Cellular behavior after mechanical stimulation on biofunctionalized polylactic acid nanofibers for tendon tissue engineering

Abstract

Recently, tissue engineered solutions have been investigated to cure rotator cuff tears, whose curing is often long and painful. Scaffolds mimicking the natural structure and composition of natural tendons have been found to be a promising alternative. Tendons are typically composed of aligned collagen nanofibers on which tendon cells called tenocytes lay and produce their extracellular matrix. As a consequence, electrospinning was chosen as a technique to produce aligned nano-scaled fibers of poly(lactic acid), a biocompatible polymer. In the next steps, the surface of the fibers was functionalized with collagen and the resulting scaffolds were seeded with tenocytes isolated from tendon biopsies. The behavior of the cells was evaluated with and without mechanical stimulation of the scaffolds.

First, the fibers were mechanically characterized and their diameter was indeed calculated to be in the nano-range, proving the efficiency of the electrospinning. The functionalization process was also characterized. Collagen immunofluorescence showed that covalent bonding of collagen on the fibers with carbodiimide chemistry (EDC/NHS) was more effective than the physical adsorption of collagen.

Later, tenocytes isolated from a tendon biopsy were seeded on functionalized and non-functionalized scaffolds as well as on glass and a cells immunofluorescence was performed to verify the attachment of the cells on the different substrates. The area of the cells was higher on glass than on other substrates, however the cells were much more elongated on fibrous scaffolds and were all aligned in a single direction. It is very likely that the cells aligned along the fibers direction which would demonstrate that the scaffolds had a positive effect on the cells. Indeed, this cellular behavior is very close to what can be observed on natural tendons. The influence of collagen functionalized on the scaffolds was yet not clear. Although cells had a higher area and elongation on the first day of culture on functionalized scaffolds, the values of non-functionalized scaffolds became extremely similar after the third day of culture.

A stimulation test on a scaffold seeded with tenocytes had to be ended prematurely due to experimental issues. The sample eventually was 75% stiffer but its yield strain was four times lower. Physical ageing was considered as the most consistent explanation over collagen production on a short stimulation period.
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Glossary

BCA: Bicinchoninic Acid
BSA: Bovine Serum Albumin
DAPI: 4',6-diamidino-2-phenylindole
DMSO: Dimethylsulfoxide
DPBS: Dulbecco’s Phosphate Buffered Saline
EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
FBS: Fetal Bovine Serum
MSC: Mesenchymal Stem Cells
NHS: N-hydroxysuccinimide
PBS: Phosphate Buffered Saline
PDLLA: Poly(DL-Lactide)
PFA: Paraformaldehyde
PLA: Poly(Lactide) or Poly(Lactic Acid). To simplify, PLA will be used in the Materials and methods part as well as in the Results and discussion part to mention the polymer fibers used in this project (fibers of Poly(L-Lactide-co-DL-Lactide) 70/30)
PLDLA: Poly(L-Lactide-co-DL-Lactide)
PLGA: Poly(L-Lactic-co-Glycolic Acid)
PLL A: Poly(L-Lactide) or Poly(L-Lactic Acid)
PVA: Poly(Vinyl Alcohol)
SEM: Scanning Electron Microscopy
TE: Tissue engineering
TFE: 2,2,2-trifluoroethanol
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1 Introduction

1.1 Introduction to tissue engineering

The term “tissue engineering” (TE) was first used at a National Science Foundation (NSF) sponsored meeting in 1987. It was later defined as “the application of principles and methods of engineering and life sciences toward fundamental understanding and development of biological substitutes to restore, maintain and improve human tissue functions” [1]. This definition embraces methods where the biological substitutes are cells that may be implanted on a scaffold such as natural collagen or as synthetic and biocompatible polymers to form a newly grown tissue.

Besides having applications for enhancing the curing of certain diseases, in which the tissue is grown inside a patient or outside a patient after transplantation, this field can be an efficient diagnostic tool where the tissue is made in vitro and can be used for testing toxicity, pathogenicity or drug uptake. [2]

Tissue engineering is a multidisciplinary field whose research includes various areas such as:

- Biomaterials: providing physical and chemical characteristics to improve and direct the proliferation, the growth or the differentiation of cells in order to form a functional tissue.
- Cellular science: giving methodologies to attest for the proliferation or the differentiation of cells and providing appropriate knowledge concerning the type of cells to be used.
- Biomechanical design: providing knowledge about properties of native tissues such as their composition in order to attest for the efficacy and safety of engineered tissues. [2]

Tissue Engineering is a fast expanding field of applied biology and biomedical engineering that aims to create artificial organs for transplantation, basic research, or drug development. Therefore it is needed to integrate knowledge of organic chemistry, cell biology, genetics, mechanics and transport processes to create functional tissue engineered constructs.
Some tissue engineering methods are now in medical use. Among them, skin tissue replacement for ulcerations and scaffolds allowing a slow release of anticancer agents to combat a form of brain cancer. The research goes on to develop new methods, in the fields of bone tissue engineering or tendon tissue engineering for instance. [1]

1.2 Rotator cuff tears

In this project, the attention will mainly be focused on rotator cuff tears. The rotator cuff is a group of four muscles and four tendons that act to stabilize the shoulder. The muscles comprised in the rotator cuff are the Supraspinatus, the Infraspinatus, the Subscapularis and the Teres minor muscles. These muscles are linked to the head of the humerus via four tendons.

Rotator cuff tears are some of the most common injuries affecting shoulders, with more than 75 000 repair procedures every year in the United States [3]. In that case, tendons are torn and this can affect the mobility of the whole arm. This condition occurs mostly as a consequence of aging or in some cases, intense activity of the shoulder with sport for instance. The Supraspinatus tendon is the most frequently torn due to its position in the shoulder. Rotator cuff tears can be divided in two categories: partial tears and complete tears, crossing the whole tendon from one side to another. An example of a partial rotator cuff tear is shown on Figure 1.1.

Figure 1.1: Example of partial rotator cuff tear on the supraspinatus tendon. [3]
Operative and non-operative treatments exist for rotator cuff tears. The non-operative treatment is based on rest of the arm in the first place. After the inflammation is gone, the patient can start a gentle range-of-motion program to maintain a good mobility and range of motion of the arm. As soon as the pain is gone, the patient starts a strengthening program in order to restore the full power of the tendon.

On the other side, the operative treatment is called arthroscopy. Small tools are inserted via small incisions that make it possible for the surgeon to reinsert rotator cuff tendons. This treatment is less time-consuming and is recommended for younger and active patients. In every way, the treatment never allows the tendons to recover their full strength and chronic tears are extremely frequent. Failures as high as 94% have been reported after primary repair of chronic cuff injuries.

1.3 State of the art of tendon tissue engineering

Since the recovery in the case of tendon tears is long and painful, it is of primary importance to improve the healing of such conditions. For this purpose, tissue engineered solutions are being investigated. These studies focus on various scaffolds able to support cellular adhesion, proliferation and sometimes differentiation. The ultimate goal of these projects is the production of extracellular matrix with sufficient mechanical properties to be able to replace torn tendons. [4]

As tendons are mostly made of collagen, some studies started using gel-collagen sponges as scaffolds and examined the effect of MSC post-surgery. Juncosa-Melvin et al. seeded mesenchymal stem cells (MSC) on collagen sponges and compared them to acellular sponges [5]. These scaffolds were implanted in rabbit tendons containing defects and the scaffolds were analyzed after 12 weeks post-surgery. Mechanical properties for the cellular scaffolds were between 50% and 75% those of a healthy tendon whereas the mechanical properties for the acellular scaffolds were twice as low. Moreover, both repairs showed staining for key proteins as well as good cellular alignment. In brief, these scaffolds showed good mechanical properties and histology and gave very promising results.

Juncosa-Melvin et al also studied the influence of the cell-to collagen ratio on the biomechanics and histology of the repair [6]. They implanted seeded tissue engineered
constructs in rabbit tendon defects, using different cell-to-collagen ratios and different seeding densities. The repairs were assigned for biomechanical and histological analyses after 12 weeks post-surgery. They concluded from this study that high cell-to-collagen ratios do not improve biomechanics or histological appearance of the repairs. Lower cell-to-collagen ratios showed no formation of ectopic bone and similar biomechanics and histology.

More recent studies emphasized new ideas for tissue-engineered constructs for tendons repairs. It is now known that cells can sense the mechanical efforts undergone by tendons. As a consequence, to recreate in vivo conditions, the effect of mechanical stimulation on the scaffolds has been studied by Abousleiman et al [7]. MSC were seeded on decellularized human umbilical vein embedded in collagen gel and stimulated the construct with a mechanical stimulator. The stimulation took place during up to 2 weeks and the results were compared with non-stimulated constructs. The results were quite promising since the stimulated constructs showed way higher (more than twice as high) mechanical properties as well as increased cell number. Moreover, the cells demonstrated parallel alignment with the collagen fibers and cellular morphology close to the native tendons. Their results proved that mechanical stimulation has a very positive effect on the mechanical properties of the constructs as well as on the cellular behavior.

Pursuing in an effort to increase the mechanical properties of the scaffolds, new studies investigated the effect of scaffold stiffening. According to the previous results, increasing the mechanical properties of the constructs could have a positive effect on the repairs biomechanics. For this purpose, Nirmalanandhan et al. created collagen type I sponges stiffened by crosslinking and seeded MSC [8]. Crosslinking lead to a high increase in the mechanical properties of the sponges. The constructs were then stimulated with a mechanical stimulator using two different frequencies (100 cycles/day and 3000 cycles/day). The results were compared with non-crosslinked stimulated samples. Surprisingly, non-crosslinked constructs showed higher mechanical properties than the crosslinked constructs. Moreover, no significant difference was observed between the two stimulation intensities. These results were explained by the fact that the scaffolds were stiff to such an extent that the cells could not sense the mechanical stimulation. This phenomenon is called stress shielding: the material absorbs all the mechanical energy without transferring it to the cells. This study helped to
design future scaffolds in the sense that they must be stiff enough to handle the mechanic efforts but not too stiff to avoid stress shielding.

