Chapter

Mechanical Methods of Producing Biomaterials with Aligned Collagen Fibrils

Shunji Yunoki, Eiji Kondo and Kazunori Yasuda

Abstract

Collagen has been used in various therapeutic medical devices, such as artificial dermis, bone, and cartilage, wherein the effectiveness of collagen mainly depends on its biological features of biocompatibility, biodegradability, bioresorbability, cell affinity, and weak antigenicity. Collagen is the main structural protein in the human body and is responsible for the mechanical properties of tissues and organs. The fundamental structural component of tendon tissue is uniaxially aligned collagen fibrils that run parallel to the geometrical axis. Thus, the fabrication of artificial tendons is an excellent example of developing biomaterials using collagen as a structural backbone. Previous attempts to construct aligned fibril-based biomaterials involved electrospinning, freeze drying, using a strong magnetic field, and mechanical methods, including shearing and tension during wet extrusion. Among these, mechanical methods have been extensively studied owing to their simplicity and effectiveness suitable for mass production. However, few review articles have focused on these mechanical methods. Thus, this article reviews the mechanical methods for creating biomaterials from aligned collagen fibril while discussing the other fabrication methods in brief.

Keywords: tendon, collagen, fibril, alignment, shearing

1. Introduction

Since the research and development of collagen-based artificial dermis began in the 1980s [1, 2], many biomaterials using collagen as the base have been developed and clinically applied [3, 4]. Currently, many advanced collagen-based biomaterials have been developed for cellular or acellular tissue engineering and cell therapies [5, 6], and collagen remains one of the most essential biomaterials. Collagen is useful as a base material for therapeutic biomaterials due to its excellent biochemical properties (biocompatibility, biodegradability, and bioabsorbability) [3, 4] and cell affinity [5]. These properties enable the resultant biomaterials to be decomposed through biological activity, absorbed and metabolized at the damaged sites, and eventually be replaced with normal tissues. The effectiveness of collagen in such biomaterials primarily depends on the abovementioned biological features as well as

its weak antigenicity. Its excellent moldability and low cost have further facilitated the development of sheet-shaped artificial dermis [7], porous artificial bones [8], and hydrogel-based artificial cartilages [9].

However, the mechanical properties of such collagen-based biomaterials and artificial tissues are significantly inferior to those of living tissues. Collagen is the main

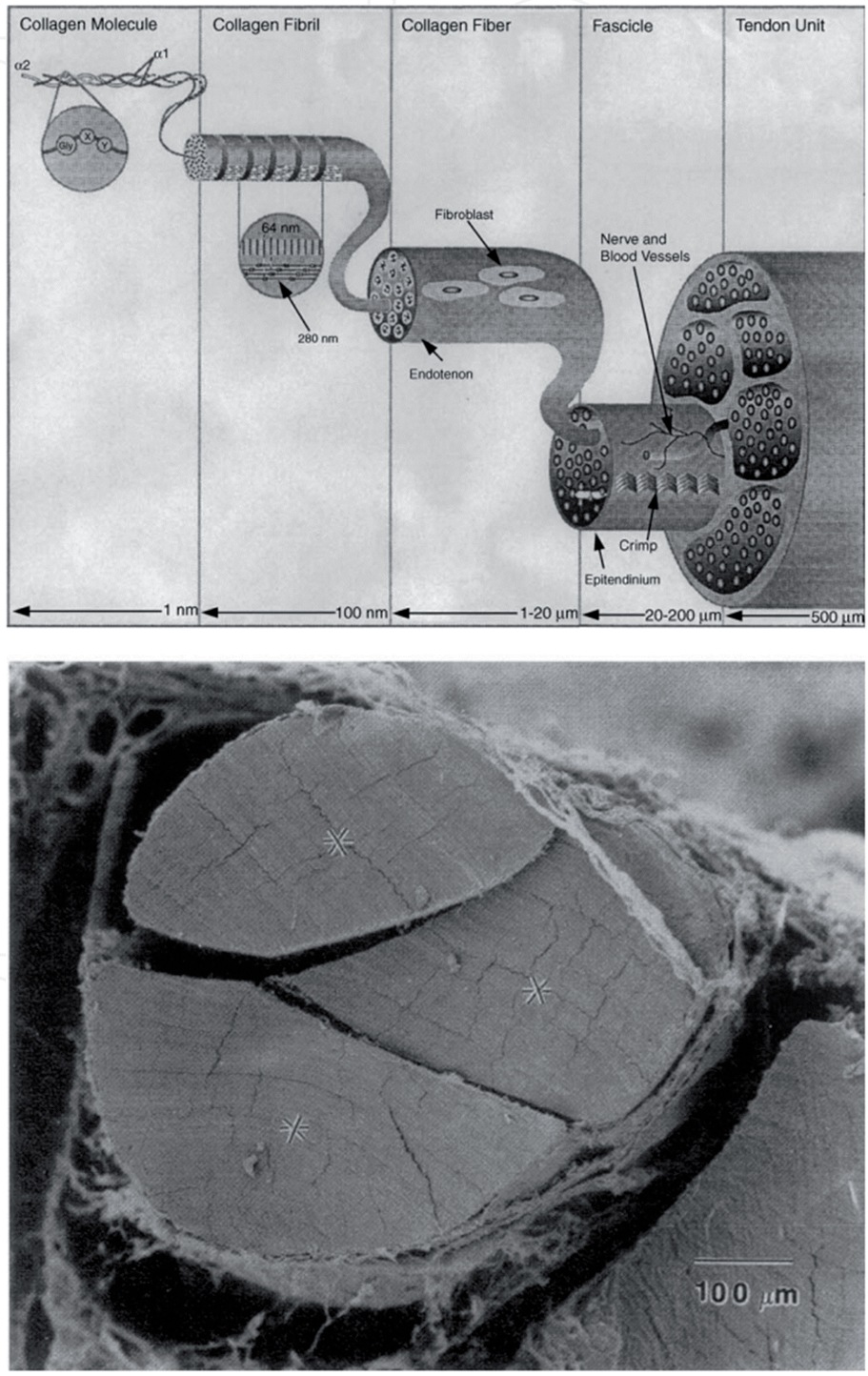

Figure 1.
Structural hierarchy in the tendon. Diagram illustrating the relationship between collagen molecules, fibrils, fibers, fascicles, and tendon units (top). Although the diagram does not show the fibril subunits, the collagen fibrils appear to be self-assembled from intermediates that may be integrated within the fibril. Scanning electron micrograph of rat tail tendon showing fascicle units (asterisk) making up the tendon (bottom). Reproduced from Ref. [11] with permission from Elsevier.

structural protein in the human body and is responsible for the mechanical features of tissues and organs [10]. Among all the various tissues and organs, tendons comprise collagen fibrils with unique hierarchical structures (**Figure 1**) that are responsible for human motor function [11]. Therefore, the fabrication of artificial tendons is a good example of biomaterials developed using aligned collagen fibrils as a structural backbone. As stated in this review, research on artificial tendons made using collagen has rapidly increased in the last decade to meet the clinical needs of replacing autologous tendon transplants.

Artificial tendons must have uniaxially aligned collagen fibrils running parallel to the geometrical axis; this characteristic collagen structure is responsible for the excellent mechanical features of live tendons [12]. Collagen fibrils can be simply prepared by well-known *in vitro* fibrillogenesis. Collagen molecules are stable in acidic solutions at low temperatures and are capable of self-assembling nanofibrils that respond to body temperature and neutral pH [13, 14]. The fibrils exhibit amorphous networks. Previous attempts to produce aligned fibril-based biomaterials used electrospinning, freeze drying, strong magnetic fields, and mechanical methods, such as shearing and tension during wet extrusion. Of these, only mechanical methods demonstrated the potential for use in the industrial production of artificial tendons by showing the ability to maintain and hierarchize collagen fibril structures.

Although many reviews on the fabrication of aligned collagen fibrils have been published in recent years [15–17], they do not focus on mechanical methods and the fabrication mechanisms therein. Here, we introduce the various mechanical methods of producing biomaterials with aligned collagen fibrils and discuss their mechanism and limitations in detail. This review will also act as a significant introduction for researchers willing to apply collagen-based artificial tendons in clinical practice.

2. Requirements of collagen-based artificial tendons

Tendon tissues connect muscles to bones and influence the transmission of mechanical loads between them [12, 18]. Ligament tissues connect bone to bone and stabilize joints [12, 18]. These two types of tissues are similar in structure and comprise uniaxially aligned collagen fibrils [19]. The characteristic structure of tendons and ligaments is a multi-unit hierarchical structure comprising longitudinally aligned collagen molecules (approximately 1 nm in diameter), fibrils (approximately 100 nm in diameter), fibers (1–20 μm in diameter), and fascicles (20–200 μm in diameter) [11]. This hierarchical organization of collagen fibrils is crucial to the nonlinear and viscoelastic mechanical properties of collagen-based organs [20]. Tendons and ligaments are remarkable for their superior tensile strength and stiffness; the tensile strengths of the Achilles' tendon and anterior cruciate ligament (ACL) are 54 ± 20 MPa [21] and 24 ± 9 MPa [22], respectively, and their tensile moduli are 212 ± 109 MPa [21] and 113 ± 45 MPa [22], respectively.

Tendons and ligaments are tough tissues; however, ruptures of these tissues are common traumas among athletes [23]. The ACL is a part of a pair of cruciate ligaments (the other being the posterior cruciate ligament) in the human knee that connects the femur to the tibia to stabilize knee joint movements. ACL is the most frequently injured knee ligament [24]. Once the ACL ruptures, it can rarely connect end-to-end through conservative treatments. The poor healing capacity of ACL, particularly after rupture, is clinically common, although the underlying reasons for this remain unclear [25]. ACL reconstruction surgeries are required for such

traumas; ACL injuries are among the most common among the athletic populations, with nearly 130,000 ACL reconstructions performed in 2006 in the USA alone [26]. Although there are no published survey results, ACL construction surgeries in Japan are estimated to exceed 17,000 per year.

In recent years, autogenous tendon tissues have been frequently used as substitutes for human tendon grafts (allografts) to reconstruct torn ligaments [27]. One notable advantage of this reconstruction surgery is the remodeling property of the autogenous tendon [28, 29], called ligamentization. Although the process of biological remodeling remains incompletely understood, clinicians agree that the strength of an autogenous tendon graft reduces soon after reconstruction and gradually increases with time, accompanied by structural changes in the collagen fibers [30]. Intrinsic fibroblasts in the tendon graft undergo ischemic necrosis followed by extrinsic cell infiltration with graft revascularization [31]. After remodeling implanted autogenous tendon tissues, patients can return to their daily activities as before. However, autogenous tendon grafting inevitably results in damage and consequent morbidity at the donor site, necessitating a second invasive procedure [32]. Although allogenous tendons are considered an alternative graft material, they have their disadvantages, including disease transmission risk and slow graft remodeling [33]. Currently, tendon xenografts cannot be used in a clinical setting; accordingly, synthetic tendons have been studied to avoid these disadvantages [16]. Various synthetic materials, such as polyethylene and polytetrafluoroethylene, have been used previously to create artificial tendons. However, they have not been clinically used as they fail after implantation because they undergo biodegradation without any remodeling [34]. Therefore, the fabrication of artificial tendons showing hierarchical structures of uniaxially aligned collagen fibrils seems to be the most promising approach as they are expected to undergo remodeling in the human body after implantation in a manner similar to that of autogenous tendon tissues [16, 19].

