Chapter

Advances in Tendon and Ligament Tissue Engineering: Materials Perspective

Feras Alshomer[1,2]*, Camilo Chaves[1,3] and Deepak M Kalaskar[1,4]

[1]UCL Centre for Nanotechnology and Regenerative Medicine, Division of Surgery & Interventional Science, University College London, UK
[2]King Khalid University Hospital Surgery Department 37, Plastic and Reconstructive Surgery Section, KSA
[3]Université Paris Sud, 63 Rue Gabriel Péri, France
[4]Institute of Orthopaedics and Musculoskeletal Science, Division of Surgery and Interventional Science, University College London, Royal National Orthopaedic Hospital, UK

***Corresponding Author:** Feras Alshomer, UCL Centre for Nanotechnology and Regenerative Medicine, Division of Surgery & Interventional Science, University College London, London NW3 2PF, UK

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Abstract

Introduction: Tendons are specialised, heterogeneous connective tissues, which represent a significant healthcare challenge after injury. Primary surgical repair is the gold standard modality of care; however, it is highly dependent on the extent of injuries. Tissue engineering represents an alternative solution for good tissue integration and regeneration. In this review, we look at the advanced biomaterial composites employed to improve cellular growth while providing appropriate mechanical properties for tendon and ligament repair.

Methodology: Comprehensive literature searches focused on advanced composite biomaterials for tendon and ligament tissue engineering. Studies were categorised depending on the application.

Results: In the literature, a range of natural and/or synthetic materials have been combined to produce composite scaffolds tendon and ligament tissue engineering. In vitro and in vivo assessment demonstrate promising cellular integration with sufficient mechanical strength. The biological properties were improved with the addition of growth factors within the composite materials. Most in vivo studies were completed in small-scale animal models.

Conclusions: Advanced composite materials represent a promising solution to the challenges associated with tendon and ligament tissue engineering. Nevertheless, these approaches still demonstrate limitations, including the necessity of larger-scale animal models to ease future clinical translation and comprehensive assessment of tissue response after implantation.

Introduction

Traumatic tendon and ligamentous injuries represent significant healthcare and economic challenges for the future. Notably, these injuries are estimated to affect 110 million people in the United States [1], and incomplete repair is associated with variable disabilities and chronic sequelae [2,3].

Tendon represents a specialised connective tissue in which collagen type I accounts for ~80% of the net dry weight. In combination with proteoglycans and elastin, collagen permits high mechanical strength in tendons [4,5]. Furthermore, tendons display a unique structural hierarchy where collagen molecules produce collagen fibrils, which group together to form collagen fibres. The multicomposite tendon units are composed of several collagen fibres, known as tropocollagen (Figure 1) [3,4,6].

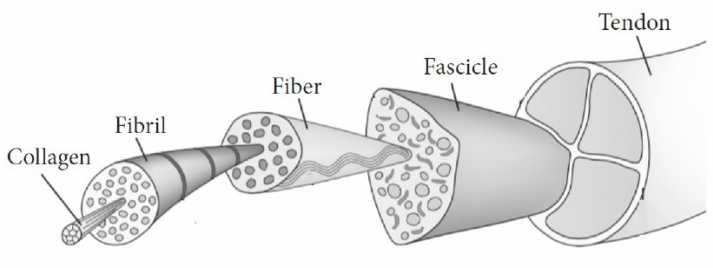

Figure 1: Complex structural hierarchy of tendon. Unique structural hierarchy in which collagen molecules represent the simplest forming structure of tendon with complex arrangement up to tendon fascicles producing the final tendon tissue.

Ligaments are another form of viscoelastic connective tissue, with a highly organised composition where collagens (types I, III, and V) constitute the bulk. Proteoglycans and chondroitin sulfate are also expressed, allowing the ligament tissue to swell in aqueous environments [7]. In the body, the attachment of tendons and ligaments to bone involves a transition zone with unmineralised and mineralised fibrocartilage [3,8]. Tendons can further attach to muscles through fascia [4]. Defining the structural organisation of tendons and ligaments has improved understanding of the way these heterogeneous tissues function in synergy [9].

Surgical repair of tendons and ligaments via primary suture or autologous transfer techniques are considered the gold standard modality of care. However, these solutions are often associated with challenges including reduced mechanical strength due to scar tissue formation, infections, donor site morbidity, and limited availability of autografts [10]. Furthermore, specialised physiotherapy protocols are required to improve the repair-site properties and limit scar tissue formation [11].

To overcome this clinical issue, additional therapeutic options involving the use of synthetic prosthesis or tissue engineered biomaterial constructs have been investigated in the literature [12]. Ranges of synthetic prosthetic devices have been described as tendon and ligament tissue substitutes. Nonabsorbable and biocompatible polyester polyethylene terephthalate (PET) was investigated as a potential tendon and ligament tissue prosthesis material with suitable mechanical and tissue integrative properties [13,14]. Other materials like polytetrafluoroethylene (PTFE) were also investigated as ligament tissue substitutes or in tendon augmentation grafts, for their biologically inert and strong mechanical characteristics [15,16]. However, limitations such as graft failure, poor durability, poor tissue integration, and foreign body synovitis were observed with these materials [17]. Tissue engineering represents an alternative option with potential for proper tissue integration of implants.

Different synthetic or naturally derived materials have been investigated as scaffolds, which permit cell integration and subsequent matrix deposition. Among the naturally derived materials, collagen and chitosan have been extensively investigated for scaffold development due to their optimum biocompatibility and tissue integration potential [18]. Nevertheless, these materials exhibit limitations such as low mechanical strength, batch variability, and difficult processing with latent immunogenicity [19].

Synthetic materials, including polylactic acid (PLA), polyglycolic acid (PGA), and polylactic-co-glycolic acid (PLGA) have been extensively investigated for tendon and ligament repair [20]. Synthetics exhibit several advantages linked to large-scale manufacturing, limited disease transmission, controlled degradation, and better host tissue integration. There are also limitations associated with synthetic biomaterials; cell integration is challenging without further material treatment, degradation-related products can be cytotoxic, and the materials are mechanically weaker than healthy musculoskeletal tissues [18,21].

Novel tissue engineering approaches using composite materials have also been described to mimic the complex, nonhomogenous environments in tendons and ligaments. In these composite materials, specialised cells have been combined with biomaterials to produce complex, heterogeneous scaffolds with controlled mechanical properties [1, 9]. This review will present an overview of the different approaches described to address the use of composite scaffolds for tendon and ligament tissue engineering. An in-depth critique of the mechanical properties, cell/ tissue integration, success criteria, and limitations is discussed in detail.

