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Review article

Tendon tissue engineering: Cells, growth factors, scaffolds and production techniques

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ABSTRACT

Tendon injuries are a global health problem that affects millions of people annually. The properties of tendons make their natural rehabilitation a very complex and long-lasting process. Thanks to the development of the fields of biomaterials, bioengineering and cell biology, a new discipline has emerged, tissue engineering. Within this discipline, diverse approaches have been proposed. The obtained results turn out to be promising, as increasingly more complex and natural tendon-like structures are obtained. In this review, the nature of the tendon and the conventional treatments that have been applied so far are underlined. Then, a comparison between the different tendon tissue engineering approaches that have been proposed to date is made, focusing on each of the elements necessary to obtain the structures that allow adequate regeneration of the tendon: growth factors, cells, scaffolds and techniques for scaffold development. The analysis of all these aspects allows understanding, in a global way, the effect that each element used in the regeneration of the tendon has and, thus, clarify the possible future approaches by making new combinations of materials, designs, cells and bioactive molecules to achieve a personalized regeneration of a functional tendon.

1. Introduction

Nowadays, tendon injuries are a health problem that annually affects millions of people around the world, involving a great clinical burden on health systems that have to face a high cost associated with operations, rehabilitations and infiltrations, among others [1,2]. In addition, the number of people who will suffer from this type of injury is expected to rise as the life expectancy is continuously increasing and the number of people who do sports continuously, as well [3]. Currently, the therapies that are used to treat this type of injury range from surgical treatments to conservative treatments, or even treatments using the infiltration of cells or growth factors [4,5]. However, these therapies are not entirely effective as reinjures are very frequent [2]. The great scientific advances that have occurred in recent years in fields such as materials

Abbreviations: 3DP, 3D-printed scaffolds; ACL, Anterior cruciate ligament; ADSC, Adipose-derived stem cells; BAFS, Book-shaped native fibrocartilage tissue decelularizaded scaffold; bFGF, Basic fibroblast growth factor; BMP, Bone morphogenetic protein; BMSC, Bone marrow stem cells; b-TCP, b tricalcium phosphate; COL1, Type I collagen; COL1A1, Type I collagen alpha 1 chain; CS, Chitosan; ECM, Extracellular Matrix; EDC, 1-ethyl-3-(3- dimethyl aminopropyl) carbodiimide hydrochloride; E-Jetting, Electrohydrodynamic jet printing; ESC, Embryonic stem cells; GAGs, Glycosaminoglycans; GelMA, Gelatin Methacryloyl; HA, Hyaluronic acid; HAp, Hydroxyapatite; HBDS, Heparin/fibrin-based delivery system; hUCB-MSC, Mesenchymal cells of human umbilical cord blood; HY, Hybrid yarns; IGF-1, Insulin-like growth factor 1; iPSC, Induced pluripotent stem cells; MSC, Mesenchymal stem cells; MY, Microfiber yarns; NCSCs, Neural crest stem cells; NHDF, Normal Human Dermal fibroblasts; NHS, N-hydroxysuccinimide; NSAIDs, Nonsteroidal anti-inflammatory drugs; PA6, Polyamide 6; PCL, Polycaprolactone; PCNA, Proliferating cell nuclear antigen; PDGF, Platelet-derived growth factor; PDGF-BB, Platelet-derived growth factor-BB; PECAM-1, Platelet endothelial cell adhesion molecule; PEO, Poly (ethylene oxide); PET, Polyethylene terephthalate; PGA, Polyglycolic acid; PGS, Poly (glycerol sebacate); PLA, Polylactic acid; PLGA, Poly(L-lactic-co-glycolic acid); PLLA, Poly-L-lactic acid; PRP, Platelet-rich plasma; RCA, Rotator cuff augmentation; SDF-1α, Stromal cell-Derived Factor 1α; TDSC, Tendon-derived stem cells; TGF-β, Transforming growth factor beta; TN-C, Tenascin C; TNMD, Tenomodulin; TSPCs, Tendon stem/ progenitor cells; Tβ4, Thymosin beta-4; VEGF, Vascular endothelial growth factor.

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engineering, biochemistry and physicochemistry have allowed the development of another very promising type of therapy for instance tissue engineering, also known as regenerative medicine [6]. Tissue engineering applies the knowledge generated from engineering and life sciences to obtain structures similar to those present in the body, formed by the combination of different elements (scaffolds, cells and growth factors, generally) that, when used in the organism, allow to recover, maintain or improve the function of various organs and tissues [7]. Therefore, to understand the techniques and elements used in tissue engineering applied to a certain organ or tissue, it is necessary to understand the physiological nature of that organ or tissue. In other words, before studying tissue engineering applied to the tendon, it is necessary to know what tendons are, what structure and composition they have, what tendon injuries are and how they occur and the mechanisms that the organism itself has for tendon regeneration. All of these aspects are discussed below.

1.1. Tendon structure, composition and biomechanics

Tendons are fibrous connective tissues whose main function is to connect and transmit forces from muscles to bones [8]. They act as energy storage sites and help to maintain posture and joint movement [9,10], what implies that tendons have to suffered great tensile strengths and high compressive forces [8]. Its functions are associated with unique physicochemical and mechanical properties that make this tissue very different from other tissues in the body.

Macroscopically, tendons have a hierarchical structure (Fig. 1) [11]. As already mentioned, they are continuously stretching and contracting, suffering tensile forces of different magnitudes. This type of movement is possible, mainly, thanks to the oriented arrangement of the collagen fibers that make up the tendons, their hierarchical organization (microfibril, subfiber, fiber and fascicle), the composition of their extracellular matrix and the membranes or sheath that cover the different structures. These last ones, allow the fibers to glide along each other without producing friction [8]. With respect to the vascularisation of this tissue, high variability of blood supply is found between the different tendon types. In all cases, tendons are considered a poorly vascularised tissue. The vascularisation is predominantly concentrated on the external surface of the tendons. In addition, the blood flow is very slow. Therefore, as studied later, this limited blood supply contributes to slow healing after injury.

From a biochemical perspective, tendons have low cellularity and consist primarily of a water-rich extracellular matrix (ECM) (55-70%) [8]. In addition to water, the matrix also has different compounds such as proteoglycans and glycosaminoglycans (GAGs) (1-5% of the dry weight), elastin (1-2% of the dry weight) and collagen fibrils (60-85% of the dry weight). Type I collagen constitutes around 80-90% of the overall collagen profile, and it is the main responsible of the properties of the tendon. The basic unit of collagen I is a hetero-polymeric triple helix formed by two α1 chains and one α2 chain [13]. In addition to collagen I, many minor collagen types play vital roles in proper tendon development and function. For example, type II collagen (2%) and type III collagen (1-10%) are present in tendon tissues in much lower proportions. Elastin, on its behalf, is responsible for providing part of the characteristic flexibility of the tendon. The proteoglycans, GAGs, glycoproteins and other small molecules play specific roles in the tendon. They slow the deformation of the tissue, add viscoelasticity, act as a lubricants, give integrity to the ECM occupying the intra-fibrillar space and preventing their collapse, among other functions [14].

In regard to the cell population, several cell types with similar characteristics are present in tendons among which tenocytes and tenoblast are the most abundant (90-95% of the cells in tendons are tenocytes) [8]. Tenocytes are specialized fibroblast cells with an elongated form and stellate in cross section. They usually lie sparingly in rows between the collagen fibrils. They synthesize the components of the ECM and release signals to regulate the formation and development of tendons [15]. Another important cell type present in tendons are tenoblasts. These cells are immature tendon cells. Tenoblasts are motile and highly proliferative. Initially, they are different in size and shape but with the aging of the individual, the morphology of the cells changes and they become longer, more slender and more uniform in shape, and transform into tenocytes [14,16]. The remaining 5-10% of the cells are a combination of progenitor cells (tendon-derived stem cells, TDSC), chondrocytes (found in the bone junction area), vascular endothelial cells (around the vascular network), lymphocytes or other immune cells (for example, mast cells, neutrophils, macrophages), nerve cells and smooth muscle cells (located near the junction with the muscle) [10].

All the aforementioned structural characteristics are ultimately responsible for the unique mechanical properties of tendon tissues, to mention viscoelasticity, nonlinear elasticity and anisotropy [17]. The

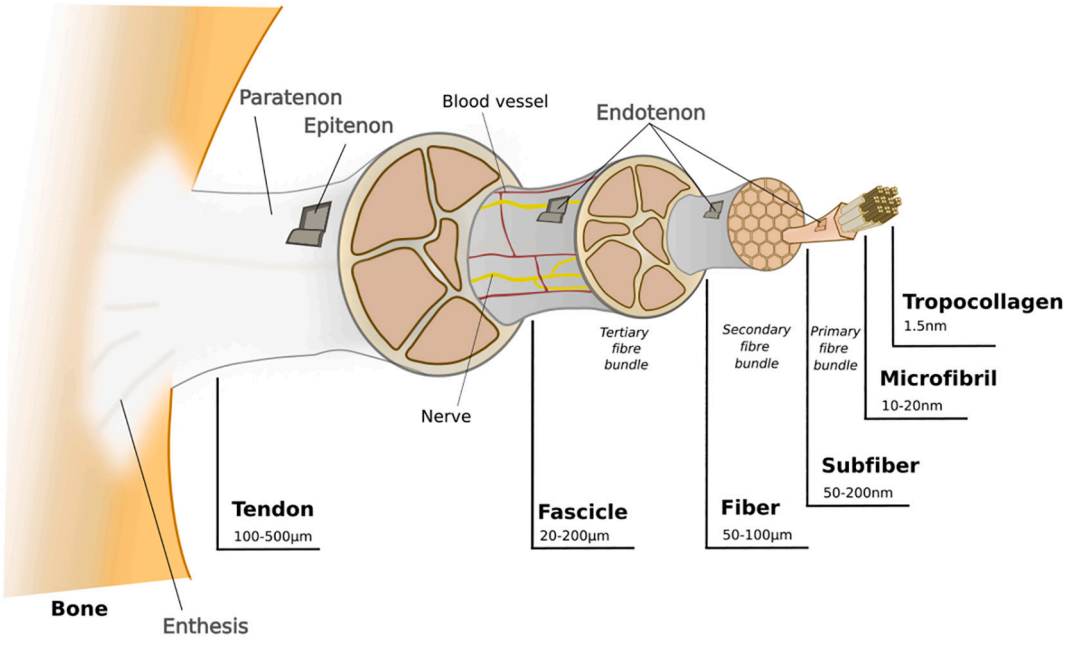


Fig. 1. Tendon hierarchical structure. Based on [12].

first mention property is the *viscoelasticity* of the tendon. This property allows it to recover its original shape when the load that has caused a deformation is removed. This phenomenon can occur because tendon tissues have a high degree of resilience [18]. The second property, *nonlinear elasticity*, refers to the stress-strain curve that is obtained when applying different stress values to the tendons. Three distinct regions can be distinguished in a strain-stress curve owing to the nonlinear characteristics of the tendon (Fig. 2) [19]. The first one is the toe region and defines the behaviour of tendons at deformations up to 2% of strain (low deformation). As deformation increases, the tendon passes from the toe region into the linear region (up to 4% of strain). In this region, the tendons show elastic and reversible behaviour. If deformation continues to increase, the tendon reaches the yield point (6% of strain) and the failure region (8% of strain). In these cases, the tendon extends beyond its physiological limit [20]. The deformation can reach a point, the failure point, where even macroscopically ruptures occur. The third property, *anisotropy*, refers to the difference tensile strength that can tolerate the tendons depending on the direction of the applied force (directionally dependent) [17,21].

1.2. Tendon healing and regeneration

Tendon injuries, known as tendinopathies, are common musculoskeletal disorders and a principal cause of functional disability. These kind of injuries affect mainly to athletes, active working people and the elder population worldwide [23–27]. Tendon injuries affect to nearly 30 million people and a significant amount of loss of productivity and morbidity is registered each year due to these injuries all over the world [28–31]. All tendons can suffer injuries, being some of the most famous ones the rotator cuff injury, the tendon-to-bone junction injury, and the Achilles tendon injury. These pathologies influence the quality of life of the affected population as they cause matrix disorganization, scar tissue formation and loss of mechanical properties [32].

Tendon injuries may be caused by acute or chronic changes or a combination of both. Chronic injuries are more often associated with intrinsic factors as genetics, sex, age, nutrition or general health, while acute injuries are more often associated with extrinsic factors, as excessive or absence of mechanical loading. Acute injuries are more commonly related to sports injuries and have been increased considerably in the last decade due to the increase in the number of people who practise sports in a professional and semi-professional way [33].

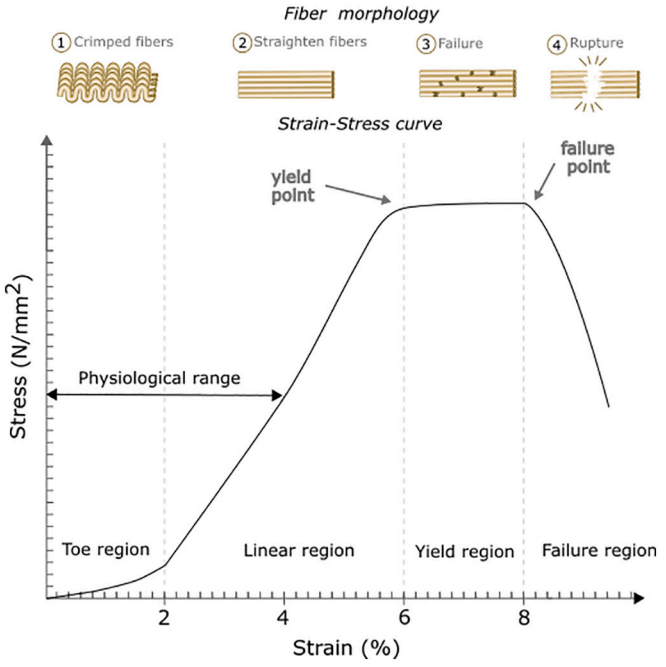


Fig. 2. Strain-stress curve of tendons. Four different regions can be observed; toe region (<2% strain), were the fibers are crimped; linear region (2% strain-6% strain), were the fibers are straighten; yield region (6% strain-8% strain), were irreversible damage is produced; and failure region (>8% strain), were rupture is produced. Physiological range is considered to reach to 4% strain. Based on [20] and [22].

Tendon recovery after injury is extremely poor due to low cellularity, hypovascularity and low metabolic activity of tendon tissue. Furthermore, in most patients, the healed tendon does not regain the mechanical properties of the original healthy tissue and, in a significant percentage of them, there is a recurrence of the rupture [34]. This problem of prevalence of reinjury is related to inadequate tissue regeneration in which the molecular and histological structure of the new formed tendon is different from the original one. This situation may well occur because the cells present in the regenerated tendon are not tenocytes (that are predominant in the healthy tendon), the composition or arrangement of the ECM is not adequate to meet the mechanical and physiological characteristics this tissue needs, or the vascularization turns out to be much greater or less than necessary. All these defects cause weakness, pain, fibrous adhesions, eventual tear or the aforementioned complete tendon rupture [35–38].

Natural tendon injury regeneration is characterized by three main stages: inflammation, proliferation and remodelling (Fig. 3) [4,39]. *Inflammation* is the first phase that occurs after tendon injury. It usually lasts between 3 to 7 days [10]. In this first phase, different chemotactic factors are liberated mediating the inflammatory response, stimulating the proliferation of fibroblasts and tenocytes, stimulating the angiogenesis processes [40–42]. Type III collagen and other matrix components are synthetized. In the second phase, the *proliferative phase*, that last approximately until the third- fourth week, there is a great increase in cellularity (mainly fibroblasts) and synthesis and deposition in a random way of materials of the ECM (mainly type III collagen). The final stage, *remodeling*, is further divided into the consolidation and maturation periods. During the consolidation period, the synthesis of collagen and GAGs is decreased, and tenocytes and collagen fibers align themselves along the longitudinal axis of the tendon, in the direction of stress, and, eventually, become capable of supporting load [43,44]. Cellularity decreases during this stage. Conversely, type I collagen production increases. The last phase, maduration, is a long process that can take even years [45]. During maturation, the scar tissue begins to acquire a histological appearance more similar to that of the healthy tendon. However, the biomechanical properties of the healed tendon are weaker than the uninjured tendon, with less crosslinking and smaller diameter collagen, what makes it more susceptible to reinjury [46].

1.3. Methods for tendon repair

As it has been described, the natural healing process of the tendon is very complex and not fully understood. Furthermore, natural healing is very slow and usually results in fibrous tissue that would not regained completely the function and strength of the undamaged tendon [32]. To solve this problem, traditionally, different treatments have been proposed: conservative treatments [49,50], surgical treatments (suture or autografts, allografts or xenografts) [49,51–53], non-steroidal anti-inflammatory drugs (NSAIDs) [54,55], cell infiltration [56], growth factor infiltration [57,58], and treatments using gene therapy [59,60]. All of these treatments are further discussed below. A summary of the advantages and disadvantages of each of them is also included (Table 1).

Conservative treatment aims to control pain and reduce the inflammation produced at the injured area [1,61]. The approaches that are carried out in this type of treatment range from rest and cryotherapy to the use of extracorporeal shock wave therapy [62,63], ultrasound [64–66], electrotherapy [67], and the performance of strengthening and balancing exercises [12,67–69]. Combined treatment regimens frequently reveal better tendon regeneration than individual treatments

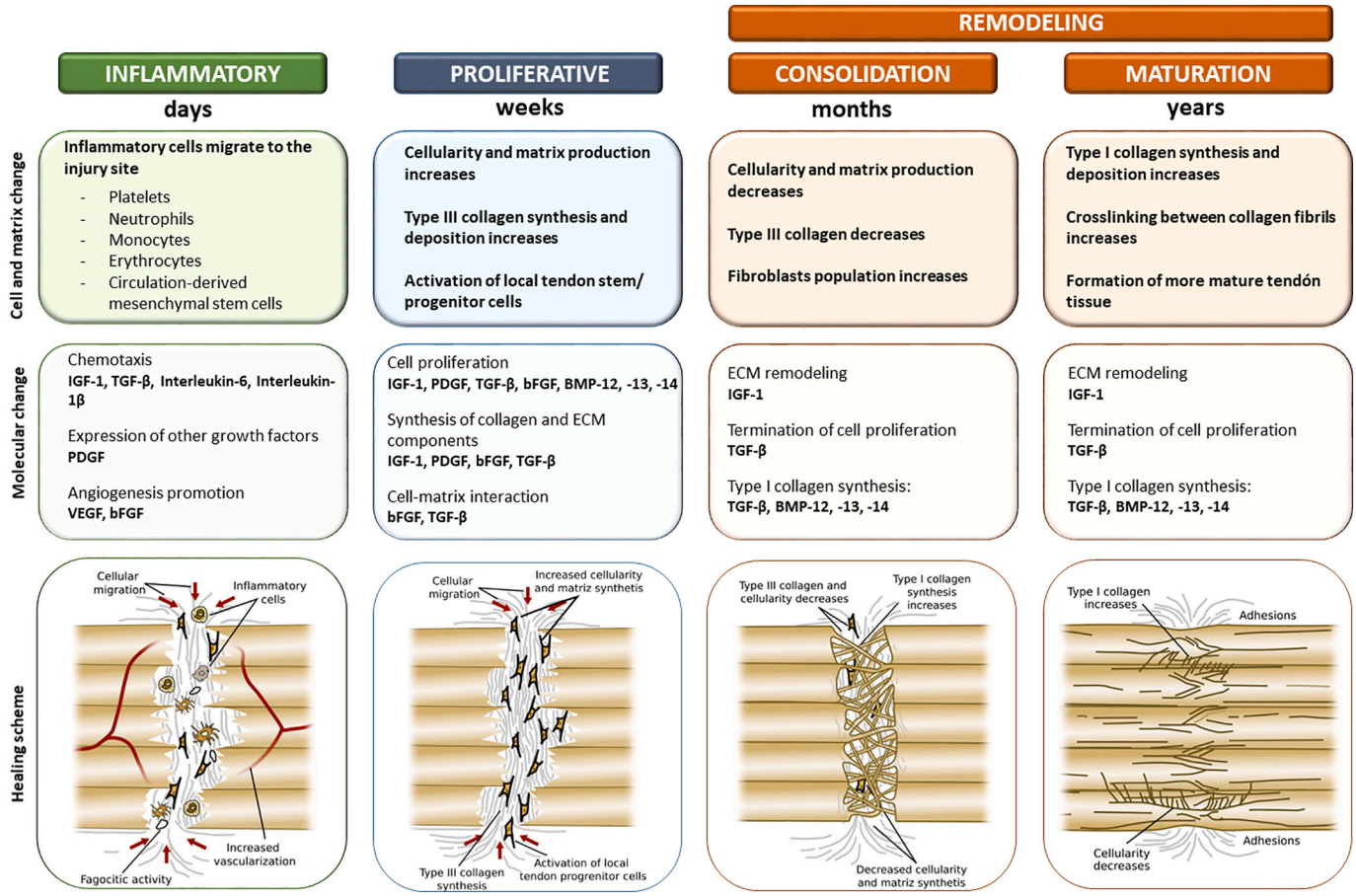


Fig. 3. Main changes produced during the different phases of tendon regeneration: inflammatory phase, proliferative phase and remodeling phase. Changes are organized based on their nature in: cellular and ECM changes and molecular changes. Fig. based on [47] and [48].

[70].

The frequent failure of non-surgical approaches is the reason why *surgical intervention* remains the treatment of choice [70]. Especially the combination of surgery and early movement of the injured tendon stands out as the most used therapy [71]. Approaches that are carried out in this type of treatment range from the removal of damaged tissue and suture between the not damaged ends, to the application of autografts, allografts and xenografts. Despite clinical advances, this treatment has still lots of limitations such as: (i) creation of scar tissue or adhesions, (ii) fail to regenerate tissue, (iii) loss of tissue mechanical properties, (iv) risk of damage and infection, (v) functional disability of donor tissue (autografts), and (vi) need for immunosuppressive drugs to prevent tissue rejection (allografts) [72,73].

NSAIDs are among the most used drugs for the treatment of tendon injury by professional athletes [70,74]. This type of treatment is controversial since its effectiveness has not been proven [75]. Different reports indicate that its effectiveness depends on the time when the drug is used. Factors that can influence the results obtained are the analgesic agent used, the anatomic site of injury, the treatment durations and the dosage, among others [76].

Another therapy consist in the infiltration of *growth factors* [12,77]. Growth factors play a very important role in the natural regeneration of the tendon since they participate in cell recruitment and in the stimulation of ECM synthesis, among others [78]. For this reason, it has been extensively studied how to incorporate them into the damaged tissue and the effect they produce on it [58]. Among the growth factors that have been studied it should be mentioned the vascular endothelial growth factor (VEGF) [79,80], PDGF [81,82], bFGF [83,84], BMP-12 [83,85], TGF-β [86–88], or IGF-1 [34,89], among others. The platelet-rich plasma (PRP) should be also pointed out as it has been applied in numerous cases and its effectiveness seems evident [90]. This treatment has still lots of limitations. Little is known about the needed dosage, the time the growth factors reside in the damaged tissue and the timing of injection [91,92].

Cell therapy is another studied therapy [93]. It consists in the use of cells from other parts of the body or from allogenic sources and injecting them in the site of injury. For this purpose, it can be used two types of cells: differentiated cells (tenocytes and fibroblast) or stem cells [48,94]. Each kind of cell has its own positive and negative effects on tendon healing.