3. Overview of the fabrication methods used for aligned collagen fibrils

3.1 Electrospinning

Before focusing on the mechanical methods, we present an overview of the fabrication methods used for aligning collagen fibrils. Electrospinning has been widely considered an efficient method for fabricating polymer nanofibers, and several studies have described this fabrication technique [35, 36]. Briefly, the system comprises three elements—polymer solutions dissolved in volatile solvents, a high voltage supplier, and a metal target. The high voltage supplier provides electric potential differences in many kV between the polymer solutions and the target. The polymer solutions are then gradually extruded through a needle, and the electrically charged polymer solution is ejected from the tip of the needle which then reaches the target while being spun into thin threads (in the order of nm to μm in diameter). The volatile solvents evaporate during the interim, resulting in the collection of the polymer nanofiber mesh.

When the target is rotated during collagen electrospinning, each nanofiber tends to be aligned uniaxially [37]. Such materials can be helpful in *in vitro* experiments that evaluate the effects of scaffold alignments on the biological behaviors of cultured cells [37–39]. However, electrospinning appears to be an ineffective option as a fabrication method for artificial tendons due to the inevitable collagen denaturation. Fluoroalcohols

are used as solvents to dissolve the collagen as this hydrophilic polymer shows little or no solubility in conventional volatile solvents for electrospinning, such as dichloromethane and chloroform. Zeugolis et al. demonstrated that collagen in electrospun nanofibers was denatured to gelatin almost completely using fluoroalcohols as solvents [40]. This event can be explained by the ability of fluoroalcohols to break hydrogen bonds among proteins. A pioneering study of collagen electrospinning [41] demonstrated a characteristic cross-striated pattern of collagen fibrils in electrospun nanofibers fabricated using hexafluoroisopropanol as a solvent; however, the debris of collagen fibrils is presumed to have remained according to the findings of Zeugolis et al. The nanofibers prepared using electrospinning collagen solutions cannot be defined as collagen nanofibers.

3.2 Freeze drying

Freeze drying (also called freeze-casting) is one of the most critical industrial processes used to preserve heat-sensitive biological materials, including food, pharmaceuticals, microorganisms, and plants, with minimal deterioration of their intrinsic chemical and physical properties during the drying processes [42]. The fundamental principle of freeze drying is sublimation, that is, the direct shift from a solid state to a gaseous state [43]. The freeze drying process can be explained through a characteristic phase diagram of solid (ice), liquid (water), and gas (vapor). When water-based slurry, suspension, or solution is frozen at atmospheric pressure, the contents in water are separated from ice crystals and concentrated. If we increase the temperature of the frozen material above 0°C while keeping the atmospheric pressure below 0.06 atm, the ice turns into a gas without going through a liquid phase in accordance with the phase diagram of water [43]. Generally, dried materials thus obtained have microporous structures, whereas the contents eliminated from the ice crystals had thin walls and pores, which was similar to that of ice crystals [44].

Based on the above freeze-drying principle, Schoof et al. fabricated collagen sponges using aligned structures of pores and thin walls using the unidirectional solidification technique [45, 46]. Briefly, a cylindrical container filled with a collagen suspension was sandwiched from the top and bottom using a pair of copper blocks and then cooled with liquid nitrogen. Plate-like ice grew in the collagen suspension along the depth of the cylindrical container, by which collagen molecules are eliminated from the unidirectional solidification of the growing ice crystals. Freeze drying the frozen suspension created unidirectional thin collagen walls and interconnected pores. The pores had a width of 20–40 μm and were alternately separated by much thinner walls; this structure can be considered as collagen fibers with wide gaps. Some researchers have thus used unidirectional solidification to fabricate fibers from suspensions of collagen molecules or fibrils [47–49]. Additionally, a modified technique has been developed to concentrate collagen axially and form a fiber-like construct [50]. As a result, those macromolecules are likely to be partially aligned because of the high aspect ratios. However, there is little evidence of the unidirectional alignment of collagen fibrils in the walls after freeze drying, whereas aligned thin walls or fiber-like structures were observed microscopically [45–49]. The authors believe that collagen in the micro structures is almost amorphous on a fibrillar scale, affecting cellular responses and morphologies.

3.3 Exposure to a strong magnetic field

Based on the fact that some proteins in solutions exhibit birefringence under strong magnetic fields, Torbet et al. demonstrated for the first time that collagen

fibrils are magnetically aligned [51]. A neutral collagen solution (0.6 mg/mL) was heated from 4°C to 27.5°C to induce fibrillogenesis under a strong magnetic field (13T), resulting in the formation of aligned collagen fibrils. Collagen molecules have a negative diamagnetic anisotropy and they lie perpendicular to the magnetic field. Many researchers have applied this method to fabricate aligned collagen fibril hydrogels and used the gels for *in vitro* examinations to assess the effects of collagen fibril alignments on cell behaviors [52–56].

The notable advantage of this strong magnetic field is that it is noninvasive to living cells and organisms. However, the disadvantage is the lack of mass productivity of tough collagen fibers. The starting substance of the fabrication process is a neutral collagen solution, which is set in a narrow chamber (a few cm) with a strong magnetic field generator. This small-batch process is not suitable for the mass production of biomaterials. Further, the concentration of the collagen solution has to be low enough (≤10 mg/mL) to allow the rotation of the molecules due to magnetic force, preventing the production of high-density collagen fibrils. Recently, new methods have been developed wherein magnetic substances (beads or rods) are added to collagen solutions to mechanically pull or assist in the alignment of collagen fibril under magnetic fields [57–60]. A challenge associated with these manufacturing methods is that the magnetic substances are retained in the collagen gel.

3.4 Electrochemical method

Electrochemical fabrication for assembling aligned collagen bundles was first reported in 2008 [61]. This method is substantially different from the previous method using strong magnetic fields in that the physical force does not directly affect the collagen molecules. When the parallel set anode and cathode electrodes are soaked in a shallow pool of collagen solution, a pH gradient perpendicular to the electrodes is generated by the migration of electrolytes. Collagen molecules with a low pH are positively charged, whereas those with high pH are negatively charged. Therefore, all the collagen molecules migrate toward the isoelectric point (pH 8.2), congregate, and form fibrils under neutral conditions. The electrochemically aligned collagen (ELAC) threads with diameters of 50–400 μm and lengths of 3–7 cm were prepared depending on the electrodes used [61]. Although electron microscopies have not yet visualized uniaxial alignments of collagen fibrils, it is reasonable that collagen fibrils tend to align uniaxially by the electrochemical compaction to a bundle.

Continuous molding of ELAC threads was successfully performed using a rotating electrode electrochemical alignment device [62]. The main parts of the device include a power supply for providing voltage for the electrochemical cell, a syringe pump, a rotating electrodes wheel, and a collection spool. A collagen solution is extruded onto the caved edge of the rotating electrodes wheel placed vertically. The electrodes are placed parallel on the wheel's edge, allowing continuous ELAC formation synchronized with the rotation speed. Furthermore, ELAC threads were twisted to form yarn, and the yarn was pin-weaved toward a highly porous scaffold as an artificial tendon. The ELAC scaffolds showed ultimate stress and tensile modulus comparable to the natural tendon [62]. Furthermore, the biological effects of collagen fibril alignments in ELAC threads have been assessed *in vitro* [62–66] and *in vivo* [67].

The electrochemical method is the first to continuously produce aligned collagen fibril threads. ELAC threads (diameters 50–400 μm) in the yarns seem to correspond to collagen fibers (diameters ~20 μm [11]) in living tendons, although the diameters

of the former are much larger. Further studies are required for ELAC thread-based biomaterials to provide the tendon-like hierarchical structure of collagen fibrils.

3.5 Mechanical methods

The main purpose of this paper is to describe the mechanism and challenges associated with manufacturing tendon-like bundles of uniaxially aligned collagen fibrils through mechanical methods. Briefly, mechanical methods involve the use of mechanical force (shearing or tension) to align collagen fibrils. Mechanical forces can be generated before, during, or after the fibrillogenesis of collagen molecules.

4. Solution extrusion methods

Among the various aligned collagen fibril fabrication methods, mechanical methods have been extensively studied because of their simplicity and effectiveness as well as mass production suitability. These mechanical methods can be generally categorized into the following based on their fabrication mechanisms: solution extrusion methods (wet spinning and others), shear flow deposition, flow-induced crystallization, and gel-extrusion method. Herein, fabrication mechanism, effectiveness, and challenges of solution extrusion methods are discussed.

4.1 Wet spinning

Wet spinning is a typical example of a solution extrusion method that was first developed to produce collagen threads for artificial tendons [68]. A collagen solution is extruded from a narrow channel directly into a coagulation bath to form a cord-like gel through collagen fibril formation [69]. **Figure 2** shows a schematic illustration of a typical experimental setting for wet spinning. Neutral buffers such as phosphate buffer saline (PBS) containing polyethylene glycol (PEG) have been frequently used as coagulation baths for wet spinning as PBS provides suitable conditions for collagen fibrillogenesis [14] and PEG dehydrates the collagen molecules to promote fibrillogenesis. In Kato's method [68], the acidic collagen solution is filled in a reservoir, such as a syringe, and is extruded through a narrow tube (≤1 mm diameter) at a constant speed using a pump. The tip of the tube is submerged in a coagulation bath, and the collagen solution stream is immediately gelled due to fibrillogenesis. As a result, the cord-like collagen gel is continuously molded. Finally, drying the cord-like gel results

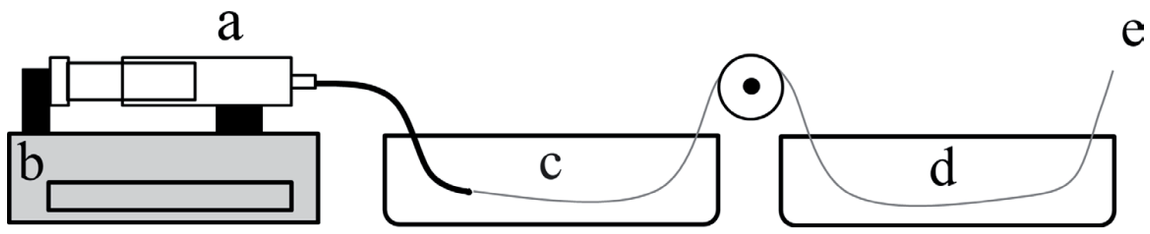

Figure 2.
Schematic illustration of the typical experimental setting for wet spinning. An acidic collagen solution in a syringe (a) is loaded in a syringe pump (b) and infused via a narrow tubing into a coagulation bath (neutral buffer containing PEG is frequently used) heated at 37°C (c). As a result, the cord-like collagen gel is continuously molded and sequentially introduced into an ethanol bath (d) to promote dehydration. The cord-like gel is then wound up and air-dried to produce a collagen thread (e).

in a tough thread with a diameter of 20–300 μm [70]. Cavallaro et al. succeeded in continuously processing dried collagen threads through a sequence of conventional wet spinning and subsequent drying using a ventilation-type cabinet [71]. Acetone was also used as a coagulation bath, allowing the fabrication of narrow collagen threads with an approximate diameter of 15 μm [72].