Creating polymer fibrous scaffolds can also mimic the natural structure of tendons. The electrospinning technique allows the fabrication of such scaffolds since it is able to create aligned nanofibers with a similar size to the collagen fibers in tendons. Moffat et al. designed poly(L-lactic-co-glycolic acid) (PLGA) nanofibers scaffolds via electrospinning and seeded human fibroblasts in order to attest for the cellular behavior of the cells on the scaffolds. They also compared the cellular behavior on aligned and unaligned scaffolds [9]. The analyses showed that cells attached along the nanofibers axis on aligned scaffolds where cells did not show a preferential orientation on randomly oriented scaffolds. Moreover, cell alignment; distribution and matrix deposition were higher in the case of aligned scaffolds as well as mechanical properties that were naturally higher. This study proved that aligned nanofibers scaffolds could recreate the natural architecture of the tendons and be used as scaffolds for tendon tissue engineering.

Different fibers organization and polymers were also considered. These fibrous polymeric scaffolds were as well tested for cellular differentiation of MSC. Barber et al. studied the cellular behavior of human MSC on nanofibrous electrospun scaffolds using poly(L-lactide) (PLLA) [10]. They designed braided nanofibers bundles in order to increase the mechanical properties of the scaffolds and mimic the actual structure of tendons. Their results showed that the mechanical properties of the scaffolds were higher for 3-bundle scaffolds (compared to 4 and 5-bundle). They observed that human MSC adhered and aligned well along the fiber axis and also that the scaffolds supported cellular proliferation and were responsible for the upregulation in the expression of key genes. Under mechanical stimulation with tenogenic growth factors, the human MSC differentiated well into the tenogenic lineage, proving that these scaffolds provide a good environment for cellular differentiation.

In this trend, Surrao et al. designed aligned electrospun scaffolds using different polymers: PLGA, poly(L-lactide-co-DL-lactide) (PLDLA) (98kDa and 250kDa), poly(DL-lactide) (PDLLA) and PLLA [11]. They also introduced a crimp-like pattern as it is present in natural tendons. All the fibrous scaffolds were mechanically tested and fibroblasts were seeded. PLDLA scaffolds exhibited the highest modulus and the lowest degradation rate. The
cellular analyses showed good attachment, proliferation and deposition of extracellular matrix on these PLDLA fibers. The extracellular matrix deposition resembled closely to the organization of natural tendons. This study proved that PLDLA can be a first choice polymer for tendon tissue engineering and that mimicking the tendon structure is the key to a good cellular behavior.

1.4 Description of the project

In this project, a biological graft will be developed using aligned nanofibers scaffolds. A biodegradable and biocompatible copolymer, poly(L-lactide-co-DL-lactide) 70/30, will be electrospun with a custom electrospinning device. Properties such as the average fibers diameter will be characterized. The electrospun fibers will be functionalized with collagen type I, which is the most abundant protein in the tendon extracellular matrix, using carbodiimide chemistry. Moreover, the scaffolds will be seeded with tenocytes isolated from a tendon biopsy. The material-cell construct will be mechanically stimulated to match the in-vivo conditions and the results will be compared to non-stimulated scaffolds in a mechanical and histological way. Every step of the process will be accordingly characterized.
2 Theoretical background

2.1 Generalities about tendons

Tendons are soft connective tissues with limited vasculature. Their role is to transmit mechanical stresses between muscles and bones. Therefore, they play a significant role in musculoskeletal biomechanics. A good knowledge of tendons structure and functioning is a must in order to mimic the most efficiently its organization and discuss the results appropriately. [12]

2.1.1 Structure and composition of tendons

Briefly, tendons are collagenous constructs, which means that collagen is the protein responsible for the structural integrity of tendons. They are also built in a hierarchical organization as shown in Figure 2.1: a tendon is made of several fascicles, each fascicle is made of many fibrils which can be divided in sub-fibrils and micro-fibrils, finally these micro-fibrils are composed of numerous collagen fibers. This hierarchical configuration ensures that minor damages do not spread to the entire tendon, providing a very high structural strength. [12, 13]

Figure 2.1: Hierarchical structure of a tendon [13]
Concerning its composition, a tendon contains approximately 55% to 70% of water. Between 60% and 85% of its dry weight is composed of collagen organized in crimped fibers. This crimp feature or waviness gives even more elongation efficiency to the fibers. More than 60% of the whole collagen content is type I collagen organized in fibers composed of two $\alpha_1$ and one $\alpha_2$ chains. The rest of the collagen content is mostly composed of type III collagen and low percentages of type IV, type V and type VI collagen. Elastin fibers are also present inside tendons with a percentage of 2% of the dry weight. These fibers provide more elasticity to the tendons and allow them to resume their initial shape after being stretched. All the tendon components are surrounded by a proteoglycan matrix, which binds all the fibrous elements in one cohesive structure. [14]

2.1.2 Tendon cells and mechanotransduction

Collagen and other proteins are synthesized by fibroblasts, the most abundant cells in tendons. In the case of tendons, these fibroblasts are called tenocytes and are elongated along the collagen fibers. They lie in longitudinal rows and also have sheet-like extensions far into the extracellular matrix. Tendons also have actin based cell-cell interaction: actin stress fibers run along the rows of tenocytes. Moreover, it is now known that tendons have a high extracellular matrix turnover, meaning that the extracellular matrix is entirely re-created after a while but also that damaged fibers can be quickly replaced with fresh collagen fibers produced by tenocytes. [14]

Recent studies have demonstrated that the matrix production rate is also dependent on the efforts undergone by the tendon. The more intense and the longer the effort is, the higher the production rate will be. This process is called mechanotransduction: the cells sense mechanical stresses and the cells adapt accordingly.

This means that a signaling path is created from the extracellular matrix to the inside of the cells. It is known that integrins play an important role in the mechanotransduction process. Indeed, integrins are proteins acting as receptors since they are linking the extracellular matrix to the cytoskeleton of the cells. They create a continuum between the outside and the inside of the cells. It is well believed that they are acting as sensors for tensile strains in the same way. They perform an outside-in signaling but also in an inside-out mode.
As a consequence, they are partly responsible for the cellular response to mechanical stresses such as cells growth, survival, morphology or proliferation. [14]

2.1.3 Mechanics of tendons

Knowing the biomechanics of tendons is also of a great importance to study this subject. The hierarchical structure and the waviness of tendons fibers play an important role in their mechanical behavior. Indeed, they exhibit a non-linear behavior to tensile forces. On a typical stress/strain curve (Figure 2.2), a toe region appears at low forces. This region corresponds to the uncrimping of the fibers.

Since it is easier to uncrimp the fibers than stretching them, this region has a lower slope than the elastic zone. The following region is the linear elastic region, followed by the yield region and finally failure. In general, the mechanical behavior of tendons must be seen as the individual uncrimping and failure of collagen fibers. Moreover, the hierarchical structure of tendons gives them a hyperelastic behavior. This is characterized by a low yield region and a large elastic region, meaning that the material will suffer plastic deformation only in very extreme cases. [12]

![Figure 2.2](image)

*Figure 2.2: Typical curve for a stress-strain test of a tendon, showing the toe region, the linear region and yield and failure region. [12]*

The second important feature is the viscoelastic response of tendons, which means a time dependent mechanical behavior. In brief, the relationship between stress and strain depends on the time of displacement or load and this can be seen with two important features.
of viscoelasticity: creep and stress relaxation (Figure 2.3). Creep means that, under a constant load, the deformation increases over time. On the other side, stress relaxation is the decrease of load under a constant deformation. [12]

![Figure 2.3: Typical curves of a stress relaxation test (left) and a crimp test (right) [12]](image)

Finally, if a viscoelastic material is loaded then unloaded several times, the material will display energy dissipation. In other words, the curve corresponding to unloading will not follow the loading curve, and this difference shows the amount of dissipated energy during the test. During cyclic loadings, the stress-strain curve slightly shifts to increased strains with each cycle. The curve eventually becomes steady after a certain number of cycles, usually 10 in the case of tendons. [12]

![Figure 2.4: Stress-strain curves showing energy dissipation after loading-unloading cycles in a viscoelastic material [12]](image)
2.2 Biomaterials

2.2.1 Generalities about biomaterials

In the early 1900’s, artificial materials were developed to replace various components of the human body. A biomaterial is now defined as “a non-drug substance suitable for inclusions in systems which augment or replace the function of bodily tissues or organs”. These materials must be capable of being in contact with human tissues or fluids without negative effects or reactions during long periods of time. [15]

These materials can be classified according to their composition, in polymers, metals, ceramics or composites formed with a combination of different materials. In this report, the attention will be focused on biopolymers. As any sort of biomaterial, they can also be classified according to their interaction with human bodies or human fluids.