Materials and Methodology

Comprehensive literature searches were conducted on the PubMed, Medline, Web of Science, and Google Scholar databases. Various combinations of keywords were used in the search process, including “tendon”, “ligament”, “tissue”, “engineering”, “hybrid”, “composite”,

"scaffold", "material", "biomaterial", "graft", and "polymer". Only publications in English were included and there were no restrictions on year of publication since no previous reviews were found covering the use of composite materials in tendon and ligament tissue engineering simultaneously. All relevant manuscripts were accessed and articles that combined tissue engineering approaches for both tendons and ligaments were included. Articles that discussed different tissue engineering approaches of the tendon/ligament to bone interface were also excluded due to the broadness of the topic and the important number of review articles on the subject [8,22–24].

Results and Discussion

Composite Scaffolds for Tendon and Ligament Tissue Engineering

Tendon injuries present significant healthcare challenges due to slow healing rates, loss of function, and scar tissue formation around trauma sites [12]. In severe cases, tissue engineered scaffolds have been fabricated to replace the lost tissues. These artificial grafts can be composed of natural or synthetic biomaterials [49]. The ideal scaffold in tendon tissue engineering should exhibit specific properties such as controlled degradation rates, appropriate mechanical properties, nonimmunogenicity, and suturability. Additionally, easy mass processing and fabrication, while mimicking the native tissue environment, are desirable [12].

Synthetic Composite Materials

One example of materials used for tendon tissue engineering involved the fabrication of composite, heterogeneous scaffolds from polyglycolic acid (PGA), and polylactic acid (PLA) [44]. The outer surface was composed of a knitted scaffold made from a 4:2 mixture of PGA and PLA fibres. Internally, the construct contained longitudinally arranged, unwoven PGA fibres. The whole construct was folded and secured with sutures at each end to produce a cord structure and was tested both in vitro and in vivo. Results showed that the scaffold

seeded with adipose-derived stem cells (ADSCs) promoted matrix deposition and the formation of mature collagen fibrils. These scaffolds presented subphysiological mechanical properties. Although the study showed promising results, assessment of tenogenic differentiation of ADSCs was not elaborated.

Another manufacturing technique was proposed by Baker B. M. et al., who investigated the use of coelectrospinning technique of different polymers to yield a composite scaffold with variable degradation techniques [28]. They evaluated the use of PCL fibres as slow absorbing elements, while PLGA (50:50 polylactic/ glycolic acid) or PCL/ PLGA fibres were applied for intermediate degradation rates in a single scaffold. A water-soluble polyethylene oxide (PEO) was as a sacrificial fibre element with fast absorption rates and aimed to increase the scaffold porosity with minimal influence on scaffold integrity. Mechanical assessment of PCL/ PLGA/ PEO constructs showed a maximum stress yield of nearly 3.5 MPa at 0.08% strain. The modulus was around 100 MPa and the yield strain was 0.026. PCL/ PLGA-PCL/ PEO scaffolds showed a maximum stress yield of 2 MPa at 0.12% strain. Initial modulus of the material was 25 MPa and dropped to 12.5 MPa after 63-day incubation in culture media. Strain increased from 0.065% to 0.12% after incubation. A 22% mass decrease of the composite scaffold after hydration was caused by the dissolution of the PEO component. However, the mechanical properties change after hydration and dissolution of PEO are worth further explanation.

It has been suggested that woven scaffolds are superior for tissue integration due to their interconnected porous structures. Nevertheless, they require challenging cell seeding techniques and complex cell delivery systems [4,5]. Sahoo S. et al. investigated the use of woven scaffolds made from PLGA or PLLA [29]. Both f scaffolds were coated with PCL, PLGA nanofiber, or collagen type I to yield composite scaffolds and were seeded with porcine bone marrow-derived MSCs. Some collagen-coated scaffolds were seeded with human dermal fi-

broblasts to test cell seeding and integration efficiency. Results showed that PLLA-based woven scaffolds performed inferiorly in terms of cell attachment. This was linked to the hydrophobic nature of the PLLA material. Furthermore, PCL coating of both types of knitted scaffolds was associated with higher mechanical strength but reduced cell attachment. This was also linked to greater hydrophobicity in PCL than in the other polymers.

As mechanical strength is particularly important, an approach using PLA with graphene nanoplatelets (GNP) and PLA with carboxyl functionalized carbon nanotubes (CNT-COOH) has been proposed by Pinto et al. [25, 26]. In vitro assessment of the composite was nontoxic to human fibroblasts. In vivo assessment using mouse model did not show any toxicity or local or systemic inflammatory response. The addition of nanofillers mentioned enhanced the mechanical properties of PLA polymer films reaching Young's modulus of 4.86±0.47 GPa for CNT-COOH scaffolds and 4.92±0.15 GPa for GNP scaffold groups. The tensile strength however was mostly enhanced in the PLA/ CNT-COOH scaffolds reaching up to 72.22±1.52 MPa. The authors did not investigate, however, the effect of in vivo implantation on the associated mechanical properties mimicking real clinical settings.

Biological Composite Scaffolds

Collagen type I composite scaffold was also investigated to incorporate resilin-like protein. Resilin is an arthropods protein with elastic and highly stretchable structure. Sanami, M., et al. [27] investigated the fabrication of such composite. The scaffold was made through extrusion process of composite solution containing Collagen and Resilin at different concentration into polyethylene glycol buffer. Fibres were subsequently cross-linked using 4-arm poly(ethylene glycol) ether tetrasuccinimidyl glutarate solution. Mechanical assessment showed that resilin in non-cross-linked collagen scaffold sig-

nificantly reduced stress at break and Young's modulus values, while significantly increasing break strain. Cross-linked collagen/resilin scaffold showed significant increase in stress and strain values and a significantly decreased Young's modulus values. This shows an interesting effect of resilin exhibiting its natural properties. In vitro, the scaffold produced supported 100% fibroblast proliferation and alignment compared to 80% in collagen-fibre control.

Scaffolds made from collagen and glycosaminoglycan (GAG) were recognised for their role in supporting cellular proliferation and differentiation. However, this type of scaffold lacks the mechanical characteristics required for tendon tissue engineering applications. Caliari et al., 2011, have proposed the concept of developing composite materials in a core-shell fashion with the necessary mechanical properties [30]. The group fabricated scaffolds composed of highly porous, aligned isotropic GAG cores surrounded by strong, high density isotropic GAG membranes. The core-shell design was proposed to increase the mechanical strength of the scaffolds. The material was fabricated using an evaporation technique to create membranes and freeze-drying to incorporate the core within the membrane shell. Dehydrothermal (DTH) cross-linking was used to increase material integration, as well as the mechanical properties. In vitro assessment using horse-derived tenocytes demonstrated good cell attachment, proliferation, and cell viability up to 14 days after seeding. Scaffolds exhibited high porosity and appropriate mechanical properties depending on the membrane thickness. Several factors however that need further investigation like cellular functional assessment (e.g., protein expression), nature and content of collagen after cell seeding, and the mechanical properties of the scaffold at different states (e.g., wet versus dry).