Tenocytes are very interesting cells to use in tendon regeneration since, as already mentioned; these cells are the most abundant ones in healthy tendons [2,29,95,96]. They are capable of producing growth factors and the materials necessary to repair the ECM. Another differentiated type of cells are the fibroblasts [93,97,98]. Their main advantage is that they are very abundant. Both, tenocytes and fibroblasts, will not produce teratoma and, in this regard, they are better than stem cells [99–102]. Nowadays, the use of stem cells is very popular in different fields and their capacity for regeneration of different tissues is very promising [103–105]. Some of the stem cell types that are being used in tendon regeneration are embryonic stem cells (ESC) [106–108], induced pluripotent stem cells (iPSC) [18,29,107,109,110], mesenchymal stem cells (MSC) [25,26,35,111,112], bone marrow stem cells (BMSC) [31,113–115], adipose-derived stem cells (ADSC) [37,116,117], and TDSC [24,30,31,118], among others. Stem cells have many interesting characteristics for their use in cell therapy, however, they also have some important drawbacks. On the one hand, it is important to note that both multipotent and pluripotent stem cells, especially iPS cells, are

Table 1
Summary of the main advantages and disadvantages of the treatments used for tendon regeneration.

Methods for tendon repair	Advantages	Disadvantages	Ref
Conservative treatment			
Extracorporeal shock wave therapy	• Generally save • Simple to apply • Good success rate in some tendon injuries	• Mechanism not fully understood • The effects on healing are unclear. Too much diversity of results • Not clear pressure application necessities	[62,63]
Ultrasound	• Short-term pain relief • Adhesion prevention • Reduction of the amount of inflammatory infiltrate • Generally save • Simple to apply	• Mechanism not fully understood • The effects on healing are unclear. • Too high-intensity ultrasound can have tissue destructive effects • Cannot be used as a sole treatment	[65,66]
Exercise	• Decreased tendon volume • Increased synthesis of type I collagen • Improved tendon gliding and repair strength • Adhesion prevention	• Risk of re-injuring • Most appropriate exercises for each type of injury are unknown	[12,69]
Surgical intervention	• Alternative when other techniques have not worked or in cases of total tendon rupture • Remove the damaged part of the tendon	• Loss of tissue mechanical properties • Generation of scar tissue or adhesions • Unable to regenerate completely the injured tissue • Risk of damage and infection	[72,73]
NSAIDs	• Short-term analgesic effect	• The effects on healing are unclear. Little evidence showing a benefit to long-term symptomatology • Negative effects when given postoperatively: decreased failure loads and increased rates of failure • Gastrointestinal, cardiovascular and renal risks	[70,74,76]
Growth factors	• Simplicity of the injection • Well-studied targets • Demonstrated effect on tendon regeneration: • Increasing load to failure and elongation values, stimulating collagen and matrix production, inducing tenocyte differentiation and tendon-specific gene markers, inducing cell proliferation, etcetera	• Short half-life in the damaged tendon (requires repeated injections) • Costly • Little-known about the dosage and timing of injection • Depending on the origin can provoke immunogenicity	[12,91]
Cell therapy	• Demonstrated effect on tendon regeneration • Improved clinical outcome scores, improved biomechanical testing, increased collagen production and alignment, etcetera	• An important part of the implanted cells leave the injected site • Risk of unleashing an immune response	[93,94]
Tenocytes	• Mimics the natural tendon cell population • No risk of teratoma	• Difficult to harvest. Scarce • Risk of injury of the donor tissue • Difficult to expand and maintain their characteristic features of the tenocytes over the passages	[29,96]
Fibroblasts	• Can differentiate into tenogenic linages • Molecular expression conditioned by the environment • Very numerous in number • Easy to obtain with minimal injury into the donor site • No risk of teratoma	• Differences between fibroblasts obtained from different sources. Different expression profiles • Differences in healing dynamics	[93,98]
Multipotent stem cells	• Can differentiate into tenogenic linages • High proliferative capacity • Easily harvestable without injury of the donor site • High synthetic activity • Angiogenic and anti-inflammatory effects.	• Risk of tumorigenicity • Risk of differentiation into unwanted cell linages	[104,105]
Pluripotent stem cells	• Can differentiate into tenogenic linages	• Risk of tumorigenicity • Ethical controversy • Risk of differentiation into unwanted cell linages	[18,110]
Gene therapy	• Sustained and targeted production of growth factors and additional molecules • Can avoid immunogenicity	• Expensive • Complicated to manufacture • Gives raise safety issues when using viral vectors • Still not well developed technique	[12,119]

associated with risk of teratoma development after transplantation [8,48]. For this kind of therapy, further investigation is needed to develop optimal methods that ensure the maintenance of the viable cells within the injury site.

The last mentioned therapy, *gene therapy*, consists in the treatment of a disease by introducing ~~of~~ a foreign therapeutic gene into living cells [23,59,119]. In this way, it can be obtained a sort of transcriptional change so that more cell adhesion or ECM molecules, neurotrophic

factors and transcription factors are produced. There are two ways to carry out this type of therapy: *in vivo* or *ex vivo* [12].

Nowadays, conventional methods for treatment of tendon injuries are not fully effective. In addition, most of these treatments have high rates of reinjury or re-tear, treatment failure, or re-rupture. Therefore, there is a clear need to seek improvements in current treatments. Many researches and health professionals are trying to develop treatments that are capable of restoring the normal structure and functionality of tendon post-injury. In this sense, recently, tissue engineering has provided new hopes for complete and efficacious treatments for tendon injuries.

The advantages that tissue engineering would provide over current clinical therapies are multiple. It is the only approach that would provide a structure (scaffold) that helps maintaining the biomechanical functions of the tendon. Additionally, this scaffold also serves as a skeleton for the regeneration of the new tendon. It allows the incorporation of cells, growth factors and genes that are used in the aforementioned clinical therapies. However, in the case of tissue engineering, by using scaffolds, the incorporation of cells, growth factors and genes would be much more controlled. In this way, the problems of diffusion to unwanted locations far from the damaged area would be avoided. On the other hand, when required, the diffusion rate of these factors can be controlled. Another advantage of this approach is that it can be controlled (mainly by adjusting the used materials) how long the scaffold is going to be in the body before it degrades. In conclusion, tissue engineering allows greater control of tendon regeneration, thereby increasing the effectiveness of the treatment. The characteristics of tissue engineering for tendon regeneration are going to be further discussed in the next section.

2. Tissue engineering applied for tendon regeneration

Tissue engineering is an emerging discipline that combines the fields of biomaterials, bioengineering and cell biology to repair or regenerate biological tissues [120]. Tissue engineering involves the choice or development of materials that are used to create scaffolds. These scaffolds are combined together with cells (generally stem cells) and biologically active molecules to give rise to structures that serve to renew, regenerate or replace parts or whole body tissues (Fig. 4) [121]. The emergence and development of this new discipline has been possible due to the scientific advances in biomaterials, cell isolation and cultivation, and the production and isolation of growth factors and other bioactive molecules. Thanks to all these advances, the structures obtained are able

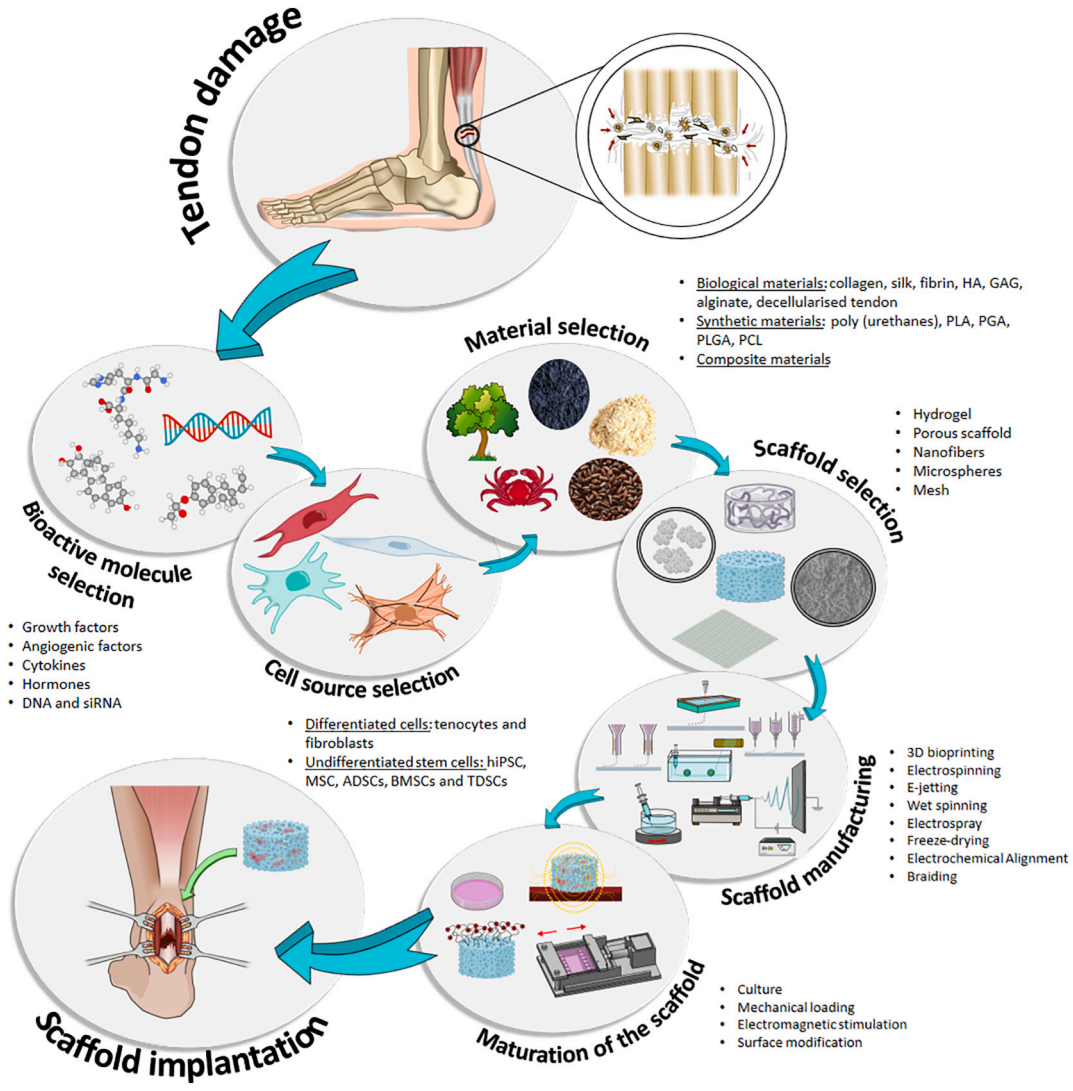


Fig. 4. Diagram of the main elements used in tissue engineering: scaffolds, cells and bioactive agents. The steps of the more usual approximation for the application of tissue engineering are indicated. hiPSC: induced pluripotent stem cells; MSC: mesenchymal stem cells; ADSCs: adipose-derived stem cells; BSCs: bone marrow stem cells; TDSCs: tendon-derived stem cells; HA: hyaluronic acid; GAG: glycosaminoglycans; PLA: polylactic acid; PGA: polyglycolic acid; PLGA: poly(L-lactic-co-glycolic acid); PCL: polycaprolactone.

to create biomimetic structures with characteristics very similar to the original tissue [122,123].

It is a very complex discipline in which many aspects must be taken into account, such as the type of tissue to be replaced or healed, its location, structure, physicochemical properties, the cell types present and their functions, the molecules that are part of the ECM, etcetera. All these aspects are just some of the ones that have to be considered when designing the scaffold, determining its structure, its materials, the type or types of cells that will be included, the bioactive molecules that may be necessary in the structure and many more [124,125]. Among the major challenges now facing tissue engineering is the need to create much more complex structures whose functionality, and biomechanical and structural behaviour is more similar to the biological structures they replace or heal [126,127].

In this sense, tissue engineering has positioned itself as a promising alternative for promoting the regeneration of damaged tissues, including tendons. Tissue engineering applied for tendon regeneration pretends to create new, healthy tissue to replace or restore the damaged tendon [128]. It is mainly based on (i) establishing an appropriate scaffold with physicochemical and biomechanical properties as close as possible to the ones of the original tendon, (ii) using different types of cells (tenocytes, fibroblast and differentiated cells) to imitate the tendon cellular composition, and (ii) creating an environment that helps tenocyte survival and ECM synthesis within the scaffold [129,130].

2.1. Selection of growth factors for achieving molecular changes within the healing tendon

Bioactive molecules are key components to achieve tissue regeneration. A bioactive molecule is a compound or molecule (including angiogenic factors, growth factors, cytokines, DNA, siRNA and hormones) synthetized and secreted by cells to produce an effect on the organism, the tissue or other cells [131]. The function of these bioactive molecules is to interact with and modulate the activity of the cells. For instance, theses bioactive molecules can stimulate cell differentiation, migration and proliferation. Over the years, more and more is known about them, having studied such decisive aspects as their molecular structure, their location, concentration, function in the organism, synthesis process and kinetic profiles. This knowledge has turned out to be very important for tissue engineering, since by means of a precise control of the signals carried out by these molecules, the tissue regeneration processes can be controlled much more precisely [132].

The aim of incorporating molecules with biological activity to scaffolds in tissue engineering is to achieve a strategy in which the positive effects of each of its elements are synergistically added. The scaffold serves as a vehicle for bioactive molecules and cells, but it also provides physical and mechanical support, guides the development of new tissue, and provides elastic properties to tissue while it is regenerating [125]. For its part, bioactive molecules have different functions [133], so depending on the molecule used it could be achieved a cellular effect, for example, differentiation to tenocytes [134], increased vascularization [135], or increased synthesis of ECM [136].

Incorporating these bioactive molecules into the scaffold is relatively simple. There are different strategies to do it. They can be incorporated into the structure of the already synthesized scaffold or to the biomaterial and later, through manufacturing technology, lead to a scaffold with the bioactive molecules already incorporated [137]. In addition, scientists have developed different mechanisms to ensure that these bioactive molecules have their effect at a certain point in time, that is, they have adjusted their release profile depending on when and for how long their activity is necessary [138,139]. Thus, the bioactive molecules have been encapsulated using different nanoparticles or they have been retained in the scaffold for longer using different biomaterials [140–142].

Recently, one of the most proposed approaches is the use of several growth factors simultaneously [143,144]. This approach is based on the fact that, naturally, growth factors are not synthesized in an independent way, but in the form of “cocktails” working in concert during tendon repair [134]. This approximation allows obtaining complex scaffolds more similar to the original tissues. It seems that there is still a long way to be able to apply this approach in the clinic. However, the great interest aroused by the good results makes this application closer to becoming a reality.

Although more and more of their synthesis, structure and how they work is known, it is true that there is still a lack of knowledge on how to use them in tissue engineering especially when more complex approaches are proposed, such as the mentioned use of different growth factors at the same time. This is the main reason why the number of applications carried out in tissue engineering is still reduced. The same happens with tendon tissue engineering. However, the studies in which active biomolecules have been incorporated into scaffolds to try to improve tendon healing have shown very promising results with hardly any adverse effects.

The growth factors with greater importance for tendon tissue engineering are: PDGF-BB [145], IGF-1 [146], BMP-7 [147], BMP-12 [148], VEGF [149], SDF-1α [150], bFGF [110], TGF-β1 [151] and TGF-β3 [152]. Table 2 summarizes some of the studies that involve these growth factors and the main results obtained.

In general, all these studies demonstrate that the incorporation of molecules with biological activity to the scaffolds allows a more sustained and controlled release of them, thus, achieving a longer effect over time. The results are, therefore, better than those achieved with injections of the same molecules with biological activity [143]. It is expected that these bioactive molecules will increasingly be incorporated into scaffolds in order to achieve much more complex and natural tendon-like structures that will reduce the rates of reinjury.

Acronyms. PDGF-BB: Platelet Derived Growth Factor-BB; IGF-1: Insulin-like Growth Factor 1; BMP-7: Bone Morphogenetic Protein 7; BMP-12: Bone Morphogenetic Protein 12; PCL: Polycaprolactone; PA6: Polyamide 6; GelMA: Gelatin Methacryloyl; TN-C: Tenascin C; VEGF: Vascular Endothelial Growth Factor; SDF-1α: Stromal cell-Derived Factor 1; BAFS: Book-shaped native fibrocartilage tissue decelularizaded scaffold; bFGF: basic Fibroblast Growth Factor; TGF- β1: Transforming Growth Factor beta1; TGF-β3: Transforming Growth Factor beta3.

2.2. Selection of cell source for optimal matrix development and recovery

The limited regenerative capacity of tendons has often been associated with their low cellularity [153]. These cells are ultimately responsible for the synthesis of the materials that make up the ECM, in addition to producing molecules with biological activity capable of regulating a wide variety of physiological processes. Thus, the infiltration of cells in the damaged area has been considered an important approximation to treat tendon injuries [93,94]. However, as previously mentioned, cell infiltration in humans has not been as effective as postulated, mainly due to the low cell permanence at the initial location [154]. Tissue engineering is, therefore, an advance in this regard, since cells are incorporated into a scaffold. This represents the main advantage of this technology, which permits, among other things, to retain cells in the place where the damage has occurred avoiding them to migrate to other tissues where they could even produce a negative effect [155].

However, it should be considered that determining the combination of these elements is not entirely easy since the scaffold (its composition and structure) can influence the behaviour of the cells [156]. The ideal scaffold should (i) avoid the cell membrane rupture during the process of making the scaffold and during its implantation in the body, (ii) allow the exchange of nutrients, waste substances, molecules with biological activity and gases through their structure, (iii) allow rapid integration into the native tissue, and (iv) permit the cells survival while maintaining their characteristics and functionality [157–159].

The type of chosen cells and the source from which they are isolated

Table 2
Summary of the main growth factors used in tendon TE. An example of each growth factor is analysed indicating the type of scaffold, type of tendon injury and main results obtained.

Growth factor	Scaffold	Injury	Main results	Ref
PDGF-BB	Double-layered emulsion and coaxially electrospun scaffolds made from polyester urethane	Achilles tendon full laceration	• Increased tensile stress, failure stress, stiffness and elastic modulus of treated tendons • Upregulated expression of collagen I and III • Thickening and enlargement of the cross sectional area of the tendons • Decreased cellularity (maybe due to the dosage)	[145]
IGF-1	Autogenous cell-free tendon scaffold	General tendon injury	• Increased cellularity • Increased collagen synthesis in TDSC • Increased glycosaminoglycan synthesis	[146]
BMP-7	Gelatin hydrogel scaffold	Rotator Cuff tear	• Favourable orientation of rotator cuff collagen fibers • Helps tendon-to-bone maturing • Improves the ultimate force-to-failure	[147]
BMP-12	Electrospun nanofibrous scaffold PCL- PA6 coated with a thin layer of cell-laden GelMA hydrogel	General tendon injury	• Increased cell proliferation • Upregulated expression of key tenogenic markers (collagen I, tenomodulin, scleraxis, TN-C • Improved cell alignment	[148]
VEGF	Collagen/ mesoporous silica nanoparticle scaffold	General tendon injury	• Increased rMSC growth • Increased capillary branches and newly formed blood vessel complexes • Good biocompatibility	[149]
SDF-1α	BAFS	Bone-tendon insertion	• Chemotactic ability • SOX9 and AGG overexpression • Thickening of the fibrocartilage • Increased chondrocyte density • Stretching of the new bone area • Increased load-to-failure and stiffness values	[150]
bFGF	Hidrogel loaded with MSC and bFGF incorporated into a knitted PLGA scaffold	Achilles tendon defect	• Increased tendon-related gene expression • Improvement of the biomechanical strength • Stimulate MSCs tenogenic differentiation	[110]
TGF- β1	Alginate scaffold	Rotator Cuff tear	• Increased failure load • More prevalent midsubstance tear • Heightened modified total Bonar score • Better collagen orientation, continuity and organization	[151]
TGF-β3	Electrospun PCL Fiber Scaffolds	Rotator Cuff tear	• Increased maximum force values of the repaired tendons • Scattered mast cells visible in the granulation tissue surrounding the original scaffold	[152]

also might determine the effectiveness of the bioactive construct [109]. In general, cells that are associated with the damaged tissue are often used. Thus, in tendon TE, the cells selected are usually tenocytes [160] and fibroblasts (differentiated cells present in the tendon) [153] or undifferentiated stem cells (iPSCs [110], MSCs [161], ADSCs [162], BMSCs [163], TDSCs [164]) that, after differentiating, can also give rise to tenocytes or fibroblasts [165]. Table 3 shows some of the studies carried out for tendon tissue engineering, indicating the cells used, the type of scaffold and the main results.

It should be noted that the vast majority of tissue engineering studies in tendon that have been carried out to date involve the use of tenocytes or BMSCs [93]. Tenocytes are the cells that arise most interest since the ultimate objective of tendon tissue engineering is to mimic the natural composition of the tendon as much as possible, trying to achieve mechanical and biological properties very similar to those of the tendon (tenocytes represent about 90–95% of the cells in tendon). The results obtained with this type of cell are very interesting and promising [160,166–168]. However, tenocytes are difficult to obtain, generally causing damage to the donor tissue, and hard to grow, as they have a low duplication rate [29,96]. This is one of the main reasons why these approaches are taking time to reach the clinics. A widely used alternative to these tenocytes are BMSCs [163,169–171]. These cells do not present the problems mentioned for tenocytes [105]. As they are stem cells, it is important to provide them with the appropriate signals so that their differentiation is towards a tenocytic line (the areas adjacent to the scaffold implantation site already do so), and they do not differentiate towards an osteogenic line, producing an ECM characteristic of bone tissue instead of tendon tissue [172]. Despite the fact that there are many questions that still remain to be clarified about the best cells to be incorporated in the scaffolds for tendon tissue engineering, it is evident that their use is essential to improve the results of regeneration by obtaining scaffolds with properties more similar to those of healthy tendon.

2.3. Design and development of scaffolds with good biocompatibility and mechanical properties

Scaffolds are 3D structures that have been designed and fabricated to mimic the shape and function of the ECM of the native tissue and cause desirable cellular interactions (adhesion, proliferation and differentiation) contributing in that way to the formation of new functional tissue [173]. Usually, they have at least one of these functions: (i) allow the cells to attach and migrate, (ii) deliver and retain cells and molecules with biological activity, (iii) facilitate the diffusion of nutrients and products of interest, and (iv) modify the cells behaviour by influencing them mechanically and/or biologically [174].

The ideal scaffold for tissue regeneration should meet some specific requirements: (1) be biocompatible, (2) be able to hold and support cells and bioactive molecules, (3) have good structural and dimensional characteristics meaning that it should have high porosity and adequate pore size to facilitate the diffusion of nutrients and molecules with biological activity, (4) minimize the host immune inflammatory response, (5) be biodegradable, this usually is an crucial factor since scaffolds should rather be absorbed by the organism than removed with necessity of surgical intervention, and, related with this point, (6) be clinically easy to use, and (7) be cost effective. For the regeneration of

Table 3
Summary of the main cell lines used in tendon TE. An example of each cell line is analysed indicating the type of scaffold, type of tendon injury and main results obtained.