- Inert biomaterials: there are no interactions between the tissue and the implant, which also means no response of the tissue in contact with the material. This is the case of high molecular weight polyethylene for instance. [16]
- Bioactive materials: the material shows the possibility to create bonds with the surrounding tissues. Polymers are typically not bioactive; bioactive materials are almost exclusively ceramics materials. [16]
- Resorbable materials: they are deeply incorporated into the surrounding tissue, and may degrade in order to let freshly grown tissue expand. Polylactic acids, for instance, are resorbable materials. [16]

Some polymers are also biodegradable (Figure 2.5). A biodegradable material is defined as a material “capable of undergoing decomposition into carbon dioxide, methane, water, inorganic compounds and biomass” (according to ASTM standard D-5488-94d and European norm EN 13432). Testing a material for biodegradation is a complex procedure. However, the main mechanism is the enzymatic action of microorganisms. The tests are generally carried under different media: liquid, inert or compost medium and the resulting products are analyzed. Few rules can be used to attest for the evolution of the biodegradability of a material, such as the hydrophobicity, the molecular weight or the crystallinity. [17]
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2.2.2 Polylactic acid

Polylactic acids or polylaactides are aliphatic polyesters commonly used in tissue engineering. The main advantages of these polymers are their biodegradability and biocompatibility. The biodegradation process is done by hydrolysis as water diffuses into the material. The resulting products of the biodegradation are lactic acid and glycolic acid, which are further metabolized into water and carbon dioxide. The consequence of this hydrolysis is the breaking of polymer chains, leading to a loss of chemical stability and mechanical integrity. [18] The main drawbacks of poly(lactic acid) (PLA) are its brittleness and its poor thermal stability. [19]

Prior to 1950s, it was impossible to achieve high molecular weight polylactic acid. The key to this improvement had been the discovery of specific products, called lactides, coming from the controlled depolymerization of low molecular weight polylactic acid. These lactides can be re-polymerized into high molecular weight polylactic acid in a ring-opening fashion. [20]
Moreover, two configurations, L and D, exist for lactic acid (Figure 2.6). Since lactides are cyclic diesters, they come with three different configurations: L-lactide, D-lactide and DL-lactide (or meso-lactide) (Figure 2.7). Poly-L-lactide or PLLA is semi-crystalline and thus has a low degradation rate: the full degradation can take place over years, depending on the size. [20] This crystallinity can also lead to not-so-ideal biocompatibility. Copolymers of L-lactide and D-lactide have then been created in order to increase the degradation rate and reduce the crystallinity. However, such copolymers lack mechanical properties that are necessary in certain applications. Poly(DL-lactide) is, on the other hand, an amorphous polymer with a rather high degradation rate. Copolymers of L-lactide and DL-lactide show good mechanical properties as well as acceptable degradation rate for tissue engineering purposes. These properties can obviously be modified changing the proportions of the two monomers to fit the best to the application. [22]
transformed into lactic acid after fermentation performed by microorganisms. A direct and cheap source of glucose can be found in corn which is now widely available. Lactic acid produced via fermentation is almost exclusively in the L configuration. In order to produce racemic mixture, a chemical synthesis is necessary. [23] The process of synthesis of lactic acid is shown in Figure 2.8.

Figure 2.8: Process of synthesis of lactic acid via enzyme hydrolysis of starch produced via photosynthesis then fermentation of the glucose into lactic acid [21]

The first process used to produce PLA is the direct polycondensation of lactic acid. However, this process is unable to produce relatively high molecular weight polymers. Thus, a second route is being used to produce higher molecular weight chains. In the first step, lactic acid is polymerized via standard polycondensation. This polycondensation leads to low molecular weight polymers or oligomers. In the next step, dimers called lactides are obtained thanks to a controlled depolymerization of the oligomers. During this step, water is removed from the system without the use of solvent then the dimer is purified under vacuum distillation. In the last step, heat is applied in order to perform the ring-opening polymerization of the lactides, still without the need of solvent. The purity of the lactides is extremely important as it will have influence on the molecular weight of the final polymer.
[24] The whole process for the synthesis of high molecular weight PLA is shown in Figure 2.9.

![Chemical structures](image)

**Figure 2.9:** Synthesis of high molecular weight polylactic acid via ring opening polymerization of lactides [21]

Buchatip et al. studied the thermal and mechanical properties of poly(L-lactide-DL-lactide) containing different mol% of the two monomers. They synthesized these copolymers by ring opening of the corresponding lactides. As expected, the 70/30 PLDLA copolymer was entirely amorphous and its glass transition temperature was around 54°C. Concerning mechanical properties, both the tensile strength and modulus decreased as the percentage of DL lactide increased. On the other hand, the fraction of DL lactide had the opposite effect on the elongation at break. As more DL lactide were introduced into the copolymer, the elongation at break increased and reached an optimal value at 30% DL lactide. As a consequence, it appears that the copolymer used in this project should be less strong than standard PLLA but a way higher elongation at break. In the case of polymeric films, the Young’s modulus was twice as high for PLLA, the tensile strength was nine times higher for PLLA and the elongation at break was 25 times higher for 70/30 PLDLA. [25]
2.3 Polymeric fibers

2.3.1 Electrospinning process

Electrospinning is a method used for the production of nano-scaled to micro-scaled fibers. It has recently gained interest due to the increasing need of polymer nanofibers scaffolds for tissue engineering. This process is attractive in the way that it is easy to setup and quite inexpensive. A custom setup is often used, which shows its simplicity. A second positive aspect is that it can be used with a wide variety of polymers.

In order to produce fibers from a polymer solution, the process uses an electrical voltage to draw very fine fibers from a polymer solution. The polymer solution is typically injected inside a syringe and a voltage is applied at the needle of the syringe. The applied voltage induces charge within the polymer, creating repulsion forces in the solution. The electrical forces exceed the surface tension of the solution and create a cone named Taylor’s cone at the end of the needle. As the solvent evaporates, fibers are formed and cast on a grounded collector. The whole electrospinning process can be seen in Figure 2.10.

![Electrospinning Process Diagram](image)

**Figure 2.10:** Process of fibers production via electrospinning showing the syringe pump, the polymer solution inside the syringe, the power supply, the collector [26]
2.3.2 Electrospinning parameters

Controlling the process parameters such as the feed rate, the applied voltage, the polymer concentration is very important to obtain the desired fiber diameter and avoid the formation of beads. Many parameters will have an influence on the resulting fiber mats. Such parameters include: the viscosity of the solution (linked to the polymer concentration), the conductivity of the solution, the feed rate, the applied voltage, the distance between the needle and the collector as well as the collector composition and its geometry. [27] [28]

First of all, while elaborating the polymer solution to be electrospun, two parameters can be modified. The first is the concentration of the polymer in the solvent which can also be considered as the viscosity of the solution. This parameter can have a major importance in most cases. Correlations have been tried to be found between the concentration and the intrinsic viscosity of the polymer. However, it has been found that these correlations depend highly on the type of polymer. [28] For instance, Koshi et al discovered that the product $[\eta]c$ (where $\eta$ is the intrinsic viscosity and $c$ the polymer concentration) must exceed 5 in order to obtain a solution able to be electrospun. [29] In general, it has been found that increasing the polymer concentration increases the fibers diameter. For instance, PLLA fibers of less than 300nm were produced with a 1%wt solution whereas a 5%wt solution produced fibers of diameter higher than 800nm. [27] [28]

The second parameter taking place in the preparation of the solution to be electrospun is the choice of the solvent and thus the conductivity of the solution. Nonetheless, the choice of the solvent is mainly driven by the need to effectively dissolve the polymer. It has been found that a higher conductivity in the solution leads to smoother fibers and the formation of fewer beads. The addition of salts, such as NaCl, is a common method to increase the conductivity of the solution and thus, to obtain smooth fibers without beads. [27]
Figure 2.11: SEM photograph showing fibers and beads formed during the electrospinning process inside the fibrous mat.

The feed rate is the rate at which the polymer solution flows out of the needle. It is another parameter whose influence has been widely studied. However, its effect on the diameter of the fibers is not precisely known. A trend indicates that lower flow rates yield fibers with smaller diameters, but in some cases, no actual influence had been noticed. [28] An assured observation shows that the feed rate must be high enough to produce fibers. On the other hand, if this parameter is set too high, the jet will be too thick and the solvent will not entirely evaporate. This can cause a partial melting of the fibers due to the high temperature of the solvent as it leaves the needle and leads to the formation of beads. [27] [28]

In this process, the voltage applied at the needle of the syringe is used to draw polymer fibers from the solution. It is a key feature of the process and thus, this parameter has also been studied in many ways. First, it must definitely be high enough to overcome the surface tension of the solution. The relation between the fibers diameter and the voltage is not clearly defined. With some polymers like PDLA or poly(vinyl alcohol) (PVA), higher voltages yielded larger fibers although the opposite effect was observed with other polymers like bisphenol-A-polysulfone. Still, a common observation proved that very high voltages led to
the formation of many beads inside the fibrous mat. [27] [28] Figure 2.12 shows the influence of the voltage and the polymer concentration on the fiber diameter in the case of polycaprolactone.

![Graph showing the influence of the polymer concentration and the voltage used during electrospinning on the diameter of polycaprolactone fibers](image)

**Figure 2.12:** Graph showing the influence of the polymer concentration and the voltage used during electrospinning on the diameter of polycaprolactone fibers [28]

A particular attention must be paid to the distance between the needle and the collector. If the collector is too close, the solvent is not entirely evaporated and can cause melting of the fibers and forms beads. In the opposite case, the fibers might not all reach the collector, resulting in an important loss of material. [27]

Moreover, the composition of the collector can influence the aspect of the fibers. Metallic collectors typically lead to smooth fibers whereas porous collectors lead to porous. Finally, a rotating collector can be used in order to obtain aligned fibers. A stationary collector will typically lead to random oriented fiber mats. [27]

### 2.3.3 Characteristics of polymeric fibers

In this project, the choice of electrospun fibers is of primary importance. Indeed, polymeric fibers offer many advantages over polymeric films for instance. First of all, they mimic the natural structure of tendons and it is known that cells need a familiar environment to proliferate. Nanoscaled fibers, moreover, offer even more advantages. Nanofibers have a
very large surface area to volume ratio and a large weight to mechanical properties ratio. Their surface can also be modified easily according to the desired expectations. All of these properties are emphasized in this project, making such fibers a first class choice for tendon tissue engineering.

2.4 Functionalization process

In order to mimic also the composition of the collagen fibers, it is necessary to attach collagen on the surface of the fibers. This is done in this project thanks to a functionalization step.

On the one hand, the surface of PLA can be hydrolyzed in order to reveal carboxylic groups. On the other hand, it is common to take advantage of the primary amino groups present on proteins such as collagen. The best way to attach such molecules on hydrolyzed PLA surfaces is using carbodiimide zero-length crosslinkers.

