotoxicity experiment was set. The purpose of this test was the evaluation of cell behaviour in the presence of the degradation products that the layers might have released once immersed in culture medium. The experiment was performed by culturing hMSCs in standard 24 multiwell plates with the presence of the substrates floating in the culture medium.

Three different ES PCL layers were selected: two of them were embedded with the two different NPs types (MgO and MgCO$_3$) whereas one was normal ES PCL. The latter was used as a control, since it was already revealed as cytocompatible. The three matrix types were chosen with fibre diameter, a parameter that might affect the release kinetic, not significantly different among these samples. For each type, portions of layers were cut into a size suitable for the well and weighed to control the mass of material in each well (1.7±0.1 mg). HMSCs were expanded as per standard protocol (see section 4.1.1) and 2 x 10$^4$ cells were pipetted into each well and let adhere to the bottom for 24h.
The viability AB assay was performed in order to assess the initial cell viability (see section 4.1.1). Immediately after the test, the ES PCL layers were placed into the multiwell plates. Considering the aim of the experiment was to evaluate any effect of released Mg NPs on cells, it was decided to adopt two different strategies; one is a total medium change every 2-3 days and other is three-quarter medium change with the same pace. The rationale for this latter approach was to replicate more closely the local accumulation and transportation of products released from the matrices. Hereafter, the aforementioned strategies will be referred to respectively as “normal” and “partial change”. AB test was performed after two days after soaking the ES PCL layers and then every 5 days until day 18.

9.3.2 Cytocompatibility test on Mg NPs embedded ES PCL

After having verified that Mg NPs embedded matrices have no adverse effect on hMSCs in the time span studied (see section 9.4.5), it was decided to culture cells directly on their surface. The samples studied were ES PCL layers embedded with MgO or MgCO$_3$ NPs either; as control normal ES PCL layers and a standard tissue culture plate were used. hMSCs were cultured as per standard protocol and the layers treated as reported in section 4.1.1. Cell viability was assessed using AB assay (see section 4.1.1) 24h after seeding and then on weekly basis up to 21 days.

9.3.3 Fluorescence OM imaging

Immediately after the last viability evaluation, two layers were fixed with formalin and stained with Hoechst 33342 and Alexa Fluor-555 according to the protocols described in section 4.1.1. Images were acquired with Nikon Ti Eclipse fluorescence microscope.

9.3.4 SEM imaging

One sample per type that was not subject to formalin treatment was fixed with glutaraldehyde and dehydrated following the protocol described in section 4.1.1. One sample fragment was mounted on
the pin stub facing the cell seeded side upwards, whilst the other faced downwards. This procedure ensured at least one matrix exposed the cells to the detector.

9.4 Results

9.4.1 Dynamic light scattering

DLS analysis demonstrated that the particles dispersed within the solvents formed aggregates of diameter in the range from few hundred nanometres to few micrometres. Trials with Mg NPs dissolved with PEG or PVP and sonication of Mg NPs suspension, led to no or little improvement when compared with the negative control. Due to limited amount of time, an extensive study contemplating all the combinations between solvent, concentration, capping and treatment could not be performed.

9.4.2 Wettability

Wettability tests were conducted on different ES PCL substrates, evaluating the effect of Mg NPs addition. Figure 9.1 reports the contact angle values of the ES PCL layers with and without Mg NPs. The difference between the two ES PCL layers loaded with Mg NPs is significant (p<0.05) compared with unloaded ES PCL.
9.4.3 SEM characterisation

SEM investigation was conducted in the same way as for normal ES PCL layer; albeit with the specific aim of confirming the presence of Mg NPs. Mg NPs loaded layers were compared with specimens fabricated with the same process parameters but without Mg NPs. The first matrix fabricated by adding 0.05% wt MgCO₃ NPs (Figure 9.2) showed a different fibre morphology compared with the control sample. Along each fibre, the diameter was sometimes irregular with knots.

Figure 9.1: Chart relative to contact angle results of Mg NPs loaded and unloaded ES PCL layers. * indicate significant difference (p<0.05) compared with ES PCL.
Further investigation was conducted after manufacturing other two matrices with increased Mg NPs concentration (ten times, from 0.05 to 0.5% wt). Compared with the matrix shown in Figure 9.2 aggregates were detected more easily within the fibres. In fact the presence of more occurrences of knots on the fibres (Figure 9.3) is clear. Fibre structure is also more irregular with almost inconstant diameter along each fibre. No noticeable difference between the two Mg NPs types was detected. Eventually, the qualitative evaluation of the knots size revealed it to be between hundreds nanometres to few micrometres.

Figure 9.2: Comparison between a sample loaded with 0.05% wt MgCO\(_3\) NPs (A and B) and without (C). On normal ES PCL layer (C) the bulging structures found on the loaded matrix could not be individuated. Magnification 10kx, scale bar 2 µm.
Figure 9.3: MgCO$_3$ (A) and MgO (B) NPs loaded ES PCL layers. The arrows highlight the aggregates embedded in the polymeric fibres. Magnification 5kx, scale bar 10 µm.
9.4.4 EDX spectroscopy

Energy dispersed X-ray (EDX) technique was exploited initially to assess the presence of Mg NPs when added to ES PCL fibres. In Figure 9.4 an image of Mg NPs loaded ES PCL is revealed along with the overlapped map of Mg. Mg concentration is proportional to the blue intensity. The image shows the co-localisation of Mg with the presence of knots on the fibres. In some cases, Mg was identified in areas where there is no evidence of aggregates emerging from the fibre, possibly because of Mg NPs, or Mg NPs small aggregates completely embedded in the polymer. Some areas were also selected for a more detailed analysis and comparison.
Figure 9.4: A) Original SEM image of Mg NPs loaded fibres; B) EDX analysis of nine different specific areas. Blue regions identify Mg presence proportionally to the intensity. Quantification of Mg concentration is shown in Table 9.1.
9.4.5 Cytotoxicity of Mg NPs embedded ES PCL matrix

Cytotoxicity of the ES PCL matrices with Mg NPs incorporated was studied through hMSCs culture on standard tissue culture plates in medium to which the said specimens were added. ES PCL layers with two different Mg NPs types were tested, along with two different medium change schedules. Viability assay, performed up to 18 days, demonstrated a positive trend for all the samples (Figure 9.5).