Chitosan is a naturally derived polysaccharide with excellent potential for tissue engineering applications. Due to the biocompatible and cell adhesive properties of chitosan, the polysaccharide has been investigated for tendon tissue regeneration [9–11]. In one example,

composite scaffolds made from chitosan and alginates were produced through a spinning/coagulation technique to yield an alginate-0.1% chitosan scaffold. Alginate is an anionic polysaccharide with calcium chain in which the integration of chitosan improves its biocompatibility and cell adhesive potential and decreases its degradation rate. In vitro assessment using rabbit patellar tendon fibroblasts showed that alginate-0.1% chitosan scaffolds had significantly higher cell adhesion and matrix deposition compared to alginate-only and polyglactin 910 controls. The alginate-0.1% chitosan material was evaluated mechanically and exhibited lower tensile strength and strain at failure than the polyglactin group [33]. Chitosan was also fabricated with hyaluronic acid followed by wet spinning and hybridisation to increase the mechanical properties of the construct. Different hyaluronic acid concentrations were investigated and showed that the combination of chitosan with 0.1% hyaluronic acid showed the best cell attachment. The final composite constructs were sterilised using ethylene oxide gas prior to in vitro evaluation with rabbit patellar fibroblasts. Mechanical properties of the materials were reduced within the first 2 hours after seeding but the consequent modulus was maintained for 28 days of culture. Cell proliferation quantification using DNA content analysis showed significant improvement in chitosan-0.1% hyaluronic acid composites compared to other chitosan-based scaffolds. To determine the clinical potential of the composite scaffolds, authors assessed the chitosan-0.1% hyaluronic acid composites in vivo by treating rabbit rotator cuff injuries with cell-seeded scaffolds [31]. The scaffolds were cultured with rabbit patellar tendon fibroblasts for 4 weeks prior to implantation. Additionally, authors tested the potential for ligament tissue engineering using a rabbit medial collateral ligament injury model. They used scaffolds seeded with fibroblasts adapted from rabbits Achilles tendon for 2 weeks prior to implantation. For the tendon model, results indicated collagen deposition in cell-seeded scaffolds with significant improvement in the mechanical properties from 4 to 12 weeks after implantation. For the ligament-engineering model, authors showed a lack of tissue integration with the bony tunnel attachment with 60% recovery in failure load compared to healthy liga-

ment. Additional research on the chitosan-hyaluronic acid scaffolds has aimed to understand the effects of mechanical stimulation on fibroblasts response [35]. It was found that the application of 90-degree rotations and 5% stretch at 0.5 Hz was associated with increased expression of fibromodulin, and collagens I and III. No further assessment of the overall mechanical influence of the cultured scaffolds under dynamic conditions was made.

Additionally, composite scaffolds made from extruded, cross-linked bovine type I collagen and chondroitin-6-sulfate were fabricated [36]. The collagen-based constructs underwent a series of cross-linking steps using carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and N-hydroxysuccinimide (NHS). Cross-linking was combined with freeze-drying to incorporate a porous chondroitin-6-sulfate. The final constructs had open and interconnected porosity with an average pore size of 100 μm and axially aligned collagen fibres. Mechanical assessment showed that the cross-linked composite scaffolds could withstand 61.94±15.54 N, compared to 11.75±2.62 N without cross-linking. The maximum strain was shown to be 30.17±7.17% with cross-linked fibres, while it was 15.40±3.22% without. Composites with cross-linked fibres presented a tensile strength of 1.55±0.30 MPa compared to 0.24±0.08 MPa for non-cross-linked fibres. It is important to mention that scaffold porosity is inversely related to mechanical strength and further studies are required to evaluate the cell response of such construct for application in tendon and ligament tissue engineering.

Gelatin was also utilised to fabricate a composite scaffold for tendon tissue engineering applications. Coelectrospinning of aligned poly-ε-caprolactone (PCL) and methacrylated gelatin (mGLT) followed by photo-cross-linking was used in the fabrication process [34]. Scaffold films were first produced and then were seeded with ADSC, after which photo-cross-linking of 5 layers using UV radiation was made to produce a multilayered scaffold mimicking the natural tendon structure. In vitro assessment showed biocompatibility of pro-

duced composite in which ADSCs were oriented along the longitudinal access of aligned fibre construct and expressed tendon-related markers (scleraxis and tenascin-C) after being stimulated with TGFβ-3. Mechanical assessment of cell-seeded cross-linked scaffolds however was far inferior for potential clinical application (details in Table 1).

Other groups have combined collagen type I with silk to improve scaffold properties for tendon applications [37]. Sericin protein was extracted from silk fibres produced by Bombyx mori, and mixed with collagen solution to fill the gaps in the silk fibre net. Furthermore, adherence and mechanical strength were increased though DTH cross-linking. Mesenchymal stem cells derived from human embryonic stem cells (hEC-MSCs) were used in in vitro and in vivo models and showed that the mechanically loaded knitted silk/ collagen microsponge scaffolds can induce tenogenic differentiation in mesenchymal stem cells and support tendon regeneration up to 360 days after implantation (Table 1).

In an additional study, the same composite scaffolds were tested with the supplementation of recombinant human stromal cell-derived factor-1 alpha (rhSDF-1 alpha), to the collagen type I sponges [38]. SDF-1 is a chemokine that promotes cell recruitment and enhances tissue regeneration. In a murine Achilles tendon model, the authors showed that this approach permits higher expression of collagen one week after implantation, indicating an accelerated onset of tendon healing. Further, the mechanical properties were marginally higher than in scaffolds without rhSDF-1 alpha treatment.

Top 5 Contributions in Materials Science

Table 1: Summary of the literature related to the applications of hybrid biomaterial in tendon tissue engineering.* indicates that approximate numbers were extracted from supplied figures since no exact reference values are mentioned in the text. PDS: poly-dioxanone; SCX, scleraxis; TGFβ, transforming growth factor beta; hECS-MSCs, mesenchymal stem cells derived from human embryonic stem cells; rhSDF1, recombinant human stromal cell-derived factor 1; PGA, poly-(lactide-co-glycolide); PCL, poly-caprolactone; bFGF, basic fibroblast growth factor.