2.4.1 Hydrolysis of the PLA surface

Carboxylic groups must be created on the surface of the PLA fibers. This can be done with a hydrolysis of the fibers with a strong alkali like sodium hydroxide. The action of the alkali is to break the polymer chains and to yield a carboxylic group on the end of one chain (Figure 2.13). The intensity of the hydrolysis can be controlled by modifying the concentration and the exposition time. The longer the exposition time and the more concentrated the solution, the more carboxylic groups are formed. However, the hydrolysis has also a negative effect on the fibers because polymer chains are broken during the step, possibly decreasing the mechanical properties. As a consequence, it is important to measure the effect of the hydrolysis and determine an adequate combination of exposition time/concentration. [30]
2.4.2 Covalent attachment of collagen

Carbodiimide chemistry and more specifically carbodiimide zero-length crosslinkers are often used to create amide bonds between carboxylic groups and primary amino groups. This type of crosslinking is often used to bond proteins like collagen since they contain several amino groups.[33]

The carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Figure 2.14) is the main crosslinker used in such cases. A non-negligible drawback of this technique is the unstable intermediate formed during the reaction. Indeed, the reaction of EDC and the carboxylic group forms an active ester, o-acylisourea, which is very reactive towards primary amines. This intermediate will react with any primary amine group and form an amide bond between the PLA surface and the collagen molecule. An isourea by-product is created at the end. A consequence of this is the formation of poly-proteins due to the presence of several amino groups in proteins. [31] Figure 2.15 shows the action of EDC on the coupling of a carboxylic acid and a primary amine.
It is necessary to obtain a more stable intermediate in order to avoid this undesirable effect. The addition of N-hydroxysuccinimide (NHS) (Figure 2.16) has been found to have a positive effect on the reaction. Indeed, the NHS molecule couples directly to the unstable intermediate to form a much more stable intermediate that will not produce undesirable reactions. Moreover, the use of the couple EDC/NHS makes it possible for the reaction to be carried at physiologic pH or in a phosphate buffered saline solution, while EDC-direct coupling is only possible at acidic pH.

**Figure 2.15:** Action of EDC coupling of a carboxylic acid with a primary amine [31]

**Figure 2.16:** Chemical structure of NHS and its molecular weight [33]
3 Materials and methods

3.1 Preparation of the polymer solution

In this project, the considered polymer is 70/30 L-lactide/DL-lactide copolymer (Purasorb PLDL 70/30, IV midpoint 3.8 dl/g, Mw=850kDa, © Purac). A 50mL solution of 4% w/v of polymer in 2,2,2-trifluoroethanol (TFE) was prepared for electrospinning. This polymer has been chosen for its well-known biocompatibility and biodegradability, which makes it a first class polymer for tissue engineering applications.

3.2 Production of nanofibers via electrospinning

![Figure 3.1: SEM photograph of electrospun PLA fibers](image)

The aligned nanofibers scaffolds were made via the electrospinning technique. This was carried out thanks to a custom electrospinning setup. The previously mentioned polymer solution was inserted in a 20mL syringe pump of 20,3mm diameter with a needle of 25GA (0,5mm) diameter. A voltage of 7,5kV to 10kV was applied at the needle and the polymer solution was fed at a rate of 0,5mL/h to 1mL/h. A rotating collector at 1000rpm was placed at 13cm from the needle and used for collecting aligned fibers. A 21cm x 28cm mat of polymer nanofibers was obtained. The 50mL of polymer solution prepared previously were entirely electrospun.
The parameters used in this project are listed in Table 3.1.

<table>
<thead>
<tr>
<th>Electrospinning parameters used in this project</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter of the needle</td>
</tr>
<tr>
<td>Diameter of the syringe</td>
</tr>
<tr>
<td>Distance between needle and collector</td>
</tr>
<tr>
<td>Rotating speed of the collector</td>
</tr>
<tr>
<td>Applied voltage</td>
</tr>
<tr>
<td>Feeding rate</td>
</tr>
</tbody>
</table>

### 3.3 Fibers characterization

#### 3.3.1 Fibers diameter

Samples of 1,5cm x 1,5cm were cut and prepared for scanning electron microscopy (SEM) analysis. Fibers diameters were measured using ImageJ. A mean value was calculated out of 300 measurements to ensure statistical distribution.

#### 3.3.2 Porosity of the fibrous scaffolds

When considering polymeric fibers scaffold, the porosity is an interesting parameter to determine. Therefore, 4 samples of 1,5cm x 1,5cm were cut and their mass was measured
thanks to precision scales. The samples were then entirely dipped in an ethanol solution 70% and gently mixed during 1h to ensure optimal soaking. After this period, samples were retrieved from ethanol and weighted. The porosity is given by the following formula:

$$\text{Porosity} = \frac{V_{\text{ethanol}}}{V_{\text{ethanol}} + V_{\text{polymer}}} = \frac{\Delta w}{\frac{\Delta w}{0.869 \text{g/cm}^3} + \frac{w_i}{1.3 \text{g/cm}^3}}$$

With $\Delta w$ the mass difference after and before soaking with ethanol

$w_i$ the initial mass of the samples

Polymer density 1.3g/cm3

Ethanol density 0.869g/cm3

### 3.3.3 Thickness of the electrospun mat

The thickness of the fibrous mat was calculated according to the following formula.

$$V_{\text{sheet}} = 21 \text{cm} \times 28 \text{cm} \times \text{thickness} = \frac{2.87 \text{g}}{1.3 \text{g/cm}^3} \times \frac{1}{1 - \text{porosity}}$$

$$\text{thickness} = \frac{2.87 \text{g}}{1.3 \text{g/cm}^3} \times \frac{1}{1 - \text{porosity}} \times \frac{1}{21 \text{cm} \times 28 \text{cm}}$$

### 3.3.4 Mechanical properties of the scaffolds

Mechanical properties of the fibers were tested using a Bose Electroforce 5100 Biodynamic equipped with a 200N load cell installed inside an incubator. Appropriate grips for fiber mats testing were used.
Samples of approximately 30mm x 10mm were cut, weighted and measured. Moreover, notched paperboard reinforcements of approximately 10mm x 7mm were put on each extremity of the samples using double-sided tape to ensure optimal adhesion with the grips. The cross-sectional area of the samples was calculated using the following formula:

\[ A = \frac{m}{\rho L} \]

Where m is the mass of the sample, \( \rho \) the density of the polymer and L the gauge length.

The following procedure was used to test the mechanical properties of the samples. The samples were first subjected to a preload of 0.5N in order to have them very slightly stretched before starting the test. The samples were then preconditioned with 10 cycles of 0.1mm at 0.1Hz. Finally, the samples were subjected to a ramp until 6mm or failure at 0.1mm/s. Stress-strain curves were drawn for these samples and mechanical properties were calculated. The elastic modulus was calculated as the slope of the elastic region of the curve. The yield strain...
and yield strength are respectively the strain and the stress just before the transition of the elastic region to the yield region.

Moreover, the elastic behavior of the material was tested thanks to a stress relaxation test and a creep test. For the stress relaxation test, samples were subjected to a displacement of 1mm and the evolution of the load was observed during 40 seconds. For the creep test, samples were subjected to a load of 14N and the evolution of the displacement was observed during 150 seconds.

### 3.4 Surface hydrolysis

The surface of poly(lactic acid) fibers is highly hydrophobic. In order to effectively attach collagen and cells to the surface of the scaffold, a hydrophilic surface is essential. This is an important drawback that can be avoided by appropriate chemical treatment, such as hydrolysis. A strong alkali like sodium hydroxide can break the polymer chains at the surface and reveal carboxylic functions, giving hydrophilic properties to the surface. However, it is yet unknown what concentration and exposition time can give the best results.

In order to attest for the effect of the hydrolysis on the fibers diameter and shape, 12 samples of 1.5cm x 1.5cm were cut and treated with NaOH in 12-well plates. Teflon rings of 1cm diameter were put on top of the samples and the NaOH solution was poured inside the rings to avoid leaking. The NaOH solutions were made at three different concentrations (0.1M, 0.05M and 0.01M) and the samples were exposed during various exposition times (2 minutes, 3 minutes, 4 minutes and 5 minutes). After this exposition period, the liquid was removed from the Teflon rings and the samples were washed several times with water and prepared for SEM analysis. Fibers diameter were measured using ImageJ. A total number of 300 measurements were made for each concentration/time combination.

All the other samples treated with NaOH for the next steps were hydrolyzed the same way using a 0.1M solution and were exposed during 5 minutes.

### 3.5 Activation of the carboxylic groups

The next step of the functionalization is the activation of the carboxylic groups formed via hydrolysis. This can be done using the EDC/NHS couple (EDC solution 97%, © Acros
Organics; NHS powder 98%, © Aldrich chemistry). Therefore, samples of 1,5cm x 1,5cm dimensions were cut and hydrolyzed with NaOH at 0,1M concentration during 5 minutes then placed in 12-well plates. A 0,1M/0,02M EDC/NHS solution in phosphate buffer saline (PBS) was prepared and 2mL of this solution were inserted in each well. After 1h, the liquid was removed and the samples were then washed several times with PBS.

### 3.6 Covalent attachment of collagen type I

![Figure 3.4: Photograph of PLA samples in 12-well plates during functionalization](image)

As soon as the carboxylic groups are activated, it is possible to add collagen type I on the surface of the scaffolds. Therefore, a 100µg/mL collagen type I (rhCollagen type I bulk solution, © Fibrogen) solution in PBS was prepared and 2mL were inserted in each well during 24h. Moreover, the same treatment was done to 6 clean samples (non-hydrolyzed, non-activated) to attest for the physical adsorption of collagen onto the fibers. The samples were then washed several times with PBS.

![Figure 3.5: Functionalization process with concentrations](image)
3.7 Collagen immunofluorescence

To verify the adhesion of collagen on the fibers, samples were tested with the immunofluorescence technique. A total of 12 samples with collagen were tested: 6 fully functionalized and 6 attesting for physical adsorption of collagen. The samples were dipped in a PBS/glycine/bovine serum albumin (BSA) 6% solution for 30 minutes then washed several times with a PBS-glycine solution. A 1:500 primary antibody solution (mouse anti-collagen type I, Santa Cruz) in PBS-glycine-BSA 3% was added during 1h at 37° to 3 fully functionalized samples and 3 samples attesting for physical adsorption of collagen. The samples were then washed several times with PBS-glycine. A 1:500 secondary antibody (fluorescent goat anti-mouse, Santa Cruz) solution containing fluorochromes in PBS-glycine-BSA 3% was added during 1h at 37° to all the 12 samples. The samples were then washed several times with PBS-glycine. The samples exposed only to the secondary antibody will be used as control samples. Since the secondary antibody contains fluorochromes, it was important to wrap the samples in aluminum paper in order not to degrade the fluorescence process.