After Kato's pioneer study, many researchers have applied wet spinning to fabricate collagen threads for different biomaterials [69, 73]. However, the nanostructures of the collagen threads fabricated by wet spinning are far from those of tendon unit structures. Pins *et al*. revealed that collagen fibrils in wet spun threads were amorphous, prompting alignment by stretching the wet spun threads [74]. Despite many studies on wet spinning, the molecular events in the thread-making process have not yet been clarified, but it is likely that oriented collagen molecules under shearing in a narrow channel immediately changed to amorphous after extrusion into a coagulation bath (discussed in Section 4.5).

Assuming that the rapid relaxation of the oriented collagen molecules occurs, some treatments for delaying relaxation effectively promote fibril alignments in wet spinning. The addition of viscous materials or increase in collagen concentration to delay collagen molecule relaxation prior to fibrillogenesis has been investigated. Nerger et al. investigated 3-D bioprinting of collagen ink containing LAPONITE® (a type of layered silicate), Pluronic® F-127 (a type of polyethylene glycol), or Matrigel® (extracellular matrices of sarcoma) as rheology-adjusting agents [75]. The cord-like collagen gel extruded from a conical nozzle comprised of incompletely but preferably aligned collagen fibrils. Lai et al. prepared 30 mg/mL of rat tail collagen solution by dialysis against PEG and used it for fabricating tubular collagen gels with a custom-made syringe [76]. The fibrils on the surface of the collagen gels were aligned almost uniaxially, whereas the alignments of interior fibrils were not observed.

4.2 Modified wet spinning

In 2010, Caves et al. attempted to increase the fibril alignment of wet spun fibers by dropping the extruded cord-like collagen gels vertically with a coagulation buffer [77]. The extrusion of the collagen solution into a coagulation bath was performed in the same manner as wet spinning, resulting in the continuous formation of a cord-like gel. The bath was a long column through which the coagulation buffer was circulated to generate a vertical flow for carrying the collagen gel downwards along the column while simultaneously stretching it. The fibril alignment in the dried collagen thread was higher than that obtained using conventional wet spinning [73]; however, mechanical stretching (strain ratio of 10–20%) was required to achieve uniaxial alignments.

Recently, an extrusion method has been developed that incorporates the sequential stretching process of extruded gels to overcome the lack of fibril alignments [78]. This experimental setting is illustrated in **Figure 3A**. The collagen solution was continuously introduced into a flat flow channel (1-mm thick and 35-mm wide) with a pair of buffers containing PEG to ensure the three-layer of buffer-collagen-buffer. During co-extrusion, the collagen solution could be coagulated to some extent by dehydration with PEG. A sheet-shaped stream of partially coagulated collagen solution was extruded from the outlet into a coagulation buffer, resulting in the continuous production of a collagen gel sheet. Subsequently, the gel sheet was stretched along the machine direction with a rotating mandrel, thus enhancing the alignment of the collagen fibrils. Finally, wet collagen sheets as thin as 1.9 μm were obtained

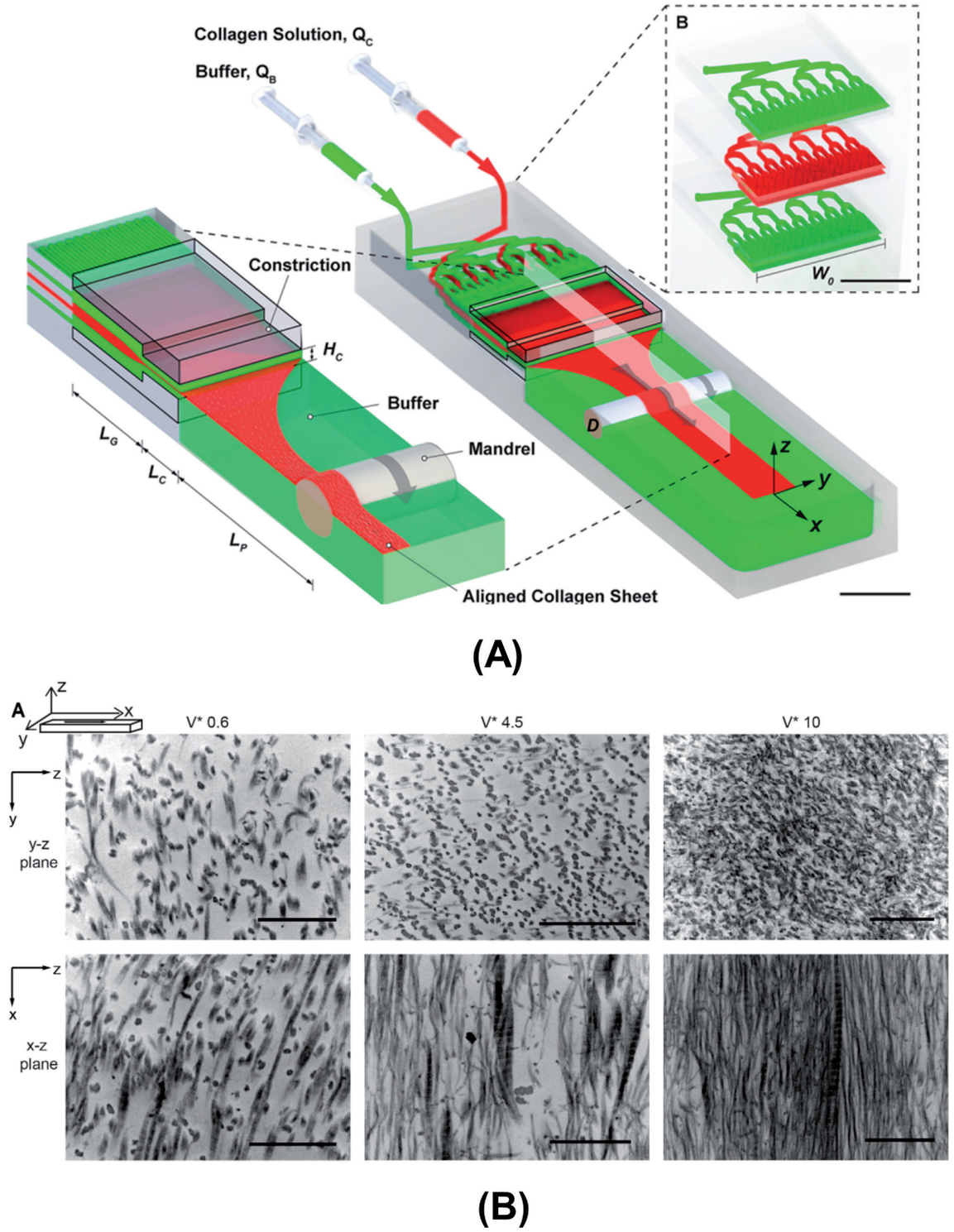

Figure 3.
Schematic illustration of the extrusion method incorporating sequential stretching of extruded sheet-shaped gels (A) and nanostructure of collagen gels observed on transmission electron microscopy (TEM) (B). (A) Collagen (red) and buffer solutions (green) are delivered to a three-layered microfluidic device. An emerging collagen sheet then undergoes fibrillogenesis and is strained by passing over a rotating mandrel. (B) TEM images of collagen sheets were produced at V of 0.6, 4.5, and 10 in (y – z) and (x – z) planes (depicted in (A)). V* = (Vp – VT)/VT, where Vp is the velocity of the rotating mandrel and VT is the total bulk velocity of the solutions passing through the flow constriction. Reproduced from Ref. [78] with permission from ACS Publication.*

which exhibited good mechanical qualities (tensile strength, 0.5–2.7 MPa and elastic moduli, 3–36 MPa). The alignment of collagen fibrils along the machine direction was enhanced depending on the rotating speed of the mandrel (**Figure 3B**).

Malladi's study indicates that the stretching process of extruded collagen gels can be incorporated into the conventional extrusion processes, including wet spinning. The stretching of gels was effective for enhancing the alignments of collagen fibrils, whereas fibrils extruded in the gels were almost amorphous. A parameter $V^* = (Vp - VT)/VT$ was used, where Vp is the velocity of the rotating mandrel and VT is the total bulk velocity of the solutions passing through the flow constriction. The elastic moduli increased as V^* increased from 0.1 to 10; this was explained by the fibril density and degree of fibril alignment increase. As per the authors' experience, collagen fibrillar gels are less stretchable. The excellent stretchability in this case ($V^* \leq 10$) could be due to the use of acid-solubilized rat tail tendon collagen [77] with intact intermolecular crosslinking. The type of collagen used in the experiment also affects the molding propriety.

4.3 Other extrusion methods

Lai et al. reported a fabrication method for cord-like collagen gels with longitudinally aligned fibrils effectively using shear force compared with a conventional wet spinning [79]. This method would result in fibrillogenesis [13, 14] before the relaxation of the shear force-induced orientation of the collagen molecules. An acidic collagen solution of rat tail tendon collagen (30 mg/mL) was continuously extruded from a syringe with a 22-gage needle onto a glass slide and submerged in a coagulation bath of 10× PBS. In this process, the syringe and glass slides were moved in opposite directions, thus generating shear forces on the extruded collagen solution, which immediately initiated fibrillogenesis while maintaining alignments of collagen molecules due to the solution's high viscosity, resulting in a cord-like collagen gel with aligned fibrils. When the human dermal microvascular endothelial cells were cultured on the gels, the cells exhibited elongated morphologies along the alignment direction of fibrils.

A method for producing edible collagen casings, that is, artificial intestine for sausage, [80] has been applied for manufacturing tubular gels comprising aligned collagen fibrils through a counter-rotating extrusion method [81, 82]. The experimental setting and appearance of the material obtained are shown in **Figure 4** [81]. This method does not include collagen fibrillogenesis but uses a fibril-rich collagen dough made from living tissues as a starting substrate. Briefly, the homogenized collagen dough (5% [w/v]) was fed to a metering pump and then into a counter-rotating extruder using a piston stuffer. This unique extruder comprises two coaxial cylinders rotating in the opposite direction. The collagen dough is continuously introduced into the gap (0.5 mm) between the larger and smaller cylinders along the axes of the cylinders so that the rotation in the opposite direction generates a shear force on the collagen dough in the gap. Consequently, tube-shaped collagen gels are extruded in which the collagen fibrils are preferably aligned in the circumferential direction. Thus, the tubes must be cut in the circumferential direction to fabricate an artificial tendon with longitudinally aligned fibrils.