On the last day cells in all the wells had viability significantly higher compared with the first assessment. Though with low statistical significance, ES PCL with MgCO₃ embedded NPs behaved better than ES PCL when medium was totally changed (p<0.1).
Cytocompatibility was studied after it was observed that hMSCs were able to grow in medium conditioned with Mg NPs embedded layers. Cells were cultured on ES PCL matrices with MgO and MgCO$_3$ NPs embedded. As a control, an equivalent number of cells was seeded on normal ES PCL, with the same morphological features, and on standard tissue culture plates.

Cells cultured on Mg NPs embedded layers had better performances than the controls (Figure 9.6); in particular cells on MgCO$_3$ matrix demonstrated to be more viable than those on PCL matrix (p<0.05). No significant difference was detected between the two types of control specimens.

**Figure 9.5: Viability of hMSCs cultured in presence of Mg NPs embedded ES PCL matrices. Bar represents significant difference (p<0.1).**

### 9.4.6 Cytocompatibility assessment of Mg NPs embedded matrices

Cytocompatibility was studied after it was observed that hMSCs were able to grow in medium conditioned with Mg NPs embedded layers. Cells were cultured on ES PCL matrices with MgO and MgCO$_3$ NPs embedded. As a control, an equivalent number of cells was seeded on normal ES PCL, with the same morphological features, and on standard tissue culture plates.

Cells cultured on Mg NPs embedded layers had better performances than the controls (Figure 9.6); in particular cells on MgCO$_3$ matrix demonstrated to be more viable than those on PCL matrix (p<0.05). No significant difference was detected between the two types of control specimens.
9.5 Discussion

9.5.1 Fabrication and characterisation

Embedding Mg NPs into ES PCL layers was undertaken to fabricate a dual functional substrate able to release Mg with time; thereby enabling the exploitation of the Mg biological response in a manner analogous to controlled drug release from polymer matrix. The incorporation of Mg NPs in the fibres needed to firstly disperse the NPs in a suitable solvent to be successfully electrospun. The major challenge when handling the NPs is how to avoid aggregates formation. The more the NPs form big aggregates the more likely is that the release will not be even, due to localised burst release. Two approaches were therefore identified; one was based on finding the most suitable solvent (or solvent system) that allows NPs not to aggregate and, subsequently, adapt the same solvent+NPs system to PCL electrospinning technique. The other approach was to dissolve NPs in the already established PCL+solvent solution (i.e. THF+DMF or CF+DMF). In case of incompatibility with the said solvent systems, more homogenous dispersion could be facilitated with the aid of techniques such as...
as sonication or capping. The tests showed that some combinations of solvent with varied NPs concentrations led to smaller aggregates (e.g. IPA) compared with others. However, no solvent was able to prevent the formation of aggregates smaller than hundreds nanometres. Conversely, the efforts to dissolve NPs in known solvent systems by using sonication and mixing NPs with small molecular weight PEG did not significantly limit aggregates formation. Thus, due to time constraints, it was decided to adopt the second approach by mixing Mg NPs in the established standard electrospinnable solutions.

Characterisation of surface properties of Mg NPs loaded ES PCL was performed by means of contact angle. A statistically significant difference was detected between Mg NPs loaded and unloaded ES PCL samples. The range of Mg NPs concentration adopted was not sufficient to detect any influence of Mg NPs amount on wettability. It has been revealed that the incorporation of TiO₂ (Gupta et al. 2012) and tricalcium phosphate (Erisken et al. 2008) NPs in ES PCL fibres is able to modify the matrix wettability. In the aforementioned studies the NPs concentration adopted was higher than that used here; therefore, it is likely that an additional increment in Mg NPs concentration will induce further wettability increases.

The ultimate proof of the success of the embedding process was provided by imaging and spectroscopy techniques. SEM images revealed the presence of embedded aggregates of size consistent with that measured by means of DLS, though this test had been conducted without the presence of PCL. PCL presence, along with the electrospinning process, did not apparently affect aggregates size. Eventually EDX analysis confirmed that Mg was actually co-localised with the aggregates. NPs incorporation, in terms of mere presence, has been proved by means of EDX by other researchers for a range of polymers and NPs combinations, including Mg (Sridhar et al. 2013; Sundarraj & Ramakrishna 2007; Fereshteh & Mozaffarinia 2014). EDX mapping has been used to locate selected elements in ES PCL fibres and their influence on fibre morphology (Kim & Choi 2013;
Miao et al. 2013; Tran et al. 2011). However, in the present work, this versatile technology has been used for the first time, to the author’s knowledge, to demonstrate the successful combination of MgO and MgCO$_3$ NPs and PCL to prepare enhanced ES matrices. Likewise, in a recent publication, Fereshteh et al. incorporated fluoroapatite NPs doped with Mg into ES PCL fibres for bone regeneration purposes (Fereshteh & Mozaffarinia 2014). The biological properties of such substrate still need to be evaluated.

Considering the good correlation between the size of the aggregates identified through DLS and SEM, future efforts should focus on preventing Mg NPs aggregation; thereby achieving a more homogeneous distribution within the fibres.