COMPOSITE/HYBRID	MODEL	SCAFFOLD PROPERTIES	CELL/TISSUE INTEGRATION
PGA/PLA composite. [24]	In vitro (ADSCs) In vivo (rabbit)	After implantation in Achilles tendon, Tensile strength: 4.88±8.07 MPa No report of other mechanical properties.	(i) Grossly, the implanted cell-seeded scaffold was integrated with the native tissue interference, with a smooth surface cord-like shape with less noticeable remaining material after 45 weeks. (ii) Tissue adhesions, described grossly to be less compared to control. (iii) Parallel and more mature collagen fibers and longitudinally aligned cells are presents than control.
P (LLA-CL)/Collagen I. [25]	In vitro (Tenocytes)	Young's modulus Nonseeded about 2.2 MPa. Cell seeded about 3 MPa. Tensile strength Nonseeded about 3 MPa. Cell seeded about 4.5 MPa.	(i) Significantly higher cell proliferation in the nanoyarn scaffold compared to other scaffolds and control. (ii) SEM showed spindle-shaped cell in both nanoyarn and aligned nanofiber scaffold, while polygonal and random pattern cells are found in the random oriented fibers. (iii) Expression of tendon specific ECM (type I collagen, type III collagen, decorin, tenascin-C, and biglycan) was significantly higher at 14 days in the nanoyarn group.
P (LLA-CL)/ Collagen I nanoyarn. [26]	In vitro (TDSCs) In vivo (mouse)	Mechanical stress: Dynamic group was 59.58 ± 7.81 MPa Static group was 43.18± 6.58 MPa Control group was 32.43± 5.27% MPa Young's modulus: Dynamic group was 51.99±7.16 MPa Static group was 34.76 ± 4.75 MPa. Control group was 23.30 ± 3.83 MPa	TDSCs were used in scaffold seeding with both dynamic and static culture conditions. In vitro: (i) TDSCs showed elongated fibroblast-like morphology with a significant increase in cell count in dynamic group at 14 days. (ii) More cell infiltration and dense matrix in dynamic group. (iii) PCR confirmed Tendon related mRNA expression more in dynamic group. (iv) Western blotting showed significant increase in the protein expression levels of Collagens I and III, and tenascin-C in the dynamic group. In-vivo: (i) Significantly lower number of cells at 12 weeks with greater matrix deposition and longitudinal spindle-shaped cells in dynamic group compared to others. (ii) Collagen content was highest in dynamic group reaching 77.76 ± 6.82% of normal rabbit patellar tendon (174.31 ± 13.89 μg/mg). (iii) Collagen I expression was Significantly higher in dynamic group.
PLA / Collagen-I electrospun bundles [27]	In Vitro (Tenocytes)	Composite scaffolds were made from blends containing PLA/ Coll-75/25 (w/w). Young's modulus was 98.6 ± 12.4 MPa Maximum stress was 14.2 ± 0.7 MPa	(i) Seeded Tenocytes shown to exhibit good cell adhesion profile and more elongated morphology that was better over PLA/ Coll-50/50 blends than other.
PLA/ graphene nanoplatelets (GNP) and PLA/ carboxyl-functionalized carbon nanotubes (CNT-COOH) [28, 29]	In vitro (fibroblast) In vivo (mouse)	Young's modulus PLA control: 3.99 ± 0.42 GPa PLA/ CNT-COOH: 4.86 ± 0.47 GPa PLA/ GNP: 4.92 ± 0.15 GPa Tensile strength PLA control: 59.90 ± 4.93 MPa PLA/ CNT-COOH: 72.22 ± 1.52 MPa PLA/ GNP: 58.56 ± 3.99 MPa	(i) Both produced scaffolds supported fibroblasts metabolic activity and proliferation till final assessment point (72 hrs). (ii) In vivo assessment showed lack of any local or systemic inflammatory response using N-acetylglucosaminidase (NAG) and nitric oxide (NO) serum levels. (iii) No associated hepatotoxicity in histologic assessment. (iv) Histologic assessment of explanted scaffold showed formation of thin capsule around the implant with homogenous granulation tissue.
PCL/collagen-PLLA/collagen [30]	In vitro (myoblasts, fibroblast)	Tensile strength was 0.5058 ± 0.2130 MPa Maximum strain was 18.49% ± 8.210 Young's modulus was 7.339 ± 2.131 MPa	(i) Statistically higher viability of both myoblast and fibroblasts in all regions of the scaffold. (ii) Scaffold could support the formation of myotubes that is essential for normal muscle-tendon junction formation.
Aligned PLLA nanofiber/ Layered chitosan-collagen hydrogel/Alginate outer coating. [31]	In vitro (Tenocytes)	Maximum Force to break For uncoated 2 and 3 layers scaffold: 7.89±1.5N and 7.45±0.3N, For gel coated 2 and 3 layers scaffolds: 4.76 ± 0.23N and 6.49 ± 0.09N. No report of other mechanical properties.	(i) Alginate coating was associated with significantly less attached proteins than control. (ii) Both coated and uncoated scaffold maintain 50% of their substance after incubation with PBS containing 10^4 units/ml lysozyme solution. (iii) Alamar blue and DNA concentration assessment showed high cellular viability, metabolic activity, and proliferation up to 7 days after seeding. (iv) Seeded scaffolds were shown to support cellular alignment.

Table 1: Contd

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Top 5 Contributions in Materials Science