![Figure 3.6: Immunofluorescence samples distribution](image)

3.8 Micro BCA protein assay

The micro bicinchoninic acid (BCA) protein assay (© Thermo Scientific) is used to measure the protein concentration of a sample. This assay takes advantage of the reduction of Cu2+ ions into Cu+ ions by proteins. The first part of the assay consists in building a calibration curve (Figure 3.7). For this purpose, six different solutions of concentration 25,
12.5, 6.25, 3.125, 1.5625 and 0 µg/mL of collagen type I in PBS were prepared. For each solution, one milliliter was put inside a test tube, along with one milliliter of the working reagent (containing 25:24:1 reagents MA, MB and MC, provided in the assay box). Two replicates were made to ensure statistical distribution. The tubes were then incubated at 60°C during 60 minutes. The absorbance of the final products was measured in a spectrophotometer at 562nm and plotted against the concentration in the calibration curve.

Four samples functionalized with collagen type I were cut and their dimensions measured. Each sample was put inside a test tube along with 1mL of PBS and 1mL of the previously mentioned working reagent. The tubes were then incubated at 60°C during 60 minutes. Their absorbance was then measured in a spectrophotometer at 562nm and the results were reported on the calibration curve to determine the actual concentration.

![Calibration curve for the micro BCA protein assay](image)

**Figure 3.7: Calibration curve for the micro BCA protein assay**

### 3.9 Mechanical properties of functionalized scaffolds

The properties of dry scaffolds compared to those of functionalized scaffolds, which are wet could be quite different. This test was meant to get mechanical properties as close as possible to functionalized scaffolds bearing cells and thus dipped in medium at 37°C.
For this purpose, samples of approximately 60mm x 15mm were cut, weighted and measured. Both ends were folded several times over 20mm to create reinforcements, similar to the paperboard pieces used for dry samples. Such samples can be seen in Figure 3.8. This resulted in samples of approximately 20mm x 15mm in size. The samples were functionalized as it is explained in the paragraphs 3.4 to 3.6 and left immersed in PBS until testing. The same formula was used to calculate the cross-sectional area of the scaffolds and a similar testing procedure was used. The samples were mounted between the grips of the machine and the incubator closed. When the temperature reached 37°, the same procedure as for dry samples was used to test the samples. The procedure is explained in the paragraph 3.3.4.

### 3.10 Cell culture of MSC and tenocytes

#### 3.10.1 Cell types and mediums composition

Two types of cells were used in this project. Rat MSC were first used for the live cell staining in order to verify that the scaffolds were able to support cells. Later, human tenocytes were acquired thanks to a tendon biopsy and these cells’ behavior on the scaffolds was studied as it is explained in the following parts.

Dimethylsulfoxide (DMSO) medium or freezing medium, used for freezing cells:

- 90% PBS
- 10% DMSO
  A cryoprotective agent that reduces the freezing point of the medium and also allows a slower cooling rate, greatly reducing the risk of ice crystal formation, which can damage cells and cause cell death. [34]

Culture medium for rat MSC:

- Advanced DMEM (Gibco)
  A widely used basal medium that allows the culture of mammalian cells with reduced Fetal Bovine Serum (FBS) supplementation. [35]
- 1% L-Glutamine
  An amino acid required for cell culture. [36]
- 1% Penicillin/Streptomycin
  The combination of the antibiotics penicillin and streptomycin are used to prevent bacterial contamination of cell cultures due to their effective action against gram-positive and gram-negative bacteria, respectively. [37]
- 15% FBS

Culture medium for tenocytes:

- DMEM F12 (containing glutamine) (Gibco)
  A widely used basal medium for supporting the growth of many different mammalian cells. Cells successfully cultured in DMEM/F-12 include MDCK, glial cells, fibroblasts, human endothelial cells, and rat fibroblasts. [38]
- 1% Non-essentials amino acids 100x (Gibco)
  Is used as a growth supplement for cell culture medium, to increase cell growth and viability. [39]
- 1% Penicillin/Streptomycin
- 10% FBS
- 0.1% Ascorbic acid (0.5M)

3.10.2 Unfreezing cells

Frozen cells with DMSO medium in 1mL cryotube were taken out of the freezer and heated. A culture flask was filled with 14mL of culture medium and the content of the cryotube was poured inside the flask. The content of the flask was looked at under a microscope to verify the homogeneous repartition of the cells. The flask was then left inside an incubator at 37°C.

3.10.3 Changing culture medium

After removing the old medium, 5mL of PBS were introduced in the flask to wash out the dead cells. The PBS was then removed and 10mL of fresh medium was poured
on the side of the bottle in order not to touch the cells with the flow of liquid. The flask is then put back in the incubator at 37°C.

### 3.10.4 Trypsinization and freezing cells

When confluence is reached, which means when the surface of the flask is entirely covered with cells, trypsinization must be done to separate cells and let them grow freely with more space. At the end of this step, fewer cells will be seeded in new flasks and the excess of cells will eventually be frozen.

After removing the old medium and washing the cells with PBS as it is explained in 3.9.2, 5mL of trypsin were added in the flask. The flask was then gently shaken to detach the cells from the surface and the flask was placed in the incubator at 37°C during 5 minutes. After checking that the cells were all detached from the surface, medium was added and the content of the flask was poured into a 5mL tube. The tube was placed in a centrifugation device during 5 minutes at 1000rpm to split the cells from the medium/trypsin mix. Afterwards the mix of medium and trypsin was removed from the tube and replaced with fresh medium. 10μL of this solution were used to count an average concentration of cells and several flasks were seeded with 500 000 cells in each. The flasks were then placed in an incubator at 37°C. The step 3.9.2 was the repeated for these new flasks. The excess of cells was inserted in cryotubes with 1mL of DMSO medium with an average number of one million cells per cryotube. The cryotubes were then placed in the freezer.

### 3.11 Cell seeding on scaffolds

Fully functionalized scaffolds were placed in 24-well plates and ultraviolet sterilization was performed during 15 minutes. The samples were rinsed twice with sterilized PBS then culture medium was added. The plates were incubated at 37°C during 24h. The next day, rat MSC were counted and seeded on the scaffolds at a density of $2 \times 10^4$ cells/cm². The cells were allowed to attach during 2h then culture medium was added. The scaffolds then underwent a live cell-staining assay to quickly attest for the viability of the cells on the scaffolds. The same procedure was used for the seeding of tenocytes.
3.12 Live cell staining

This type of staining uses Calcein-AM in order to make cells visible through fluorescence. The medium was removed from the plates and a Dulbecco’s phosphate buffered saline (DPBS) 1X solution in MilliQ water was used to wash the samples twice. After washing, a solution containing Calcein-AM at 2µM in DPBS was added on top of the fibers. Since Calcein-AM is sensible to light, it is necessary to wrap the plates in aluminum paper. The plates were then incubated during 20 minutes at 37°C. After this duration, the samples were washed twice with the previously mentioned DPBS 1X solution. The scaffolds were then observed under a fluorescence microscope (Nikon Eclipse E600).

3.13 Cells immunofluorescence

This immunofluorescence technique uses the conjugation of the 4',6-diamidino-2-phenylindole (DAPI) and the phalloidin molecules. The cells nuclei can be observed thanks to DAPI and the cells cytoskeletons can be observed thanks to phalloidin. Samples seeded with cells as it is explained in paragraph 3.10 were retrieved from culture medium after 1 day, 3 days and 6 days and underwent the following procedure. Three different types of samples were compared: glass, functionalized PLA and PLA.

The samples were washed twice with PBS-gly and then covered with a fixative solution during 10 minutes at 4°C. A 4mL fixative solution contains: 760µL of paraformaldehyde (PFA) 16%, 240µL of sucrose 1M in MilliQ water, 2mL of PBS and 1mL of MilliQ water. The samples were then washed twice with PBS-gly. The samples were then permeabilized during 10 minutes with a Triton X-100 solution at 0,05% in PBS-gly. The samples were then washed three times with PBS-gly. The next steps must be done covering the samples with aluminum paper to avoid the decomposition of the fluorescent compound (DAPI and phalloidin). During 8 minutes, the samples were covered with a phalloidin solution 1/500 in PBS-gly. After the 8 minutes, a 1/250 solution of DAPI in PBS-gly was introduced on top of the samples. The samples were then washed three times with PBS-gly. The samples were finally observed under a fluorescence microscope (Nikon Eclipse E600). Images of the cells nuclei and cytoskeletons were taken using different filters and composite images of the samples were obtained using ImageJ. The same software was used to calculate the area as well as the roundness of the cells.
3.14 Mechanical stimulation of seeded scaffolds

Samples of approximately 60mm x 15mm were cut, weighted and measured. Both ends were folded several times over 20mm to create reinforcements. Those samples were functionalized as it explained in parts 3.4 to 3.6 and seeded with tenocytes as it is explained in part 3.11. After seeding, the samples were incubated one day at 37°C to let the cells attach before starting the stimulation. The next day, the samples were placed between the grips of the Bose Electroforce Biodynamic 5100 and the chamber was filled with culture medium pre-warmed at 37°C as it shown in Figure 3.9. The incubator containing the machine had been previously heated to 37°C. Maximum care was taken to avoid contamination while placing the samples in the machine. Most of the procedure was done under a fume hood when it was possible. The samples were then stimulated 8 hours a day with 8% strain at 0.5Hz. At the end of the stimulation process, the samples underwent a ramp to 6mm at 0.1mm/s in order to calculate the final mechanical properties of the scaffolds.

Figure 3.9: Setup of a sample mounted in the Bose Electroforce Biodynamic 5100 ready for mechanical stimulation.
Cellular behavior after mechanical stimulation on biofunctionalized polylactic acid nanofibers for tendon tissue engineering
4 Results and discussion

4.1 Fibers characterization

4.1.1 Fibers alignment and presence of beads

SEM images were taken to look at fibers alignment and to verify the presence of beads (Figure 4.1). While fibers seem much disorganized on the top of the samples, they look well aligned in the bulk. White arrows are drawn to show the direction of alignment. A small number of beads can also be seen. The size of these beads is typically comprised between 1µm and 5µm. Red arrows are drawn to show some of the beads that can be seen in the samples.