### 9.5.2 HMSCs response to Mg NPs loaded ES PCL matrix

Cytotoxicity and cytocompatibility of hMSCs seeded on Mg NPs embedded ES PCL layers were tested. For cytotoxicity test, cell culture, carried out using conditioned medium, was performed by adopting two different medium change strategies. This experiment design was implemented with the purpose of simulating the behaviour of composite materials, characterised by controlled release of molecules embedded in the matrix, for application in tissue engineering. Composite materials, once in the human body, release matrix degradation by-products and loaded materials, like Mg NPs as in this study, over time. Hence, by-products are removed from the tissues nearby the implants and excreted from the body mainly through liver and kidneys (Sun et al. 2006; Liberman et al. 2014). However before being excreted, degradation products may accumulate in the vicinity of the implant and in organs, more commonly liver, kidney and spleen (Liberman et al. 2014). Degradation products and NPs are anticipated to be harmful depending on the dose, which, subsequently, may depend on degradation rate, and organs and body clearance capability (Liu et al. 2011; Heidemann et al. 2002; Heidemann et al. 2001; Martin et al. 1996). Higher degradation rate, leading to release of high NPs amount over time, along with insufficient clearance capabilities, is a factor that can favour
accumulation. Thus, changing the medium in two different ways aims to simulate two different kinetics of Mg NPs accumulation.

For both MgO and MgCO$_3$ embedded layers the total medium change strategy was more beneficial for cells compared with partial change, in terms of viability, though not significant. In addition, no significant difference was found when comparing all the combinations of medium change strategy and NPs type except for MgCO$_3$ embedded layers that demonstrated higher viability with low statistical significance. Nevertheless overall better performance of Mg NPs embedded layers in respect to unloaded ES PCL was obtained, considering the outcome of both cytotoxicity and cytocompatibility tests.

### 9.5.3 Remarks on Mg NPs release from ES PCL layers

The actual concentration of Mg released from ES PCL matrices is unknown; however, from tests using flame atomic absorption spectrometry (FAAS), there is evidence that over the duration of the cell culture experiment Mg is actually released. In Figure 9.7, the concentration of Mg released by PCL matrices soaked in PBS over 3 months is reported. These preliminary results indicate that the amount of Mg released from ES PCL incorporating MgCO$_3$ was higher than the amount estimated to be present in the ES PCL matrix. Despite the assessment needs to be repeated, the release of Mg of few mg/L order of magnitude seems reasonable. Therefore, even though MgO and MgCO$_3$ have low solubility in water, equal to 86 and 139 mg/L respectively (Anatolievich 2015b; Anatolievich 2015a), the chance of Mg precipitation in the concentration detected upon release from ES PCL scaffold is low.
The chemical composition of interstitial fluid is rather complex; nonetheless considering that body fluids are all based on water and that MgO and MgCO$_3$ are ionic compounds (Chambers & Holliday 1975), it is reasonable to predict the dissociation of MgO and MgCO$_3$ to give raise to Mg ions, which is the most common form in extracellular environment, upon release from the ES PCL matrix. MgO has been administered orally to patients as Mg diet supplement (Shechter et al. 2012; Tatsuki et al. 2011) and injected in mice (Jahangiri et al. 2013) proving its safety. MgCO$_3$ can also be ingested to control phosphate serum level (Tzanakis et al. 2008) and finds application in bone scaffolds as it can be included in apatite (Iafisco et al. 2014).

The release of Mg is the key feature of this novel type of matrix. The amount and kinetic of Mg released depends on a number of factors. One is the Mg amount that can be embedded in the ES PCL matrix. The concentration adopted during the fabrication phase was 0.5% wt compared with the solvent (3.3% NPs/PCL ratio) obtaining slightly cloudy suspension. In a different organic solvent-
polymer system, MgO has been suspended at 15% wt concentration in respect to the polymer (Dadvar et al. 2011). Additional tests will be required to assess the maximum Mg NPs quantity that can be suspended in the solvent system adopted here.

Further to Mg amount, the release depends on ES PCL degradation kinetic. The results demonstrated that ES PCL fibres are subject to bulk degradation rather than surface erosion (section 5.3). This is an unfavourable condition because surface erosion would allow a continuous Mg NPs release; in contrast, bulk erosion causes sudden bulk breakdown hence Mg NPs burst release. However, the degradation behaviour of ES PCL may turn into surface erosion once in-vivo, as also predicted by in-vitro tests in accelerated degradation condition (Lam et al. 2008).

This thesis demonstrates that Mg NPs are dispersed in the bulk of the ES PCL fibres but are also present in the form of aggregates. This configuration would probably induce local occasional rapid increase of Mg concentration, upon detachment of aggregates from the fibres, along with a more constant release due to the Mg NPs evenly distributed in the bulk of fibres. To achieve a sustained Mg release, which is desirable for an implanted device designed to induce long-term remodelling processes (Chew et al. 2005), the homogeneity of embedded Mg NPs distribution must be improved. As discussed previously, the combinations of process parameters tested in the present project did not allow the achievement of such even distribution. Nevertheless, a large number of combinations between parameters were left unexplored; for example, solvent system type, ultrasound application pattern, capping polymer or surfactant type, and concentration.

From the perspective of adopting Mg NPs embedded ES PCL for AF tissue engineering, an adequate cell source must be individuated. Mg is considered a promising material for the fabrication of scaffolds for bone regeneration due to its mechanical properties and high concentration in bones compared with other tissues (Li et al. 2014). Mg has been revealed to enhance bone formation and differentiation of MSCs towards osteoblastic lineage (Li et al. 2014; Scaglione et al. 2012). Conversely,
opposite bone matrix inhibitory effect (Zhang et al. 2014) and chondrogenesis (Shimaya et al. 2010) in the presence of Mg have also been demonstrated. In addition, by providing opportune biochemical and topographical cues, hMSCs differentiation towards endothelial cells, has been proved to be achievable (Valarmathi & Fuseler 2011). The latter finding makes hMSCs an attractive cell source since, in view of the aim of the present project, a unique cell source may be utilised to regenerate both the AF collagen-rich tissue and induce vasculogenesis at AF periphery.
Appendix II: study on the applicability of ES PCL for tendon regeneration purposes

10.1 Introduction

Within the present project, the author had the opportunity to collaborate with the research group of Prof. J. Cornish and Dr. D. Musson at the University of Auckland (UoA). The initial aim of the visit to the UoA was to study the response of tenocytes seeded on mechanically-stimulated ES PCL layers. Nonetheless, the bioreactor able to exert uniaxial stretching, developed at the UoA, broke down before the visit and could not be fixed before the end of the collaboration. Therefore, the initial experimental plan became an investigation into tenocytes response to ES PCL matrices in static conditions.