The solution extrusion methods are summarized as follows: collagen molecules can be oriented using shear force in a narrow channel, resulting in the production of cord-like collagen gels with nearly amorphous fibrils. This is probably due to the immediate relaxation of the molecules after extrusion from the tips of the channels. Additional mechanical stretching is required to improve the alignment. Thus, suppression of molecular relaxation appears to be effective for fabricating collagen gels with longitudinally aligned fibrils. The use of collagen fibril dough as starting substances or the sequential stretching of gels is also effective.

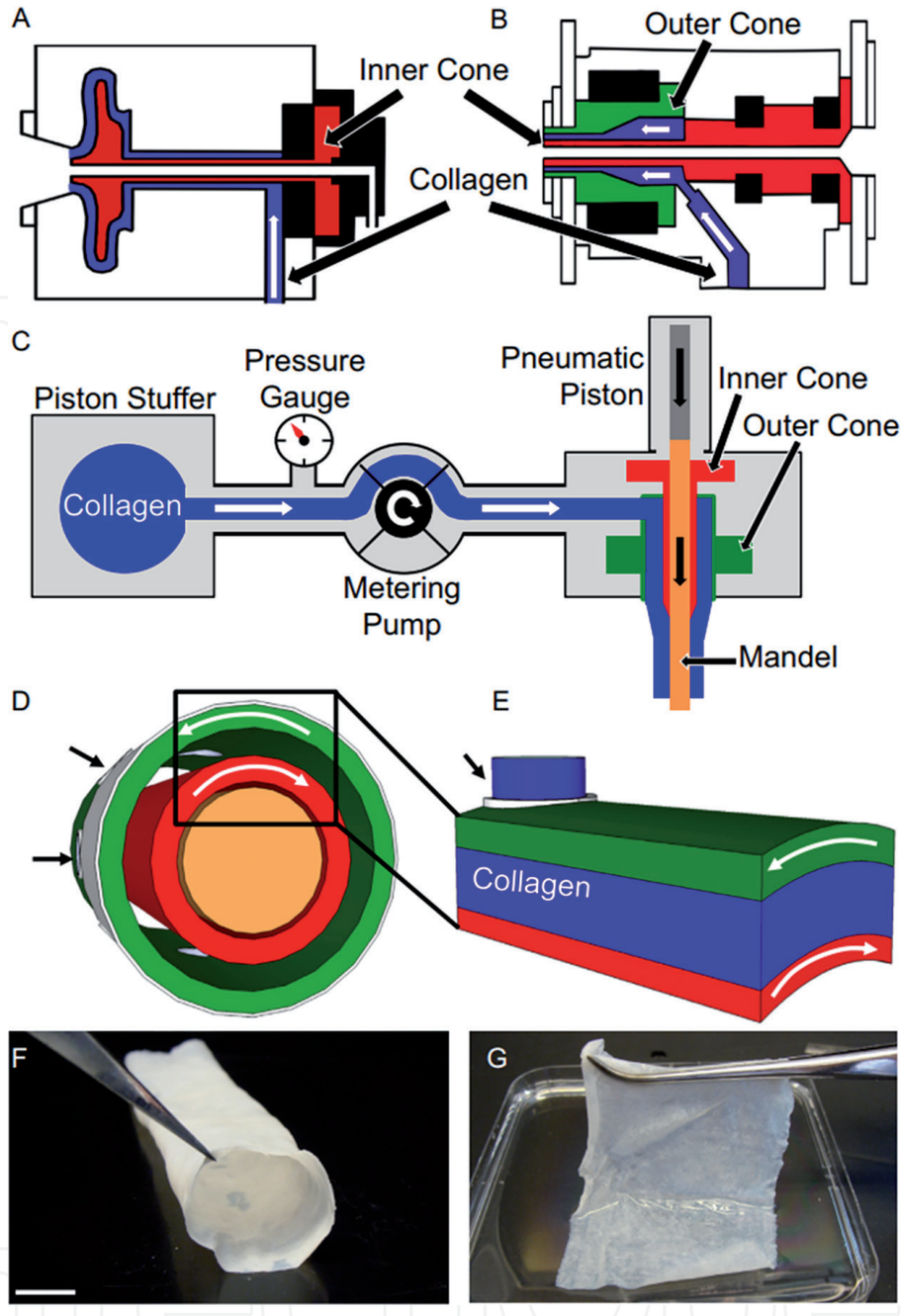

Figure 4.
Overview of counter-rotating extrusion method. (A–E) Schematic illustrations of the method. (F and G) Appearances of tube-shaped collagen gels obtained using this method. Reproduced from Ref. [81] with permission from Elsevier.

4.4 Limitations of solution extrusion methods

As described in Section 4.1, wet spinning has a limited capability of producing threads with well-aligned collagen fibrils, especially in the interior of threads. Although the mechanisms of solution extrusion have not been described in detail compared with those of shear flow deposition, it is obvious that rheological features of collagen solutions play a predominant role in the alignment of collagen fibrils. Here, rheological data of collagen solutions are introduced in the next paragraph to discuss the presumed mechanisms of solution extrusion.

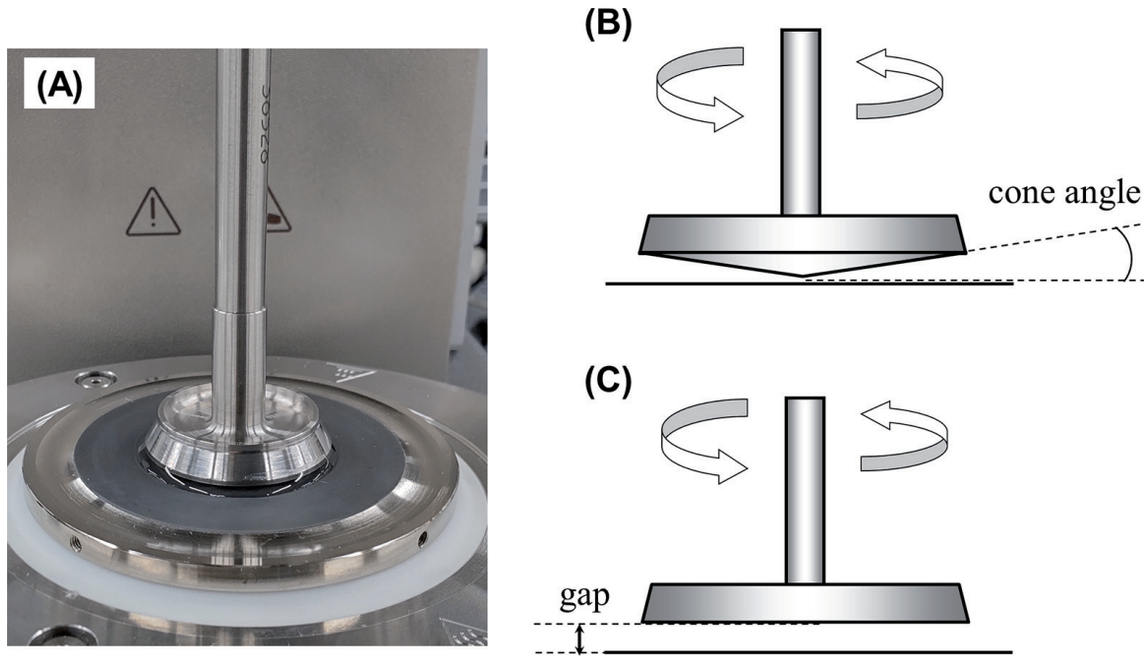

Figure 5.
Sensors of the rotational rheometer. (A) Appearance of a parallel plate sensor. (B) and (C) are schematic illustrations of cone plate and parallel plate sensor, respectively. A sample solution is placed on the Peltier-controlled bottom plate and the movable upper sensor is positioned to achieve a pre-set gap. The sensor is rotated unidirectionally to obtain rotational measurements. Oscillational measurements are obtained by sinusoidal oscillation with extremely small shear deformation (usually ≤1%).

For the rheological measurements, a rotational rheometer was used (MCR 502; Anton Paar, Ostfildern, Germany). This apparatus is effective for simultaneously evaluating the viscosity and gelation features of low viscous biopolymer solutions [83]. A collagen solution was filled in a gap between a Peltier-controlled bottom plate and a movable upper sensor (cone plate sensor, diameter, 35 mm; cone angle, 1°; parallel plate sensor, diameter, 50 mm) (**Figure 5**). This apparatus can conduct rotational as well as oscillational measurements. Rotational measurements measure the flow and viscosity curves of the specimen, providing information about reductions in increased shear stress (shear thinning) and thixotropic properties under shearing. Conversely, oscillational measurements are helpful in tracking the changing rheological properties of a collagen solution (in this case, recovery of rheological properties just after shearing). Two types of collagen were used, acid-solubilized collagen from the porcine tendon (designated ASC) (Cellmatrix® type I-A; 0.3% solution, Nitta Gelatin, Osaka, Japan) and pepsin-digested collagen from the porcine dermis (designated PC) (Collagen BM; 0.53% solution, Nitta Gelatin, Osaka, Japan). ASC remains intermolecular crosslinking, and the physicochemical qualities can be considered as similar to those of a conventional rat tail tendon collagen. PC is a representative of pepsin-digested collagens which are generally used for commercial biomedical devices.

Rotational measurements simulated the behaviors of collagen molecules during wet spinning processes. **Figure 6** presents the viscosity curves of collagen solutions obtained by reciprocal rotational measurements at shear rates 0.1–100 s^{-1}. Both the collagen solutions showed a shear rate-dependent decrease in viscosities (non-Newtonian behavior) during the shear rate-rising process, suggesting molecular alignments along the flow direction. The viscosity curves obtained from the falling of shear rates overlapped almost entirely in both the collagens, suggesting that the alignments of collagen molecules under shearing are not hysteresis. A sequential

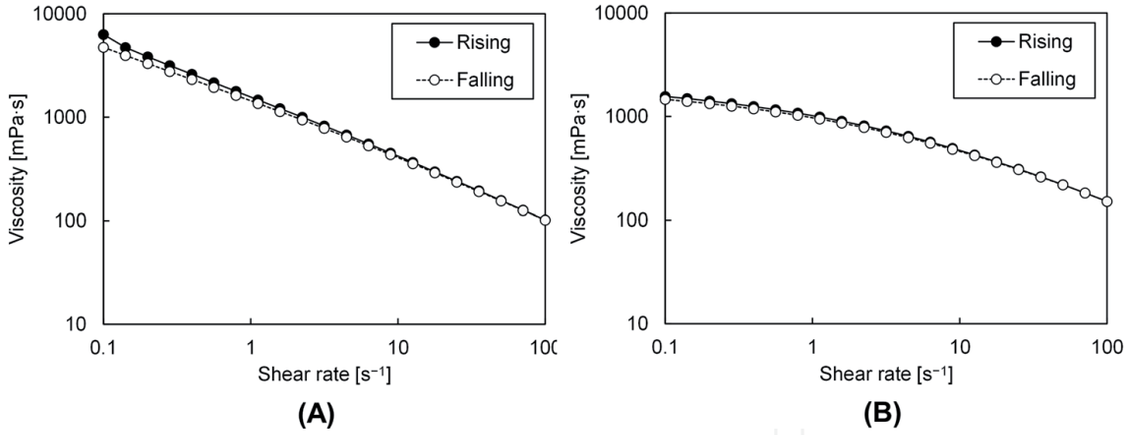

Figure 6.
Viscosity curves of collagen solutions were obtained by reciprocal rotational measurements at shear rates of 0.1–100 s^{-1}. (A) Acid-solubilized collagen from porcine tendon (0.3%) and (B) pepsin-digested collagen from porcine dermis (0.53%). Both the solutions showed a shear rate-dependent decrease in viscosities (non-Newtonian behavior) during the shear rate-rising process. The viscosity curves obtained from the falling of shear rates overlapped almost entirely in both the collagens.