Figure 4.1: SEM photographs of electrospun PLA fibers. White arrows indicate the alignment direction of the fibers. Red arrows indicate beads formed during the electrospinning process.

The few manipulations performed on the samples may have affected the surface of the samples and may have changed the alignment of the fibers on the surface. This could have some importance when cells will be seeded on the scaffolds, since they will attach mostly on the surface and may not find a unidirectional geometry. However, they are indeed aligned in the bulk meaning that this will not affect the mechanical properties of the scaffolds. Moreover, the presence of beads is almost insignificant and will not have any effect on any property of the scaffolds.
4.1.2 Properties of the electrospun fibrous mat

Average fibers diameter was calculated out of 300 hundreds measurements. It was calculated that fibers have an average diameter of 700nm. The porosity of the scaffolds was calculated by measuring the difference of weight before and after dipping the samples in ethanol. It was calculated that the average porosity of the scaffolds is 77%. Out of this result, it is possible to calculate the thickness of the electrospun fiber which is 163µm in average. These results are summarized in Table 4.1.

<table>
<thead>
<tr>
<th>Fibers diameter</th>
<th>Porosity of the mat</th>
<th>Thickness of the mat</th>
</tr>
</thead>
<tbody>
<tr>
<td>700nm</td>
<td>77%</td>
<td>163µm</td>
</tr>
</tbody>
</table>

4.1.3 Mechanical properties of the scaffolds

Mechanical tests were performed on PLA samples at room temperature. All these tests were tensile tests performed in the direction of alignment of the fibers. In order to compare the results, all the loads (in Newtons) and displacements (in millimeters) were converted respectively into stresses (MPa) and strains (%) according to the following formulas:

\[
\text{Stress (MPa)} = \frac{\text{Load (N)}}{\text{Cross-sectional area (mm}^2)}
\]

\[
\text{Strain (%) = } 100 \times \frac{\text{Displacement (mm)}}{\text{Gauge length (mm)}}
\]

The elastic behavior of the material was tested using a stress relaxation test and a creep test (Figure 4.2). In the stress relaxation test, the samples were subjected to a constant displacement and the evolution of the load was observed during a certain time. When the strain of approximately 6.7% was applied, the stress instantly increased to approximately 58MPa and then slowly decreased to a lower value under 20MPa. In the creep test, the samples were subjected to a constant load and the evolution of the displacement was observed during a certain time. When the stress of approximately 47MPa was applied, the strain rapidly increased to approximately 8% then kept increasing less rapidly to a higher value over 15%.
Figure 4.2: Load and displacement against time curves for stress relaxation test and creep test. These tests were performed on PLA samples at room temperature.

Stress-strain curves were drawn for non-functionalized samples (Figure 4.3). As it can be seen on the presented curve, the results from one sample to another were quite similar. The curve can be divided in three parts. The first part, called the toe region, can be seen between strains of 0% to 2,5% in average. The second part, called the elastic region, can be seen between 2,5% and 10% in average. The last part, called the yield region, can be seen after 10% of strains. It was impossible to reach the failure of the samples with the machine that was used. The maximum displacement of the machine was 6mm.
Few experimental issues were encountered. The first issue encountered was an insufficient preload. The preload was used to very slightly stretch the samples before the test, in order to have them straight. If the preload was insufficient, the samples were not correctly stretched before the test. This resulted in much longer toe regions. The other experimental issue encountered was the sample slipping out of the grips. This was due to the grips not tightening up enough the samples. The use of the reinforcements was meant to solve this problem but it still happened in rare cases. The consequence of this was a premature stop of the test, occurring at relatively high stress or strain.
Cellular behavior after mechanical stimulation on biofunctionalized polylactic acid nanofibers for tendon tissue engineering

Figure 4.4: Example of stress-strain curve used to calculate the mechanical properties of the sample. The black line shows the linear tendency of the elastic region and the corresponding equation is also shown. The blue lines show where the yield strain and the yield stress coordinates are taken.

Mechanical properties of the samples can be calculated out of the strain-stress curve. Figure 4.4 shows a brief summary of how the properties were determined. The elastic modulus was calculated as the slope of the elastic region. It was determined that the elastic modulus of the samples was 9,1MPa in average. The yield strength and the yield strain were respectively determined as the coordinates of the curve just before the transition between the elastic region and the yield region. It was determined that the yield strength and the yield strain of the samples were respectively 73,6MPa and 9,7%. A summary of these properties as well as the dimensions of the samples can be seen in Table 4.2.

Table 4.2: Mechanical properties of non-functionalized scaffolds.

<table>
<thead>
<tr>
<th>Size (mm x mm)</th>
<th>Cross sectional area (mm²)</th>
<th>Elastic modulus (MPa)</th>
<th>Yield strength (MPa)</th>
<th>Yield strain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean 30,82 x 10,73</td>
<td>0,191</td>
<td>9,1</td>
<td>73,6</td>
<td>9,7</td>
</tr>
<tr>
<td>±2,77 x ±0,57</td>
<td>±0,033</td>
<td>±1,1</td>
<td>±4,1</td>
<td>±1,3</td>
</tr>
</tbody>
</table>

The creep and the stress relaxation tests exhibited a very time-dependent behavior to stresses and strains. This phenomenon is called viscoelasticity. This is an important result as it shows that the scaffolds are viscoelastic just like natural tendons and it is a step forward to the process of mimicking natural tendons properties.
On the stress-strain curves can be seen the toe region, the elastic region and the yield region. The toe region can be seen at low deformations and has a very low slope compared to the elastic region. This means that the resistance of the material to the deformation is low at this point. Indeed, this region is typically seen in the case of fibers and corresponds to the uncrimping (or stretching) of the fibers. After this region, the fibers are completely stretched and start to deform. The elastic region corresponds to the reversible deformation of the fibers themselves and the yield region to the irreversible deformation of the fibers.

These three regions are also present in the case of natural tendons, although they are present in different proportions. The toe region is much longer for tendons as the collagen fibers are naturally wavy whereas they are slightly crimped in the case of electrospun scaffolds. Moreover, the yield region is much longer for PLA fibers because they don’t exhibit a hyperelastic behavior as natural tendons do (as explained in part 2.1.3).

The mechanical properties of natural rotator cuff tendons have been studied by Halder et al [40]. Elastic moduli of these tendons were found to be between 100MPa and 200MPa and ultimate stresses between 10MPa and 30MPa. However, the yield and rupture behaviors of tendons are unique because of its hierarchical structure and cannot be compared accurately with other materials. Moreover, tendons typically start to break at a micro-scale between 5% and 10%. In the case of PLA, the yield stress and strain are higher than the rupture values for tendons although the elastic modulus is one order-of-magnitude lower. When looking at these values, it is important to keep in mind that a tendon is a 3D component whereas the PLA samples in this test can be considered as 2D components. This statement can explain the huge differences of modulus between the two. It is expected further in this project that the cells produce extracellular matrix that will increase the elastic modulus of the material, but might make it more brittle.

4.2 Effect of the hydrolysis step

4.2.1 On the appearance of the fibers

Tests were done to check the possible effect of the hydrolysis step on the fibers. Several tests were done, using various concentrations of NaOH (0.1M, 0.05M and 0.01M) and
exposition times (2 minutes, 3 minutes, 4 minutes and 5 minutes). SEM images were taken for some concentrations/exposition time combinations (Figure 4.5).

**Figure 4.5**: SEM images of fibers hydrolyzed with NaOH at different concentrations during different exposition times. Top left: 0.1M during 5 minutes. Top right: 0.01M during 4 minutes. Bottom left: 0.05M during 3 minutes. Bottom right: 0.1M during 2 minutes.

The top left picture (0.1M during 5 minutes) shows a quite important effect of the hydrolysis. It appears that some fibers are stuck together forming larger fiber bundles with diameters over 10 times the diameter of one fiber (up to 10µm). Being the hydrolysis with the highest concentration/exposition time, the top left picture shows the most important effect of hydrolyzation. On the top right picture (0.01M during 4 minutes), the effect is less visible. Fewer fiber bundles can be seen and their diameter is less than 5 times the diameter of one fiber. The bottom left picture (0.05M during 3 minutes) shows a moderate effect of
Cellular behavior after mechanical stimulation on biofunctionalized polylactic acid nanofibers for tendon tissue engineering

hydrolyzation. Fiber bundles can be seen, with their diameter being 5 to 10 times the diameter of one fiber. The bottom right picture (0,1M during 2 minutes) shows absolutely no effect of hydrolyzation.

**4.2.2 On the diameter of the fibers**

The hydrolysis could have also had an effect on the fiber diameter, which would have rather important consequences. As a consequence, the diameter of the fibers was measured on samples that underwent hydrolysis at different concentrations and different exposition times. The values for the fibers diameter are the following: 701nm for PLA without hydrolysis, 673nm for NaOH at 0,1M during 5 minutes, 673nm for NaOH at 0,01M during 4 minutes, 741nm for NaOH at 0,05M during 3 minutes and 661nm for NaOH at 0,1M during 2 minutes. These values are all very similar, slightly higher for the treatment at 0,05M during 3 minutes which is an intermediate combination of concentration/exposition time. Standard deviations are also very similar in magnitude. These results can be seen on Figure 4.6.

**Figure 4.6:** Effect of hydrolysis on the diameter of the fibers for non-hydrolyzed samples (PLA) and samples exposed to NaOH at different concentrations during different exposition times. Standard deviations are also represented.

As a conclusion of these results, it is clear that the hydrolysis only had an effect on the appearance on the fibers and not on their diameter. This experiment was used to determine what concentration/exposition time combination needed to be used for the further steps of the functionalization. The purpose of the hydrolysis is to create carboxylic groups on the surface of the fibers. It was a certain fact that the higher the concentration and the longer the
exposition time, the more carboxylic groups were created. Thus it was chosen to continue the functionalization using the highest concentration/exposition time combination (0.5M during 5 minutes). Although the effect on the appearance of the fibers was not negligible, the hydrolysis had an actual effect with these parameters and many carboxylic were definitely present on the surface of the fibers. The choice was mainly driven by this objective.