The adoption of ES PCL layers for tissue engineering of tendon, along with the AF, arises from the similarity between these two tissues. Tendon and AF lamella share, in fact, two key features; namely, the anisotropy and the collagen composition (Wang 2006).

This chapter illustrates the experiment relative to primary rat tenocytes culture on ES PCL layers. The investigation focused on the assessment of cell viability, morphology and gene expression.

10.2 Literature review

Tendon is a crucial component of the musculoskeletal system as it bridges muscle and bone; thereby transmitting the force between these two components (Kannus 2000). High rupture incidence involves mainly the flexor digitorum, the Achilles and the supraspinatus, which are located respectively in the hand, the calcaneus and the rotator cuff (Yang et al. 2013). The supraspinatus and Achilles tendons, in particular, are subject to high tensile loads. Therefore, it is fathomable that the rupture of such relevant tendons impacts profoundly on mobility and the quality of life.
With regard to the supraspinatus tendon, it has been revealed that diseases of the rotator cuff account for 16% of the total musculoskeletal ailments (Longo, Berton, et al. 2012). Only in the US, 75,000 operations on the rotator cuff are carried out every year (Vitale et al. 2007). Such a high number of interventions leads to an estimated annual expenditure of about 30 billion US$ (Chen et al. 2009). This translates into relevant indirect costs, such as absenteeism from work and a high rate of applications to the worker’s compensation scheme (Harryman et al. 2003).

The tendon is a high anisotropic tissue composed largely of collagen arranged in a well-defined hierarchical structure (Kannus 2000). The composition of the tendon is reported in Table 10.1.

*Table 10.1: List of the main tendon components and relative amount.*

*Adapted from (Wang 2006; Kannus 2000; Kjaer 2004).*

<table>
<thead>
<tr>
<th>Component (% of dry weight)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Water)</td>
<td>(55-70%)</td>
</tr>
<tr>
<td>Collagen</td>
<td>60-85%</td>
</tr>
<tr>
<td>Type I</td>
<td>60%</td>
</tr>
<tr>
<td>Type III</td>
<td>0-10%</td>
</tr>
<tr>
<td>Type IV</td>
<td>~2%</td>
</tr>
<tr>
<td>Type V</td>
<td>0-5%</td>
</tr>
<tr>
<td>Elastin</td>
<td>1-2%</td>
</tr>
<tr>
<td>Proteoglycans</td>
<td>0.2-3.5%</td>
</tr>
<tr>
<td>GAGs</td>
<td>0.2-5%</td>
</tr>
</tbody>
</table>
Tendon is characterised by low density population of cells (Pingel et al. 2014), called tenocytes, interspersed in collagen fibres. Tenocytes, which are fibroblast-like cells, are responsible for tendon homeostasis by synthesising collagen in response to mechanical loads according to Buchanan et al. (Buchanan & Marsh 2002). Tenocytes are arranged in rows parallel to the tendon axis with cytoplasmic processes associated with collagen fibre bundles (Kannus 2000; McNeilly et al. 1996). The tendon is not highly vascularised, with few blood vessels forming a “web-like network” underneath the paratenon from which they penetrate into the ECM (Petersen et al. 2002).

Tendon failure may be the result of a traumatic event or spontaneous; in the latter case, it has been demonstrated to be preceded by degeneration (Järvinen et al. 1997; Fenwick et al. 2002). The healing process of ruptured tendon can be divided into different stages (Sandrey 2002). In the first few days post-trauma, macrophages and neutrophils are recruited and migrate in the injured tissue surroundings with inflammatory and angiogenic factors release. This phase is followed by the recruitment of tenocytes, which intensify collagen III synthesis (Sharma & Maffulli 2005). After about six weeks, the remodelling phase occurs with the conversion of disorganised collagen type III into physiological type I (Sharma & Maffulli 2005; Sandrey 2002), followed by gradual collagen synthesis reduction (Russell & Manske 1990).

To date, the outcomes of surgical treatments upon tendon rupture are unsatisfactory as demonstrated by the high failure rate estimated to be 11% for flexor tendon (Wong & Peck 2014; Dy et al. 2012). After primary rotator cuff surgery, a failure rate of 20-90% has been reported (Zhang et al. 2012; Galatz et al. 2004) and Duquin et al. re-tear rate has been assessed to be between 7 and 69% depending on the repair technique and torn size (Duquin et al. 2010).

Currently, the performances of a number of commercially available devices designed for tendon augmentation have been evaluated. It has been revealed that a porcine intestine submucosa
augmentation device, used to cure chronic rotator cuff tears, leads to no significant improvement compared with no augmentation control (Iannotti et al. 2006). Similar commercially available devices have been found to have Young’s modulus one order of magnitude lower than the reference tendon (Coons & Alan Barber 2006), to be subject to incomplete decellularisation (Derwin et al. 2006) and be the cause of non-specific inflammation after rotator cuff surgery (Malcarney et al. 2005). At the academic level, a thriving research is advancing in parallel with the aim of developing alternative strategies to restore the physiological tendon tissue, otherwise weak due to the formation of disorganised scar tissue after surgical repair (Miyashita et al. 1997; Lee & Schuberth 2012). A number of 3-dimensional structures have been studied to achieve tendon mechanical properties and supporting cell proliferation (Xu et al. 2013; Qiu et al. 2013; Yang et al. 2014; Chainani et al. 2013). Dynamic culture techniques have also been attempted (Bosworth et al. 2014; Teh et al. 2013).