Cell type	Scaffold	Injury	Main results	Ref
Tenocytes	Collagen scaffold	Infraspinatus tendon defect	• Healed tendons had a similar appearance compared to the thickness and width of the healthy tendons • Less tissue dedifferentiation, enhanced fiber orientation, greater synthesis of proteoglycans and increased genesis of collagen I and III • Improved breaking stress and tensile strength of the tendon • No signs of tissue infection	[160]
Fibroblasts	CS-based hyaluronan hybrid scaffold	Infraspinatus tendon defect	• Increased collagen I synthesis • Higher tensile strength and tangent modulus • Crimp patterns more regularly arranged	[153]
BMSCs	Decellularized tendon matrix scaffold	Achilles tendon defect	• TNMD and COL1A1 expression upregulated (differentiation toward tendon) • Cells spindle-shape, elongated and aligned parallel to tendon longitude axis (after 12 weeks) • Better structural restoration and maturation of the healing tendon • Better mechanical properties: higher failure load, stiffness, ultimate strength and Young's modulus	[163]
ADSCs	Scaffold composed of an external part of a net knitted with PGA/ PLA fibers and an internal part of PGA unwoven fibers	Achilles tendon defect	• Produced ECM in vitro • At the interface, engineered tendons healed well to the host tendons • Level of tissue adhesion reduced • Increased tensile strength, but only 60% of the normal tendon's tensile strength • After 10 months, the structure was comparable to that of native tendons. Elongated cells aligned longitudinally with the paralleled collagen fibers and good cellular density were achieved.	[162]
hUCB-MSCs	3D bioprinted PCL scaffold with an HA entrapped cell-laden hydrogel	Chronic full-thickness rotator Cuff tear	• Increase in the number of cells positive for, PCNA, PECAM-1, COL-1 and VEGF. • Improve regeneration of tendon fibers • Decreased tear size compared to the control • Improvement of motion parameters: mean walking speed, walking distance and fast walking time.	[161]
iPSC-NCSCs	Fibrin gel	Patellar tendon window defect model	• Improved connective tissue and more ECM deposition • Alignment of greater number of spindle-shaped cells along the longitudinal axis of the tendon • Increased collagen synthesis • Better failure load than non-treated injury • Increased expression of tendon-related genes (suggesting differentiation into tenocytes)	[110]
TDSCs	Fibrin gel	Patellar tendon window defect model	• Hiher ECM deposition • Increased collagen fibers • Higher ultimate stress and Young's modulus	[164]

Acronyms. CS: Chitosan; BMSCs: Bone Marrow Stem Cells; TNMD: Tenomodulin; COL1A1: type I collagen alpha 1 chain; ADSCs: Adipose-derived stem cells; PGA: polyglycolic acid; PLA: polylactic acid; ECM: extracellular matrix; hUCB-MSCs: mesenchymal cells of human umbilical cord blood- mesenchymal stem cells; PCL: polycaprolactone; HA: hyaluronic acid;COL-1: type I collagen; PCNA: Proliferating cell nuclear antigen; VEGF: vascular endothelial growth factor; PECAM-1: Platelet endothelial cell adhesion molecule; iPSC-NCSCs: induced pluripotent stem cells- neural crest stem cells; TDSCs: tendon-derived stem cells.

the tendons, in addition to the previous mentioned characteristics, scaffolds should have good mechanical properties. These mechanical properties of the scaffold are very important since they will have to withstand great efforts (they have to modify their structure to be deformed and regain their shape depending on the stress they suffer). Additionally, the rate at which the degradation occurs should be as similar as possible to the rate of tendon formation. If the scaffold degrades before the tendon fully regenerates, the new formed tissue could be too weak compared to the original tissue and, thus, be much more likely to re-injury (Fig. 5) [175,176].

Generally, the classification of the scaffolds is made based on the material used to elaborate them. They can be biologic scaffolds,

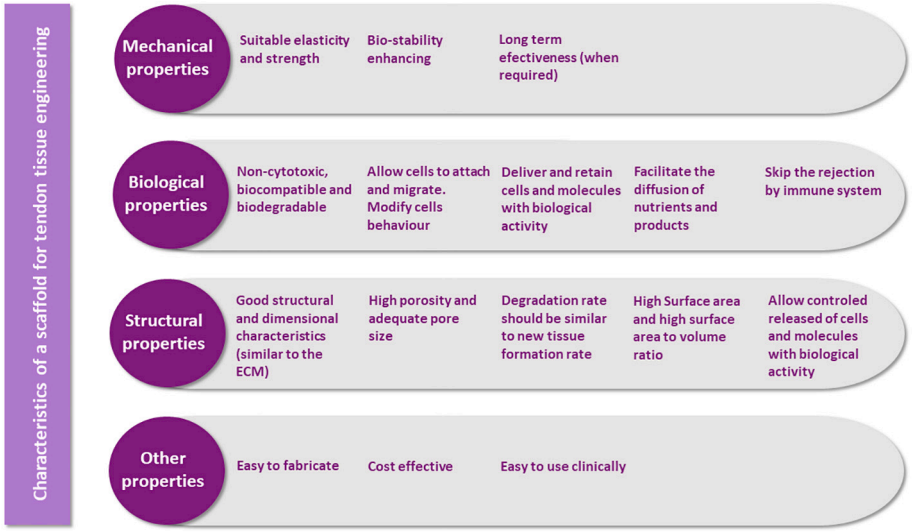


Fig. 5. Schematic resume of the main characteristics that a scaffold for tendon tissue engineering should fulfil. These characteristics are classified into four groups: mechanical, biological, structural and other properties.

synthetic scaffolds or composite scaffolds [82,177].

Biologic scaffolds include decellularized native tendon matrices and naturally occurring polymers [159]. Generally, they do not produce an immunogenic response, they are not toxic for the organism and they are biodegradable [178]. One of their main advantages is that they can easily mimic the biochemical composition of the native tissue because of their biologic origin, especially the decellularised native scaffolds [179]. By resembling the architecture and signals of the native tissue, the response of the cells is usually much faster [180]. This is clearly beneficial because a much quicker adaptation of the scaffold to the host tissue is going to be achieved, thus, increasing the chances of success. However, their availability is reduced in comparison with that of the synthetic materials. In fact, they have to be harvested from the biological source using, in most cases, destructive techniques that affect the donor tissue and the characteristics of the harvested material. In addition, it is difficult to achieve good reproducibility with this type of materials [165,177]. *Synthetic scaffolds* usually consist of new-engineered materials that have been fabricated in order to have the desired properties (usually polyester derivatives) [181]. They have two main advantages. First, they can be synthesized with the most convenient architecture for each type of tissue and patient [182]. Although with natural materials good structures can also be obtained, in general, synthetic materials allow obtaining more defined, stiffer and more consistent structures. Second, by choosing their characteristics, such as their chemical structure, size and shape, many parameters of the scaffold (mechanical properties, degradation characteristics, etcetera) can be adjusted [183]. In this sense, it is very interesting for some tissues, like the tendon, to modify the properties of the synthetic material in order to obtain a scaffold with adequate physicochemical and biomechanical characteristics [178]. However, as their main drawback, we can mention that synthetic materials and their degradation compounds may potentially generate an unwanted host response [183].

The main properties and characteristics of biologic and synthetic scaffolds are summarized in Table 4. A third group of scaffold materials can also be considered, the *composite scaffolds* [184]. These are the result of the association of two or more materials. The most used approach implies the use of natural and synthetic materials in the same scaffold. Lately, this approach is the most used one since it allows combining the advantages that each type of material offers, achieving a more complex structure similar to the natural tendon [82]. Therefore, the resulting properties of the composite scaffold depend on the properties of the used biologic and synthetic materials [10]. There are multiple combinations of materials that have been studied so far. Among them are

Table 4
Comparison of the main characteristics of biologic and synthetic materials used for scaffold development.

Properties	Biologic materials		Synthetic materials
Availability	Difficult		Easy
Structure	No homogeneous. Limit control over shape		Tailored and homogeneous architecture
Mechanical properties	Poor		Good. Can be adjusted
Reproducibility	Difficult		High
Immunogenicity	As they are obtain from biological sources they may contain antigens that produce an immune response		As they are not naturally present in the organism they may produce an immune response
Bioactivity	High. Natural interaction with cells and tissues through receptors and signals. Promote cell adhesion, proliferation and differentiation		Poor. Lower interaction with cells or tissues
Biocompatibility	Different depending on the source		Very poor. Risk of rejection
Biodegradability	High. Over time, they allow host cells to produce their own EM		Poor. Less susceptible to enzymatic hydrolysis (tend to degrade by simple hydrolysis) Potential toxic effects of the degradation products
Degradability	High variability in degradation rates		Generally, low degradation rate. Can be controlled by making modifications of the polymer itself.
Examples	Proteins:	Polysaccarides:	• Poly(α-hydroxy esters) • PLGA
	• Collagen • Gelatin • Silk • Fibrinogen	• Agarose • Alginate • Cellulose • CS • HA	• Polylactid acid • PGA • poly(ε-caprolactone) • Poly(vinyl alcohol) • Polyurethane

* Used references: [177, 180, 183, 184, 191]

polycaprolactone (PCL) with chitosan (CS)/ hyaluronic acid (HA) [185], PCL with collagen I [186], methacrylated gelatin with PCL [187], poly (L-lactic-co-glycolic acid (PLGA) with fibrin [38,188,189], or silk/ fibroin with PLGA and collagen [190].

2.3.1. Natural materials for scaffold development

Natural materials have many times been selected to construct

scaffolds; in particular, different components of the ECM of tendons have been considered to stablish their capacity to support cell growth, for example fibrin, collagen and polysaccharidic materials, like CS or GAGs [183]. Most of the mentioned materials have proved cell compatibility (as they are naturally present in the ECM of the tendons), but in some cases, problems with potential immunogenicity still remain [192].

Among natural biopolymers, the most studied ones for tendon tissue engineering have been those systems based on *collagen* since it is the main component of the ECM of the tendons and has unique chemical, physical and biological properties. Its way of use has evolved over time [193]. The first approximations consisted in the use of randomly oriented collagen type I fibers to form porous scaffolds [10]. These first scaffolds were used without cells and did not present good mechanical properties compared to those of the native tendon tissue [194]. Since then, in order to overcome this limitation, other approaches have been tried.

One of the proposed alternatives was the use of gels seeded with MSCs or fibroblasts. The obtained results were interesting. The effectiveness of MSC-collagen composites was analysed *in vivo* in a created patellar tendon defect in New Zealand white rabbits. The recovery with and without the use of the composite was studied with biomechanical, histological and morphometric analyses. Results showed that the patellar tendons treated with collagen composites developed higher maximum stresses and moduli. No differences were observed in the cellular disposition and histological structure of the repaired tendons with and without collagen composites. Despite the good results, it was not possible to obtain values similar to those of native patellar tendon, indicating that it was not a complete recovery [195]. A similar study using collagen scaffold seeded with tenocytes instead of MSCs showed better results. In this case, the effectiveness of the scaffolds was proved *in vivo* in a defect created over a tendon-bone junction of the infraspinatus tendon in sheep. After 12 weeks, the histological analysis revealed that, compared to the control, the threated sheep presented higher production of proteoglycans, better fiber patterns and increased genesis of collagen III. Biomechanically, the tensile strength was just a 10% lower than the natural tendon and the breaking stress was very similar between these two groups as well (no significant difference) [160]. The key to the good results of this approach seems to be the material used (collagen), the design of the scaffold (a sponge-shaped phase on a basement membrane) and the cells used (autologous tenocytes).

Another approximation consisted on the mechanical stimulation of the developed cell-seeded collagen scaffold. These gels, unlike the first scaffolds, presented *in vitro* significantly better mechanical properties, higher stiffness and tensile strength, in part due to the properties provided by the collagen fibers themselves (aligned due to cyclic tensile strain applied), and the cells seeded on them [196,197]. Since those initial results, mechanical stimulation of the scaffold has been considered important to achieve good mechanical properties and adequate cell adaptation and differentiation [175,198,199]. Currently, in addition to this technique (mechanical stimulation), manufacturing technologies are also used to achieve correct fiber alignment [200]. An example of these technologies has been the use of counter-rotating extrusion to manufacture an aligned collagen membrane from insoluble collagen. This membrane was seeded with BMSCs. The tensile strength was similar to that of the native tendon. Its biological effect was studied *in vitro* and *in vivo*. In the *in vitro* studies, it could be observed that a higher tenogenic differentiation of BMSCs could be promoted using the aligned collagen membrane compared to that of the randomly oriented collagen membrane. The *in vivo* studies were used to analyse the in-situ tendon repair capability of the developed scaffolds. To do so, Achilles tendon defects were produce in Sprague-Dawley rats. The results indicated that the orientated fibers of collagen allowed better regeneration of the damaged tendon (better thickness and weight, fiber alignment, density and state of cells and increased expression of tendon related genes and proteins). Nevertheless, these properties were far from the ones of the normal tendon. Another in vivo studied parameter was the Achilles functional index value. In this case, both scaffolds allowed recovering almost completely the normal values of this parameter showing a good repair performance. Other tensile mechanical properties analysis, as maximum load, stiffness, maximum tensile stress and Young's modulus, were, again, quite far from the ones of the autogenous tendon, which indicates that there are still modifications to do in order to achieve a complete tendon recovery [201].

Nevertheless, collagen scaffolds not only can be loaded with cells, they can also be loaded with molecules with biological activity or other biopolymers. For example, mentioned collagen sponges have been already loaded with GAGs, growth factors, and various cell types. These structures were able to maintain the cell phenotype *in vitro*, as well as to increase the expression of collagen levels in small animal models [144,202–204]. Another study showed that the collagen scaffolds have more advantages than those previously discussed. Kishore and others (2012) explained that collagen fibers also promote TDSC migration and BMSCs differentiation towards the tenogenic line *in vitro*, even in the absence of biological signals [205]. Since these first approximations, other fabrication techniques have been used for obtaining aligned collagen fibers: extrusion [201], electrochemical [206,207], or microfluidic methods [208] (Fig. 6). The alignment of collagen fibers has been established to be as important as the material itself. As it has been seen, the use of this well studied material has many advantages but it is not without drawbacks, which reduce its more widespread use. The main one is the need for processing the collagen in order to remove foreign antigens and potential donor pathogens specially when it is derived from animal tissues. These processes reduce the biomechanical strength and makes it more susceptible to rapid degradation *in vivo* [65,66,209].

Another studied natural protein is *fibrin*. This protein is very interesting as it can crosslink with the thrombin giving rise to fibrinogen. This crosslinking strategy has been studied using different proportions of fibrinogen and thrombin. The *in vitro* results indicated that high concentrations of both molecules led to better mechanical properties (failure loads, tensile stiffness and compressive stiffness). Nevertheless, these concentrations also were related with reduced cell migration and more rounded cell morphology, indicating that intermediate concentrations were the best option to create a cell delivery scaffold for tendon regeneration [210]. Another group also used fibrin to make a matrix in which BMSCs were seeded. In this case, the effectiveness of the fibrin matrixes was proved on full-length defects created in the central part of patellar tendons of 96 rats. The used analysis were histology, collagen gene expression and Young's modulus. The results *in vivo* were promising: increased collagen deposition, bigger collagen fibril diameter, upregulated collagen I gene expression and improved mechanical properties (compared with fibrin matrix without BMSC). Nevertheless, they were still unable to achieve the ideal mechanical properties (similar to the ones of the original tendon) suggesting that additional modifications should be made in order to achieve functional replacements [211].

GAGs can also be used as natural material for scaffold generation. Among them, *HA*, possibly in combination with crosslinking agents (e.g. glutaraldehyde, water-soluble carbodiimide, etc.), is one of the most studied options [212]. This compound is widely distributed throughout the human body. It is especially important in tendons as it maintains the viscoelasticity of the ECM, supports the cellular structure and growth, and functions as lubricant (allows collagen movement without damaging the fibers) [213]. The use of this compound has two additional advantages: it is anti-adhesive and antimicrobial [214]. One example of HA scaffold was the one used by Vindigni et al. (2013). They used biodegradable HA-based scaffolds, HYAFF-11, to seed human ADSCs. Subsequently, mechanical stress was applied to the structure for 15 days to see the effect it had *in vitro* on cell growth and scaffold development. The results showed that cells were able to adhere to the entire surface of the biomaterial fibers, indicating that it was

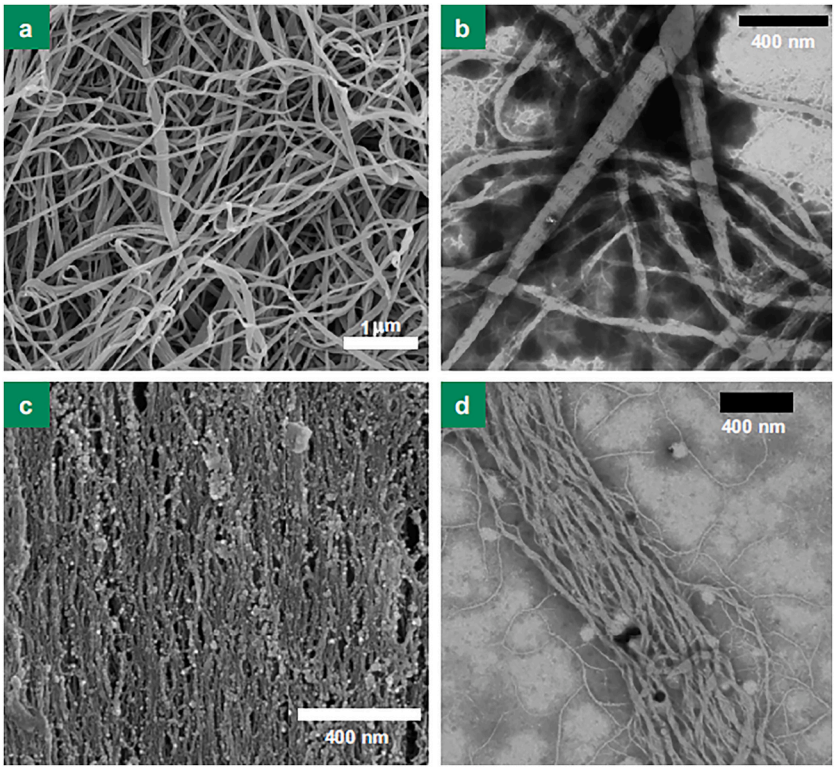


Fig. 6. Aligned and not aligned collagen fibers. In (a), SEM image, and (b), TEM image, no electrochemical process was employed to obtain the collagen fibers. In these cases, a randomly orientated network was achieved. The fibers were big in size. On the contrary, in (c), SEM image, and (d), TEM image, collagen fibers were obtained via the electrochemical method. In these cases, aligned collagen bundle was achieved. Opposite to the first ones small orientated fibers could be observed. Reproduced with permission [207].

biocompatible. Once mechanical stress was applied, ADSCs were lined up in parallel to the traction direction and synthesized type I collagen, also aligned with the direction of the movement. In addition, ADSCs were able to reconstruct a well-defined microcapillary network inside the tendon like structure. In the mentioned case, the group made some modifications in the HA structure. These modifications were responsible for the improved mechanical properties of the scaffold [215]. However, the natural HA molecule has low mechanical properties. For this reason, it is used mainly to produce hydrogels [216], but randomly used alone for rigid scaffold development. Another approximation is to use it in combination with other materials for creating scaffolds more complex and with better mechanical properties [161,171,185].

Another material widely used in tissue engineering is *alginate*. In this case, although it has proved its good properties for being used in scaffold development, there is still little investigation done for tendon tissue engineering [217]. This anionic polymer is of great interest due to its biological properties (low toxicity, biocompatibility and structural similarity to ECM) and for its capacity of gelling using different methods (ionic crosslinking, covalent crosslinking and thermal gelation) [218]. Alginate scaffolds can incorporate different cell lines and bioactive molecules, which can be released with precise kinetic profiles adjusting the crosslinking types and methods [219,220]. There are some studies carried out incorporating biomolecules in alginate scaffolds to see their effect on tendon regeneration. In one of these studies, the molecule with biological activity that was incorporated was TGF-β1. *In vitro*, the release profile of the TGF- β1, the cytotoxic effect of the alginate scaffold on human fibroblast cells and the activity of the growth factor were studied. Good results were obtained: high cell viability, not cytotoxic effect and increase levels of growth factor over time on the scaffold. After these analysis, the effectiveness of the scaffolds were analysed *in vivo* in bilateral supraspinatus tendon injuries performed in 48 rabbits. Results showed that the tendon of the rabbits treated with this scaffold presented better biomechanical properties (ultimate failure load) that the ones naturally healed. In addition, this scaffold achieved better collagen orientation, continuity and organization of the new generated tissue [151]. Another group also reported the proper properties of alginate for being used in tendon tissue engineering. Their objective was to determine the best concentration of alginate to produce a scaffold with good biochemical and biomechanical characteristics. The results *in vitro* indicated that the mechanical strength of the scaffolds and the degradation time increased with increasing alginate concentration. The interconnected porosity had an opposite behaviour. In fact, it was greater with lower alginate concentrations. Cells could attach and grow on the surface and in the pores of the scaffolds. Moreover, scaffolds were safe and had no cytotoxic effect. The good results obtained from alginate scaffolds demonstrate that it is a suitable material for tendon regeneration [221]. The vast majority of approaches advocate its use in combination with other biological materials (composite scaffolds) such as CS [222], or with synthetic materials such as PLGA [223,224], or poly-L-lactic acid (PLLA) [225].

From the natural scaffolds discussed here, *silk* is the material reported as the one with best properties. It is biocompatible, provides good mechanical stability, promotes cell adhesion and proliferation, and deposition of materials that make up the ECM [226]. It is generally believed to be a non-degradable material since it presents a very small loss of tensile strength *in vivo*. However, it is degradable as it can be enzymatically degraded at a relative slow rate [197]. Different structures have been achieved using this material (Fig. 7). Silk has shown tenogenic potential [227,228], as well as regenerative potential at the tendon level [229]. Furthermore, promising results were achieved when silk scaffolds were used for the replacement of the anterior cruciate ligament (ACL) in large animal models [230]. Although it has gained a lot of interest in recent years, the use of silk in tendinopathies is not something new; indeed, it had already been widely used for a long time in the repair of tendon tears, mainly as a suture material [231]. Furthermore, silk scaffolds have shown promising results in tendon regeneration as they achieve comparable mechanical properties to those of the native tendon [232]. Its main drawback is the concern it generates about the adequate elimination of contaminating proteins and its associated immunogenic risk, since silk is not a protein naturally found in humans, even though it is a natural material [233].