4.3 Collagen immunofluorescence

Collagen type I was marked via immunofluorescence to detect its presence after the functionalization process (Figure 4.7). The control samples exhibited no fluorescence (bottom picture). The samples containing collagen covalently attached to the fibers showed an important fluorescence, proving the presence of collagen type I (top left picture). The samples attesting for the physical adsorption of collagen exhibited moderate fluorescence, which demonstrate the presence of lower quantities of collagen compared to the samples containing covalently bonded collagen.

![Image of immunofluorescence marking for collagen type I on PLA fibers. Top left: covalently attached collagen with EDC/NHS. Top right: collagen attached by physical adsorption. Bottom: no primary antibody. (Nikon Eclipse E600).](image)

**Figure 4.7:** Immunofluorescence marking for collagen type I on PLA fibers. Top left: covalently attached collagen with EDC/NHS. Top right: collagen attached by physical adsorption. Bottom: no primary antibody. (Nikon Eclipse E600).
This experiment was intended to mark collagen functionalized on the fibers via immunofluorescence. In this test, the first antibody (or primary antibody) is an anti-collagen antibody and normally attaches to collagen. The second antibody (or fluorescent antibody) is an anti-primary antibody and normally attaches to the primary antibody. However, it is possible that the second antibody attaches directly to collagen or PLA which would give wrong observations. To verify that this does not happen, control samples containing only the second antibody were also studied. These control samples showed absolutely no fluorescence, proving that it does not attach to collagen or to non-functionalized PLA without the presence of the primary antibody. Concerning the samples containing both antibodies, they actually exhibited fluorescence. The functionalized samples showed a much higher fluorescence than the non-functionalized samples. This proves that high quantities of collagen are attached to the functionalized fibers whereas physical adsorption does not enable a good attachment of collagen. The shape of the fibers can be clearly seen in both cases.

### 4.4 Collagen concentration with micro BCA assay

The collagen concentration of functionalized samples (covalently attached collagen) was measured using the micro BCA assay. The samples had an average surface of 0,92cm². On these samples, the collagen concentration was measured as an average 18,15µg/mL. Therefore the efficiency of the grafting of collagen on the fibers is 18,15%. The results can be seen in Table 4.3.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Surface (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0,92</td>
</tr>
<tr>
<td>±3,65</td>
<td>±0,06</td>
</tr>
</tbody>
</table>

**Table 4.3**: Collagen type I concentration of functionalized samples. The initial concentration of collagen that was incorporated onto the samples was 100µg/mL.

### 4.5 Mechanical properties of functionalized scaffolds

Typical stress-strain curves for non-functionalized at room temperature (results from 4.1.3) and functionalized samples at 37° were drawn on the same graph in order to compare them (Figure 4.8). The overall shape of the curve is similar: the three regions, toe, elastic and yield, can still be seen. However, the toe region is much longer for functionalized samples and
goes to strains of almost 7% whereas for the non-functionalized samples, it only goes to a maximum of 2.5%. The slopes of the elastic region of the two curves are very close from each other. This will result in very close elastic modulus also. Moreover, the yield region seems to start at a much lower stress but at a quite higher strain in the case of functionalized samples.

![Stress-strain curves (non-functionalized and functionalized)](image)

**Figure 4.8:** Comparison of stress-strain curves for functionalized samples tested at 37°C (red) and non-functionalized samples tested at room temperature (blue).

Following the same procedure as explained in paragraph 4.1.3, the mechanical properties of the samples were calculated for the functionalized samples tested at 37°C. The elastic modulus of these samples was 8.8MPa in average whereas it was 9.1MPa. The yield strength and the yield strain were respectively 54.5MPa and 10.85% for the functionalized samples at 37°C whereas they were 73.6MPa and 9.7% for the non-functionalized samples. As expected from the curve, the yield strength is higher for non-functionalized samples although the yield strain is higher for functionalized samples at 37°C. A summary of the properties of the functionalized samples tested at 37°C can be seen in Table 4.4.

**Table 4.4:** Mechanical properties of functionalized samples tested at 37°C compared with dry samples tested at room temperature.

<table>
<thead>
<tr>
<th></th>
<th>Elastic modulus (MPa)</th>
<th>Yield strength (MPa)</th>
<th>Yield strain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Functionalized scaffolds at 37°C</td>
<td>8.8 ±0.5</td>
<td>54.5 ±0.7</td>
<td>10.85 ±0.49</td>
</tr>
</tbody>
</table>
This experiment was meant to recreate the testing conditions of the mechanical stimulation of the samples that will be performed. Some parameters of the mechanical stimulation such as the stimulation strain will be eventually deduced from this experiment. The functionalized samples were left dipped in PBS until testing and the testing was done at 37°C. Temperature and humidity typically have the same effect: they give more elasticity to the samples. In this case, the yield strain was improved by these parameters and the elastic modulus was unchanged. However, the yield strength suffered a major drop, from an average 73,6MPa to 54,5MPa. Indeed, this can be partly due to the temperature. It is also possible that such a loss of property was caused by the effect of humidity on the polymer. Since PLA degrades with water, it is possible that the chemical treatments and the liquid in which the samples were dipped in degraded the polymer, causing a loss of mechanical properties. The aspect of the degradation of PLA has not been studied in this project, although it might have a non-negligible effect on the integrity of the samples.

The choice of the strain for the mechanical stimulation was done thanks to this study. This strain must not damage or deform plastically the samples but must be high enough to be sensed by the cells. As a consequence, the value of 8% was chosen in the elastic region of the curve, a few percent under the average yield strain to prevent the statistical differences between the samples (10,85%).

### 4.6 Live cell staining for cellular viability

A live cell staining was performed to verify the ability of the scaffolds to support cellular attachment. Images were taken with a fluorescence microscope (Figure 4.9). Cells were clearly visible on the samples and adopted different shapes. Some cells still had a rounded shape, although others showed a more elongated shape. The elongated cells all pointed toward a unique direction. Moreover, the surface being fibers, it was not possible to focus on many cells at the same time since the cells lie on different layers.
The cells were only left few hours on the samples before the observation. However, they seem to attach well to the samples. A decent density of cells can be seen as well as elongated cells all pointing in one direction. It is very likely that this particular direction is the direction of alignment of the fibers. This preliminary result is a very good indication that such scaffolds can support cells and guide their behavior in the way it is designed to.

### 4.7 Cells immunofluorescence

Tenocytes were seeded on glass, functionalized PLA and non-functionalized PLA samples and were observed via immunofluorescence after 1, 3 and 6 days of culture. The area and the roundness of the cells were calculated using ImageJ.

The culture duration had no influence on the cells area for the functionalized PLA samples. The values were very similar for all the time points with an average area between 1100µm² and 1200µm². Concerning PLA, the area of the cells was an average 874µm² on the first day and increased then stabilized the next time points between 1100µm² and 1200µm². The cells present on glass samples had an area of 2200µm² the first day, decreased on the third day to 1784µm² and slightly increased the sixth day to 1894µm². These results can be seen on Figure 4.10.
Figure 4.10: Cells area calculated for tenocytes seeded on glass, functionalized PLA and PLA during culture periods of 1 day, 3 days and 6 days. The values were calculated using ImageJ.

On the first day, tenocytes seeded on PLA had a roundness of 0.333. The value decreased and stabilized the next time points around 0.200. Concerning functionalized PLA, the roundness was 0.256 for the first day, then decreased to 0.217 the third day and finally to 0.202 the sixth day. Cells seeded on glass samples had an average roundness of 0.522 the first day, then decreased to 0.485 the third day and finally 0.326 the sixth day. These results can be seen in Figure 4.11.

Figure 4.11: Cells roundness calculated for tenocytes seeded on glass, functionalized PLA and PLA during culture periods of 1 day, 3 days and 6 days. The values were calculated using ImageJ.
Figure 4.12: Fluorescence images of tenocytes after 1 day of culture on different materials.

Blue: cells nuclei (DAPI)
Red: cytoskeletons (phalloidin)

Top: glass 
Middle: functionalized PLA 
Bottom: PLA 
(Nikon Eclipse E600)
Figure 4.13: Fluorescence images of tenocytes after 3 day of culture on different materials.

Blue: cells nuclei (DAPI)
Red: cytoskeletons (phalloidin)

Top: glass
Middle: functionalized PLA
Bottom: PLA
(Nikon Eclipse E600)
Figure 4.14: Fluorescence images of tenocytes after 6 day of culture on different materials.

Blue: cells nuclei (DAPI)
Red: cytoskeletons (phalloidin)

Top: glass
Middle: functionalized PLA
Bottom: PLA
(Nikon Eclipse E600)
Figure 4.12, Figure 4.13 and Figure 4.14 represent tenocytes seeded on glass, functionalized PLA and PLA after one, three and six days of culture respectively.

On glass after one day, the cells looked large and very slightly elongated without showing any preferential orientation. On functionalized PLA, the cells looked much smaller and very elongated in one unique direction. On PLA, the cells had a similar size and elongation to the functionalized PLA cells and were also orientated in one direction, although it was not as obvious as for functionalized PLA. Many cells could still be seen as very small and round on PLA.

On glass after three days, the cells had approximately the same size and shape as for the first day. On functionalized PLA, their shape and size also looked very similar to the first day. They were still clearly elongated in one direction. On PLA, less small round cells could be seen and the alignment direction was more obvious.

On glass after six days, the cells were more elongated than on the previous days. However, they still did not show any orientation. On functionalized PLA, the cells still had a similar shape, size and preferred orientation. Moreover, they were more densely packed than on the previous days. On PLA, the observations were very similar to the one made on the previous days.