Replication, especially of the mechanical properties, proved challenging as the tendon is a demanding tissue concerning modulus and strength. Tests on cadaver assessed the modulus to be up to 822 MPa for the Achilles tendon (Wren et al. 2001) and in the range 10-165 MPa for the supraspinatus (Lake et al. 2010; Itoi et al. 1995).

Further optimisation is undoubtedly needed, along with a more comprehensive knowledge of the tendon diseases and degeneration process upon rupture. In this respect, trials of implantation of acellular fibrous devices in animal models have been undertaken; however, moderately positive results (Beason et al. 2012) were achieved or the outcome assessment was restricted to the mechanical properties (Taylor et al. 2010). Conversely, cellularised PGA sheet seeded with MSCs was tested in a rabbit model demonstrating inadequate restoration of mechanical properties after 16 weeks (Yokoya et al. 2012). Thereby, the insufficient and unreliable data collected thus far proves that a well-established and safe solution is required, but indeed is not ready available, with a view to clinic translation (Zhang et al. 2012).
10.3 Materials and methods

10.3.1 Biological experiments. Techniques

Primary rat tenocytes extraction and standard culture procedure

Human primary tenocytes were extracted from rat tails obtained from the animal facility of UoA as follows (in each session two rat tails were processed simultaneously):

1) Two rat tails are soaked in EtOH 70% solution for 30’ to sterilise the skin and the wound.
2) The tails are soaked in PBS to remove EtOH traces and to keep the tissue in proper environment.
3) The tails are placed on a 24 multiwell plate lid; the skin cut through from the base to the tip and peeled off with forceps.
4) Tails are cut in three pieces and, with tweezers, the tendons are pulled out and collected in a Petri dish. PBS with 10% FBS is used to keep the tendons wet.
5) The operation is repeated after cutting each tail piece in smaller fragments to facilitate the collection of tendons not otherwise accessible.
6) All the tissue fragments looking as non-tendon tissue are removed to prevent contamination of cells of other types (i.e. muscle and bone).
7) Tendons are weighed and subsequently chopped into small fragments.
8) The so obtained mass of tendons is transferred to a large bottle containing 80 ml filtered medium (DMEM F12/10% FBS, Sigma-Aldrich) with 40 mg collagenase (Sigma-Aldrich) and 40 mg dispase (Sigma-Aldrich).
9) The bottle is placed in a 37 °C oven equipped with a shaker and left overnight until all the tissue bits are digested.
10) The solution is strained using a 70 µm strainer to remove the tissue fragments and split into twelve 15 ml tubes.
11) The tubes are centrifuged (Biofuge, Thermo Fisher) at 1500 rpm for 3’.
12) Pellets are resuspended and pooled into two 15 ml tubes.
13) Tubes are centrifuged again (1500 rpm for 3’) and resuspended in 6 ml to perform cell counting with a haemocytometer.
14) Cells are then seeded in a suitable number of T75 flasks (Corning) with 5 to $7.5 \times 10^5$ cells each. 30 ml medium (Table 10.2) is added to each flask and changed every 3-4 days.

15) When tenocytes are nearly confluent they can be harvested through trypsinisation and reseeded or frozen.

Trypsinisation and freezing process are carried out when reaching between 80 and 90% confluence in the same way as for hMSCs. Note that the medium used to grow cells on ES PCL samples had only 5% FBS to prevent too fast cell proliferation and enhance any effect of culture condition.

Table 10.2: Composition of the medium used to expand primary rat tenocytes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s Modified Eagle Medium F12 (DMEM F12)</td>
<td>89%</td>
</tr>
<tr>
<td>FBS</td>
<td>10%</td>
</tr>
<tr>
<td>Antibiotic and antimycotic</td>
<td>100 and 0.25 µg/ml</td>
</tr>
</tbody>
</table>

10.3.2 Characterisation techniques

Viability assessment

Viability assay was performed by pipetting 5% AB solution in each well. After 4h incubation, 200 µl per well were pipetted in 96 multiwell plates for plate reader measurement. A preliminary test guaranteed that the presence of the metal ring that holds the ES PCL matrix in place did not affect the AB reduction.

Live and dead assay

Visual confirmation of cell viability was carried out by means of calcein AM and ethidium homodimer-1 (EthD-1) (Life Technologies) that stain cytoplasm of living cells and nuclei of dead cells respectively. Samples were washed with PBS once in presence of the metal rings and then once
again after their removal. Hence samples were treated according to the protocol established for rat
tenocytes. Staining solution was prepared by adding 0.5 µl EthD-1 and 1 µl calcein AM to 1 ml
PBS/FBS 10%.

At the end of the staining process, ES PCL samples were turned over on a glass slide and observed
with fluorescence microscope (CKX41, Olympus).

**Cell morphology evaluation**

Images of tenocyte stained by means of live and dead assay were further analysed in order to
investigate the elongation degree of cells. The procedure was established using Image-j built-in
functions on the basis of the protocol adopted in section 4.1.1 for cell nuclei and published studies
(Musson et al. 2015). The contrast of green channel images was enhanced to have a stronger signal
against the dark background. Images were then converted into greyscale and the threshold value
adjusted to obtain binary pictures of cells black bodies on a white background. The cells were
analysed to measure their circularity value, which likewise for nuclei analysis, one represents a circle.
Two images per sample type were analysed by evaluating approximately 600 cells per sample.