Another form of scaffold under investigation is the one based on

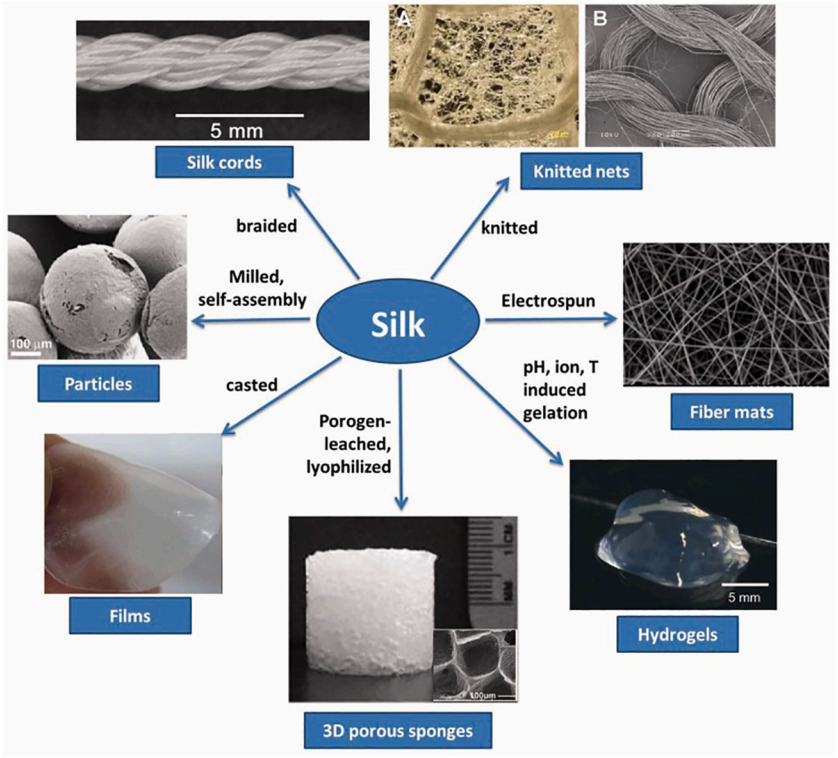


Fig. 7. Scaffolds developed using silk and applied for tendon tissue engineering. Among them: knitted silk nets, braided silk cords, silk fibroin films, electrospun silk fibroin nanofibers, silk fibroin microparticles,3D porous silk fibroin sponges and silk fibroin hydrogels. Reproduced with permission [232].

decellularised tissue extracts (allografts and xenografts). Theoretically, decellularised scaffolds are considered able to preserve the biochemical and biomechanical properties of the natural tendon since they are directly obtained from this same tissue type [234]. Nonetheless, , scaffolds made of this kind of natural material (including allografts and xenografts) must be decellularized using different protocols to ensure that no cell-associated immunogenic antigens remain before implantation. These decellularization techniques must be soft enough to allow for general preservation of chemical and mechanical properties, but robust enough to eliminate all the cellular material (Fig. 8) [235]. One of the best advantages of this technique is the obtaining of a scaffold with the architecture and orientation of the collagen fibers identical to those of the original tissue [179]. However, obtaining healthy tendons that can be decellularized to be used as scaffolds is very restricted (low bioavailability), which is a very important limitation for using this type of scaffold [236]. Despite these drawbacks, many groups have used this approach to obtain scaffolds with relative good properties for tendon regeneration [143]. The approaches used are different; there are groups that directly use the decellularized tendon as scaffold to regenerate the tendon [237]. Other groups, once decellularized, section the tendon in sheets [238,239], or mill the tendon to obtain a powder that serves as a material to develop scaffolds [240,241]. Decellularised tendons can be used together with bioactive molecules [242], or cells [163,243]. As already mentioned, the decellularization processes can cause the tendon to lose mechanical and structural properties, making it different from the natural tendon. Nevertheless, it is an excellent scaffold with good properties that once implanted *in vivo* allows the tendon to regenerate its structure. In other words, the approach of all these groups is not to use

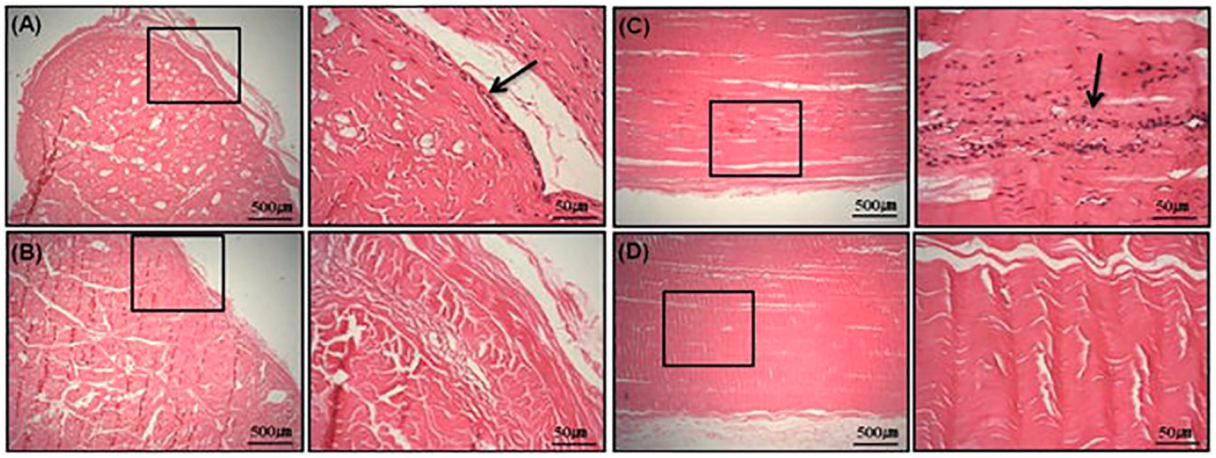


Fig. 8. Vertical histological cross sections of (A) normal tendon and (B) decellularized tendon and horizontal sections of (C) normal tendon and (D) decellularized tendon. Arrows indicate fibroblasts. Reproduced with permission [235].

the decellularized tendon as a replacement structure but as a support structure for regeneration.

2.3.2. Synthetic materials for scaffold development

Synthetic scaffolds, both absorbable and non-absorbable, are an alternative to biologic scaffolds. They are being used extensively in the field of tendon regeneration, since they allow to have a better control over the molecular weight, hydrophobicity and degradation time, among others [244], and offer high reproducibility [245]. With this kind of materials, it is possible to achieve mechanical properties more similar to those of native tissue [179].

As it has happened with the biologic scaffolds, the synthetic scaffolds have evolved over time. The first synthetic scaffolds were inert materials, which had limited success. Initially, they were able to restore the function but they failed to simulate the biomechanical properties of native tendons [246]. These first inert grafts were designed as a physical support to fill the gap left by the damaged part of the tendon. They were not thought for covering different biological functions and, thus, they failed to integrate with the host tissue [247]. Furthermore, these materials also exhibited poor mechanical properties: degradation, low extensibility and permanent deformation, among other [248,249]. Some of these first materials were poly(tetrafluoroethylene), poly(propylene) and poly(ethylene terephthalate).

The problems associated with the use of inert materials made it necessary the development of other types of synthetic materials. Thus, a second generation of materials emerged, characterized by having a high initial resistance and, eventually, being able to degrade in a sustained manner over time, allowing the body's natural tissue to regenerate and mature (acquiring good physicochemical and biomechanical properties). The most representative materials are poly(urethanes) [250] and poly(esters) [251], but other kinds of synthetic materials do also belong to this group.

Poly(urethanes) are a very heterogeneous and versatile group of polymers produced by the reaction of a isocyanate with a polyol in the presence of a catalyst or by activation with UV. Their physical and mechanical properties are diverse and depend, basically, on the type of isocyanate and polyol used to make the polymer [252]. One of the benefits offered by these polymers is that they easily allow modifying the physical and mechanical properties of the scaffolds by using different fabrication processes [253,254]. Among the characteristics of the scaffold that can be modified are the porosity and the degradation characteristics [255]. There are different approaches in the use of poly (urethanes) for scaffold production. For example, a poly(carbonate) –poly(urethane) patch with no seeded cells was tested to treat a supraspinatus tendon defect achieving significant tissue growth. As discussed for structures synthesized with collagen (biological scaffolds section), when seeded with fibroblasts and after applying cyclic strain, these patches showed a higher elastic modulus than that recorded for acellular and non-stimulated patches [256]. Another study also evaluated the effect of the incorporation of fibroblasts in scaffolds of poly(urethane) after a cyclic strain regimen. Authors found an increased cell proliferation and matrix accumulation [255]. Poly (urethane) scaffolds can also be obtained using electrospinning (Fig. 9). These scaffolds showed good biological properties allowing the seeded cells to attach and mature in the tissue. In addition, the collagen deposition was enhanced [257]. Nevertheless, poly(urethanes) are still unable to achieve the robust mechanical properties (similar to the ones of the original tendon) and they may release some degradation products that are potentially toxic for the organism [258].

Other synthetic materials widely used are the poly(esters) [259]. They are a group of polymers whose main chain is composed of the ester functional groups. They are hydrolytically degradable via their ester bounds, but only a few of synthetic poly(esters) are biodegradable, the aliphatic poly(esters) [260]. When used in tissue engineering, preferably biodegradable poly(esters) are selected. When poly(esters) are used for tendon regeneration the scaffolds are produced in a fibrous form in order to mimic the natural structure of tendons. Those forms can be processed later to form higher structures as sheets [261,262], meshes [263], and braids [264]. The most commonly used poly(esters) are homopolymeric poly(esters): polylactic acid (PLA), polyglycolic acid (PGA), PLGA and PCL. Different applications of these materials and their results are discussed hereafter.

One of the mentioned polymers is the PLA (Fig. 10) [265]. This polyester is found naturally in the body and its degradation leads to lactic acid units. This implies that when it is used as the main material of the scaffolds, its degradation in the organism will not produce chemicals that generate an immune response or damage; on the contrary, waste products will be able to be easily removed [266]. The effectiveness of knitted PLA scaffolds has been analysed finding that when seeded with MSCs they upregulated collagen I, TN-C, decorin, integrins, and matrix metalloproteins after two weeks in culture. This effect was increased by adding different growth factors: TGF-β, platelet-derived growth factor-BB (PDGF-BB), and BMP-13 [263,265].

Other similar materials that have also been used frequently for tendon regeneration are PGA, PLGA and PCL. Their degradation mechanisms are similar to that of PLA, but PGA and PLGA exhibit faster degradation rate (2 weeks and 12 weeks respectively, in comparison to 6 months to 2 years of PLA), while PCL exhibits slower degradation rate compared to PLA (3 years) [267].

Various studies have evaluated the properties of the scaffolds obtained from PGA or PLGA. They found that when these scaffolds were seeded with fibroblasts, cell alignment, distribution and deposition of the ECM *in vitro* were adapted to the fiber alignment, thus resembling the original tendon tissue. As was the case with collagen scaffolds, the mechanical properties were improved by applying cyclic mechanical strain [268]. When braided, PLGA scaffolds exhibited *in vitro* better viscoelastic properties and mechanical strength. The large pore diameter that was left between the fibers allowed more collagen deposition *in vitro*

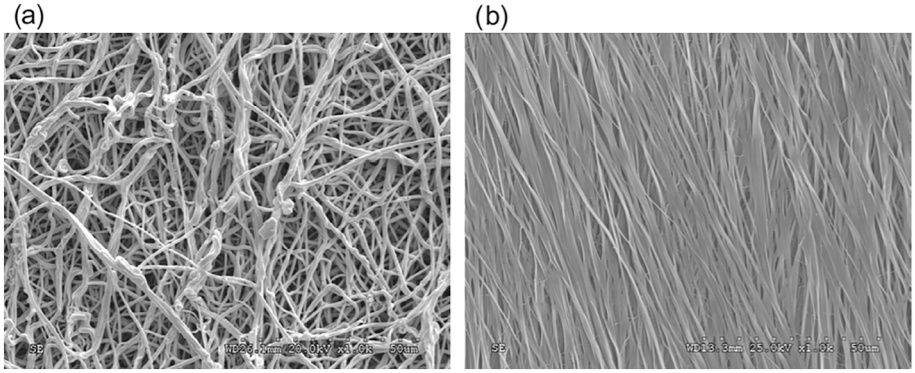


Fig. 9. (a) Random and (b) aligned electrospun fibers of biodegradable polyurethane. Reproduced with permission [257].

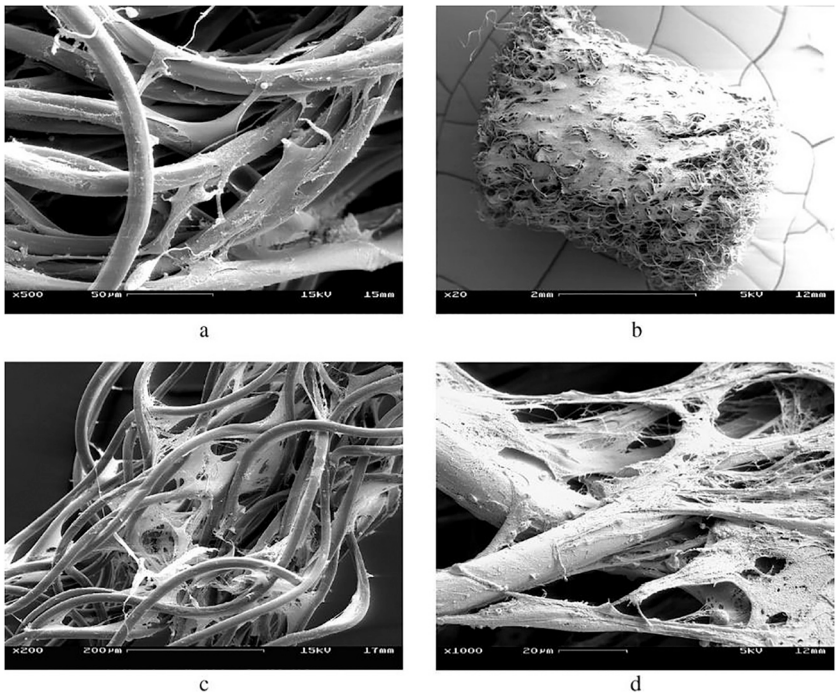


Fig. 10. Scanning electron micrographs of PLA scaffolds at different times after seeding. Not aligned fibers were obtained. Good biocompatibility could be observed as cells could adhere and form three dimensional networks. Reproduced with permission [265].

throughout the construct [269]. In addition, these types of constructs are capable of releasing therapeutic agents and biological molecules at the site of injury to enhance the formation of new tissue [270,271]. For example, *in vitro* studies using PLGA scaffolds loaded with bFGF showed to increase the proliferation of MSCs and their differentiation to tenocytes. An increase in the expression of collagen type I and TN-C was also observed [272]. The same enhancement in collagen deposition, both *in vivo* and *in vitro*, was observed when seeding braided scaffolds with BMSCs under static loading conditions or after adding GDF-5 and TGF-β [273–275].

The advantages of synthetic scaffolds consist on a higher safety profile (as they reduce the risk of disease transmission), a greater potentiality to control their characteristics, and the ability to carry out terminal sterilization. Furthermore, these materials have demonstrated good mechanical strength and great structural integrity [276]. However, they are not yet capable of meeting the requirements for functional remodeling of the tendon tissue. Part of their drawbacks are associated with their reduced biosecurity and their hydrophobic nature and their lack of signs of cellular recognition [245]. When it comes to safety, synthetic materials have a lower risk of disease transmission in comparison with natural materials [276]. As mentioned above, synthetic materials do not have the risk of containing previous biological material that can generate a reaction in the recipient organism. Likewise, they are easily sterilized, which reduces the chances of transmitting some type of pathogenic organism [277]. However, since they are not materials that are found naturally in the body, there is a very high risk of immune response [278]. Furthermore, the solvents used to dissolve these type of materials, as well as the products that are produced during their degradation within the body entail very important safety risks [258]. The different groups that advocate the use of this type of materials have tried to study and reduce the immune response generated by synthetic scaffolds. The approximations that can be use are multiple [279]. In this sense, the low biosecurity of these materials greatly hinders their application to the clinic. Another very important drawback of synthetic materials is their hydrophobic nature, which greatly reduces their biocompatibility. This is translated into a poor tendon cell adherence, low proliferation rates, phenotypic changes, and limited survival compared to biological scaffolds [280]. All of this represents a great problem for its application in tendon regeneration since the effectiveness of the scaffold developed is seriously diminished, (its function would be reduced to a simple structural support). One of the main approaches that has been taken to improve the biocompatibility has been the combination of these materials with more hydrophilic and biocompatible hydrogels. The latter ones have by themselves less structural integrity than PLA, PCL, PGA and PLGA. In this sense, when combining them, the aim is to achieve a synergy that allows obtaining scaffolds with good structural integrity and high biocompatibility [184]. However, there is still a lot of research to be done to find a synthetic material or a combination of materials that will reproduce the mechanical properties of the tendons overcoming the aforementioned biosafety, biocompatibility and final efficacy problems of the developed scaffold.

2.3.3. *Composite materials for scaffold development*

Until a few years ago, the general trend has been to use materials individually and independently. However, in recent years the study of composite scaffolds that are the result of the combination of natural and synthetic materials is gaining interest [82]. The objective of using these combined materials is to achieve a synergy in which each material contributes to the scaffold adding its own benefits, and its problems are diminished by the contribution of the other materials. Generally, the approach that has been made so far is the combination of fibrous scaffolds with hydrogels. The first would contribute on aspects more related to the structure of the tendon, such as promoting the alignment of cells and ECM in the gel. For its part, the hydrogel would contribute more on aspects related to cell survival and the transport of nutrients and molecules with biological activity [281,282].

Despite the fact that by means of this approach the problems of each type of material are reduced, it cannot be guaranteed that they will disappear completely. That is, the presence of high concentrations of natural materials can greatly reduce the mechanical properties provided by synthetic materials. In the same way, the presence of synthetic materials (no matter how much their concentration is reduced) can generate immune responses in the body, thus reducing the biosecurity of

the obtained scaffolds. The biocompatibility of these material combinations is lower than that of natural materials individually. Depending on the arrangement of the two or more types of materials in the final scaffold (especially if the synthetic material covers the entire external part of the system), the biocompatibility of the natural material can be totally reduced. That is why, although it is tremendously complex, it is necessary to adjust the parameters, concentrations, layout, etc. as much as possible to achieve the best possible mechanical resistance, biosafety and biocompatibility. To date, different combinations of biological and synthetic materials have been studied, getting disparate results. Although there have been many advances, the results obtained have not yet been sufficiently effective to be able to carry out their study at a clinical level.

An example of composite scaffolds is the blending of human hair keratin with a small amount of poly(ethylene oxide) (PEO) (Fig. 11). The resulting fibrous matrix showed proper qualities for the attachment and proliferation of fibroblasts *in vitro* [283]. In this case, keratin is a very interesting material since it is very abundant and has many bioactive properties [284].

Another scaffold that enabled homogeneous seeding of MSCs was the one made of silk cable-reinforced with gelatin/silk fibroin. Cells were able to proliferate, differentiate into fibroblast-like cells and secret ECM components *in vitro* [281]. Fibroblasts also secreted ECM components *in vitro* in a composite scaffold containing aligned, electrospun poly(e-caprolactone-co-D,L-lactide) fibers embedded in a photocrosslinked N-methacrylated glycol chitosan hydrogel [285]. Molecules with biological activity can also be incorporated in this kind of scaffolds. For example, bFGF was loaded in a gelatin hydrogel. Next, this hydrogel was incorporated into a braided PLLA scaffold and wrapped with a collagen membrane. For the *in vivo* assays, the ACL of healthy rabbits was removed and the developed scaffolds were incorporated. Different groups were created for the study of the effectiveness of the scaffolds: without surgery, with different times of implantation, with the implantation of scaffolds with and without bFGF, and with and without the collagen membrane wrapping. The comparison of the results observed in the histology analysis, mechanical test and the assay of collagen production, allowed to stablish that the PLLA scaffold loaded with bFGF in a gelatin hydrogel enhanced mechanical strength, collagen deposition and vascularization when compared with the PLLA alone [286]. Another group fabricated a hybrid (natural-synthetic) composite that apart from delivering growth factors (PDGF-BB) also delivered cells (ASCs). Both, PDGF-BB and ASCs, were incorporated to a heparin/fibrin-based delivery system (HBDS). Then, this hydrogel was coated with an electrospun PLGA nanofiber. The natural part of the scaffold allowed the delivery of PDGF-BB and ASCs, while the synthetic part provided structural integrity. *In vitro* good viability and sustained PDGF-BB release was observed. *In vivo* studies were perform in the defects made in the intrasynovial flexor tendons of adult mongrel dogs. Cellularity and vascularity were analysed to determine the effect of the scaffold, the PDGF-BB and ASCs in the damaged tendon. The most outstanding results were that the incorporated ASCs remained viable after 9 days from the implantation, that a sustained release of the growth factor was achieved and that the scaffold was biocompatible up to 9 days (although an increase of inflammation gene expression was observed). No studies on the effect on tendon regeneration were performed. Nevertheless, the results indicated that this might be an appropriate approach to deliver growth factors and cells to the damaged area [287]. The combination of PCL electrospinning membrane and CS/HA multilayers film showed also good results (Fig. 12). Thanks to the biological materials, stromal cell-derived factor-1 α (SDF-1α) and BMP-2 could be locally delivered. This membrane could promote cell proliferation and recruitment *in vivo*. To do these experiments, the native ACL from 48 female New Zealand white rabbits was removed and injured. Afterwards, it was reintroduced with the developed multilayer films incorporated at its end. The

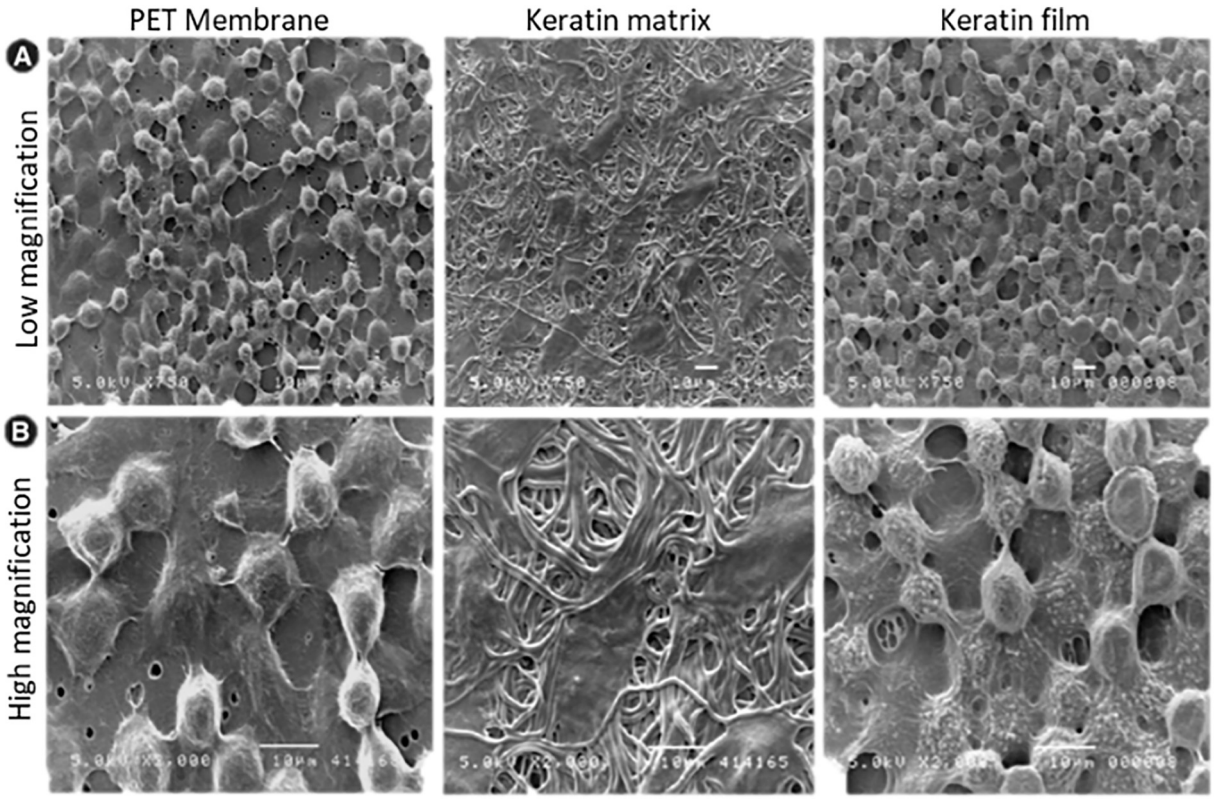


Fig. 11. Representative SEM images of L929 murine fibroblasts cultured for 7 days on polyethylene terephthalate membrane, keratin matrix and keratin film. (A) Low-magnification images and (B) high magnification images. Cells on a 2D PET membrane and keratin film were predominantly rounded while electrospun keratin supported the typical spindle morphology expected of fibroblasts. Scale bars: 10 μm. Reproduced with permission [283].