test of oscillation-rotation-oscillation was used (**Figure 7**) to simulate conditions of collagen molecules in wet spinning. The first step is the oscillational measurement at constant shear deformation (1%) and frequency (1 Hz) to test the viscoelastic qualities of the collagen solution as a starting substance wherein collagen molecules are dispersed amorphously. The rapid rotation (shear rate, 100 s^{-1}) as the second step

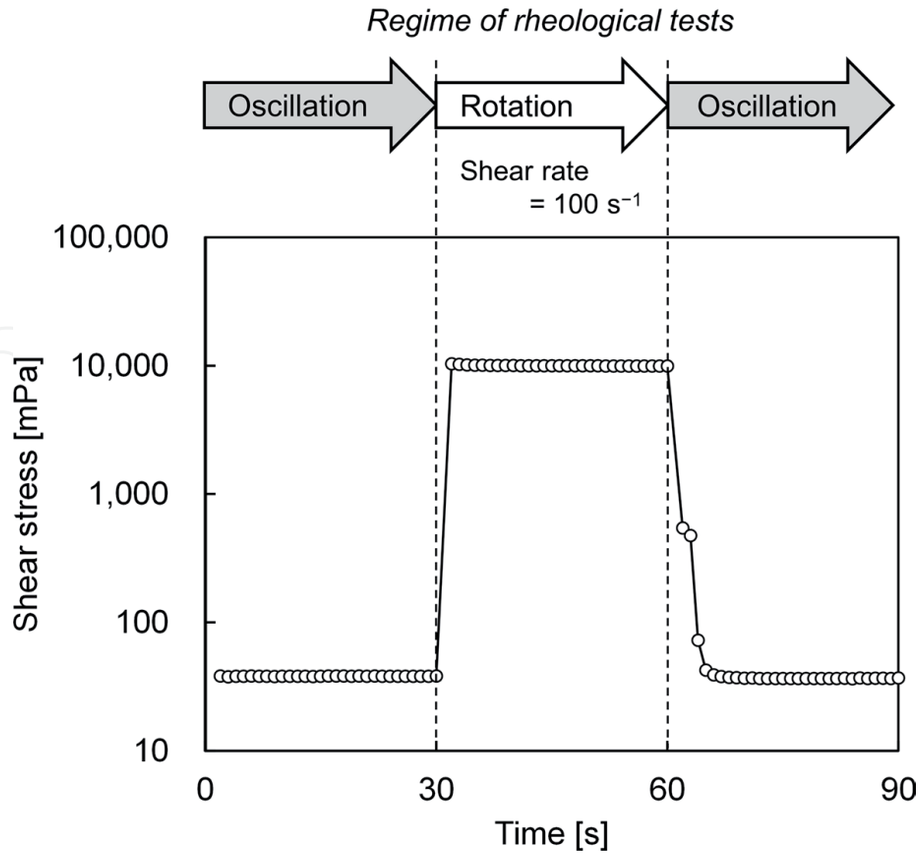

Figure 7.
Scheme and results of sequential testing of oscillation–rotation–oscillation for evaluating the relaxation of collagen molecules.

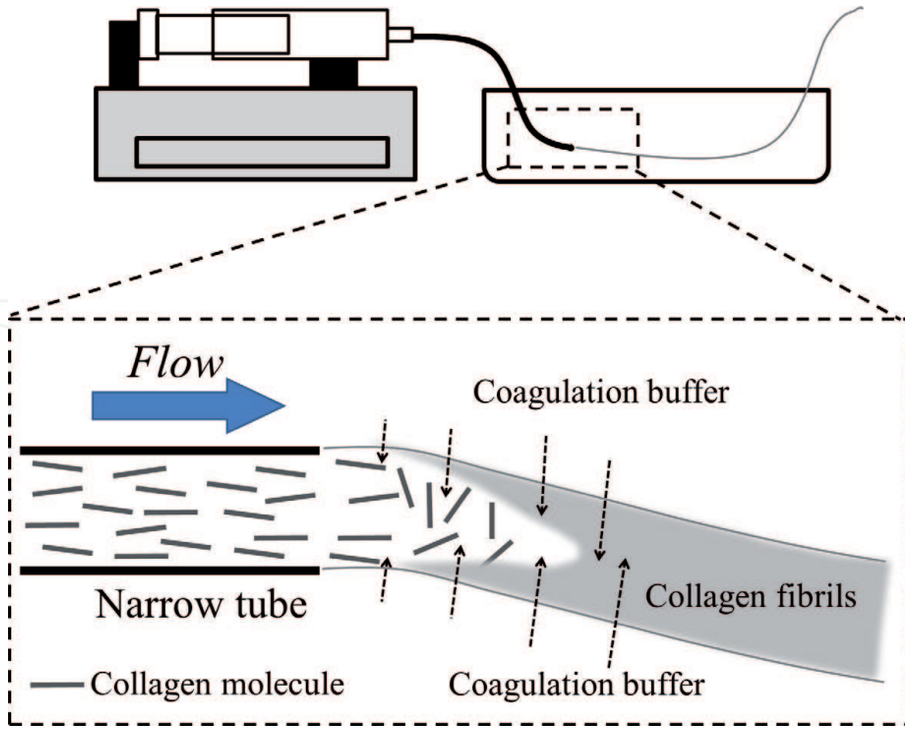

Figure 8.
Schematic illustration of a conceivable scenario in the thread-making process of wet spinning. An acidic collagen solution is extruded through a narrow tube, in which collagen molecules should be oriented along the flow direction. The stream of the viscous collagen solution extruded from the tip of the tube should immediately coagulate to form fibrils from the surface layer. If the coagulant penetrates the stream of the collagen solution before the molecular orientation is relaxed, the collagen fibrils are aligned. However, the alignments of collagen molecules would be immediately relaxed and become amorphous.

simulates strong shearing on the collagen solution introduced into a narrow tube. Oscillational measurement as the last step monitors the recovery of shear stress after the collagen solution is released from the strong shearing, which can simulate the recovery of amorphous dispersion of collagen molecules just after extrusion into a coagulation bath. **Figure 7** indicates the results of the sequential test for ASC. The shear stress sharply decreased by more than one order of magnitude (from 9940 mPa to 540 mPa) only in 2 s after the rotation was terminated and subsequently became identical to that obtained at the first step (before rotational shearing). The small delay in the recovery of shear stress could be due to the inertial force of the flowing collagen solution.

Considering this rapid recovery of shear stress and no hysteresis of viscosity curves, the following scenario is conceivable in the thread-making process of wet spinning (**Figure 8**). An acidic collagen solution is extruded through a narrow tube wherein the collagen molecules should be oriented preferably along the flow direction, as proposed from the non-Newtonian behavior of an acidic collagen solution (**Figure 6**). The stream of the viscous collagen solution extruded from the tip of the tube should immediately coagulate to form fibrils on the surface layer. If the coagulant penetrates the stream of the collagen solution before the molecular orientation is relaxed, the collagen fibrils are aligned. However, the alignment of collagen molecules with an approximate molecular weight of 300,000 will be immediately relaxed and become amorphous, as suggested by the stress-relaxation curves of an acidic collagen solution (**Figure 7**). The above scenario somewhat explains the mechanism of collagen fibril alignments in the solution extrusion methods.

5. Other mechanical methods

The previous paragraph described wet spinning and other solution extrusion methods derived from wet spinning. In the last decade, unique mechanical methods were newly developed to fabricate biomaterials with aligned collagen fibrils. Herein, the fabrication mechanism, effectiveness, and challenges of other mechanical methods (shear flow deposition, flow-induced crystallization, and gel-extrusion method) are discussed.

5.1 Shear flow deposition

This section focuses on the methods of applying shear force during collagen fibrillogenesis, called shear flow deposition. When some part of a collagen fibril is anchored onto a substrate under a strong shear flow, the fibrils are aligned in the direction of flow. This investigation is conducted using a thin collagen solution with low viscosity and a thin flow channel to induce a uniform and fast flow.

In 2009, Saeidi et al. reported the effects of shear rates on fibril alignments in the shear flow deposition using a microfluidic shear flow chamber [84], which can generate a wide range of shear rates. They examined the detailed dynamics of neutralized pepsin-extracted type I collagen assembly on a glass surface under the influence of shear flow between two plates. Differential interference contrast imaging with focal plane stabilization was used to resolve and track the growth of collagen aggregates on borosilicate glass under various shear rates (500, 80, 20, and 9 s^{-1}). The nucleation of fibrils on the glass was observed to occur rapidly (~2 min) followed by the continued growth of the fibrils. The best alignment of fibrils was observed at intermediate shear rates of 20 and 80 s^{-1}, whereas the growth rates were affected by the shear rate in a complex manner. However, the investigation showed that directional fibril growth was not stable and the fibrils would often turn downstream, forming "hooks" at high shear rates.

In Saeidi's next study [85], a spin-coating technique was combined with a flow of collagen solution to produce highly aligned arrays of collagen fibrils. A chilled neutral collagen solution was introduced into the center of the spin coater, which was heated to initiate collagen fibrillogenesis. Orthogonal collagen lamellae were successfully fabricated on the coater depending on shear rates (181–2480 s^{-1}), which were adjusted by flow rates (0.1–1 mL/min) and rotation speeds (750–3000 rpm). It was possible to produce small sections (1 cm^2) of collagen fibrils with enough alignment to guide fibroblasts. However, thin-film instabilities on the coater are likely to be a significant barrier to manufacturing organized collagen fibrils over larger areas.

The effects of planar substrates with collagen-binding features on shear flow deposition were evaluated by Lanfer et al. [86]. They used a microfluidic channel system with coverslip substrates coated with poly(octadecene-alt-maleic acid) (POMA), which could bind collagen fibrils. The aligned collagen fibrils were successfully deposited on the substrates, where the degree of collagen fibril alignment increased with increasing flow rates of the solution. The matrix density increased at higher collagen solution concentrations and on hydrophobic polymer pre-coatings.