**Gene expression analysis**

The aim of this investigation was to acquire information concerning the expression of key genes
known to be relevant for maintenance of tendon cell phenotype. Gene expression investigation was
performed using six specimens per type of ES PCL matrix and time point. The genes selected to be
amplified, are: collagen type I and II (Coll I and II), Tenascin (Tnc), Tenomodulin (Tnmd). Control
genes to ensure the absence of cell differentiation into chondrocytes or osteoblasts were selected.
Aggrecan (Acan) and Sox9 are genes expressed in cartilages (Henry et al. 2012; Kiani et al. 2002)
whilst Osterix (Sp7) and Runx2 are genes typically found in bones (Komori 2006). As housekeeping
gene, 18S was chosen. All the procedures to treat the cells, from the attainment of cell lysate to
cDNA amplification, were carried out following the rt-PCR machine (QuantStudio, Qiagen) manufacturer instructions.

Rt-PCR is based on the cyclical duplication of the RNA fragments of interest until the amount becomes detectable and, therefore, quantifiable. The output of the technique, for each gene, is the Ct value which represents the number of DNA duplication cycles, after which the fluorescence signal emitted by the probe is clearly detectable over the background noise. Therefore a lower value of Ct means a higher initial amount of the RNA fragment of interest. Ct values were analysed following the widely-used $\Delta\Delta$Ct method (Livak & Schmittgen 2001; Schmittgen & Livak 2008).

According to Motulsky, ANOVA statistical test was performed before the transformation into linear scale (Motulsky 2013), comparing $\Delta$Ct values. To this purpose the Ct values of each gene of interest and reference gene were averaged across all the biological experimental repeats prior to $\Delta$Ct values calculation.

**10.3.3 Primary rat tenocytes response to ES PCL matrices**

The behaviour of primary rat tenocytes seeded on PCL ES layers with different arrangements was studied in static conditions. The test was performed on random and aligned fibres (1.59±0.49 and 1.72±0.50 µm fibre diameter respectively) using standard tissue culture 24 multiwell plates as control; the layers selected did not have a significant fibre diameter difference.

For each layer type, a suitable number of 10 mm diameter discs were cut with the aid of a punch. Sterilisation was performed by 30’ soaking in 70% EtOH followed by 30’ UV exposure per side. Prior to cell culture ES PCL specimens were preconditioned by submerging in medium for 24h inside standard tissue culture plates. Since Cell Crowns were not available, the discs were kept on the bottom of the wells by means of metal rings, previously sterilised by autoclaving.

When reaching the desired density, tenocytes were harvested from the flasks and seeded by pipetting 30 µl cell suspension with 2.5 x 10^4 cells. Cells were allowed to adhere 1h before topping up with 1 ml fresh medium. The following day, samples were moved to new multiwell plates to
remove any contribution of cells that became accidentally attached to the well bottom. Samples were analysed on days 1, 3 and 7 for AB and live/dead assays, and rt-PCR as described in section 10.3.2. All the tests were performed in triplicate to reduce the anticipated high variability as cells are primary, extracted from animals.

10.4 Results

10.4.1 Fibre arrangement influence on tenocytes metabolic activity

Over seven days, viability of cells cultured on all the substrates (random and aligned fibres, and standard tissue culture plates) showed an increase (Figure 10.1). Cells cultured in the wells revealed higher viability at all the time points compared with those on the matrices. This is due to the lower number of cells that attached on the ES PCL matrices, compared with the control. R-1.59 and A-1.72 demonstrated very close values at every time points.

![Figure 10.1: Representative chart of rat tenocytes viability over 7 day culture.](image)

10.4.2 Fibre arrangement influence on morphology and death rate

By scanning the whole surface of the specimens with the fluorescence optical microscope, at low magnification higher cell distribution homogeneity was noticed on control samples. Preliminary experiments with controls for live and dead tenocytes ensured to obtain green and red signals of
comparable intensities. On both ES PCL matrices a higher number of dead cells was found compared with the control wells (Figure 10.2, J and L). Nevertheless dead cells were in negligible number than live ones and the total number clearly increased over 7 days, consistently with the AB assay outcome, with no evident difference between the two types of layer. On the other hand tenocytes orientation demonstrated to be strongly affected by fibre arrangement. Indeed, aligned fibres induced an evident cell orientation, particularly after seven days (Figure 10.2, K).
<table>
<thead>
<tr>
<th>Day 1</th>
<th>Live cells</th>
<th>Dead cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><img src="image1" alt="Live cells" /></td>
<td><img src="image2" alt="Dead cells" /></td>
</tr>
<tr>
<td>R-1.59</td>
<td><img src="image3" alt="Live cells" /></td>
<td><img src="image4" alt="Dead cells" /></td>
</tr>
<tr>
<td>A-1.72</td>
<td><img src="image5" alt="Live cells" /></td>
<td><img src="image6" alt="Dead cells" /></td>
</tr>
</tbody>
</table>

**Day 1**

- **Control**: Live cell images (A) and dead cell images (B).
- **R-1.59**: Live cell images (C) and dead cell images (D).
- **A-1.72**: Live cell images (E) and dead cell images (F).
Cell morphology was analysed by means of imaging tools to evaluate the extent of tenocytes stretching degree. The results (Figure 10.3) demonstrated significant difference between circularity values obtained on the samples (p<0.05, values closer to one denote a more rounded shape). The lower cell circularity measured on aligned fibres compared with random arrangement and standard plate demonstrates clearly the effect of the anisotropic substrate on rat tenocytes orientation.