This test is a good tool to observe the attachment of the tenocytes on these different substrates. The area of a cell is typically considered as an effective parameter to indicate whether the cells are well attached or not. In this case, the cells area was higher on glass, which has a flat and homogeneous surface. However, the values were also quite promising for the polymer fibers, showing that the tenocytes attached well to these substrates as well. On functionalized PLA, the area of the cells was constant regardless of the culture duration. Cells might have already reached an optimum attachment on the first day. For PLA, the area of the cells was much lower on the first day but reached the same values as for functionalized PLA on the third day. These results seem to show that the collagen present on functionalized PLA offers to the cells a favorable surface to quickly attach, although after three days, the values are similar for the two materials.
The roundness of the cells had also been calculated to describe the shape of the cells. It was calculated with the following formula:

\[ \text{Roundness} = \frac{4 \times \text{Area}}{\pi \times \text{Major axis}^2} \]

A roundness of 1 indicates a perfect circle, and the value decreases as the shape changes. On glass, the roundness was relatively high on the first day and decreased after each culture period (0.522 the first day, 0.485 the third day and 0.326 the sixth day). For both functionalized PLA and PLA, the cells already had a low roundness on the first day, although the value for PLA was higher than for the functionalized PLA at this time point. For both, the value decreased on the third day and remained constant afterwards around 0.200. This value might be close to the minimum roundness for tenocytes, which typically have a very elongated shape. However, tests over longer periods should be done to confirm this hypothesis. Just like the previous results showed, functionalized PLA presents a surface that enables the cells to optimize their area and adopt a preferential shape in less than a day of culture. Nevertheless, be it the area or the roundness, these two properties reach the same value for functionalized PLA and PLA after three days of culture.

The observations made on the pictures are consistent with the values of area and roundness that were calculated. However, one very important feature can be observed on these pictures. In the case of functionalized PLA and PLA, which are composed of PLA fibers, it is clearly visible that the cells align their shape along one unique direction. Whereas cells seeded on glass do not show any preferential orientation. It is very likely that this direction corresponds to the alignment direction of the fibers. Already on the first day, the preferential alignment direction of the cells is very clear. Moreover, no matter what material is considered, it is possible to see that the cells are more and more densely packed as days go by. This could be a good indication that cellular proliferation is possible on such scaffolds. However, proliferation tests should be carried to confirm this hypothesis since such observations are purely qualitative. This whole experiment showed that functionalized PLA is a good material to support cellular attachment and that its fibrous structure has a positive effect on the cells. However, non-functionalized PLA shows similar results after three days of culture. The influence of collagen on the surface should be further investigated.
4.8 Mechanical stimulation of seeded scaffolds

Due to experimental issues encountered during the test, the stimulation process had to be stopped after one day and a half of stimulation process (8 hours of stimulation the first day and 4 hours the second day for a total 12 hours of stimulation). The sample underwent a ramp until 6mm at 0,1mm/s. The resulting stress-strain curve was similar to the curves obtained in previous tests (Figure 4.15). A short toe region can be observed at very low strains, followed by a linear elastic region and a yield region. The elastic modulus was calculated as the slope of the elastic region and its value was 15,45MPa. The yield strength and the yield strain were respectively calculated as 38,95MPa and 2,92%.

![Stress-strain curve for stimulated sample](image)

Figure 4.15: Stress-strain curve for PLA sample seeded with tenocytes stimulated during one day and a half (total 12 hours of stimulation).

As it can be seen in Table 4.5, the material properties dramatically changed after the stimulation: the material is stiffer but much more brittle. Compared to functionalized scaffolds tested at 37°C, the elastic modulus increased by 75%, the yield strength decreased by 40% and the yield strain was almost divided by 4. It was expected that tenocytes would produce collagen, eventually leading to a stiffer material. However, such an important change of
elastic modulus and yield strain after a short period of stimulation is quite unlikely to be explained only by the production of collagen.

An additional explanation could be the following. PLA is very sensitive to temperature (its glass transition temperature is between 50°C and 55°C) and the presence of liquids. In this case, the sample was dipped in culture medium at 37°C which are cell culture conditions and these parameters are known to accelerate the physical ageing process of the polymer [41]. Physical ageing of a semi-crystalline or amorphous polymer can cause undesired crystallization and thus making the material stiffer but more brittle. Buchatip et al. tested different copolymers of L-lactide and DL-lactide having different crystallinities. Materials with higher crystallinity proved to be stiffer and more brittle than amorphous copolymers in a similar fashion as it was observed in this experiment. [25]

**Table 4.5: Mechanical properties of a stimulated scaffold seeded with tenocytes tested at 37°C compared to functionalized scaffolds tested at 37°C.**

<table>
<thead>
<tr>
<th></th>
<th>Elastic modulus (MPa)</th>
<th>Yield strength (MPa)</th>
<th>Yield strain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stimulated scaffold at 37°C</strong></td>
<td>15,45</td>
<td>38,95</td>
<td>2,92%</td>
</tr>
<tr>
<td><strong>Functionalized scaffolds at 37°C</strong></td>
<td>8,8 ±0,5</td>
<td>54,5 ±0,7</td>
<td>10,85 ±0,49</td>
</tr>
</tbody>
</table>

At the moment this report was finished, it had been impossible to make other experiments. These observations should be taken into account for further experiments by decreasing the stimulation strain for instance.
5 Conclusions

The objective of this project was to design and characterize aligned nanofibrous PLA scaffolds functionalized with collagen type I and further seed tenocytes on the scaffolds to see the influence of a mechanical stimulation on the scaffold and on the cells. All these efforts were meant to create a scaffold and conditions that were as close as possible to tendons.

The electrospinning technique successfully produced aligned nano-scale fibers of PLA. Their average diameter was determined as 700nm. Further, the mechanical properties of the scaffolds were determined using the Bose Electroforce Biodynamic 5100. The scaffolds showed to be viscoelastic with typical properties and behavior of fibrous mats.

The functionalization step was divided into two parts. The first part was the hydrolysis of the scaffolds, which was meant to create carboxylic groups on the surface of the fibers. A quick study was performed to select the best concentration of NaOH and the optimal exposition time. In the next part, collagen type I was attached on the surface of the fibers using carbodiimide chemistry. The couple EDC/NHS was used to create chemical bonds between carboxylic groups created via hydrolysis and primary amino groups present on the collagen molecule. A comparison was made between covalent bonding of collagen using EDC/NHS and its physical adsorption on the surface of the fibers. Covalent bonding proved to be the most efficient as it was expected.

Finally, tenocytes were seeded on functionalized and non-functionalized scaffolds as well as glass. An immunofluorescence test was performed to observe the attachment of the cells. While cells had a larger area on glass, they did not adopt an elongated shape. On both non-functionalized and functionalized samples, cells had very elongated shapes and all pointed toward a unique direction, the alignment direction of the fibers. This behavior is very similar to what can be seen in natural tendons. No differences were noted in the area and the shape of the cells after three days of culture while comparing functionalized and non-functionalized samples.

The mechanical stimulation was not successful: the process had to be stopped after one day and a half. The stimulated sample was mechanically tested and yielded surprising results. Its elastic modulus increased by 75%, its yield strength decreased by 40% and its
yield strain was divided by 4. On such a short period, the collagen production of the fibers is very unlikely to be responsible for these changes. Physical ageing was mentioned as a probable cause of these modifications.

As a conclusion, aligned PLA nanofibers proved to be promising scaffolds for tendon tissue engineering applications. Their size and mechanical properties resembled those of natural tendons. The functionalization of the fibers with collagen was also successful although its influence on the cells has not been clearly determined. Moreover, the aligned fibrous structure of the scaffolds had a positive effect on the scaffolds as the cells adopted the same elongated and unidirectional morphology. The process of mechanical stimulation is still not functional and eventually led to incoherent results.
6 Future perspectives

Some additional experiments could be done to supplement the results presented in this report. Most of the experiments presented in this part were not done mainly because of a lack of time and in some cases experimental issues. Here are some suggestions.

The logical continuation of the project is to successfully stimulate the scaffolds seeded with tenocytes and perform a cells immunofluorescence. Experimental issues made it impossible to do on time. These tests should be done at the same time point as for the static experiments in order to make the results comparable. Testing the mechanical properties of the scaffolds at the end of the mechanical stimulation would give an idea of how much collagen is produced by the cells and its influence on the mechanical properties. The collagen production could also be seen via immunofluorescence or quantified with a micro BCA protein assay.

A proliferation assay such as the AlamarBlue assay could be performed on both the dynamic and the static experiments to quantify the cellular proliferation on the scaffolds. The observations made with the immunofluorescence pictures are entirely qualitative and would need a quantitative measurement to be confirmed. The influence of collagen has not been clearly identified in this report. However, the cellular proliferation could be affected by this parameter.

Both the dynamic and the static experiments could be done over longer periods. In this report, the experiments were done over 6 days and longer durations could reveal more interesting observations.

The aspect of the degradation of PLA has not been considered in this project. However, the degradation of PLA could lower the mechanical properties of the scaffolds which are important properties in this project.
Cellular behavior after mechanical stimulation on biofunctionalized polylactide nanofibers for tendon tissue engineering
## Cost of the project

<table>
<thead>
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<th>Product</th>
<th>Price/unit</th>
<th>Amount</th>
<th>Total price</th>
</tr>
</thead>
<tbody>
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<td>3000 €/kg</td>
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<tr>
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Few products, such as PBS-gly or PBS-gly-BSA are not listed since they belong to the common products of the lab.

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<td>2</td>
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</table>
Only the equipment which requires a reservation and are paid at the hour is listed. Most of the equipment used, such as the fluorescence microscope or the mechanical testing machine, is free or belong to the research group.

<table>
<thead>
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**Estimated total cost of the project:** 12679,94 €

**Environmental impact**

The material used in this project which is poly(lactic acid) is a biodegradable and biocompatible polymer. Thus its degradation has no negative consequence on the environment and only a small quantity of polymer was used to create the scaffolds.

Concerning the use of chemical and biological products, the laboratory has strict norms concerning the disposal of such products. All the leftovers of chemical or biological substances were poured in the appropriate recuperation containers, according to the regulations. All the materials that had been in contact with chemical or biological substances were thrown away in the appropriate trash bins.
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