Electrospun collagen I nanofiber/ collagen microfiber [32]	In vitro (fibroblast) In vivo (rabbit)	Before implantation: Maximum load was 28.33 ± 2.19 N. Maximum stress was 2.69 ± 0.47 MPa Maximum strain was 61.34 ± 4.71 % Young's modulus was 43.81 ± 4.19 KPa. 60 days after implantation to Achilles tendon: Maximum load was about 63.72 N. Maximum stress was about 9.84 N/mm. Maximum strain was about 16.35% Young's modulus was about 0.62 N/mm.	In vitro: (i) Significantly higher cell viability in the aligned hybrid scaffold compared to others. (ii) SEM and immunofluorescence proven superior alignment of fibroblasts together with cell proliferation with close cell-to-cell contact in the aligned hybrid scaffold compared to others. In vivo: (i) Significant increase in the number, density, and alignment of the collagen deposition with mature elastic fibers. (ii) Significant increase in the number and maturity of tenoblasts and tenocytes. (iii) Significantly lower peritendinous adhesions, muscle fibrosis, and atrophy and inflammatory cells found in the treated tendons compared to controls.
Electrospun collagen I nanofiber/collagen microfiber/ PDS sheets. [33]	In vivo (rabbit)	After 60 days of implantation to Achilles tendon: Maximum load was about 74.02 N Maximum stress was about 11.37 MPa Young's modulus was about 0.754 MPa	(i) Significantly higher fibrillogenesis after PDS treatment. (ii) Significantly higher collagen fibrils were found in the PDS treated scaffolds compared to the regular one. (iii) Significant increase in the load to failure and load to yield point with PDS treatment. (iv) Significantly improved peritendinous adhesions, muscle fibrosis, and atrophy scores that are comparable in the PDS treated and nontreated collagen scaffolds.
Core-shell Collagen type I/ Glycosaminoglycan. [34]	In vitro (Tenocytes)	Tensile modulus for dry membranes was about 636 ± 47 MPa to 693 ± 20 MPa Tensile modulus for hydrated membranes was 30 MPa. Tensile modulus for dry aligned core scaffold ranges from 833 ± 236 to 829 ± 165 KPa	(i) Significant increase in the number of cells at day 1 after seeding in the core-shell composite compared to core alone scaffolds. (ii) Higher metabolic activity observed at day 7 in the core alone scaffold that is statistically significant. (iii) No significant difference in the metabolic activity and cell number between the two groups at 14-day period.
Collagen-I/nanocarbon fibers [35]	-	Mechanical assessment in wet condition Young's modulus was 840 ± 140 MPa Tensile strength was 70 ± 8 MPa	(i) No cell work was presented.
Collagen type I/Resilin [36]	In vitro (Fibroblasts)	Maximum stress was 34.63 ± 9.75 MPa Maximum strain was 0.21 ± 0.04 MPa Young's modulus was 49.48 ± 10.71 MPa	(i) Human adult fibroblasts were used in the assessment process. (ii) The authors showed that addition of resilin and the use of poly(ethylene glycol) either tetrasuccinimidyl glutarate did compromise the cellular activity. (iii) The scaffold produced managed to support cellular proliferation and 100% cellular alignment after 7 days of seeding compared to 80% in collagen control.
poly--caprolactone (PCL) and methacrylated gelatin (mGLT) [37]	In vitro (ADSCs)	Maximum load was about 0.25 N. No report of other mechanical properties.	(i) Tenogenic differentiation was induced through differentiation medium having DMEM, 2% FBS, P/S, and 10 ng/ml TGF-β3 for 7 days after seeding. (ii) Seeded constructs were shown to exhibit elongated morphology as well as expression of tenogenic markers (scleraxis and Tenascin-C). (iii) Stacked scaffold was shown to have adequate porosity for cell diffusion and differentiation.
Sericin extracted knitted silk/ cross-linked collagen type I microsponges [38]	In vitro (hESC-MSCs) In vivo (mouse)	4 weeks after implantation to Achilles tendon of cell-seeded scaffolds: Maximum load was 65.24 ± 9.58 N Maximum stress was 6.72 ± 0.90 MPa Stiffness was 28.26 ± 2.95 N/mm Young's modulus was 34.91 ± 5.08 MPa	In vitro: (i) Good cell attachment, proliferation, and spreading at 14 days' period. (ii) Scx., collagen I, and collagen III expression were significantly higher in dynamic group than the control. In vivo assessment after 4 weeks of implantation: (i) Grossly, well-integrated construct with native tendons. (ii) Viable cells are presents showing spindle-shaped morphology. (iii) Significantly higher collagen I, III, Vα1, Vα2, and TGFβ1 in cell-seeded scaffold compared to cell free scaffold. (iv) Neocollagen was replacing exogenous one with more content, and mature morphology in cell-seeded scaffold compared to cell free scaffold.
Sericin extracted knitted silk/ cross-linked collagen type I microsponges/rhSDF-1 alpha [39]	In vitro (fibroblast) In vivo (rat)	4 weeks after implantation in Achilles tendon: Maximum load was 68.5 ± 18 N Maximum stress was 7.02 ± 1.7 MPa Stiffness was 39.0 ± 6.6 N/mm Young's modulus was 45.3 ± 10.4 MPa	In vivo assessment after 4 weeks of implantation: (i) More fibroblasts-like cells and less inflammatory cells are found early after implantation (4 days, 1 week). (ii) More organized continuous collagen fibers are found with a higher concentration of collagen type I. (iii) Good vascular components with less inflammatory cells are present. (iv) Significantly higher expression of Collagens I and III, decorin in the SDF-1 scaffolds at all assessment periods. (v) Large fibrils of Achilles tendons formed in the SDF-1 scaffolds (41.6 ± 5.5 nm) compared to control (37.1 ± 2.9 nm).