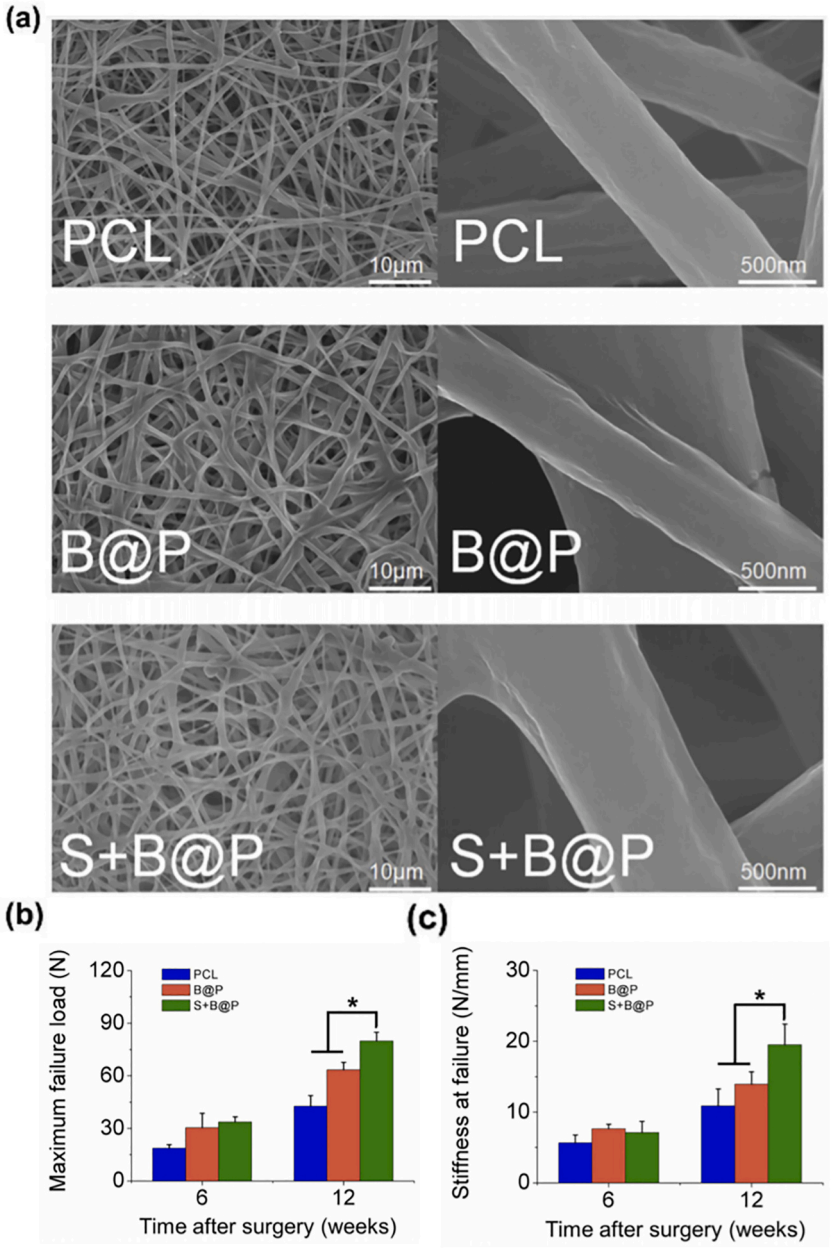


Fig. 12. A) SEM image and nanofiber diameter distribution of PCL, B@P and S+B@P. B) Maximal failure force and (F) stiffness at failure of tendon-bone interface. Adapted from [185]. Acronyms. PCL: polycrapolactone; B@P: BMP-2-loaded CS/HA multilayer-modified PCL scaffold; S+B@P: SDF-1α- and BMP-2-loaded CS/HA multilayer modified PCL scaffold.

histology revealed that the controlled delivery of growth factors from the scaffold was capable of inducing the healing of the bone tunnel and the integration of the tendon-bone interface. Moreover, the SDF-1α and BMP-2 helped, firstly, to the fusion of the membrane and, subsequently, to its degradation, what is important for the biocompatibility of the scaffold. The biomechanical tests allowed stablishing that the local delivery of the growth factors also helped to improve the recovery of the original biomechanical properties of the interface (Fig. 12. b and c) [185].

The mentioned studies focused in the regeneration of the tendon midsubstance. However, another possible approach for tendon regeneration focuses on regeneration of the tendon interfaces. This interface can be bone-tendon interface or muscle-tendon interface. In these cases, the combination of materials is different since the mechanical and structural properties sought are also different [288]. In this sense, many strategies have been proposed. One of those approaches was the use of a stratified scaffold to mimic bone-tendon interface. They used three distinct continuous phases reproducing specific regions of the interface. The used materials were poly(glactin) in a knitted mesh sheets form, PLGA in microspheres and bioactive glass. To do the *in vivo* analysis, 27 athymic male rats were used. The developed scaffolds were subcutaneously implanted and, after 2, 4 and 8 weeks, the host tissue infiltration, the scaffold cellularity, the maintenance of the matrix heterogeneity and the mechanical properties of the matrix were analysed. This scaffold showed favourable results *in vivo* as it supported proliferation, migration and interactions between cells. Additionally, the formation of continuous cellular and matrix regions (enhanced production of ECM) was observed. Furthermore, scaffold infiltration, vascularisation, and biomechanical properties were enhanced in those scaffolds that had been seeded with cells in comparison with those that had not [289]. Other approaches have been explored in an effort to achieve graded mineral deposition and, thereby, trying to mimic the mechanical

properties of this specialized bone-tendon interface [290]. In another study, muscle-tendon interface scaffolds were designed. PCL/collagen were used to mimic the tendon region, while PLA/collagen were used to mimic the muscle region. They were co-electrospun onto opposite ends of the scaffold (Fig. 13). C2C12 myoblast were seeded using a hydrogel in the muscle part of the scaffold and NIH3T3 fibroblasts in the tendon part. The *in vitro* results were promising as scaffolds exhibited variations in mechanical properties between the two sides. The strain profiles were similar to those of the natural interface between muscle and tendon (Fig. 13. (b), (c) and (d)). Scaffolds were cytocompatible and allowed cell attachment [291].

Natural and synthetic materials are potentially useful as scaffolds, and a combination of the two have an enormous potential. Nevertheless, they are still in a premature phase of development. Natural materials have shown to be suitable for the incorporation of cells and active drugs that improve tendon regeneration. However, the approaches that have been carried out using this type of materials are still very simple and far from their possible application in the clinic. Generally, the structures obtained with this type of materials have been hydrogels or membranes and, therefore, their applicability is limited to covering damaged tendons and helping for cell or growth factor delivery, but not for broken tendons. The low mechanical resistance of the structures obtained with this type of materials continues to be their main challenge to be used in tendon regeneration. Among the measures that can be proposed to improve the applicability of natural materials are: (i) the use of other crosslinking strategies that allow obtaining more rigid, resistant structures or with better mechanical properties, (ii) the use of a combination of materials instead of a unique one, and (iii) the maturation of the

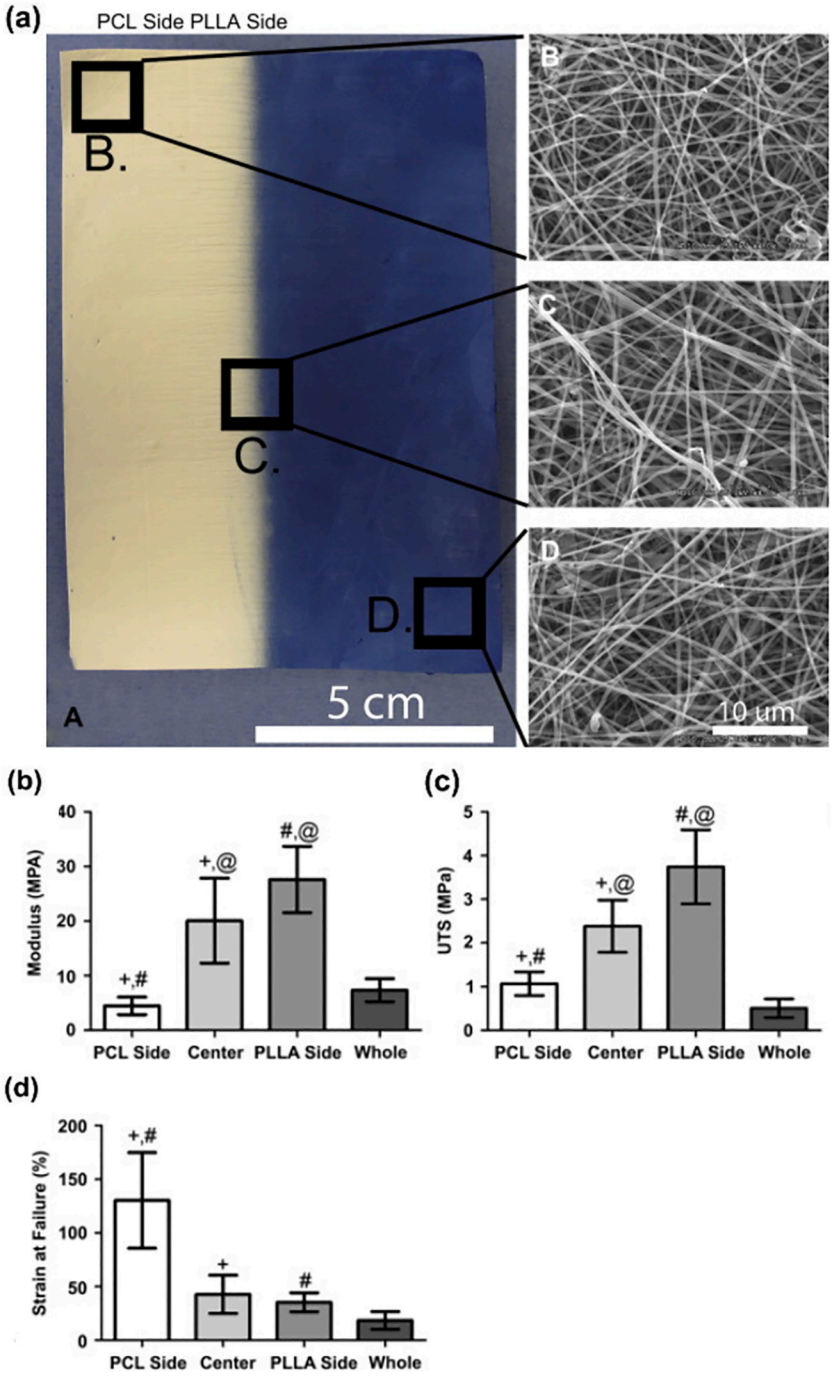


Fig. 13. (a) Picture and SEM images of the muscle-tendon scaffold. (A) Image of the three regions of the scaffold. (B-D) SEM images from the different parts and components of the scaffold (B) PCL side, (C) center, (D) PLLA side. Fibers were rounded and showed nanoscale morphology. The PLLA side had smaller fiber diameters by approximately. (b) Young's modulus, (c) ultimate tensile strength and (d) Strain at failure obtained from tensile testing to failure of each region ($n = 9$) and the whole scaffold ($n = 10$) +, #, @ indicate statistical significance with $p < 0.05$. Reproduced with permission from [291]. Acronyms. PCL: polycaprolactone; PLLA: poly-L-lactic acid.

material together with the cells and the active principles to achieve much more elastic and resistant systems, and with a morphology and composition similar to that of the natural tendon. In the case of synthetic materials, their main advantage is the resistance and mechanical properties that they provide to the scaffolds. However, these properties are not sufficient to achieve adequate regeneration. The results obtained, in most cases, show that they are effective as structural support for tendon ruptures, but they do not show adequate biological function (recovery of the extracellular matrix of the tendon, adequate arrangement of collagen fibers, recovery of an adequate cell density of metabolically active tenocytes that help tendon maintenance and function, etc.). In this case, among the measures that can be proposed to improve the applicability of synthetic materials are: (i) modification of functional groups on their surface to increase biocompatibility, (ii) elimination before implantation of any residue of solvent used to dissolve the material, (iii) elaboration of more complex designs that more closely resemble the tendon structure, (iv) modification and search for more elastic and less rigid materials, and above all, (v) suitable combination with natural materials.

The problems mentioned above are the main reason why most of these studies do not reach clinical application. However, many groups throughout the world are doing great work generating a large amount of knowledge. Ultimately, this, in combination with some of the aforementioned approximations, will be traduced in a more effective scaffold material selection and scaffold development for tendon regeneration in clinics.

3. Promising techniques for scaffolds development in tendon tissue engineering

As already mentioned, the tendon structure is very complex. It includes an essential ECM with unique physical, mechanical and morphological properties [14]. These properties allow tendons to perform their function of maintaining the position of the body and transmitting energy from the skeletal system to the muscle [9,10]. The biological properties of tendons are also very important as they allow them to be the niche of different cells, including tenocytes [8]. Precisely, one of the most important challenges in tendon tissue engineering is the creation of 3D scaffolds that can mimic these special characteristics and support tissue regeneration, while maintaining the aforementioned mechanical properties necessary to fulfil its function. Scaffolds, in addition to allowing adequate functionality, must degrade over time and must do so at an adequate speed. This speed is related to the regeneration rate of the tissue itself [175,176]. All this hints are crucial in the tendon repair process using tissue engineering. Thus, an appropriate scaffold should be designed considering the scaffold architecture, the properties of the biomaterials they are produced from, as well as the processing technique.

There are various scaffolds fabrication techniques, ranging from conventional chemical engineering to new advanced technologies. In recent years, much attention has been given to advanced techniques in tendon tissue engineering as they allow external and internal structure control of the scaffolds. Besides, they overcome some limitations of conventional methods, such as little process automation, too variable and unbendable processing procedures, and structure limits. Some of these new advanced techniques for scaffold development are 3D bioprinting, electrospinning and wet-spinning. Each of these techniques presents its own advantages, but none of them is free of drawbacks. To try to overcome their limitations, some groups have made approaches combining more than one technique. These types of approaches will also be discussed in more detail in the last section.

3.1. 3D bioprinting

3D printing allows the construction of three-dimensional objects with specific geometries using the layer-by-layer method [292]. In 3D printing, the deposited materials are known as inks. When applying this technique to tissue engineering two different approximations can be made. The mentioned 3D printing uses a biomaterial to create a scaffold, latter cells and growth factors can be incorporated. The other approach is known as 3D bioprinting. This approach is similar to the 3D printing with the difference that cells and growth factors are incorporated in the ink. This ink is known as bioink. Therefore, these bioinks combine cells and/or molecules with biological activity (generally growth factors) and biomaterials [293]. As a result, 3D bioprinting gives rise to scaffolds that appropriately mimic the characteristics of natural tissue [294]. This technology gives high expectations for its capacity to design scaffolds with very precise structural and morphological properties.

Some of the several advantages of this technology are the following: (i) control over the final geometry of the structure (macro-morphology, pore size and porosity), (ii) possibility of automation, (iii) high precision, (iii) printing capacity of a wide range of materials, (iv) ability to incorporate proteins, growth factors, drugs, DNA and other biochemical signals together with cells, (v) wide range of cell densities and possibility of introducing cell density gradients, (vi) high reproducibility, ultimately the potential to manufacture complex and sophisticated tissues much more like the natural ones [292,294,295].

These advantages are of great importance for tendon tissue engineering. To begin with, the possibility of using various materials allows printing structures with different properties. Highly biocompatible natural materials can be printed for the promotion of the regeneration of the extracellular matrix. On the other hand, synthetic materials with the ability to form more rigid structures that serve as structural support for the new tendon can also be printed. Few automated technologies allow working with such a wide variety of materials, which is a great advantage for tendon regeneration. Another advantage is the possibility of directly incorporating cells and growth factors. These elements are crucial for the regeneration of the tendon and make this technology more effective in the process of recovering the original structure and function of the tendon. In addition, it should be noted that it is a very versatile technology that offers many possibilities. The mechanical properties of tendons are being replicated using this technology by adjusting parameters such as the composition of the bioink, the printing process (the fibers could be oriented by adjusting the printing process), the infill of the printed structures, the dimensions of the scaffold, etc.

However, this technology is not without limitations. Current bioprinting processes are time- consuming and, in many cases, the applied mechanical forces during the printing process can alter the cell morphology and even provoke cell death [293,296]. Perhaps, the greatest limitation is the formation of vascular networks. Without appropriate vascularisation for nutrients delivery and waste disposal, tissues will not be able to survive. Nevertheless, improvements in this field have been achieved in the last decade [297].

3D bioprinting follows three steps: pre-processing, processing and post-processing (Fig. 14) [298]. The first step, the *pre-processing*, consists on designing a structure using CAD software that the printer will later create, designing the printing path, which guides the motions of printing heads or stages, and choosing the materials and cells that will be used. The selection of which material / materials to use and the characteristics of the designed structure are critical points in the process since they have a decisive influence on the characteristics and properties of the printed scaffold [299,300]. The *processing*, the second step, represents the authentic 3D bioprinting. The bioinks, containing cells, growth factors and selected material, are placed in a printer cartridge and, then, this cartridge in the bioprinter. A 3D construct is built by depositing cell-laden bioinks layer by layer. Since the bioprinting needs can be diverse depending on the material, the type of cells and the characteristics sought in the scaffold, different 3D bioprinting methods have been developed [301]. The last step, the *post-processing*, involves maturing the tissue or scaffold before being implanted [302]. It is a crucial step in the production of completely functional and stable biomimetic tissue. It allows cells to proliferate, differentiate and produce ECM affecting the

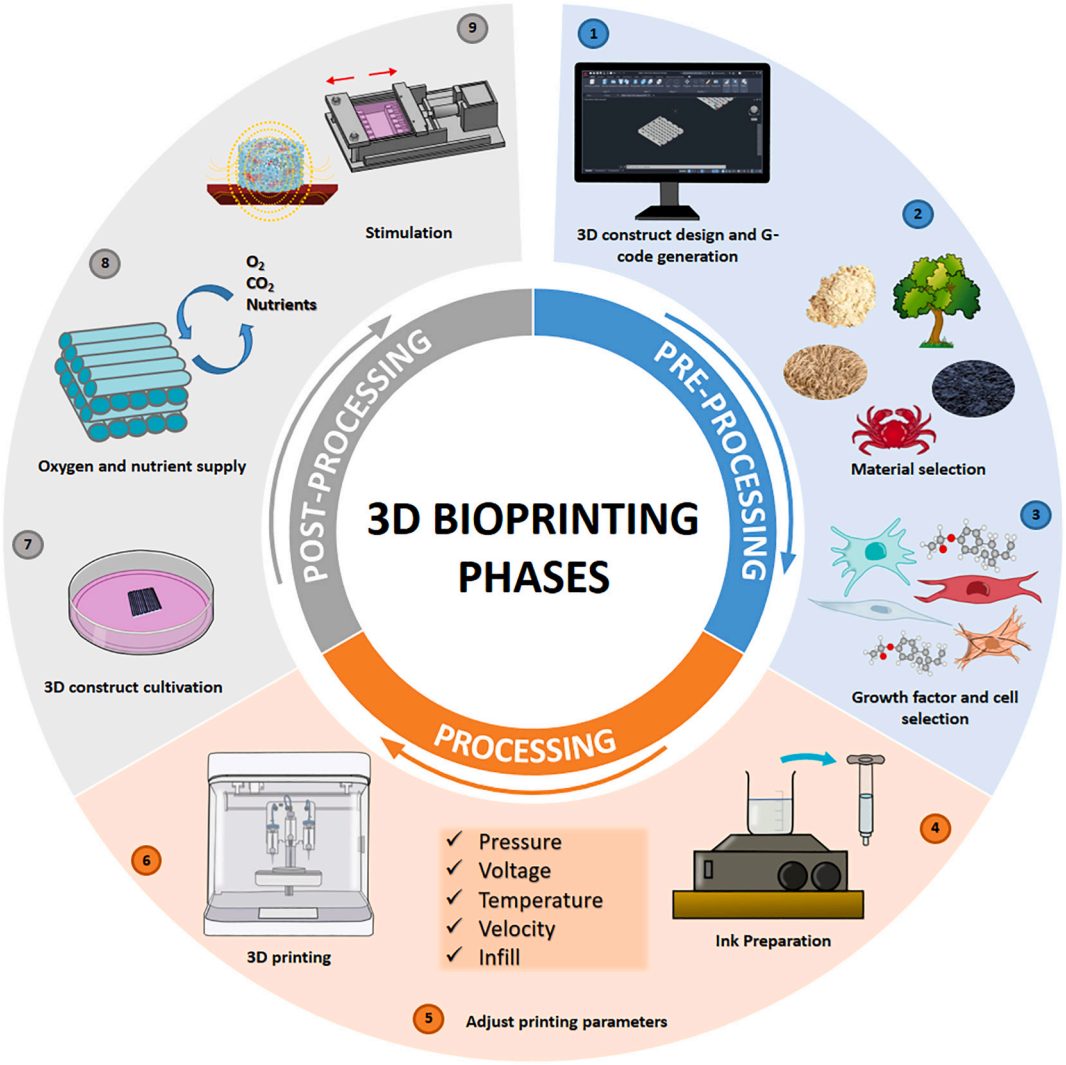


Fig. 14. Steps of a 3D bioprinting process: pre-processing (1-3), processing (4-6) and post-processing (7-9).

structural and mechanical properties of the scaffold. In some cases, mechanical stimulation is also needed. There are different methods to achieve the maturation of the bioprinted construct. The most common method is static culture in petri dishes, but in recent years, the use of bioreactors for this maturation has gained great importance [303]. Bioreactors can provide nutrients, create environments with controlled humidity and gravity, control the pressure causing solution to flow out off or through the cells, or apply compression forces for static or dynamic loading [293].

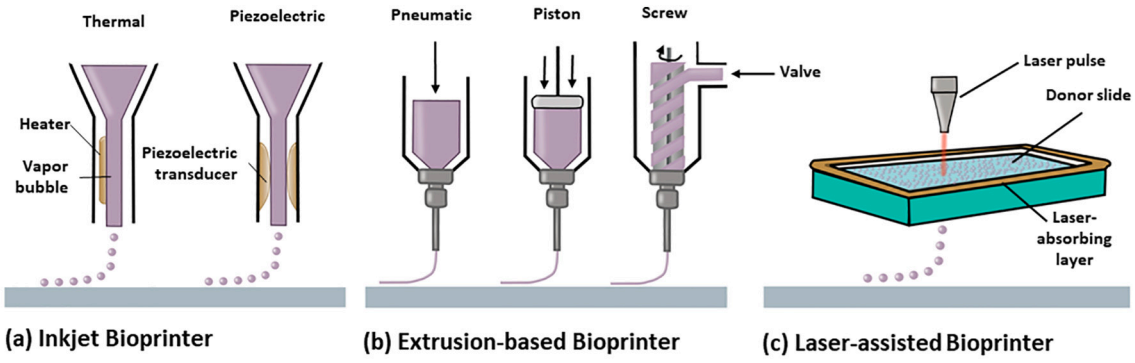
There are different 3D bioprinting techniques (Fig. 15). The most common are the extrusion-based bioprinting, the inkjet-based bioprinting and the laser-assisted bioprinting [304]. Each of these techniques is based in a different principle.