The shear flow deposition can deposit well-aligned fibrils on substrates, thus providing some insights into the fabrication conditions for achieving tendon-like collagen fibrillar gels. However, there is a limitation to fabricating thick and long products of aligned collagen fibrils. Collagen fibrils can be anchored directly to substrates at the beginning of the fabrication, promoting fibril alignments along the shear flow direction. However, in the following steps, collagen fibrils cannot be deposited due to the lack of binding features between collagen fibrils. Shear flow deposition methods

are likely to be helpful in fabricating cell culture substrates rather than therapeutic biomaterials, such as artificial tendons, to investigate the effects of collagen anisotropy on the biological behaviors of living cells [87, 88].

5.2 Flow-induced crystallization

Before describing flow-induced crystallization, the capacity of collagen molecules to form liquid crystalline should be described. At a molecular level, acid-soluble collagen molecules spontaneously assemble into precholesteric-banded patterns and cholesteric phases at concentrations above 50 mg/mL [89]. Stabilization of the liquid crystalline collagen, induced by pH modification and resultant fibrillogenesis, indicates characteristic morphologies of collagen fibril arrays in bone tissues. Furthermore, a dense gel (18 wt%) prepared by self-reassembly of collagen molecules *in vitro* shows characteristic bundles of cross-striated fibrils observed in the tendon. The qualities of collagen molecules imply that the formation of liquid crystalline at high concentrations is a key factor for manufacturing bundles of uniaxially aligned collagen fibrils.

In 2016, Paten et al. developed a novel fabrication method called flow-induced crystallization through which dense collagen molecules were microfluidically drawn to form a fiber of uniaxially aligned fibrils [90]. **Figure 9A** presents the schematic illustration of the fiber-making process. Briefly, a droplet of neutralized collagen solution was set under a flow of dry nitrogen gas, facilitating evaporation of water from the droplet surface and the formation of an enriched monomeric surface. A glass microneedle was used to pierce the droplet surface, and the dense collagen solution adhered to the tip of the needle. When the needle was drawn back to attain a low strain rate ($< 1\ s^{-1}$), the surface collagen solution was pulled up to form a thread. In this processing, flow-induced crystallization and mechanical tension-induced fibril alignment could occur. Finally, a narrow fiber as a highly aligned collagen fibrillar array was created (**Figure 9B**).

Although the flow-induced crystallization method is still a form of microfluidic examination, each event in the processing provides us with ideas for creating uniaxially aligned collagen fibrils. When a dense collagen solution with the ability to form liquid crystalline is exposed to strong shearing or tension, the collagen molecules could be ready for uniaxial fibrillogenesis. Therefore, we have to consider the possibility of the continuous heating of the dense collagen solution under strong shearing or tension resulting in uniaxial fibrillogenesis. It is expected that a continuous fabrication of a thread of uniaxially aligned collagen fibrils is developed and scaled up based on the processing of Paten et al.

5.3 Gel-extrusion method

The last mechanical method for aligned collagen fibrils is the gel extrusion recently developed by the authors' group. This method can continuously fabricate cord-like collagen fibrillar gels by incorporating the advantages of the solution extrusion method and shear flow deposition. Those are continuous extrusion of collagen solution under shearing and simultaneous stretching of fibrils by shear force.

First, we evaluated the phenomenon caused by applying shear stress to collagen during fibrillogenesis using a rotational rheometer as a measuring device and a sample fabrication device [91]. A neutral collagen solution was filled in a gap between a Peltier-controlled bottom plate and a movable upper sensor (parallel plate sensor: diameter 60 mm). Fibrillogenesis under shearing occurred by increasing the

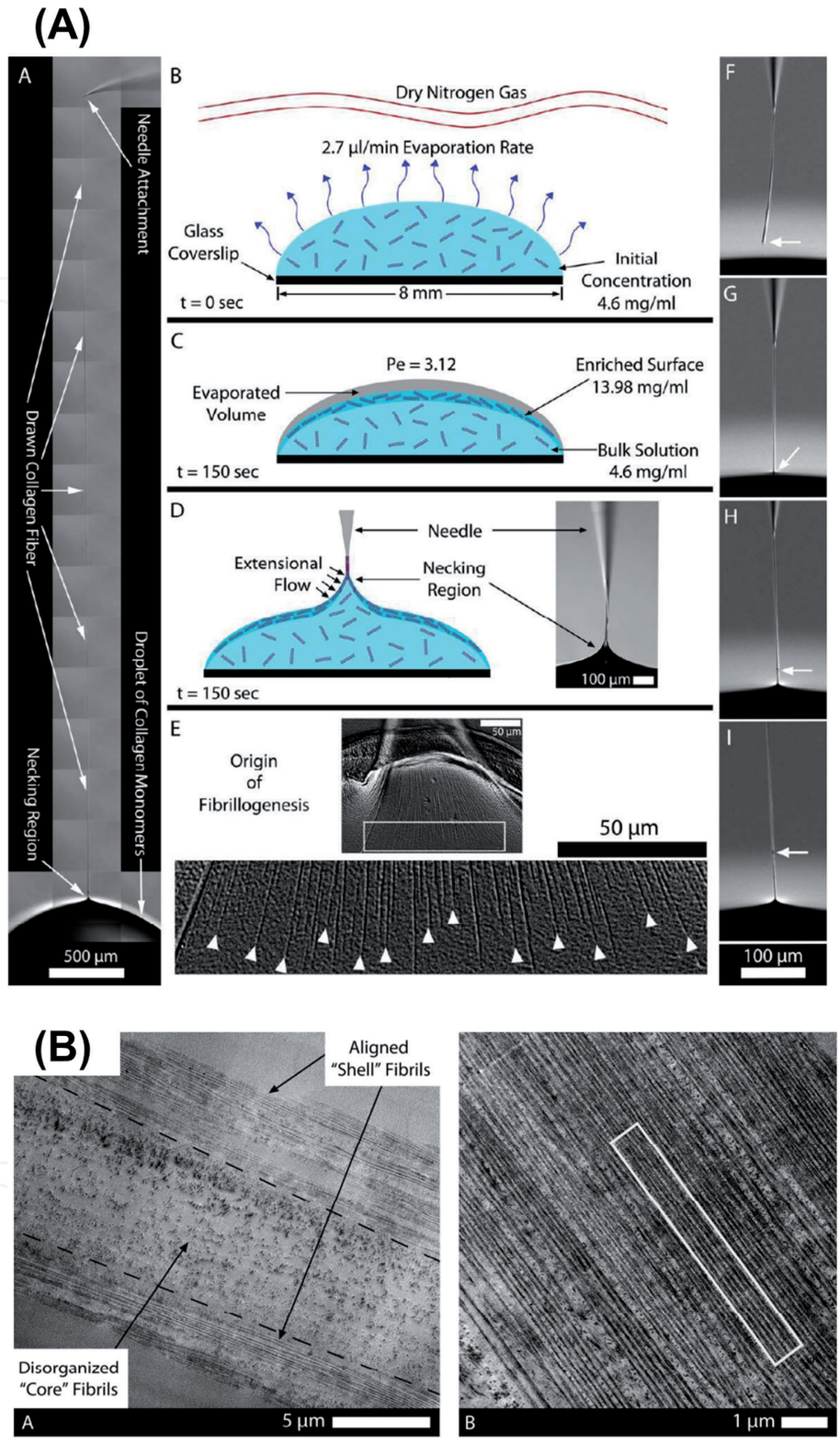

Figure 9.
Overview of flow-induced crystallization technique. (A) Schematic illustration and appearances of the fiber-making process. (B) Transmission electron microscopy images of the collagen fiber. Reproduced from Ref. [90] with permission from Elsevier.

temperature of the bottom plate (from 23 to 37°C) during rotation of the upper sensor. Wide ranges of collagen concentrations (0.1–2 wt %) and shear rates (0.1–500 s^{-1}) were preliminarily examined, but the gels were destroyed completely between the plate and sensor. The most crucial factor for successfully preparing gels under those

conditions was the rate of fibrillogenesis gelation. Increased concentrations of neutral phosphate buffer could accelerate the gelation rate, and fibril alignment occurred within 20 s during the early stage of rapid gelation. Fabrication of gels was completed with slippage between gels and the movable upper plate, and well-aligned fibrils along the rotation direction were observed in the marginal regions of disk-shaped gels. Gel thickness could be increased from 1 mm to 3 mm with the homogeneous alignment of fibrils in the entire sample. The alignment of fibrils enhanced mechanical qualities against tensile loads placed parallel to the alignment axis. The elongation of cultured fibroblasts along the alignment was observed on the gels.

Next, a continuous formation method of cord-like collagen gels comprising fibrils preferentially aligned along the geometrical axes (CCGs) was developed by transferring the events on a rotational rheometer to those in a stainless tube [92]. The experimental setting was simple (**Figure 10A**). Collagen (2.5%) dissolved in a sodium phosphate buffer containing 280 mM sodium chloride was introduced into a stainless cylinder (length 52 mm, diameter 2.0 mm) heated to 38°C at a linear velocity of 2.5 mm s^{-1}. This process caused collagen fibril alignments under acute fibril formation in the cylinder, causing the continuous formation of CCGs (**Figure 10B**). Fibril formation rate, shear rate, and shear duration were substantial factors for successful CCG formation. Advantages of this method over conventional wet spinning include the capacity of this method to form aligned fibrils in the entire gels and to control the diameter of cord-like gels over 1 mm (**Figure 10C-10F**). The air-drying of CCGs, which were cross-linked with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and N-hydroxy-succinimide, produced dry collagen fibers with cross-sectional areas of 0.0123–0.135 mm^2 (**Figure 10G**). Upon the rewetting of the fibers, they failed at a stress of 54.5 ± 7.8 MPa, which is higher than the mean failure stress of ACL tissue (13.3–37.8 MPa). These findings show that the CCG formation method enables the fabrication of collagen fibers, which are potential components of collagen-based artificial tendons.