Table 1: Contd

Top 5 Contributions in Materials Science

Sericin extracted knitted silk/ cross-linked collagen type I microsponges/SCX engineered cells [40]	In vitro (hESC-MSCs) In vivo (mouse)	4 weeks after implantation: Young's modulus was 60.63 ± 17.6 MPa Maximum stress was 8.73 ± 2.15 MPa 8 weeks after implantation: Young's modulus was 71.3 ± 12.4 MPa Maximum stress was 10.1 ± 2.2 MPa	In vitro: (i) SCX treatment increases collagen I expression with enhanced cell-sheet formation. (ii) Decreased capacity of adipogenic, chondrogenic, and osteogenic differentiation of the cells with more tenogenic differentiation. In vivo: (i) Good proliferation and early matrix deposition in the SCX cells compared to control. (ii) Increased Collagen Ia1, Ia2, and tendon related transcription factor Eya2 in SCX cells compared to control. (iii) More fibroblast-like spindle-shaped cells and fewer immunogenic cell infiltrates in the SCX cells group. (iv) Higher expression of biglycan in the SCX cells group, indicating more mature endogenous collagen.
Knitted silk coated with electrospun collagen-I / polyurethane (PU) nanofibers [41]	In vitro (Fibroblast)	Maximum stress was 13.5 ±1.5 MPa Young's modulus was 21.7±4 MPa	(i) Human fibroblasts were seeded on composite scaffold to test for viability. (ii) Assessment was made through Alamar Blue assay and showed that samples with higher collagen content had higher cellular viability profile (COL75/PU25) than other groups.
Silk coated with Polycaprolactone (PCL) or Poly(3-hydroxybutyrate) (P3HB) nanofibers [42]	In vitro (Fibroblast)	Maximum load 97.6±11.4 N for silk fibroin/P3HB 110.5±6.6 N for silk fibroin/PCL No report of other mechanical properties.	(i) Human fibroblasts were seeded on composite scaffold and showed good cellular viability and no toxicity (assessment for 3 days).
Degummed Silk-fibroin meshes/aligned Silk-fibroin [43]	In vitro (MSCs)	Maximum load for dynamic culture condition Aligned scaffold 144.44 ± 5.03 N (7 days) 172.08 ± 6.28 N (14 days) Random Scaffold 122.35 ± 3.67 N (7 days) 138.67 ± 9.22 N (14 days) Stiffness Aligned scaffold 24.33 ± 1.40 N/mm (7 days) 26.93 ± 2.40 N/mm (14 days) Random Scaffold 17.48 ± 0.93 N/mm (7 days) 23.07 ± 2.54 N/mm (14 days) No report of other mechanical properties.	(i) Highly significant cell viability in the aligned scaffold compared to the random one at both dynamic and static conditions. (ii) Consistent cellular proliferation in both aligned (dynamic and static) and dynamic random scaffold. (iii) Higher collagen deposition in the dynamic aligned scaffold compared to the static one and the random scaffold groups. (iv) Dynamic culture improved the histological assessment of both aligned and random scaffold with more cell elongation and matrix deposition. (v) Higher expression level of collagen I, tenascin-C, and tenomodulin in the aligned dynamic scaffolds compared to others.
Knitted PLGA-PLLA / coating with (1) PCL. (2) PLGA nanofiber. Type I collagen [44]	In vitro (BMSCs)	Maximum load Uncoated 68.4 ± 5.37 N. PCL coated 63.1 ± 4.52 N. PLGA coated 56.3 ± 6.61 N. Collagen coated 59.5 ± 8.3 N. Stiffness Uncoated 9.1 ± 2.38 N/mm. PCL coated 4.3 ± 0.91 N/mm. PLGA coated 5.8 ± 0.70 N/mm. Collagen coated 5.7 ± 0.48 N/mm. No report of other mechanical properties.	(i) Efficient cell seeding on PLGA nanofiber coated and collagen-coated scaffolds. (80-89% and 61-69%, respectively). (ii) Significantly higher cell proliferation between and culture days of PLGA nanofiber coated knitted PLGA scaffolds.

Table 1: Contd

Top 5 Contributions in Materials Science

PLGA nanofiber/Silk microfiber [45]	In vitro (BMSCs)	Maximum load of unseeded scaffolds 75.3 ± 4.79 N on day 0, and 61.5 ± 3.43 N on day 21 Maximum load of rolled seeded scaffolds 68.2 ± 6.72 N on day 21 Stiffness of unseeded scaffolds 4.8 ± 0.52 N/mm on day 0, and 5.9 ± 0.54 N/mm on day 21 Stiffness of rolled seeded scaffolds 5.5 ± 0.30 N/mm on day 21 No report of other mechanical properties.	(i) Dual surface seeded scaffolds showed significantly higher cell proliferation rates compared to single surface seeding. (ii) Increased cell proliferation between 14 days and 21 days after culture. (iii) Rolled-up scaffolds had nonsignificantly lower cell proliferation rates.
PLGA nanofiber/ bFGF / Silk microfiber [46]	In vitro (mesenchymal progenitor cell)	3 weeks after culture of rolled scaffolds: Maximum load Unseeded scaffolds were about 61.5 N Seeded bFGF-free scaffolds were about 68.2 N Seeded bFGF scaffolds were about 82.7 N Stiffness Unseeded scaffolds were about 5.92 N/mm Seeded bFGF-free scaffolds were about 5.53 N/mm Seeded bFGF scaffolds were about 6.97 N/mm	(i) Significantly higher cell viability in the bFGF scaffolds compared to bFGF-free scaffolds. (ii) Significantly higher collagens I and III, fibronectin, and biglycan 14 days after culture in the bFGF scaffolds compared to bFGF free scaffolds. (iii) Significantly higher collagen content in the bFGF scaffolds compared to bFGF free scaffolds by week after culture.
Alginate / 0.1% chitosan [47]	In vitro (fibroblast)	Tensile strength was 235.2 ± 8.5 MPa Maximum strain was 12.3 ± 0.3 %	(i) Significantly lower number of unattached cells in alginate–chitosan group compared to polyglactin and alginate alone. (ii) Fibroblasts were spread on the polymer fibers. (iii) Prominent collagen type I production 14 days after culture was more on the scaffold surface by immune staining with no clear visualization of both types II and III collagen.
Chitosan / 0.1% hyaluronic acid [48]	In vitro (fibroblast) In vivo (rat)	(i) In vitro: Tensile strength: Before seeding was 213.3 ± 10 MPa 2 hrs after seeding was 60 ± 6.7 MPa 14 days after seeding was 66.7 ± 6.8 MPa 28 days after seeding was 65.1 ± 6.6 MPa Maximum strain: Before seeding was 3.2 ± 0.6 % (ii) In vivo tendon model: Tangent modulus for cell-seeded scaffold: 4 weeks after implantation: about 58 ± MPa 12 weeks after implantation: about 85 ± MPa In vivo ligament model: Maximum load for cell-seeded scaffold: 12 weeks after implantation: about 110 ± 10 N	In vitro up to 28 days after cell seeding: (i) Gross observation of ECM production by light microscopy. (ii) Prominent collagen type I production 14 days after culture more on the scaffold surface. In vivo tendon model: (i) Type I collagen is seen only in cell-seeded scaffold. In vivo ligament model: None.

In order to improve the mechanical properties and tendon regeneration, Chen X. et al. utilised the same composite scaffolds with hEC-MSCs [39]. Cells were genetically engineered to overexpress the Scleraxis (SCX) gene. SCX is a transcription factor identified for its role as a tenocyte marker and upstream regulator of tendon-related genes. In vitro and in vivo results showed enhanced tendon regeneration and better quality neotendon formation, compared to the previous studies with the same scaffolds. Importantly, there was less osteogenic, chondrogenic, and adipogenic differentiation of the SCX-hEC-MSCs compared to nongenetically modified MSCs. Mechanical properties in a murine Achilles tendon eight weeks after implantation were inferior to native tendon tissue. This research highlights that scaffold properties are important for tendon tissue engineering, but factors like cell-types used can influence mechanical and histological results.