Inkjet-based bioprinting is based on the application of a heat source or an electric current that generates droplets from the bioink [305]. *Laser-assisted bioprinting* is based on the application of a laser pulse as energy source for the deposition of the material onto the substrate [306]. *Extrusion-based bioprinting* is based on the application of air pressure (pneumatic) or mechanical pressure (mechanical) to get the bioink to come out of the cartridge through a nozzle orifice or a microneedle in the form of filaments [307].

Today, not many studies have used 3D printing or bioprinting to make scaffolds for tissue engineering applied to tendons. One of the possible explanations is that many parameters must be adjusted in order to achieve the desired results [308]. As previously mentioned, the choice of the material is a key aspect. In addition, the type of 3D printing will influence the nature of the material to be used, the physicochemical and mechanical properties of the scaffold, the viability of the printed cells, etc. [309]. The combination of these two, the material and the type of 3D bioprinting, will determine the printing parameters: temperature, speed, pressure and voltage, among others [310]. Despite all this, there are different groups that have made their own approaches using 3D printing and 3D bioprinting and they have obtained very promising results.

3.2. *Examples of extrusion 3D printing and bioprinting*

One of the mentioned approaches consisted in the use of decellularised tendon as ink for 3D printing. The objective of this study was to use biomaterials and molecules with biological activity present in the decellularised tendon for the synthesis of a new scaffold whose shape and properties adjust the damaged tendon. They managed to print this biological material using a printer made by their own that was based on an aspiration and extrusion system. As a result, a structure with good biocompatibility properties was obtained. The mechanical properties were lower than those of the native tendon, so modifications of the ink are still necessary to achieve adequate properties [311]. Another group also used a printer of their own design to create a scaffold, in this case to rebuild the tendon-to-bone injury in ACL (Fig. 16.A). The materials used



Actuators	Temperature or Voltage	Air pressure, mechanical pressure or torque pressure	Laser
Ink viscosity	1-15 mPa/s	Up to $6 \cdot 10^7$ mPa/s	1-300 mPa/s
Material	Narrow range of low material viscosities	Wide range of materials	Limited ammount of materials
Cell density	Low, $<10^6$ cells/ml	No limitation	Medium, $<10^8$ cells/ml
Cell viability	70-90%	45-98%	>95%
Resolution	High (0.5-50μm)	Moderate (≈200μm)	High (≈1μm)
Printed Morphology	Droplet	Fiber	Droplet
Print speed	Fast	Slow	Medium
Cost	Low	Medium	High

Fig. 15. Main characteristics and diagram of three types of 3D bioprinting: inkjet, extrusion and laser-assisted bioprinting. Based on [304].

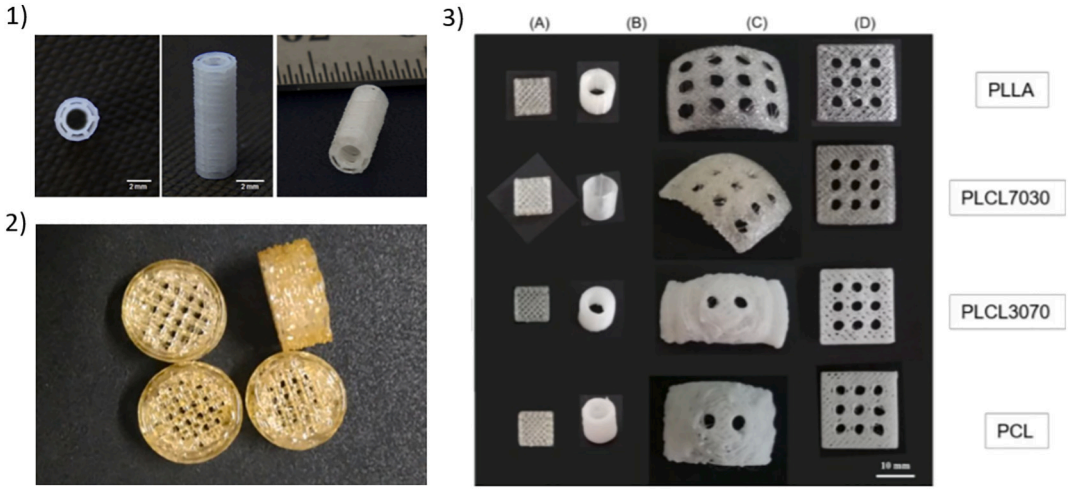


Fig. 16. Scaffolds with different sizes and shapes produced by 3D printing. 1) Printed scaffold composed of PCL, polylacticeco-glycolic acid and b tricalcium phosphate. Fully interconnected pores were achieved. Adapted from [312]. 2) Printed scaffolds composed of PLGA. The surface of the scaffold was rough. Micro-sized pores were obtained. Adapted from [188]. 3) PLLA and PCL copolymer scaffolds. Different patterns were design: small pore cubes (column A), round tubes (column B) and large pore cubes (column C and D)., Adapted from [313]. Acronyms. PCL: polycaprolactone; PLGA: poly(L-lactic-co-glycolic acid) ; PLLA: Poly-L-lactic acid.

to make the bioink were PCL, PLGA and b tricalcium phosphate (b-TCP). The hot melt-extruded method was used. The structure had interconnected pores, which helped to transport molecules (very necessary for cell survival). For the *in vivo* studies, the ACL of New Zealand white male rabbits was transected and the scaffold was placed in the created defect. The histological parameters analysed were the cellular morphology, the extent of fibrocartilage tissue around the tendon, the characteristics of the interface tissue transition from bone to tendon and the extent of matrix staining with immunohistochemical stain for type II collagen around the tendon. Results of the four studied parameters showed improvements in the repair of the damaged tissue in the group treated with the scaffold with cells, with respect to the group treated only with the scaffold. As a limitation of this study, it should be noted that the efectiveness of the scaffold for tendon regeneration was only stablished by the comparisson of the effects produce by the scaffolds with and without cells. The authors indicate that a future comparison using as control tendon grafts without the scaffold should be done [312]. More groups have studied the use of PLGA as bioink for the synthesis of scaffolds by 3D printing. Chen and others (2019) printed PLGA scaffolds with micro and macropores (Fig. 16.B). The PGLA scaffold had neither effect on cell proliferation nor was it found to be cytotoxic. The incorporation of cells transfected with BMP-12 made the group of rabbits treated with this scaffolds show that the ultimate force to failure was significantly higher than in the control group [188].

As mentioned above, both the choice of materials and design are very important for the resulting scaffold. To proof so, one group created scaffolds with different material combinations (PCL and PLA) and different designs (porous cubes with or without large holes, cuboids with

large holes and hollow cylinders) by extrusion 3D printing (Fig. 16.C). The proportions of the materials were decisive in terms of the scaffold elasticity and the ability to obtain structures more faithful to the CAD design. This test allows seeing the important relationship between the viscoelastic properties of the materials with the printing capacity and the results obtained with it. In all cases, the observed properties were adequate: high porosity, good elasticity and high biocompatibility. In this test, cells were added once the scaffold was manufactured to see cell viability *in vitro*, which turned out to be over 90% [313].

In addition to the used materials, other studies also analyse the molecules with biological activity that should be used in order to achieve a better regeneration. One example is the study carried out by Zhang et al. (2021). First, the effect of different small molecules on tendon stem / progenitor cells (TSPCs) was analysed *in vitro*. Next, they selected the most appropriate combination of small molecules to achieve greater proliferation, tenogenesis initiation and maturation and tendon-related genes upregulated expression. This small molecule's cocktail was incorporated together with TSPCs in a bioink based on GelMa and HA. 3D-bioprinting technology was used to obtain the scaffolds. The *in vivo* results were very interesting. They carried out an *in vivo* test by which the ability of ectopic tendon formation of the scaffold under *in vivo* mechanical loading was demonstrated. In addition, through histological and mechanical tests, the positive effect on the regeneration of a patellar tendon injury generated in mice could be seen. This study was very complex, not so much because of the settings used in the printing process, but because of the number of parameters studied and the elements used to make the scaffold. In future trials, this group intends to carry out approximations that are more complex as far as 3D printing technology is concerned [314].

Another currently adopted strategy is the co-printing side-by-side of an ink formed by a biodegradable synthetic material and a bioink formed by a biological material with cells (usually a hydrogel). This approach is interesting since the synthetic material confers mechanical integrity to the scaffold while the hydrogel contributes to exact cell deposition with suitable bioactivity and biocompatibility. For instance, Merceron et al. (2005) printed using four different cartridges with four different materials. In two of them, they had the synthetic materials that gave consistency to the scaffold, thermoplastic polyurethane and PCL. In the other two, they had hydrogels based on HA, fibrinogen and gelatin, with two types of cells (C2C12 and NIH / 3T3). The scaffold design consisted of successive layers up to a final thickness of 1 mm. On one side, printed lines of the polyurethane ink alternated with printed lines of the hydrogel with C2C12. On the other side, printed lines of the PCL ink alternated with printed lines of the hydrogel with NIH / 3T3. In the middle, an overlap zone was created in which the two materials and the two cell lines were printed. The obtained scaffold exhibited *in vitro* good cell viability, proper cell alignment and distinctive musculo-tendinous gene upregulation [291]. Another similar study was performed by Kwon et al. (2020). In this study, they made a scaffold to treat chronic full-thickness rotator cuff tears using extrusion 3D printing. Using a thermoplastic head, they printed a biodegradable PCL scaffold. Later, a HA hydrogel containing the mesenchymal cells of human umbilical cord blood (hUCB-MSCs) was embedded in it. Rabbits showed clear lesion improvement when treated with scaffolds (with and without cells). In addition, rabbits treated with the cell-containing scaffold presented more type I collagen in the damaged area and improved motor analyses with respect to the other conditions [161].

3.3. Examples of inkjet 3D printing and bioprinting

An example of the inkjet 3D printing applied to tendon tissue engineering is the work carried out by Wu et al. (2015). This group developed a PCL scaffold using electrodynamic jet printing. Two types of scaffold were studied: one formed by small diameter fibers (A) and the other formed by two types of fibers, one with a larger diameter and the other with a smaller diameter (B). The objective of the group was to use the larger diameter fibers to achieve structural support and the smaller diameter fibers to achieve cell alignment. The final structure had a tubular shape and a pore size between 60 and 200μm (Fig. 17). The cells that were added to the scaffold were tenocytes. The mechanical properties observed in group B were better than those of group A. However, Young's modulus and mechanical strength were still lower than those of native tendons. On the other hand, the scaffold did not showed cytotoxic effect on tenocytes indicating good biocompatibility. In addition, the *in vitro* cytotoxicity tests revealed that cells adhered to different layers of the scaffold; most of them were longitudinally aligned. Type I collagen expression in both scaffolds was upregulated [166]. This group also carried out a second study in which they fully characterized this scaffold obtained by E-jetting. Thanks to these new tests, it was found that not only the expression of type I collagen was upregulated but also the decorin, TN-C and biglycan expression within the tenocytes adhered to the scaffold. The degradation process *in vitro* was also studied and it was found that the scaffold followed bulk-controlled erosion and that the loss of material (resulting from the degradation process) occurred in parallel with the decline of mechanical properties [168].

Until now, despite the good results obtained, no more approximations have been studied using this technique applied to tendon tissue engineering. One of the possible explanations is the little existing information for controlling well the process parameters and the limitation of the materials than can be used (just liquids with low viscosity).

As conclusion, today 3D printing and bioprinting technology is presented as a promising alternative to overcome the limitations for manufacturing structures that allow a correct regeneration of the tendons. This technology allows the design of structures taking into account both the composition and the biological structure of the tendon. Thus, from the point of view of composition, 3D bioprinting can use a wide variety of materials that allow mimicking the cell and ECM composition of the tendons. From a biological structure point of view, 3D bioprinting can achieve very complex structures, from macroscale to microscale, that mimic the damaged tendon area for replacement. Despite the fact that 3D bioprinting has made great progress in recent years to achieve functional scaffolds with good biomechanical and physicochemical properties, that improve the regeneration of damaged tendons and decrease reinjury rates, there are still many issues that need to be further addressed. One of these issues is finding the combination of biocompatible materials that allow achieving robust mechanical performances and the expansion of various types of functional and supporting cells. One of the main strategies to achieve the biomechanical resistance of tendons consists in the use of oriented fibers that resemble the collagen fibers that naturally form tendons. However, with 3D technology it is difficult to work with fibers and to orient them. A possible solution to this problem consists in achieving the necessary resistance for the tendon once the scaffold has been printed by means of subsequent maturation. Another limitation of 3D printing for clinical application is the reproducibility of the shapes obtained. Many groups have managed to improve print reproducibility by adjusting the materials and concentrations used, as well as the characteristics of the 3D printers, so it is expected that this problem will be solved in a short period. Despite these challenges, the investigation already made has clearly proven 3D bioprinting to be worthy for tendon tissue engineering.

3.4. Electrospinning

Electrospinning is a robust and simple technology used for nanofiber formation. It is based on the use of electrical forces to produce polymer fibers with diameters ranging from submicrometers to nanometers [315]. One of its main strengths is the wide variety of polymer solutions of both natural and synthetic origin than can be used; PLA, polyurethanes, silk fibroin, collagen, HA, cellulose and CS/collagen, etcetera [316]. It has been widely used for different applications, and tissue engineering is one of them [317]. In this sense, it can produce a great variety of fibrous scaffolds with different properties and characteristics.

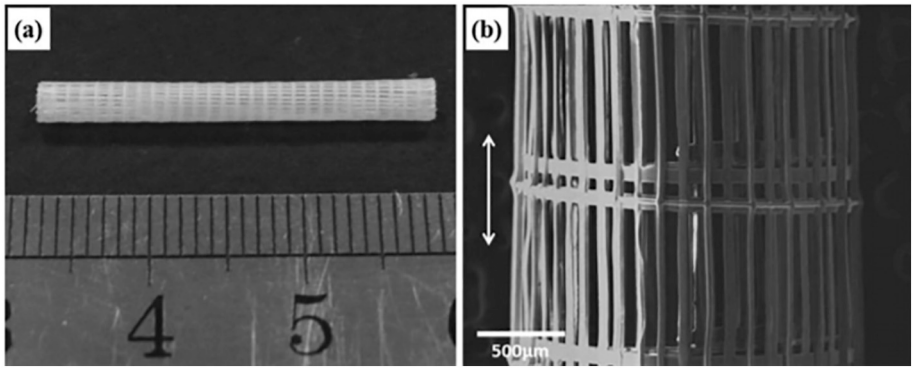


Fig. 17. (a) Tendon scaffold fabricated from E-jetting. (b) Side-view of the tendon scaffold (double-headed arrow: longitudinal direction). Adapted from [166].

Some of the main advantages of this technology are the following; (i) very high surface area per unit of volume; (ii) flexibility to produce a wide variety of scaffolds with different shapes and sizes; (iii) simplicity, it only needs four elements: a high voltage power supply, a syringe, a flat tip needle and a conducting collector; (iv) easy to variate, it can be modified the distance to the collector, the applied voltage or the solution flow, changing the scaffold properties; (v) flexibility to use a wide variety of nanofiber compositions achieving the desired results from its properties and functionality; (vi) flexibility of the surface; (vii) high porosity; (viii) interconnected pores; and (iv) better mechanical properties of the material [176,315].

Of the aforementioned advantages, the one that represents the most important advance for tendon regeneration is the possibility of obtaining perfectly oriented fibers. The great mechanical strength of tendons is partly due to the hierarchical arrangement of the oriented collagen fibers. Therefore, it is possible to think that obtaining oriented fibers of collagen, or other materials, could be a great approach to obtain scaffolds that resemble the original tendon [318]. In addition, growth factors of different nature, that help tendon regeneration, can be incorporated into these fibers. Those growth factors can help the adhesion of cells to the scaffold itself (fibroblasts or stem cells) from the patient's tendon or from other tissues.

Despite the several advantages offered by electrospinning, it has also some important limitations. One of them is that it is difficult to make a large volume scaffold using this technology [319]. Another limitation is that electrospinning has productivity problems, which limits its use in some specific applications [320]. To increase the production rate different solutions have been proposed, for example, a needle-less electrospinning system [321]. But, so far, none of them has shown to completely solve the problem by considerably increasing the productivity. Nevertheless, the major limitation of electrospinning is the limited control of pore structure, which conditions the cellular infiltration [322]. The pore size depends on the diameter of the fiber, the smaller the diameter the smaller the average pore size. This, ultimately, affects cellular infiltration as it is greatly diminished by decreasing the pore size. In some cases, infiltration can be reduced to a narrow layer on the surface of the scaffold, which reduces the advantages of 3D tissue culture [323,324]. All these limitations could potentially draw out the development and application of electrospinning for its application in tissue engineering. In this situation, it becomes essential to formulate a method allowing the fabrication of cell permeable scaffolds using techniques with better throughputs [325,326].

As it has already been said, this technology is very simple [176] (Fig. 18). Briefly, the solution of interest is driven through a capillary (a needle or a cone) by an infusion pump (vertical or horizontal systems) or by the force of gravity (horizontal systems). Once placed in the proper position, a high voltage is applied between a ground target and the tip of the capillary, what creates a charge accumulation at its surface. The buildup of electrostatic repulsion overcomes the surface tension and causes the formation of a liquid jet that forms a fiber. A mounted collector plate with an opposite or grounded charge draws the formed fibers, which comes to form a network [327].

It should be considered that electrospinning is often used to obtain structures that act as vehicles for compounds with biological activity or cells [272,328]. Depending on the technique used to incorporate those compounds, the obtained results are going to be different. Within the

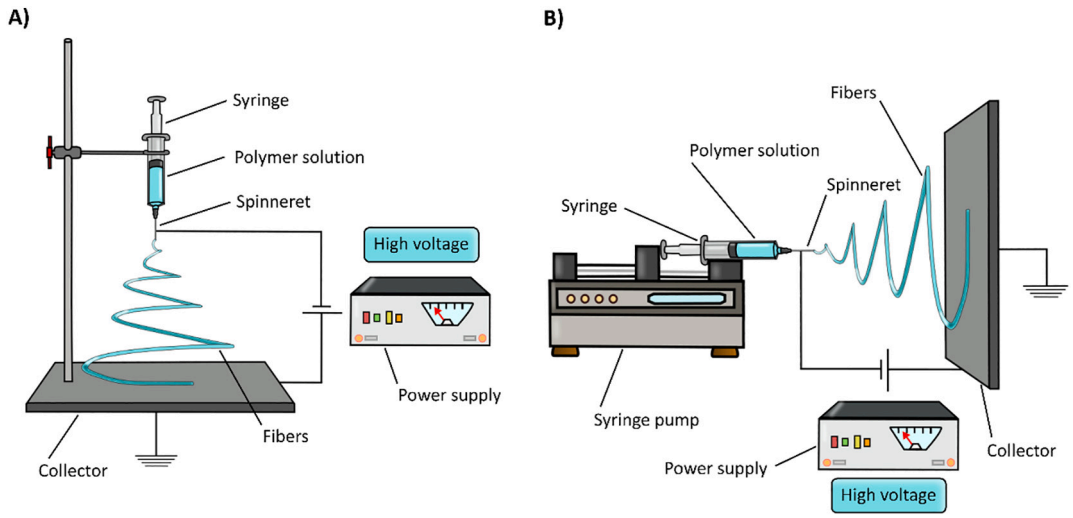


Fig. 18. Diagrams of electrospinning technique. This technique can be performed vertically (A) or horizontally (B).

different electrospinning technologies can be found: co-electrospinning, side-by-side electrospinning, multi-jet electrospinning, co-axial electrospinning, emulsion electrospinning and surface immobilization (Fig. 19) [326].

In *co-electrospinning,* the molecules are mixed with the polymer before electrospinning. In *side-by-side electrospinning*, two spinnerets are used at the same time. This option is usually used when the molecule is not soluble in the solvent that has been used to dissolve the polymer [330]. *Multi-jet electrospinning* implies using more than two spinnerets [331]. In *surface immobilization,* the molecules are covalently bonded to the structure using chemical or physical immobilization methods [332,333]. The techniques indicated so far, allow the majority of the molecules to be released shortly after being incorporated into the body (during the first 24 hours). That is why two other new alternative techniques have emerged to solve this problem: coaxial electrospinning and emulsion electrospinning. *Coaxial electrospinning* is a modification of conventional electrospinning that involves the use of multiple feeding systems that contain different solutions (different combinations of solvents, polymers and active molecules) [334]. All of these feeding systems share identical central axis allowing injection using the same needle tip. *Emulsion electrospinning* consists of the electrospinning of a mixture that contains the solvent or solvents, the polymer or polymers and the active molecule encapsulated in an emulsion [335]. Another strategy to achieve a more sustained release, regardless of the type of electrospinning used, is the development of multiple layered fiber mats. In this approach, what is done is electrospinning the molecules with biological activity in the inner layers and, therefore, they will have to go through other layers that only contain polymers before being released from the structure and reaching the tissue [336].

One of the reasons why this technique has not been used extensively so far in tendon tissue engineering is because it is necessary to control many parameters in order to carry it out; parameters that sometimes limit the ability to obtain structures with the necessary properties. Among the parameters to be controlled are the *processing parameters* such as the distance that separates the collector and the needle, the flow rate, the voltage and the diameter of the syringe. Among these parameters, *ambient parameters* also have to be considered, such as humidity and temperature, which notably influence the results obtained in the manufacture of nanofibers [323]. Other parameters that must be controlled and that greatly affect the result are the *solution parameters*. The latter include the polymer solution concentration, the molecular weight aand viscosity of the chosen polymer, the surface tension and the solvents, among others [326].