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Figure 10.2: Images of the cellularised ES PCL layers stained with live (green) and dead (red) staining, taken on day 3 and 7. Blue arrows denote ES PCL fibres direction. Scale bar 100 µm.
10.4.3 Fibre arrangement influence on gene expression

The expression of nine genes was assessed with the aim of studying the genetic profile of tenocytes seeded on ES PCL layers with different arrangements. Statistical analysis was performed on ΔCt values obtained by averaging Ct values across all the tests performed. Two-way ANOVA test with Tukey's post hoc multiple comparison revealed significant difference only for Acan gene expression level at day seven on random and aligned fibres, compared with the control (p<0.01). The small discrepancies between gene expression levels and the variability of the response of rat cells extracted from different individuals did not allow the capture of significant differences in most cases. Nevertheless, the gene expression trend across the biological repeats turned out to be fairly consistent; therefore, the results of a representative sample are shown in Figure 10.4. The charts report the fold change gene expression in respect to that obtained on standard tissue culture plate.

Figure 10.3: Circularity values for the three samples analysed (lower values indicate a more stretched morphology). Bars indicate significant difference (p<0.05).
on day 1. The following comments and the discussion take into account the results attained from all the experiments carried out.

On day 7 collagen type I expressed by tenocytes seeded on aligned and random fibres demonstrated similar level of expression as the control whereas collagen type III showed to be slightly lower compared with control day 7 even though constant for both random and aligned fibres over the culture period. Scx expression demonstrated an increasing trend for all the samples, more marked for the control. On aligned fibres Tnc exhibited upregulation too, comparable to that of the control and not significantly higher than on random fibres. Tnmd was found to be constant over time for both the ES PCL matrices at the same level of expression displayed by the control at day 3 and 7. However the comparison with the reference at day 1 demonstrated evident downregulation.

Runx2 and Sp7 are two genes involved in bone matrix synthesis; Runx2 was subject to no relevant change whilst the number of Sp7 gene copies was not sufficient to return a reliable result thereby the relative graph could not be generated.

With regard to genes that are important for cartilage development, Sox9 and Acan expression was studied. Sox9 expression displayed increasing trend on the control whereas on aligned and random fibres the expression was more stable and slightly lower than the control at day 7. Acan expression level showed a significant upregulation over time on both the ES PCL matrices, especially on random fibres whereas on the control the expression was found to remain constant.

In summary, considering the genes deemed the most important for tenocyte phenotype characterisation, Coll I, Coll III, Scx, Tnc and Tnmd, no obvious difference was found between expression levels on random and aligned fibres over the course of the experiment.
Figure 10.4: Charts representing the expression level of the genes of the most representative biological repeat.

Fold changes in respect to the reference gene, adopting the ΔΔCt method are reported.
10.5 Discussion

Two different fibre arrangements were tested and tenocytes were evaluated on the basis of their viability, with colorimetric and imaging assays, and the expression of opportunely selected genes. The AB assay revealed that rat tenocytes, similarly to what assessed with hMSCs, are able to adhere and increase their metabolic activity. The live and dead assay run in parallel allowed confirming that the viability increase was at least in part due to the increase of cell number. A similar result was obtained with tenocytes cultured on PCL blended with collagen and electrospun into random and aligned nanofibres (Xu et al. 2013). Along with the increase in cell density a clear change in morphology was observed. The orientation and morphology of tenocytes was affected strongly by the topography of the aligned fibres. Cells began changing their orientation from day 1, although some of them retained a polygonal or rounded shape; meaning that more than one day is required to let all the cells re-orientate. Rounded shape was maintained throughout the whole experiment by cells cultured on random fibres and standard tissue culture plates. Tenocytes capability to align according to the substrate has been observed on anisotropic substrates (Xu et al. 2013; Kapoor et al. 2010; Musson et al. 2015; Yin et al. 2015) and, likewise, the effect of ES PCL matrix is exerted on hMSCs, as revealed in the present thesis (section 6.2). The elongated shape of cells is a reminder of that observed in living tendons, where cells are arranged in parallel (McNeilly et al. 1996; W. Wang et al. 2013).