Synthetic and Biological Composite Scaffolds

Others have also focused on incorporating PLGA with silk derived materials for tendon tissue engineering applications [40]. A composite scaffold comprised degummed silk microfibers coated electrospun PLGA. The authors degummed silk fibres to achieve a more efficient sericin removal. This resulted in smoother fibre surfaces and preserved the general mechanical properties of the silk. In vitro studies using rabbit bone marrow-derived MSCs revealed good cell viability depending on the seeding technique applied (single or dual surface seeding) and whether scaffolds had a flat or rolled/ cylindrical morphology. Rolled structures had lower cell proliferation compared to the other constructs but, overall, the electrospun PLGA polymer provided a large surface area for cell proliferation. To increase tenocyte differentiation of bone marrow-derived MSCs, authors reported a modified protocol in which the scaffold has a 1-week release of basic fibroblast growth factor (bFGF) [45]. bFGF was blended with PLGA

and bovine serum albumin prior to electrospinning with knitted silk fibres. Results showed that incorporating bFGF was associated with upregulation of tenogenic markers during MSC differentiation. Collagen expression was also increased, and this contributed to improved mechanical properties of the scaffold. The combined effect of growth factor incorporation together with dynamic culturing conditions would be of interest for its effect over MSC differentiation and overall construct incorporation.

Sharifi-Aghdam M. et al. [46] have investigated a composite scaffold consisting of knitted silk coated with electrospun collagen-I/polyurethane nanofibres. The main aim of this approach was to incorporate a polyurethane polymer layer to increase the attachment of collagen to silk fibres. In vitro testing using seeded human fibroblast showed that composite scaffold maintained adequate cellular metabolic activity. Assessment of mechanical properties showed an ultimate stress profile reaching 13.5±1.5 MPa and Young's modulus of 21.7±4 MPa. However, further assessment of seeded constructs for their biocompatibility and cell function was not investigated.

Investigative work showed the incorporation of silk with nanofibres derived either from Polycaprolactone (PCL) or Poly(3-hydroxybutyrate) (P3HB) [41]. The composite scaffold was fabricated through electrospinning process of different polymer material over twisted silk fibroin fibres. The main aim of this approach was to incorporate nanofibrous structures onto the composite scaffold to increase the surface to volume ratio and therefore increase cellular attachment. Authors showed a composite scaffold with no toxic effect of seeded fibroblasts with good cellular viability up to day 3 after seeding. Mechanical assessment of fabricated construct showed a maximum load of 97.6±11.4 N for silk fibroin/P3HB and 110.5±6.6 N for silk fibroin/PCL with no statistical difference in between. Authors, however, did not further investigate the effect of cellular functions or matrix production or the effect of cell seeding on associated mechanical properties.

Others have focused on the modification of silk to produce scaffolds for tendon regeneration [42]. Degummed silk fibroin meshes were integrated with electrospun aligned silk fibroin cores [42]. In vitro assessment with rabbit MSCs investigated the effects of aligned fabrication techniques, compared to random fabrication, under static and dynamic culture conditions. Results showed that the effects of mechanical stimulation on MSCs was intensified during culture on aligned silk fibroin scaffolds. The dynamic effect was applied in both translational and rotational movement to mimic in vivo environment. This enhanced cellular proliferation and remodelling, with an overall improvement in mechanical properties. Apart from the material used, presence of the aligned scaffold core was proven to be essential for these positive effects when compared to the same scaffolds without alignment. Several authors have showed a similar effect when topography was introduced to the surface of a polymeric scaffold with improvement in cellular alignment and collagen content as well as the expression of different tendon-related extracellular matrix proteins [43,50].

Elsewhere, collagen has been combined with various polymers through a range of manufacturing techniques to simulate the heterogeneous nature of tendon tissues. Collagen type I was electrospun with synthetic poly(L-lactide-co-caprolactone) at a 10:90 ratio, respectively [47]. Fibres were twisted into nanoyarn to produce 150 µm thick scaffolds. Scaffolds aligned randomly or in nanofibers were tested for porosity, surface morphology, and adhesion of tenocytes. Results showed improved cell proliferation in nanoyarn scaffolds, but with poor mechanical properties not useful for future clinical applicability.

In a follow-up study, tendon-derived stem cells (TDSCs) were harvested from rabbit patellar tendons and seeded onto the fibrous scaffolds [48]. In vitro and in vivo assessment under static and dynamic conditions revealed that the composite scaffolds seeded with TDSCs were a promising means for neotendon formation. Further-

more, mechanical dynamic stimulation of the cell-seeded constructs could significantly promote tendon regeneration compared to static culture conditions.

Sensini A. et al. have investigated an electrospun bundled scaffold containing PLA and collagen type I [51]. This scaffold had sufficient mechanical properties with blends containing PLA/collagen 75:25 reaching a Young's modulus of 98.6±12.4 MPa as spun bundles and 205.1±73.0 MPa after 14 days of immersion in PBS. Maximum stress was 14.2±0.7 MPa as spun bundles and 6.8±0.6 MPa after 14 days in PBS immersion. Tenocytes were metabolically active with good cellular alignment more on blends containing PLA/collagen 50:50 ratio.

Collagen type I has been integrated also with other composite synthetic polymers blends. Polycaprolactone (PCL)/collagen and poly L-lactide (PLLA)/collagen were evaluated as potential composite materials for tendon-muscle junction tissue engineering [52]. Triphasic scaffolds were fabricated with regions primarily composed of PCL/collagen in one part, PLLA/collagen on the other part, and a mixture of both composites in the middle. All constructs had good biodegradability and biocompatibility. PCL supported myoblast growth due to its low stiffness profile and the higher stiffness of PLLA encouraged fibroblast proliferation. Scaffolds were manufactured using electrospinning technique combined with glutaraldehyde cross-linking and collagen to increase mechanical strength and cell attachment, respectively. In vitro, scaffolds presented good cell integration, viability, and formation of myotubes as those found in tendon-muscle junctions in vivo. Aligned collagen scaffolds were produced with surface cover having polydioxanone (PDS) nanoplates [32]. Assessment in rabbit Achilles tendon demonstrated the same biocompatibility as the previously tested electrospun collagen scaffolds. Further detailed assessment of the composite with and without PDS showed significant improvement in water uptake and release. Histological evaluation showed good tenocyte alignment, neotendon formation, and an initial increase in the inflammatory cell response. The addition of PDS sheets resulted in decreased peritendinous adhesions with higher

numbers of mature tenocytes and increased collagen fibril alignment. Improved mechanical properties of the construct were also observed compared to scaffolds made of collagen fibres only [32,50].

PLLA was also utilised to fabricate a composite scaffold in which electrospinning process was used to form an aligned nanofibres that was later on cross-linked to chitosan-collagen hydrogel mimicking the extracellular matrix of native tendons [53]. The scaffold was rolled and coated on the outer surface with alginate gel aiming to produce an antiadhesion layer around the construct. The scaffold supported cellular alignment and proliferation of tenocytes with no toxic effect. Additionally, adsorption tests showed significantly less attached proteins on the coated surface compared to the noncoated one. Mechanical assessment of produced scaffold showed no effect of coating process on tensile strength of produced scaffold with an effect on the layer number having a value around 2 MPa for 2-layer coated and uncoated scaffolds whereas it was around 6 MPa for uncoated and 4 MPa for coated 3-layer scaffolds. The degradation profile of the scaffold was also investigated and showed that scaffolds maintained 50% of their substance at 21 days after incubation with PBS containing 104 units/ml lysozyme solution.