A limitation of the gel-extrusion method is the incomplete alignment of collagen fibrils along the geometrical axes, especially in the core region of the gel. The mechanism of fibril alignment could explain this heterogeneity, the process of alignment

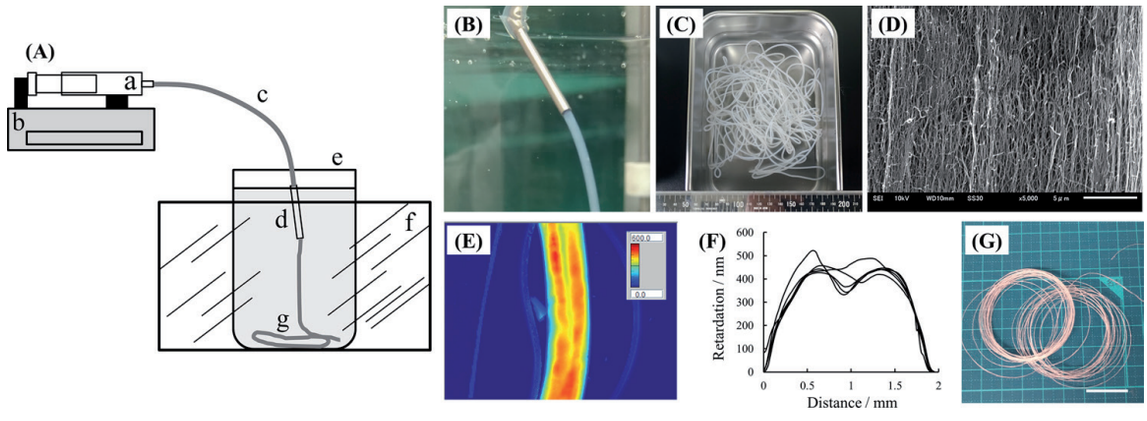

Figure 10.
Overview of the gel-extrusion method. (A) Schematic of the experimental setting. Collagen solution in a syringe (a) was loaded in a syringe pump (b) and infused via silicone tubing (c) into a stainless cylinder (d), which was immersed in a neutral buffer in a glass beaker (e) heated at 38°C in a water bath (f). A cord-like collagen gel was continuously extruded from the cylinder and accumulated on the bottom of the glass beaker (g). (B and C) A 2.0 mm diameter stainless steel cylinder during the processing and the stacked gels within. Longitudinal cross-sectional scanning electron microscopy images of the gel. Bar in figure indicates 5 μm. (E) Two-dimensional birefringence images of the gel. (F) Retardation of the gel across the perpendicular direction. (G) Appearance of dry fibers obtained from the cord-like gel. Bar in the figure indicates 20 mm.

of collagen fibrils involved their formation and then their immediate stretching by shear stress. The entanglement points act as anchors for stretching fibrils. The lengths of fibrils between entanglements were unequal, resulting in more and less stretched fibrils at a certain shear deformation. The stainless tube was heated in a water bath, causing a slower temperature elevation rate in the core region.

6. Conclusions

In conclusion, the mechanical methods for creating aligned collagen-based biomaterials are summarized. Previous attempts to fabricate uniaxially aligned fibrils have used electrospinning, freeze drying, strong magnetic field, electrochemical methods, along with mechanical methods, including shearing and tension during wet extrusion. Among the various fabrication methods, mechanical methods have been extensively studied because of their simplicity and effectiveness along with suitability for mass production. Mechanical methods can be generally divided into the following four methods depending on their fabrication mechanisms: solution extrusion methods (wet spinning and others), shear flow deposition, flow-induced crystallization, and gel-extrusion method. Solution extrusion methods can continuously mold cord-like collagen gels, from which collagen threads are prepared by air-drying. However, collagen fibrils in wet spun threads were amorphous, thus additional stretching of the threads is required to promote fibril alignments. The lack of fibril alignments is probably due to the immediate relaxation of the oriented molecules after the extrusion of collagen solutions. Additional mechanical stretching of gels or threads and delay of molecular relaxation in collagen solutions are effective to promote collagen fibril alignments. The use of collagen fibril dough as starting substance is also effective.

Shear flow deposition can deposit well-aligned fibrils on substrates. However, there is a limitation in fabricating thick and long products of aligned collagen fibrils. Collagen fibrils can be anchored directly to substrates at the beginning of the fabrication, promoting fibril alignments along the shear flow direction. But in the following steps, collagen fibrils cannot be deposited due to the lack of binding features between collagen fibrils.

Flow-induced crystallization is still a kind of microfluidic examination, combined with liquid crystallization of dense collagen solutions. This method can produce ultrathin threads of uniaxially aligned collagen fibrils. However, the production is not continuous because the starting substance is a partially dried surface of a droplet of collagen solution. It is expected that a continuous fabrication of collagen threads is developed and scaled up based on the processing of flow-induced crystallization.

The gel-extrusion method is a continuous formation method of cord-like collagen gels composed of fibrils preferably aligned along the geometrical axes in the entire gels. The feature of this method is the use of neutralized collagen sol, where the temperature-responsive fibrillogenesis is accelerated. The collagen sol is introduced into a heated channel where it can form fibrillar gels. The fibrils are aligned by shear force and stretching. A limitation of the gel-extrusion method is the incomplete alignment of collagen fibrils along the geometrical axes, especially in the core region of the gel.

Mechanical methods have recently made rapid progress. However, each of the methods cannot create artificial tendons with hierarchical structures of uniaxially aligned collagen fibrils with a capacity to undergo remodeling in the living body after implantation similar to autogenous tendon tissues. It is still challenging for biomaterial engineering to satisfy excellent mechanical and biological features. There are two promising approaches for creating an ideal collagen-based artificial tendon,

bottom-up and top-down approaches. The bottom-up approach is the creation of collagen fibers similar in size to the collagen fibers of the living tendon, followed by making them into a tight bundle (not a simple twist string). In contrast, the top-down approach is the longitudinal fragmentation of a large bundle of uniaxially aligned collagen fibrils to allow infiltration of extrinsic cells.

Recently, the performance of decellularized tendons for ACL reconstruction has been evaluated *in vivo* [93, 94]. Although there are some challenges including unevenness of material qualities, residual sources of infection, and production costs, excellent mechanical features and collagen structures similar to living tissues are suitable for ACL reconstruction. The differences between collagen-based artificial tendons and decellularized tendons should be considered in biomaterial developments.

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Conflict of interest

The authors declare that they have no conflict of interest.

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References

[1] Yannas IV, Burke JF. Design of an artificial skin. I. Basic design principles. Journal of Biomedical Materials Research. 1980;**14**:65-81. DOI: 10.1002/jbm.820140108

[2] Yannas IV, Burke JF, Gordon PL, Huang C, Rubenstein RH. Design of an artificial skin. II. Control of chemical composition. Journal of Biomedical Materials Research. 1980;**14**:107-132. DOI: 10.1002/jbm.820140203

[3] Friess W. Collagen -biomaterial for drug delivery. European Journal of Pharmaceutics and Biopharmaceutics. 1998;**45**:113-136. DOI: 10.1016/s0939-6411(98)00017-4

[4] Lee CH, Singla A, Lee Y. Biomedical applications of collagen. International Journal of Pharmaceutics. 2001;**221**(1-2):1-22. DOI: 10.1016/s0378-5173(01)00691-3

[5] Liu X, Zheng C, Luo X, Wang X, Jiang H. Recent advances of collagen-based biomaterials: Multi-hierarchical structure, modification and biomedical applications. Materials Science & Engineering. C, Materials for Biological Applications. 2019;**99**:1509-1522. DOI: 10.1016/j.msec.2019.02.070

[6] Lin K, Zhang D, Macedo MH, Cui W, Sarmento B, Shen G. Advanced collagen-based biomaterials for regenerative biomedicine. Advanced Functional Materials. 2019;**29**:1804943. DOI: 10.1002/adfm.201804943

[7] Ruszczak Z. Effect of collagen matrices on dermal wound healing. Advanced Drug Delivery Reviews. 2003;**55**:1595-1611. DOI: 10.1016/j.addr.2003.08.003

[8] Ferreira AM, Gentile P, Chiono V, Ciardelli G. Collagen for bone tissue regeneration. Acta Biomaterialia. 2012;**8**(9):3191-3200. DOI: 10.1016/j.actbio.2012.06.014

[9] Responte DJ, Natoli RM, Athanasiou KA. Collagens of articular cartilage: Structure, function, and importance in tissue engineering. Critical Reviews in Biomedical Engineering. 2007;**35**(5):363-411. DOI: 10.1615/critrevbiomedeng.v35.i5.20

[10] Fratzl P. Collagen: Structure and mechanics, an introduction. In: Fratzl P, editor. Collagen Structure and Mechanics. Boston, MA: Springer; 2011. pp. 1-14. DOI: 10.1007/978-0-387-73906-9_1

[11] Silver FH, Freeman JW, Seehra GP. Collagen self-assembly and the development of tendon mechanical properties. Journal of Biomechanics. 2003;**36**(10):1529-1553. DOI: 10.1016/s0021-9290(03)00135-0

[12] Biewener AA. Tendons and ligaments: Structure, mechanical behavior and biological function. In: Fratzl P, editor. Collagen Structure and Mechanics. Boston, MA: Springer; 2011. pp. 269-284. DOI: 10.1007/978-0-387-73906-9_10

[13] Williams BR, Gelman RA, Poppke DC, Piez KA. Collagen fibril formation. Optimal in vitro conditions and preliminary kinetic results. The Journal of Biological Chemistry. 1978;**253**:6578-6585

[14] Harris JR, Reiber A. Influence of saline and pH on collagen type I fibrillogenesis in vitro: Fibril polymorphism and colloidal gold labelling. Micron. 2007;**38**:513-521. DOI: 10.1016/j.micron.2006.07.026

[15] Dewle A, Pathak N, Rakshasmare P, Srivastava A. Multifarious fabrication approaches of producing aligned collagen scaffolds for tissue engineering applications. ACS Biomaterials Science & Engineering. 2020;**6**:779-797. DOI: 10.1021/acsbiomaterials.9b01225

[16] No YJ, Castilho M, Ramaswamy Y, Zreiqat H. Role of biomaterials and controlled architecture on tendon/ligament repair and regeneration. Advanced Materials. 2020;**32**:e1904511. DOI: 10.1002/adma.201904511

[17] Ma C, Wang H, Chi Y, Wang Y, Jiang L, Xu N, et al. Preparation of oriented collagen fiber scaffolds and its application in bone tissue engineering. Applied Materials Today. 2021;**22**:100902. DOI: 10.1016/j.apmt.2020.100902

[18] Benjamin M, Ralphs JR. Tendons and ligaments -an overview. Histology and Histopathology. 1997;**12**:1135-1144. DOI: 10.14670/HH-12.1135

[19] Kew SJ, Gwynne JH, Enea D, Abu-Rub M, Pandit A, Zeugolis D, et al. Regeneration and repair of tendon and ligament tissue using collagen fibre biomaterials. Acta Biomaterialia. 2011;**7**:3237-3247. DOI: 10.1016/j.actbio.2011.06.002

[20] Fratzl P. Collagen: Hierarchical structure and viscoelastic properties of tendon. In: Shewry PR, Tatham AS, Bailey AJ, editors. Elastomeric Proteins: Structures, Biomechanical Properties, and Biological Roles. Vol. 2003. Cambridge, UK: Cambridge University Press; 2003. pp. 175-188

[21] Birch HL. Tendon matrix composition and turnover in relation to functional requirements. International Journal of Experimental Pathology. 2007;**88**:241-248. DOI: 10.1111/j.1365-2613.2007.00552.x

[22] Chandrashekar N, Mansouri H, Slauterbeck J, Hashemi J. Sex-based differences in the tensile properties of the human anterior cruciate ligament. Journal of Biomechanics. 2006;**39**:2943-2950. DOI: 10.1016/j.jbiomech.2005.10.031

[23] Alentorn-Geli E, Mendiguchía J, Samuelsson K, Musahl V, Karlsson J, Cugat R, et al. Prevention of anterior cruciate ligament injuries in sports. Part I: Systematic review of risk factors in male athletes. Knee Surgery, Sports Traumatology, Arthroscopy. 2014;**22**:3-15. DOI: 10.1007/s00167-013-2725-3