Today, the vast majority of approaches that use electrospinning for tendon regeneration involve the development of a mesh or membrane made of one or more polymers loaded with a molecule with biological activity [337]. This mesh is used to place on or around the damaged tendon area. It should be noted, therefore, that the type of lesions for which these scaffolds are considered are tendinosis or tendinitis, but not for partial or total tendon ruptures. This is because most of the approaches of electrospinning involve obtaining small volume structures. One example of this is the work made by Weng et al. (2020). They created a membrane by means of electrospinning in which they included the active principle, doxycycline. The aim of this membrane was to release the bioactive compound adequately in the injured tendon after tendon operations. PLGA was used as the synthetic material for the electrospinning. Both the PLGA and doxycycline were dissolved in hexafluoroisopropanol. *In vivo* and *in vitro* assays were performed to determine the effectiveness of the membrane. For the *in vivo* analysis, the mid-portion of the Achilles tendon of eighteen Sprague Dawley rats was cut. For the control group, the tendon was then sutured end-to-end, while for the test group, in addition to the suture, the developed membrane was fixed around the wound. The analyzed parameters were: the post-operative activity, the doxycycline levels *in vitro* and *in vivo*, the biomechanical properties of the healed tendons and the inflammatory response. Results were promising since a large part of the doxycycline was released on the first day, but the rest was released in a sustained way over time, both *in vivo* and *in vitro*. It was also observed that there was only mild inflammation in the group of rats that underwent Achilles tendon repair with doxycycline-loaded biodegradable nanofibrous membranes, while in the control group severe inflammation was observed. In addition, the first group demonstrated higher activity levels than the control group [189]. Evrova et al. (2020) made a similar approach but with different solutions and processing parameters. One of the most interesting parts of their experiments was the use of two different electrospinning techniques: emulsion and coaxially electrospinning. The goal was to get electrospun scaffolds to use them for the delivery of PDGF-BB. The scaffolds were synthesized from polyester urethane. The two techniques gave rise to two different scaffolds that allowed the sustained release of the molecule with biological activity and with similar release kinetics. The PDGF-BB release was related to the degradation time of the scaffold. To study the effectiveness of the developed scaffolds, assays *in vitro* and *in vivo* in a rabbit Achilles tendon model were performed. The properties of these scaffolds were suitable

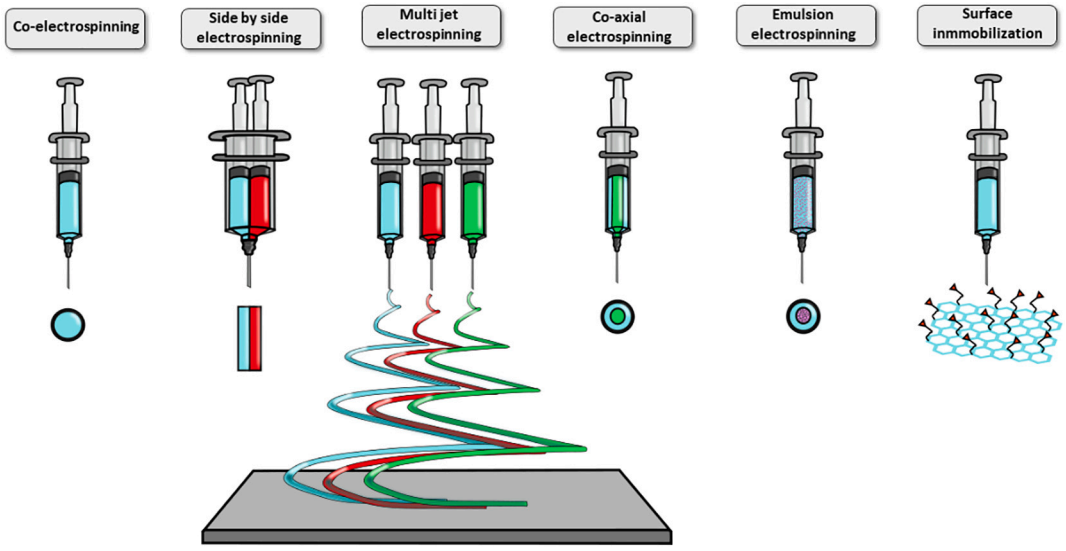


Fig. 19. Schematic representation of different electrospinning processes: co-electrospinning, side-by-side electrospinning, multi-jet electrospinning, co-axial electrospinning, emulsion electrospinning and surface immobilization. Based on [329].

for PDGF-BB delivery in tendon regeneration. On the one hand, an increase in the tensile strength of the treated tendons was observed *in vivo* without generating pro-fibrotic effects. On the other hand, a higher synthesis of collagen I and III was found compared to the native tendon, while fibronectin synthesis was lower. However, in areas away from the wound were the expression of collagen I and III was lower, an increased fibronectin expression was observed. Differences were found between the two scaffolds regarding cell adhesion. Higher cell adhesion was observed in the scaffolds obtained by emulsion electrospinning [145]. Fei et al. (2019) also used electrospinning to produce a membrane. In this case the obtained PCL membrane not only allowed to deliver the bioactive compounds, SDF-1α and BMP-2, in tendon-bone interface but also had a structural function. The first step was the development of PCL nanofiber membranes by electrospinning, followed by the layer-by-layer self-assembly loading of SDF-1α and BMP-2. Subsequently, the autologous tendon of 48 mature female New Zealand white rabbits was wrapped between two membranes and the membranes were fixed in the bone tunnel. The *in vivo* results obtained were very positive since long-term release of both growth factors was achieved, and an improvement in the mechanical properties (failure force and stiffness) of the interface, and a greater cell proliferation and recruitment were observed [185].

Although all the above mentioned electrospinning approaches only used one polymer, it is possible to use these techniques for as many combinations as necessary. For example, Wu et al. (2020) made yarns manufactured by electrospinning from two synthetic materials: PLGA and PLA. They studied the characteristics of PLA microfiber yarns (MY) in comparison with new structures made from a modified electrospinning device that allowed coating PLGA nanofibers onto PLA microfiber yarns. In this way, they could generate PLGA / PLA hybrid yarns (HY) with a well-aligned nanofibrous structure. They also incorporated thymosin beta-4 (Tβ4) into HY to study the effect this protein had on ADSC (Fig. 20). Results showed that PLA MY provided good structural integrity and mechanical properties to the HY, and PLGA nanofibers slightly improved the load resistance capacity. HY presented higher cell proliferation rate than that of the PLA MY bundles *in vitro*, showing that the combination of the two materials had beneficial effects on the resulting yarns. Its surface was remarkably effective in improving cell proliferation, growth and attachment and tenogenic differentiation *in vitro* (effect caused mainly by the action of Tβ4). Therefore, results showed that the combination of Tβ4 with the nano-topography of PLGA / PLA HY had good properties to be used for tendon tissue engineering [338].

All the studies mentioned so far obtained a membrane or a mesh. In contrast, Sensini et al. (2019) made devices whose objective was to resemble as much as possible both the structure and the normal function of the tendons using Nylon 6,6 and electrospinning. First, they created both aligned and unaligned bundles. Later, they developed a system to be able to manufacture a second hierarchical level formed by different bundles linked tightly with an electrospun sheath. They even managed to reach a third hierarchical level by grouping several 2nd-level assemblies with another electrospun sheath. The morphological results were surprising, since a structure very similar to that of the native tendons was achieved (Fig. 21). Furthermore, the results of strength and stiffness were also very promising as they were comparable to the values of the native tendons [339].

Another interesting approach to tendon regeneration using electrospinning was carried out by Yuan et al. (2021). This group made highly aligned fibers of PLLA with COL1 and CS on their surface using stable jet coaxial electrospinning. These obtained fibers were compared with PLLA fibers obtained by classical electrospinning. The fibers were deposited in a rectangular frame and then transferred to copper grids. HMSCs were seeded into the developed scaffold and then they were exposed to mechanical stimulation for 7 days. Cell morphology and proliferation, and expression of tendon regeneration genes were analysed *in vitro*. The effectiveness of the scaffolds was also tested *in vivo* in Achilles tendon injuries in rats. For this, 6 mm long lesions were created in the tendons where the scaffold was sutured. Histological, ECM protein expression and mechanical tests showed that scaffolds obtained by coaxial electrospinning (PLLA with COL1 and CS on the surface) were much more effective than those obtained by classical electrospinning (PLLA only). However, the values were much lower than those of the healthy tendon (Fig. 22). Despite the fact that with these scaffolds a total regeneration of the tendon is still not achieved, the approach that was carried out was very interesting and considerably brings the application of this technology to tendon regeneration. They were capable of using synthetic materials (PLLA), responsible of better mechanical properties, and natural materials (COL1 and CS), responsible of improving the biocompatibility (greater cell adhesion and gene expression) of the scaffold. In addition, obtaining perfectly oriented fibers considerably helped to the improvement of the mechanical properties of the scaffold [340].

In conclusion, electrospinning is a technique that is being applied in many areas, including biology, nanotechnology, and regenerative medicine. Its use in tendon tissue engineering is increasingly frequent due to its simplicity, low cost and its great ability to deliver bioactive molecules to the injured tendon. These molecules with biological

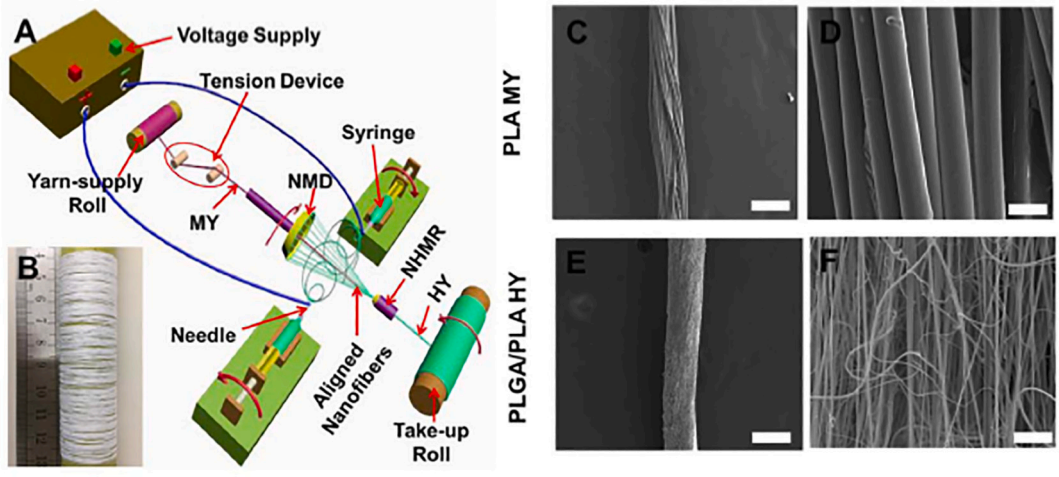


Fig. 20. PLGA/PLA nanofiber/microfiber HY. (A) Design of the modified electrospinning system. With this system electrospun PLGA nanofibers could be coated on the surface of PLA MY to generate PLGA/PLA HY. (B) Photograph of a PLGA/PLA HY package (C, D) SEM images of the original PLA MY; (E, F) SEM images of the obtained PLGA/PLA HY. Scale bars: 200 μm (C and E); 20 μm (D and F). Adapted from [338]. Acronyms. PLGA: poly (l-lactic-co-glycolic acid); PLA: polylactic acid; HY: hybrid yarns; MY: microfiberyarns.

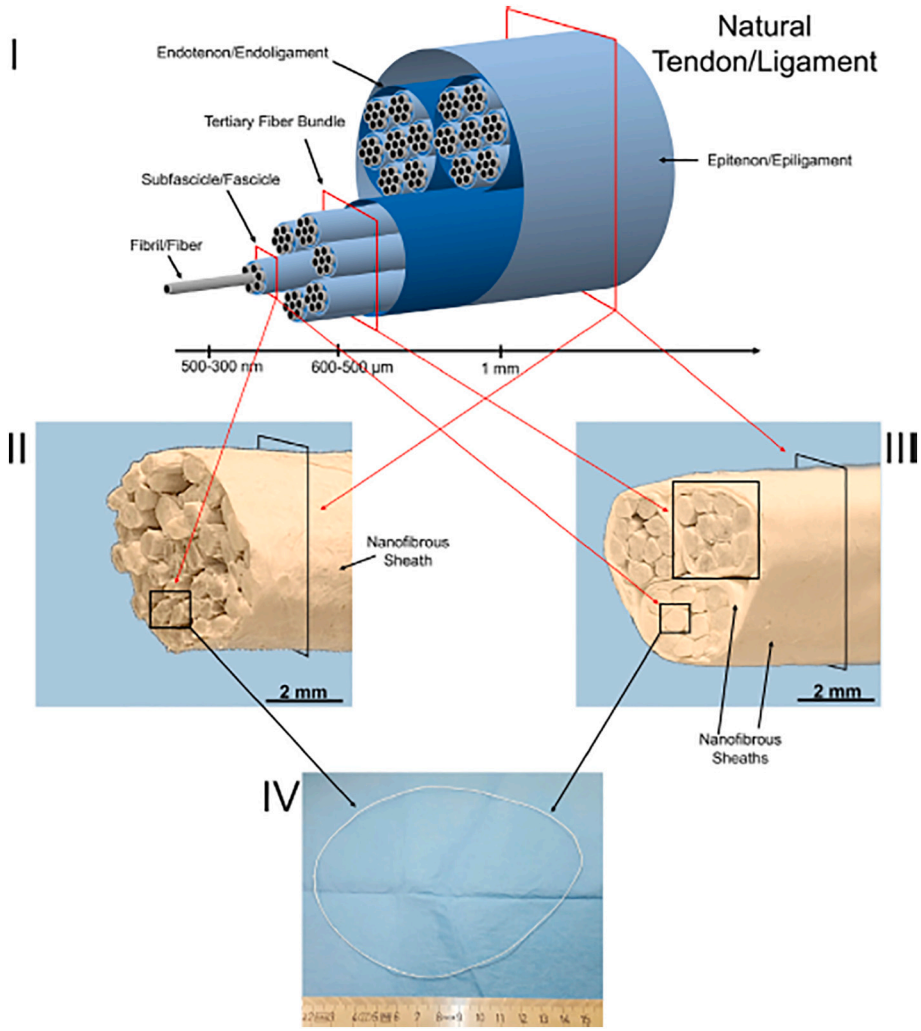


Fig. 21. Electrospun hierarchical assemblies. I) Hierarchical structure of a tendon. (II) Image of the cross-section of a 2nd-level hierarchical assembly and (III) a 3rd-level hierarchical assembly. (IV) Image of a bundle used to build the hierarchical assemblies. Adapted from [339].

activity can be incorporated in different ways (adding them directly to the polymer, introducing them in an emulsion, bonding them to the surface of the fiber by chemical or physical treatments, etc.). Despite the fact that many studies have been carried out with different polymers, controlling the final structure and achieving adequate biomechanical properties remain a challenge. In addition to the aforementioned, another limitation of this technology that takes it away from the clinic, is the inability to develop large-volume structures. This is, electrospinning is limited to the development of meshes and small-diameter fibers. This type of system can be used to transport active principles or cells and thus treat minor tendon injuries. Ongoing advances with different adjusted parameters should be focused on modifying or adapting the technique (or in combination with others) to achieve structures much more similar to the natural tendon, that allow not only delivering bioactive molecules but also providing structural support for tendon injuries.

3.5. *Wet-spinning*

Wet-spinning is a manufacturing method that allows obtaining fibers using a wide range of materials [341]. This technique has not been recently developed. Indeed, it has been in use for a long time in the textile industry. It is the oldest process within the manufacturing processes known as spinning: wet, dry, dry jet-wet, melt, gel and electrospinning. However, its application in tendon tissue engineering has been recent.

Its principle is simple. This technique is based on non-solvent induced phase separation (Fig. 23) [342]. The polymer solution is continuously extruded through the spinneret to a coagulation bath, generally, using a pump [343]. In this bath, polymer solution turns into solid polymer fibers, as the solvent is removed [341]. Finally, different procedures are carried out to collect, dry and store the fibers.

Wet-spinning is less frequently used than electrospinning. Nevertheless, it has some very important advantages compared to this technique. Some of the most important limitations of electrospinning, as previously discussed, are the inevitable use of highly toxic organic solvents, like fluroalcohols, and high voltages and temperatures [344]. The use of these production parameters are known to denature in some measure the structure of the polymers. Another limitation of electrospinning is the difficulty to control the scaffolds porosity what, at the same time, conditions the cell infiltration [342].

Wet-spinning appears as a powerful alternative tool. In contrast to electrospinning, wet-spinning does not use high voltages or temperatures reducing the probabily of denaturation the sample [345]. This gives the possibility of using highly biocompatible polymers based on ECM [341]. It uses less toxic solvents such as calcium chloride solutions, phosphate buffer solutions or acetic acid. These solvents have some advantages: they can dissolve biomacromolecules and they are easily removed from the final scaffold [344]. Another important advantage of this technique is that it allows controlling or adjusting the fibers diameter, the porosity and the pore size [346]. These controllable fiber

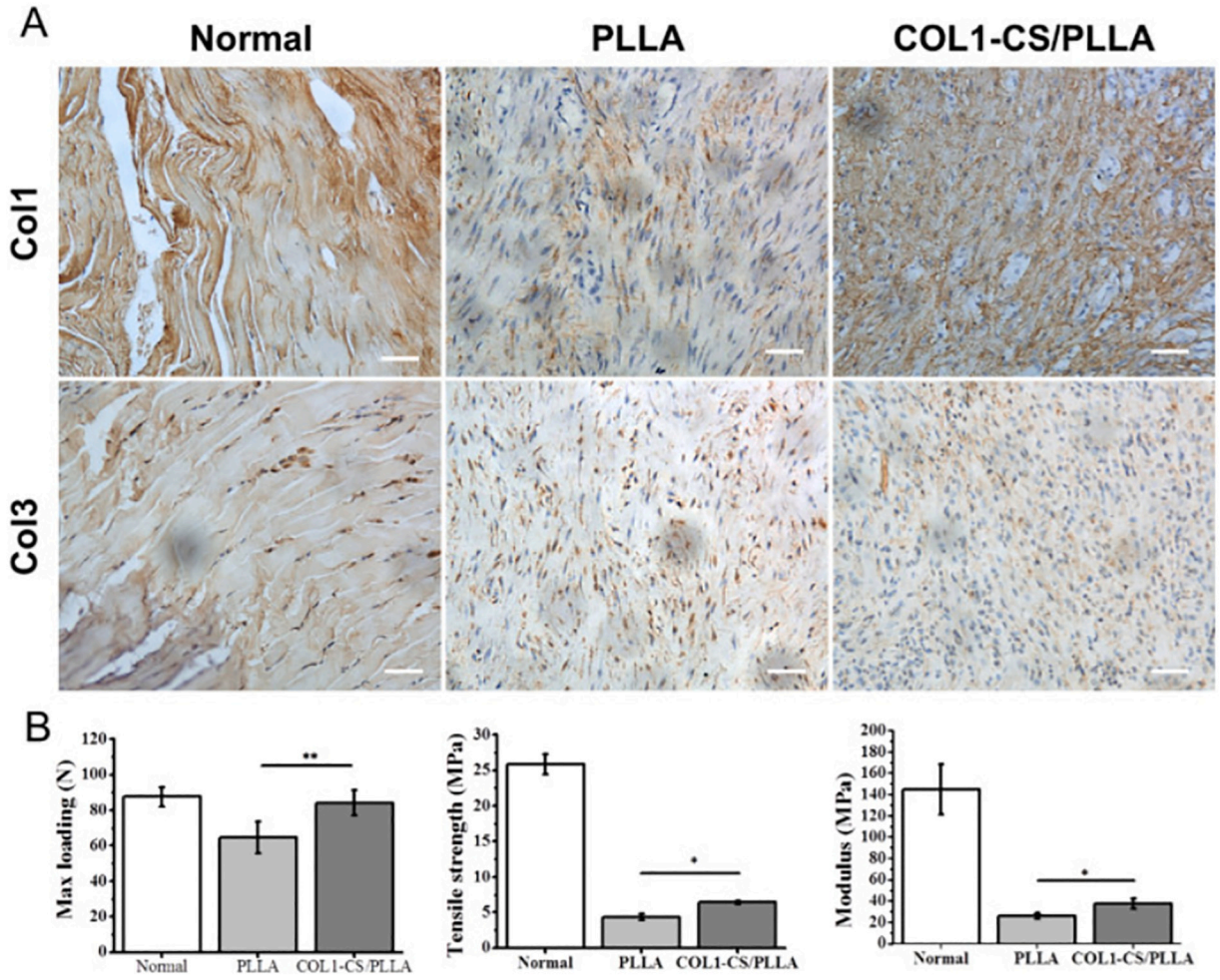


Fig. 22. (A) Immunohistochemical staining of special proteins (Col1, Col3) expressed in normal control, PLLA and COL-CS/PLLA after 8 weeks post-surgery. Scale bars = 50 μm. (B) Mechanical properties of repaired tendons after regenerated 8 weeks. *$p < 0.05$, **$p < 0.01$, $n \geq 3$, "normal" refers to native rat Achilles tendon. Reproduced with permission [340]. Acronyms. PLLA: Poly-L-lactic acid, COLI: collagen I and CS: chitosan

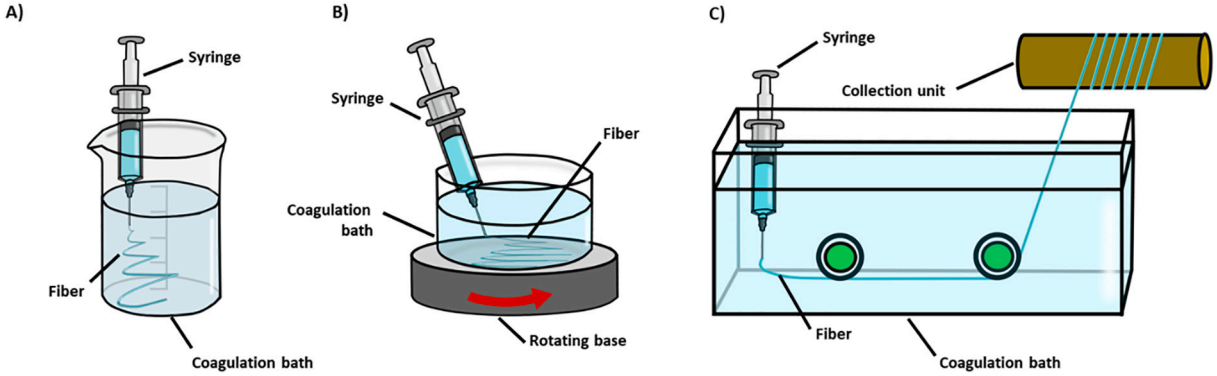


Fig. 23. Scheme of wet-spinning technique. Different approximations can be used: vertical wet-spinning setup using a non-mobile coagulation bath (a), using a rotating coagulation bath (b) and using a rotating collector (c).

characteristics, added to the use of mild conditions, makes this technique suitable for cell incorporation, as cell adhesion and proliferation are facilitated [341]. Furthermore, once produced, the fibers are easily handle and assemble. In addition to all this, wet-spinning has low cost and high yield [342].

As was the case for electrospinning, the main advantage of this technology for tendon tissue engineering is obtaining fibrillar structures. By adjusting the aforementioned parameters, it is possible to achieve the orientation of the fibers in the direction in which the scaffold will mainly suffer the stress. This orientation is much more difficult to achieve with other technologies. The materials used with this technology also allow achieving a very high resistance, similar to that of the original tendon.

The main drawback of this technique is that it only has the possibility of producing micron-sized fibers what limits its application in some cases [343].

A first approach to the application of wet-spinning to tendon

regeneration was made by Tadanao et. al (2005). This group carried out a study to apply a CS-based hyaluronan hybrid fiber scaffold synthesized by wet-spinning to the regeneration of a rotator cuff lesion. To synthesize the scaffold a CS dope was spunt through a stainless steel spinneret into three different coagulation baths. The first was a calcium coagulant solution, the second was a 50% aqueous methanol solution and the third was a 0.1% HA dissolved in 50% aqueous methanol solution (Fig. 24.A. I). The obtained fibers where subsequently twisted together forming braids. The scaffold was created from 13 braids (Fig. 24.A.II). After fabrication, the scaffold was seeded with fibroblasts. Compared to the control, the scaffolds (seeded and not seeded) showed higher tensile strength and tangent modulus. Nevertheless, the values were much lower, especially those of tangent modulus, to those of intact infraspinatus tendons. The cross-sectional area of the regenerated tendon was also higher than the cross-sectional of the control group. Positive staining for type I collagen was detected on the periphery of the implanted scaffold of the cell-seeded group but not on the non-cell seeded group [347].

Although the results obtained were not bad, this technique was no longer used for tendon regeneration for a long time. One possible explanation was the appearance of other scaffold production techniques that caught the attention of the scientific community. However, different groups have again made their approaches for tendon regeneration based on wet-spinning.