E.C. Green et al. [54] investigated the fabrication of collagen-I/nanocarbon fibres composite scaffold for potential tendon tissue engineering application. Fibres were made through gel-spinning process with the use of a filling load of 0.5 and 5 wt%. This was followed by fibre elongation at a strain rate of 0.02 mm/s and subsequent glutaraldehyde (GA) cross-linking. Material characterization showed a yielded fibre construct similar to native tendon collagen with enhanced mechanical properties.

Similarities and Dissimilarities between Tendons and Ligaments

Tendons and ligaments have similar structures with different fundamental properties and functions. Tendons are fibrous inelastic structures that connect muscles to bones within joints while ligaments are fibrous but flexible structures important for supporting bone and cartilage. On a structural level, both connective tissues are dense with variable cellular and proteomic elements. Mechanical analysis of human tendons and ligaments showed that the maximum tensile strength ranges from 4.4 to 660 MPa depending on different locations [55,56]. The maximum strain of these connective tissues was shown to range between 18 and 30%. Young's modulus was shown to range between 0.2 and 1.5 GPa [57,58]. Structurally, tendons are predominantly composed of a collagen type I matrix containing tenocytes and tenoblasts. In contrast, ligaments contain glycosaminoglycan and lower levels of collagen compared to tendon tissue, with fibroblasts being the main cellular element (Figure 2) [59,60]. Kharaz Y. et al. compared the extracellular matrix composition of both, natural and tissue engineered, tendons and ligaments [61]. Results showed that, although tissue engineered constructs share the same composition with the native tissue in variable proportions, fundamental differences exist. Specific proteins, such as asporin and tenomodulin, were limited to tendons, while versican, proteoglycan 4, and SOD3 were ligament-specific (Figure 1). Identifying differences in structural protein expression indicates that tissue engineering approaches should aim to replicate these distinctions at the proteomic level. To date, this has not always been the case, and the terms tendon and ligament are often used interchangeably in the literature. This is predominantly because the two connective tissues exhibit similar functional and mechanical properties [12]. An important concept to remember is that cell source is fundamental in dictating the type of the matrix produced [61]. This highlights the intrinsic cell memory that is different between tendon and ligament. In order to tissue engineer these specific tissues, it is important to consider their functional differences so that biomimicry

can be achieved. This will eventually help in enhancing integration and function to be restored when used to replace injured tendon or ligaments. From this, it is clear that although the two tissues share several features, a difference exist.

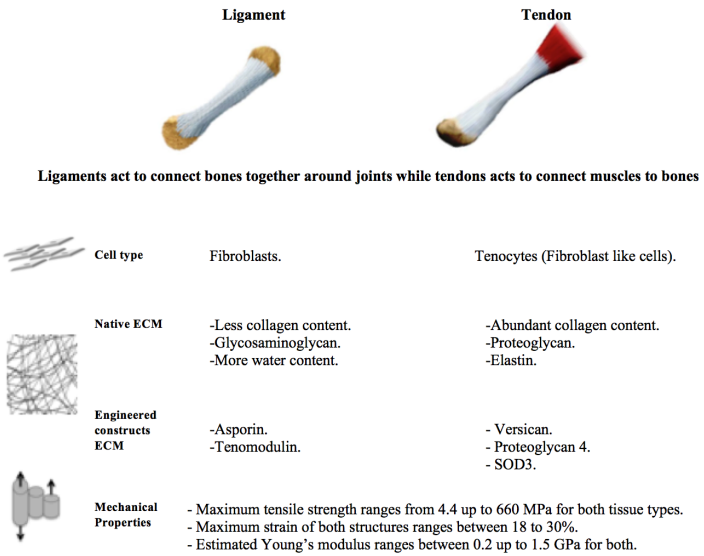

Figure 2: Comparison between natural and tissue engineered tendon and ligament constructs. Unique cellular and extracellular matrix composition that is different between the two types of tissues is important for future clinical translation of tendon and ligament research. SOD3, superoxide dismutase; MPa, mega-Pascal; GPa, giga-Pascal.

Conclusion and Future Directions

This review summarises the current strategies based on composite biomaterials for tendon and ligaments tissue engineering. This approach represents a way to address the heterogeneous nature shared between tendon and ligaments. Although both structures share similar mechanical properties, their constituting cell-types and extracellu-

lar matrix compositions are different. Currently, for tendon tissue engineering, several challenges need to be addressed. First, the necessity for a single standard evaluation identifying the mechanical requirements for successful tendon regeneration is lacking. Crucially, this should incorporate various forms of mechanical assessment (including strain and rotation) with different tissue engineering approaches to mimic the natural environment. This is difficult to achieve and implement because of the heterogeneous nature of tendons. The second challenge is to address and control the response of the surrounding tissue to the implanted scaffold. To date, this has been limited to observation of the intrinsic healing response during in vivo applications. Additionally, most of the studies investigating the use of composite materials for tendon and ligament tissue engineering utilise small animals like rabbits rather than larger models like sheep or horses [62]. Such limitations are based on significant weight and mechanical-related difference between in vivo models and native human tissues. Furthermore, the ability to fix the produced construct in situ (with suture, anchors) and shelf life availability should be considered whenever a model is being tested. In fact the interface scaffold-tendon or scaffold-bone is the place at risk of rupture after implantation rather the scaffolds itself.

The described challenges constitute a potential issue for future clinical application of tissue engineered tendon and ligament solutions. Further research is required on the roles of composite materials in order to mimic the appropriate structural, mechanical, and functional characteristics of tendon and ligaments. While synthetic materials are being used currently in clinical practice for tendon and ligament repairs, it will be essential to mechanically match their properties to native tissue. To mimic the mechanical properties of tendon is particularly important as it would allow faster physiotherapy and therefor reduce scar tissue formation which can evolve to articular stiffness. Moreover, this will enhance integration and healing at injury site. The use of certain growth factors such as basic fibroblast growth factor (bFGF) [45] can also be employed during surgery to enhance

tissue integration. However this requires a strict control and regulated environment. Since advanced composite materials can be tailored to match different mechanical properties of native tissue, they can be incorporated with growth factors and employed with and without cells, providing large number of possibilities for their clinical use based on site and extent of injuries.

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