There is evidence that cell morphology is a key feature for driving cell differentiation or phenotype maintenance according to cell potential (Gimble et al. 2008; J. Zhu et al. 2010); thereby stressing the importance of the substrate topographical features (W. Wang et al. 2013). Tnmd is one of the genes expressed by tenocytes that can be used as tendon phenotypic marker (Shukunami et al. 2006). By studying genetically modified mice lacking the Tnmd gene, it has been demonstrated that Tnmd plays a fundamental role in tenocytes proliferation and proper collagen
fibril development (Docheva et al. 2005). Zhu et al. demonstrated that Tnmd expression level drops when tenocytes are cultured on a smooth surface instead of parallel microgrooves (J. Zhu et al. 2010). In the present work, ES PCL aligned fibres did not prevent Tnmd expression decrease. However all the other important genes expressed in tendons (Coll I, Coll III, Scx, Tnc) showed similar performances compared with random fibres whilst downregulation (except Tnc) compared with the control. Tnc is an extracellular matrix protein that plays a role in matrix organisation and is found in higher amount after damages and during tendon remodelling process (Riley et al. 1996). Coll III has a similar function as it has been found to be synthesised in higher proportion in respect to Coll I, in ruptured tendons (Eriksen & Pajala 2002; Riley et al. 1994). With this regard, the small increase of Coll III expression assessed on control at day 7 and the modest downregulation on both ES PCL matrices suggest no ongoing relevant remodelling process. The fairly constant expression of Coll I and III indicates that on ES PCL layers tenocytes capability to synthesise these two structural proteins is not significantly impaired over one week culture. However, it is possible that the larger number of cells in the standard tissue culture plate control may have prevented collagen type I and II downregulation on the control. It has been revealed that tenocytes high density is an important factor for the maintenance of tenocyte phenotype (Güngörmüş & Kolankaya 2008; Schulze-Tanzil et al. 2004). Further assessment of collagen presence is necessary to evaluate that, after gene expression, the translation into collagen actually takes place. Scx increase over seven days is a positive indicator of tenocytes phenotype since this transcription factor has been found to be expressed in ligaments and tendons during embryo development (Asou et al. 2002). However Scx increasing trend observed in seven days culture, opposed to the decreasing trend of Tnmd, needs further investigation as Scx expression has been found to precede Tnmd upregulation though this has been assessed in an in-vivo model during chick embryo development (Shukunami et al. 2006). Within this experiment, the expression level of other cell markers relevant for bone and cartilage development was also evaluated. The analysis of Runx2 and Sp7, which play an important role in bone development, demonstrated a lack of relevant differences between aligned and random fibres.
Runx2 is a gene expressed in osteoblasts, in particular during bone maturation, after which its expression decreases (Komori 2010; Komori 2006). Sp7, also known as Osterix, is a marker for osteoblast differentiation and its expression is downstream to that of Runx2 (Komori 2010; Komori 2006). Sp7 was barely detectable in tenocytes seeded on aligned ES PCL fibres, which suggests that, along with no Runx2 upregulation, a differentiation process towards osteoblast is unlikely to occur. Eventually, genes screening was performed on genes expressed typically in chondrocytes; namely, Sox9 and Acan. Acan, which codifies for the ECM protein aggrecan (Kiani et al. 2002), in the test reported was expressed 125-fold on aligned fibres compared with control on day 1. On day 7, the upregulation on aligned fibres was less marked than that assessed on random fibres. Sox9, which is an essential gene for cartilage development (Henry et al. 2012; Asou et al. 2002), demonstrated no upregulation in tenocytes grown on aligned fibres. These results are apparently in contrast to the relationship between Sox9 and Acan, for which Sox9 transcription is upstream; thus, it is necessary to initiate aggrecan synthesis (Lefebvre et al. 2001). Other transcription factors, L-Sox5 and Sox-6, are required to accomplish Acan synthesis with Sox9 contribution (Han & Lefebvre 2008) but their presence was not assessed in the present work. Despite Acan upregulation, its expression was anticipated, as aggrecan is one of tendon ECM components, albeit in a small amount (Wang 2006).

The comparison of the random and aligned ES PCL scaffolds did not reveal relevant differences in the expression of the majority of genes evaluated. Yin et al. studied the response of MSCs seeded on ES PLLA scaffolds finding more marked Runx2 downregulation and Scx upregulation on aligned fibres compared with random fibres (Yin et al. 2015). This is not consistent with the results obtained herein, as Scx and Runx2 demonstrated to be expressed similarly on random and aligned fibres. However, in the study mentioned above, the phenotype was investigated only by evaluating two genes. In addition, the different polymer (PLLA) and cells (MSCs) adopted make a direct comparison with tenocytes seeded on ES PCL difficult. The role of anisotropic substrates on tenocytes was also evaluated by means of microgrooved surfaces. Zhu et al. found upregulation of Coll I and Tnmd in
cells cultured on microgrooves compared with smooth surfaces; whilst Kapoor et al., similarly to that observed in the present thesis, have measured no significant influence of aligned topography on Coll I and III expression over time (J. Zhu et al. 2010; Kapoor et al. 2010). Instead, notwithstanding the absence of Coll I and III upregulation, the direction of deposited collagen on microgrooves has been found to be consistent with the direction of the pattern (Kapoor et al. 2010). Despite the optimal scaffold being yet to be found, all the authors observed the influence of anisotropic patterns on cell morphology, consistently with the results of the present study, which is deemed a prerequisite for tendon artificial replacement development (Yin et al. 2015; Kapoor et al. 2010; J. Zhu et al. 2010).

10.5.1 ES PCL matrix for tendon tissue engineering

The modulus of the supraspinatus tendon has been evaluated in the ranges 2-5 MPa and 10-150 MPa for the toe and linear region respectively, depending on the location (Lake et al. 2010). The highest modulus measured in the present work on aligned ES PCL fibres (53.64±12.26 MPa in dry conditions, see section 5.4) is lower than the highest of the tendon.

From the perspective of exploiting the ES PCL layers for supraspinatus tendon augmentation, it must be noted that the artificial construct does not need to match perfectly the tendon mechanical properties as it would not be a total replacement but a supporting device. Despite the existence on the market of several acellular tendon augmentation grafts (Chen et al. 2009; Longo et al. 2010), the load ratio between a tendon reinserted after rupture and an augmentation device remains unknown. Aurora et al. designed a multiparametric simulation of an augmentation device application scenario and found that a device with tendon-like properties would share only 31% of the total load, with no appreciable increase in case of higher device stiffness (Aurora et al. 2012). Conversely, too low mechanical properties would make the augmentation device ineffective in respect to the sole natural tendon reinsertion (Aurora et al. 2012).
In conclusion, the experiments presented in this section yielded novel results regarding tenocytes culture on ES PCL layers. The outcome is encouraging as the aligned fibres scaffold demonstrated the fostering of cell adhesion, proliferation and inducing contact guidance phenomenon. Gene expression evaluation, in spite of Acan upregulation, demonstrated that tenocytes, on aligned fibres, maintained the capability of expressing the gene’s marker of tenocyte phenotype. The results provide a favourable starting point to develop a supraspinatus augmentation device. To achieve this goal, arranging ES PCL layers in a more complex fashion, better mimicking the natural tendon providing a 3-dimensional environment for cells and enhanced mechanical properties, is regarded as promising (Teh et al. 2013; Qiu et al. 2013; Chen et al. 2010; Musson et al. 2015; Chainani et al. 2013). In addition, usage of stem cells and providing mechanical stimulation by means of bioreactor may further improve matrix synthesis and cell differentiation for clinical application (Lee et al. 2005; Barber et al. 2013; Bosworth et al. 2014; Webb et al. 2006; Woon et al. 2011).