Calejo et al. (2019) looked for the development of gradient scaffolds to engineer tendon-to-bone interface using aligned microfibers. For this purpose, they used wet-spinning technology (Fig. 24.B.I). Two formulations were considered: PCL/gelatin, to mimic tendon tissues, and PCL/gelatin incorporating nano-to-microsized hydroxyapatite (HAp) particles, to mimic bone tissues (Fig. 24.B.II). To assess the effect of the flow rate on wet-spun fibers diameter and mechanical properties, different flow rates were tested. The results were very interesting since they allowed concluding that there is a direct relationship between the flow rates used and the properties of the obtained fibers. In addition, the difference in composition between the two formulations was reflected in the properties of the obtained fibers. It was seen how a higher speed implied a larger fiber diameter and vice versa. The incorporation of HAp also involved increasing the diameter of the fibers. The incorporation of different sizes of HAp was responsible for the presence of pores of different sizes in the PCL/gelatin/HAp fibers. The microfibers produced of PCL/geltain and PCL/ geltain/ HAp showed *in vitro* cell proliferation and adhesion. In addition, new matrix synthesis by seeded cells was observed. In the case of PCL/ gelatin microfibers, hASCs were highly aligned. In contrast, in the case of microfibers of these materials with HAp incorporated, cells presented a more random cytoskeleton orientation. Collagen type III synthesis by hASCs could be notice in the two types of scaffold [342].

All these *in vitro* results suggest that after adjusting the parameters of the wet-spinning process, fibers to manufacture scaffolds with good properties for tendon regeneration can be obtained.

Lu et al. (2020) also carried out a study of the effects that the modification of different parameters have on the fibers synthesized by wet-spinning. Specifically, they analysed the effect of the extrusion speed rate, collection speed rate and the effect of a subsequent cross-linking of the produced filaments. Briefly, the formulation was composed of a mixture of a PEG 4000 solution and an collagen I solution. The mixture was extruded using different speed rates into a coagulation bath. A rotary roller was used to collect the fibers at different speeds (Fig. 24. C.I). After the collection of the collagen filaments, they were incubated in coagulation bath for their stabilization (Fig. 24.C.II). Two

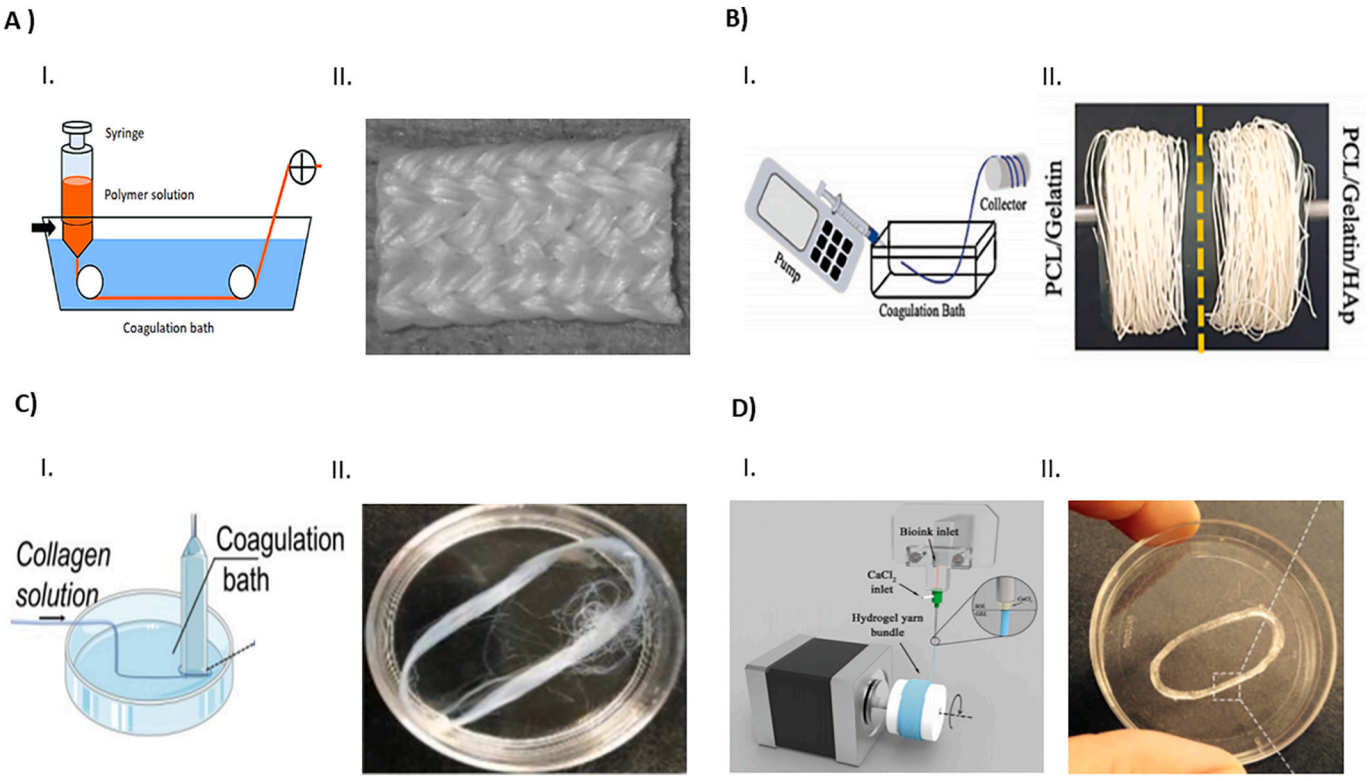


Fig. 24. Different approximations of wet-spinning technique. A) I. Vertical wet-spinning using three baths (only on represented) and a rotating collector. II. Scaffold (10 mm in length, 7 mm in width, and 0.7 mm in thickness) was created from 13 braids. Adapted from [347] B) I. Schematic representation of a continuous wet-spinning setup. II. PCL/gelatin and PCL/gelatin/HAp wet-spun fibers. Adapted from [342] C) I. Wet-spinning scheme of collagen filaments. II. Gross appearance of coiled and stretched collagen filaments. Adapted from [344] D) I. Schematic of a microfluidic chip combined with a coaxial needle extrusion system. II. Hydrogel fibers produced from GelMa, alginate and calcium chloride. Adapted from [341]. Acronyms. PCL: polycaprolactone; HAp: hydroxyapatite; GelMa: gelatin Methacryloyl.

different fiber groups were created. One group was crosslinked by 1-ethyl-3-(3- dimethyl aminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) and the other one was not. From the obtained results, different conclusions could be made. First, this method was adequate to obtain aligned collagen fibrils. Second, the swelling and degradation results indicated that the stability was improved by the EDC/NHS crosslinking method. Third, the mechanical strength could be increased by reducing the filaments diameter. Fourth, the EDC/NHS crosslinking helped to increase the mechanical strength of collagen filaments. In addition, using this method cells could adhered on the aligned collagen filaments *in vitro* showing a highly oriented cell distribution and elongated cell morphology [344]. All this conclusions can serve as a basis for future research using this same method.

However, all the aforementioned approaches are still basic studies. It is true that these studies are essential to establish the bases of the technique. Nevertheless, it is necessary to continue carrying out more complex studies, that allow testing the scaffolds obtained by wet-spinning for tendon regeneration *in vivo*.

An example of a more complex study is the one carried out by Rinoldi et al. (2019). In this work, they produced aligned cell-laden hydrogel yarns using a new system of wet-spinning. This new system was based on a co-axial extruder and a stepper motor (Fig. 24.D.I). GelMA and alginate were used as materials to produce the scaffolds (Fig. 24.D.II). Another novelty of this study was that the ink, in addition to these materials, also contained hBM-MSCs.

Co-axial extrusion allowed the first crosslinking of the pregel, formed by GelMa and alginate, to occur with calcium chloride during the spinning process. Once the crosslinking had occurred, the hydrogel fibers were collected using a rotating drum. In this way, the structures obtained were aligned. The second crosslinking was achieved by exposing the fibers to UV light.

Another very interesting aspect of this study consisted on the application of two different stimuli: mechanical stimulation (with the application of serial compressions) and biochemical stimulation (using BMP-12).

An aspect to highlight is that good viability results were obtained, demonstrating that wet-spinning is an adequate method for obtaining engineering scaffolds.

In the case of scaffolds that had been treated with BMP-12, greater cell differentiation was observed to the detriment of cell proliferation. On the contrary, in the case of the scaffolds to which mechanical stimulation was applied, it was possible to observe how the cellular proliferation was increased. Morphologically, the application of force caused the cells to be oriented longitudinally in the direction of the stretch. There was also an increase in the production of type I and III collagen over time. Another very interesting finding consisted on the significant positive regulation of SCX induced by mechanical stretching, further enhanced by the combination with biochemical stimuli. These results confirm the synergistic effect of the two types of stimuli [341].

Taking into account all the aspects discussed so far, it is expected that this technique will be used and studied in more depth for tendon tissue engineering in the coming years. However, its most widespread application is conditioned to a subsequent modification of the fibers. This is, as it has been mentioned for electrospinning, the fibers or meshes obtained with wet-spinning can be used for minor tendon injuries or for the delivery of cells and growth factors. For the regeneration of complete tendon injuries, it is necessary to develop scaffolds of greater volume that provide biomechanical resistance and structural support. One of the possible approaches is the braiding of the obtained fibers or the combination of these fibers with other techniques and materials.

3.6. Combination of techniques to obtain scaffolds with better structural and biomechanical properties

So far, few groups have studied the approach of using more than one technology to manufacture scaffolds. As discussed throughout the review, scaffolds for tendon regeneration have to fulfil, in general, two main functions. On the one hand, they should achieve a more structural and mechanical function. On the other hand, they also should reach a more biological and biochemical function. The combination of techniques for scaffold manufacture follows this idea. One of the techniques would allow obtaining a part of the scaffold with more structural functions, and the other technology would allow obtaining another part of the scaffold with more biological functions. This strategy achieves a greater complexity that is reflected in the scaffold and that makes it more similar to the natural tendon.

One example is the combination of electrospinning and electrospraying. Both are techniques based on the same principle, but in the case of electrospinning micro/nano fibers are obtained and in the case of electrospraying particles/films are obtained [345,348]. Electrospinning/electrospraying are extensively used techniques in regenerative medicine and tissue engineering. This combination has also been used in tendon regeneration. One example is the study made with the objective of synthetizing some patches that could help the healing of the Rotator cuff augmentation (RCA) tear process. To develop these patches, they merged two different techniques: electrospraying and electrospinning (Fig. 25). Electrospraying was used to produce PLC films in an organic gel, and electrospinning to produce PLA nanofibers. The layers of the two materials were combined to form a structure with good mechanical properties. This strategy was followed to achieve the desired mechanical properties and influence cell behaviour by the effect of PLA degradation products. Adult Normal Human Dermal fibroblasts (NHDF) were used for cell viability, cytotoxicity, protein quantification, and cell morphology assays. The modification of the surface of the patches was also studied by adding two types of PLA nanofibers also obtained by electrospinning (aligned and not aligned). Young Moduli of fibers produced using different time conditions was determined. Results suggested that PLA fibers layers might only affect the mechanical properties of the structure. Meanwhile, PLC film layers might affect the thickness and the inner porosity of the structure. Thus, the different combination of these two elements allow adjusting the mechanical properties and the geometry of the patches depending on the needs. Biological studies were performed *in vitro*. The designed structures turned out not to be cytotoxic. The superficial modification did not affect cell proliferation or protein synthesis, but it did affect cell morphology. Cells seeded on aligned superficial scaffolds presented aligned morphology, while cells seeded on non-aligned scaffolds presented round shape morphology [349].

Another example of combination of technologies is the assay carried out by Touré et al. (2020). In this study, they fused 3D printing technology with electrospinning. First, they created a scaffold using 3D extrusion printing. Scaffolds were shaped like square grids and had dimensions of 4x4 cm^2 (Fig. 26). They prepared three different types of inks using combinations of PCL, poly (glycerol sebacate) (PGS) and bioactive glasses. Once dried, this structure was used as a collector for electrospinning. The material used for this technique was a mixture of PCL and PGS dissolved in dichloromethane and methanol. Tensile strength and Young modulus were higher when both techniques were used instead of the scaffold obtained by 3D printing alone. Biocompatibility was studied using 3T3 cells (fibroblasts) *in vitro*. Results were good as in all cases were greater than 80% after seven days of cultivation with the scaffold. Bioactive glasses helped in that regard [350].

The results obtained in the discussed studies suggest that the combination of techniques to obtain more complex scaffolds are suitable for applying to tendon regeneration. In the near future, it is expected that more approaches of this type will be carried out.

4. Summary and future perspectives

Tendons are a tissue with very special properties and characteristics that are closely related to their function in the body. Among these characteristics stand out its elasticity and viscosity, its low cellularity, its

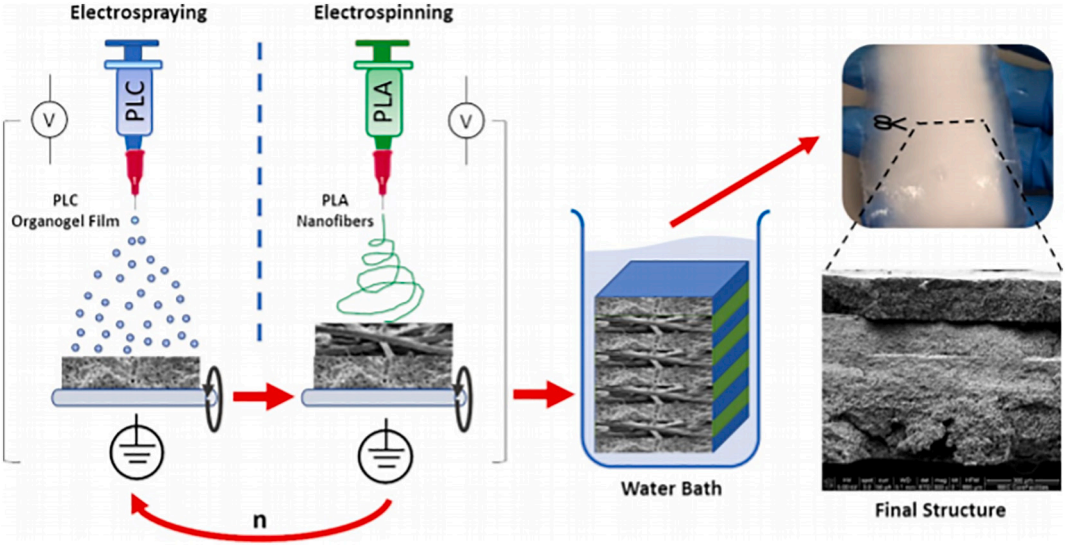


Fig. 25. Schematic workflow representation of the device fabrication. Electrospinning and electrospraying layers are alternated until the desired height is obtained. Reproduced with permission of [349].

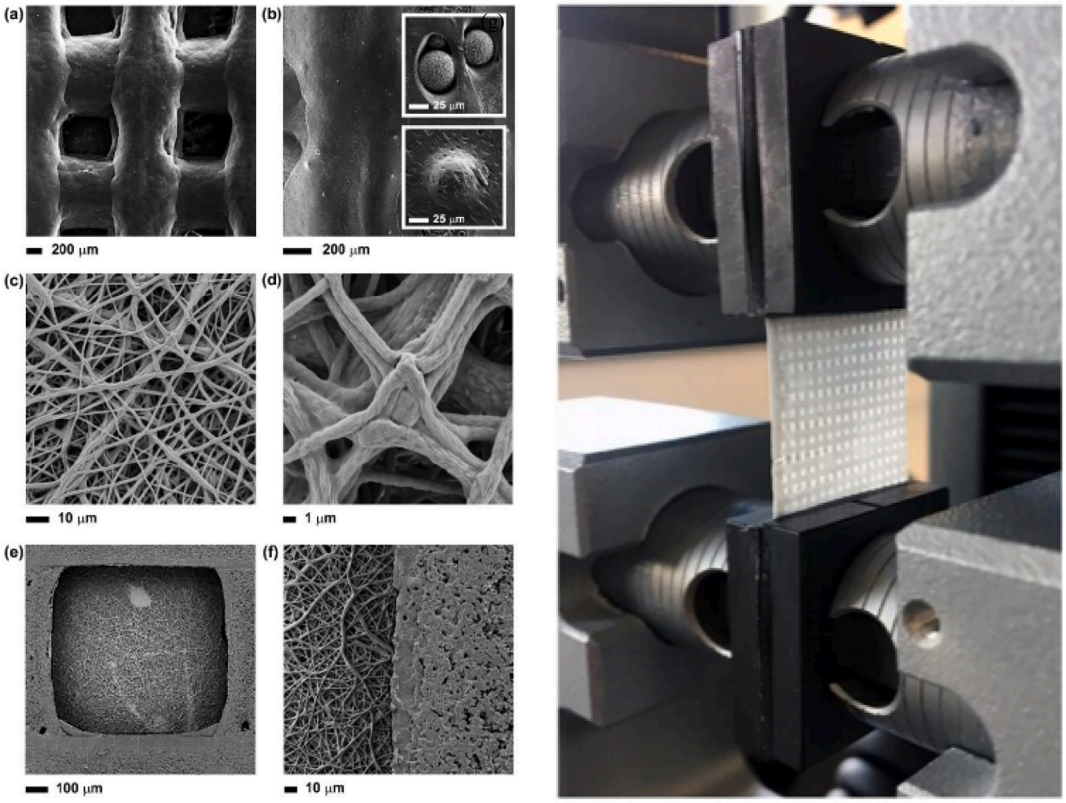


Fig. 26. SEM images at diferent magnifications of: (a) and (b) the 3DP, showing also the microspheres, (c) and (d) the surface of the composite scaffold covered with a layer of electrospun PCL-PGS mats; (e) and (f) the surface of the composite scaffold without the layer of electrospun fibres. (g) The photograph of one composite scaffold. Adapted from [350]. Acronyms. 3DP: 3D-printed scaffolds; PCL: polycaprolactone; PGS: poly (glycerol sebacate).

high degree of resilience and its low vascularity. The great efforts that these tissues are subjected to (among other factors) annually provoke millions of tendentious injuries worldwide. This is a major problem for healthcare systems around the world and for patients themselves who suffer from an injury that significantly affects their quality of life. One of the big problems with this type of injury is that its recovery is very complicated. Much of this is due to the aforementioned tendon characteristics. Besides being a complicated process, the natural regeneration of the tendons is slow and often results in non-functional or partially functional tissue.

Many treatments have been proposed to regenerate the injured tendons. Among them, conservative treatments, surgical treatments, treatments with allografts or xenografts or treatments based on cell or growth factor infiltration, nonsteroidal anti-inflammatory drugs or gene therapy stand out. Among them, the treatment that is more widely being used today in severe cases of rupture is the combination of a surgical

operation with early mobility exercises. In a relatively high percentage of injuries, it is not possible to recover the functionality or the structure that the tendon had before the injury. In order to improve tendon regeneration and to recover its functionality, the application of tissue engineering to this type of tissue has been proposed.

Tissue engineering allows performing a more complex approach to tendon regeneration. This discipline combines materials engineering, molecular biology and cell biology to obtain structures that closely resemble the original tissues. In the case of tendon tissue engineering, multiple materials can be used to obtain the designed structures or scaffolds. These materials can be classified into biological or synthetic. Each of them have their advantages and disadvantages. The main advantage of biological materials is their biocompatibility and bioactivity and their main disadvantage is their poor mechanical properties. In contrast, synthetic materials generally have good mechanical properties but they lack of good biological properties. This is why in recent years composite materials have become increasingly important as they combine the advantages of the biological and synthetic materials. Thus, many groups are using composite materials as they have good biocompatibility and bioactivity, and improved mechanical properties.

Another element used in the tissue engineering are cells. There are two different approaches for tendon regeneration: the use of differentiated or undifferentiated cells. Among the differentiated ones are tenocytes and tendon fibroblasts. These cells are used because the majority of the cells found in the tendon are of this kind and, therefore, scaffolds much more similar to those of the original tissue are achieved. Their main disadvantages are that they are difficult to obtain, difficult to expand and maintain in cultivation and that they have low activity rate. In the case of the differentiated cells, there are more kinds to choose from ESC and iPSC are hardly used due to the associated ethical problems and tenogenic risks. On the other hand, BMSC and ADSC are the most used ones, because they are easy to obtain, metabolically very active and easily differentiable to tendon lineages. As was the case for materials, different groups have established that the best approach is the combination of different cell lines.

During the natural regeneration of the tendon, many molecules with biological activity that fulfil different functions are secreted into the ECM. These molecules can also be included in the scaffolds to treat tendon injuries. Among them, growth factors have been the most used and studied. Its functions are diverse and include increasing cell proliferation, enhancing the synthesis of different components of the ECM, promoting angiogenesis or chemotaxis. The moment in which they are secreted and the role they exert in the regeneration of the tendon is increasingly understood. To date, many studies have incorporated some growth factors into scaffolds, used, in most cases, to achieve greater synthesis of type I collagen or to increase cell differentiation to tenocytes. However, it should be noted that more and more groups are proposing the approach of using more than one growth factor simultaneously. Controlling and adjusting the release of these growth factors at an appropriate time, as well as incorporating all the growth factors necessary to achieve a full recovery of the damaged tendon still seems difficult.

The technique used to obtain the scaffolds is also decisive for the characteristics of the obtained structure. The selected material is closely linked to the type of technology used. Nowadays, new techniques such as 3D printing, electrospinning or electrospraying have emerged. They allow obtaining much more complex scaffolds; more adequately control of the final structure and automate the structure development process. This allows achieving more reproducible scaffolds with properties more similar to those of the original tissue. To all this, we must add that there are different studies that combine these technologies to achieve even more complex structures.

Analysing the materials, cells, growth factors and manufacturing processes that are used in tissue engineering applied to tendon regeneration, it can be seen how those approaches that involve the combination of elements turn out to have more promising results. The most complex structures are those that best resemble the original tissue and that produce the best regeneration rates. In addition, the knowledge that is being generated in the field suggests that, in the future, the adaptation of the treatments and structures used to each type of patient and injury will be much easier.

Although great advances have been made, the approximations proposed and the results obtained so far have made it possible to obtain scaffolds with properties that are still too different from those of native tendons. The structures that have demonstrated the best results so far are mainly hydrogels or scaffolds with a structural role. The former help regeneration in cases of mild tendon injuries and their function is related to the effect that the cells and growth factors they contain have on the damaged area. The latter are used in total injuries and their function, in most cases, is limited to connect the ends of the tendon that has suffered the injury. Although either of these two approaches could be close to its application to the clinic, it is expected that in the coming years a breakthrough will be achieved in the development of scaffolds for tendon regeneration. This advance must be aimed at obtaining more complex structures, structures that replicate the biomechanical properties of the tendon. The key factors, therefore, to solve the current paradigms go through the analysis and obtaining more complex materials (with structural modifications or with suitable combinations between materials) and the development of more complex approaches with the current production systems. Using these production systems (either individually or in combination), it is intended to replicate the fibrillar and hierarchical structure of the tendons. Actually, ways of using production systems, such as 3D bioprinting, are already being studied to obtain fibers oriented in the same direction (the direction in which the effort will be made). Achieving this orientation of the fibers represents a great advance towards the improvement of the mechanical properties of the scaffolds. Undoubtedly, the reproducibility of the structures obtained and their serial production represent a key point for the approach of these techniques to the clinic. In this sense, current production systems represent a great advance since they are highly automated systems.

Taking into account the interest that is arousing in research groups around the world, everything mentioned so far suggests that tissue engineering applied to tendons will continue to evolve in the coming years towards approaches and structures more complex that finally manage to effectively and completely regenerate damaged tendons.

Declaration of Competing Interest

The authors declare no conflict of interest.

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