[24] Agel J, Arendt EA, Bershadsky B. Anterior cruciate ligament injury in National Collegiate Athletic Association basketball and soccer a 13-year review. The American Journal of Sports Medicine. 2005;**33**:524-531. DOI: 10.1177/0363546504269937

[25] Mall NA, Chalmers PN, Moric M, Tanaka MJ, Cole BJ, Bach BR Jr, et al. Incidence and trends of anterior cruciate ligament reconstruction in the United States. The American Journal of Sports Medicine. 2014;**42**:2363-2370. DOI: 10.1177/0363546514542796

[26] Levy BA, Dajani KA, Morgan JA, Shah JP, Dahm DL, Stuart MJ. Repair versus reconstruction of the fibular collateral ligament and posterolateral corner in the multiligament-injured knee. The American Journal of Sports Medicine. 2010;**38**:804-809. DOI: 10.1177/0363546509352459

[27] Ajrawat P, Dwyer T, Whelan D, Theodoropoulos J, Murnaghan L, Bhargava M, et al. A comparison of quadriceps tendon autograft with bone-patellar tendon-bone autograft and hamstring tendon autograft for primary anterior cruciate ligament reconstruction: A systematic review and

quantitative synthesis. Clinical Journal of Sport Medicine. 2021;**31**:392-399. DOI: 10.1097/JSM.0000000000000765 PMID: 31233432

[28] Pinczewski LA, Lyman J, Salmon LJ, Russell VJ, Roe J, Linklater J. A 10-year comparison of anterior cruciate ligament reconstructions with hamstring tendon and patellar tendon autograft: A controlled, prospective trial. The American Journal of Sports Medicine. 2007;**35**:564-574. DOI: 10.1177/0363546506296042

[29] Janssen RP, van der Wijk J, Fiedler A, Schmidt T, Sala HA, Scheffler SU. Remodelling of human hamstring autografts after anterior cruciate ligament reconstruction. Knee Surgery, Sports Traumatology, Arthroscopy. 2011;**19**:1299-1306. DOI: 10.1007/s00167-011-1419-y

[30] Tohyama H, Yasuda K, Uchida H. Is the increase in type III collagen of the patellar tendon graft after ligament reconstruction really caused by "ligamentization" of the graft? Knee Surgery, Sports Traumatology, Arthroscopy. 2006;**14**:1270-1277. DOI: 10.1007/s00167-006-0092-z

[31] Tohyama H, Yasuda K. Extrinsic cell infiltration and revascularization accelerate mechanical deterioration of the patellar tendon after fibroblast necrosis. Journal of Biomechanical Engineering. 2000;**122**:594-599. DOI: 10.1115/1.1319659

[32] Hardy A, Casabianca L, Andrieu K, Baverel L, Noailles T, Junior French Arthroscopy Society. Complications following harvesting of patellar tendon or hamstring tendon grafts for anterior cruciate ligament reconstruction: Systematic review of literature. Orthopaedics & Traumatology, Surgery & Research. 2017;**103**(8S):S245-S248. DOI: 10.1016/j.otsr.2017.09.002

[33] Murray MM. Current status and potential of primary ACL repair. Clinics in Sports Medicine. 2009;**28**:51-61. DOI: 10.1016/j.csm.2008.08.005

[34] Chen J, Xu J, Wang A, Zheng M. Scaffolds for tendon and ligament repair: Review of the efficacy of commercial products. Expert Review of Medical Devices. 2009;**6**(1):61-73. DOI: 10.1586/17434440.6.1.61

[35] Huang Z-M, Zhang Y-Z, Kotaki M, Ramakrishna S. A review on polymer nanofibers by electrospinning and their applications in nanocomposites. Composites Science and Technology. 2003;**63**:2223-2253. DOI: 10.1016/S0266-3538(03)00178-7

[36] Barnes CP, Sell SA, Boland ED, Simpson DG, Bowlin GL. Nanofiber technology: Designing the next generation of tissue engineering scaffolds. Advanced Drug Delivery Reviews. 2007;**59**:1413-1433. DOI: 10.1016/j.addr.2007.04.022

[37] Zhong S, Teo WE, Zhu X, Beuerman RW, Ramakrishna S, Yung LY. An aligned nanofibrous collagen scaffold by electrospinning and its effects on in vitro fibroblast culture. Journal of Biomedical Materials Research. Part A. 2006;**79**:456-463. DOI: 10.1002/jbm.a.30870

[38] Xu Y, Wu J, Wang H, Li H, Di N, Song L, et al. Fabrication of electrospun poly(L-lactide-co-ε-caprolactone)/collagen nanoyarn network as a novel, three-dimensional, macroporous, aligned scaffold for tendon tissue engineering. Tissue Engineering. Part C, Methods. 2013;**19**(12):925-936. DOI: 10.1089/ten.TEC.2012.0328

[39] Xu Y, Dong S, Zhou Q, Mo X, Song L, Hou T, et al. The effect of mechanical stimulation on the

maturation of TDSCs-poly(L-lactide-co-e-caprolactone)/collagen scaffold constructs for tendon tissue engineering. Biomaterials. 2014;**35**:2760-2772. DOI: 10.1016/j.biomaterials.2013.12.042

[40] Zeugolis DI, Khew ST, Yew ES, Ekaputra AK, Tong YW, Yung LY, et al. Electro-spinning of pure collagen nano-fibres -just an expensive way to make gelatin? Biomaterials. 2008;**29**:2293-2305. DOI: 10.1016/j.biomaterials.2008.02.009

[41] Matthews JA, Wnek GE, Simpson DG, Bowlin GL. Electrospinning of collagen nanofibers. Biomacromolecules. 2002;**3**:232-238. DOI: 10.1021/bm015533u

[42] Ciurzynska A, Lenart A. Freeze-drying-application in food processing and biotechnology—a review. Polish Journal of Food and Nutrition Sciences. 2011;**61**:165-171. DOI: 10.2478/v10222-011-0017-5

[43] Shukla S. Freeze drying process: A review. International Journal of Pharmaceutical Sciences and Research. 2011;**2**:3061-3068

[44] Wegst UG, Schecter M, Donius AE, Hunger PM. Biomaterials by freeze casting. Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences. 1917;**2010**(368):2099-2121. DOI: 10.1098/rsta.2010.0014

[45] Schoof H, Bruns L, Fischer A, Heschel I, Rau G. Dendritic ice morphology in unidirectionally solidified collagen suspensions. Journal of Crystal Growth. 2000;**209**:122-129. DOI: 10.1016/S0022-0248(99)00519-9

[46] Schoof H, Apel J, Heschel I, Rau G. Control of pore structure and size in freeze-dried collagen sponges. Journal of Biomedical Materials Research Part B: Applied Biomaterials. 2001;**58**:352-357. DOI: 10.1002/jbm.1028

[47] Zhang H, Cooper AI. Aligned porous structures by directional freezing. Advanced Materials. 2007;**19**:1529-1533. DOI: 10.1002/adma.200700154

[48] Pot MW, Faraj KA, Adawy A, van Enckevort WJ, van Moerkerk HT, Vlieg E, et al. Versatile wedge-based system for the construction of unidirectional collagen scaffolds by directional freezing: Practical and theoretical considerations. ACS Applied Materials & Interfaces. 2015;**7**:8495-8505. DOI: 10.1021/acsami.5b00169

[49] Zheng Z, Ran J, Chen W, Hu Y, Zhu T, Chen X, et al. Alignment of collagen fiber in knitted silk scaffold for functional massive rotator cuff repair. Acta Biomaterialia. 2017;**51**:317-329. DOI: 10.1016/j.actbio.2017.01.041

[50] Lowe CJ, Reucroft IM, Grota MC, Shreiber DI. Production of highly aligned collagen scaffolds by freeze-drying of self-assembled, fibrillar collagen gels. ACS Biomaterials Science & Engineering. 2016;**2**:643-651. DOI: 10.1021/acsbiomaterials.6b00036

[51] Torbet J, Ronzière MC. Magnetic alignment of collagen during self-assembly. The Biochemical Journal. 1984;**219**(3):1057-0159. DOI: 10.1042/bj2191057

[52] Dickinson RB, Guido S, Tranquillo RT. Biased cell migration of fibroblasts exhibiting contact guidance in oriented collagen gels. Annals of Biomedical Engineering. 1994;**22**:342-356. DOI: 10.1007/BF02368241

[53] Chen S, Hirota N, Okuda M, Takeguchi M, Kobayashi H, Hanagata N, et al. Microstructures and rheological properties of tilapia fish-scale

collagen hydrogels with aligned fibrils fabricated under magnetic fields. Acta Biomaterialia. 2011;**7**:644-652. DOI: 10.1016/j.actbio.2010.09.014

[54] Torbet J, Malbouyres M, Builles N, Justin V, Roulet M, Damour O, et al. Orthogonal scaffold of magnetically aligned collagen lamellae for corneal stroma reconstruction. Biomaterials. 2007;**28**(29):4268-4276. DOI: 10.1016/j.biomaterials.2007.05.024

[55] Sharma S, Panitch A, Neu CP. Incorporation of an aggrecan mimic prevents proteolytic degradation of anisotropic cartilage analogs. Acta Biomaterialia. 2013;**9**:4618-4625. DOI: 10.1016/j.actbio.2012.08.041

[56] Novak T, Voytik-Harbin SL, Neu CP. Cell encapsulation in a magnetically aligned collagen-GAG copolymer microenvironment. Acta Biomaterialia. 2015;**11**:274-282. DOI: 10.1016/j.actbio.2014.09.031

[57] Antman-Passig M, Shefi O. Remote magnetic orientation of 3D collagen hydrogels for directed neuronal regeneration. Nano Letters. 2016;**16**(4):2567-2573. DOI: 10.1021/acs.nanolett.6b00131

[58] Sharma A, DiVito MD, Shore DE, Block AD, Pollock K, Solheid P, et al. Alignment of collagen matrices using magnetic nanowires and magnetic barcode readout using first order reversal curves (FORC). Journal of Magnetism and Magnetic Materials. 2018;**459**:176-181. DOI: 10.1016/j.jmmm.2017.11.035

[59] Taufalele PV, VanderBurgh JA, Muñoz A, Zanotelli MR, Reinhart-King CA. Fiber alignment drives changes in architectural and mechanical features in collagen matrices. PLoS One. 2019;**14**:e0216537. DOI: 10.1371/journal.pone.0216537

[60] Shi Y, Li Y, Coradin T. Magnetically-oriented type I collagen-SiO_2@Fe_3O_4 rods composite hydrogels tuning skin cell growth. Colloids and Surfaces. B, Biointerfaces. 2020;**185**:110597. DOI: 10.1016/j.colsurfb.2019.110597

[61] Cheng X, Gurkan UA, Dehen CJ, Tate MP, Hillhouse HW, Simpson GJ, et al. An electrochemical fabrication process for the assembly of anisotropically oriented collagen bundles. Biomaterials. 2008;**29**(22):3278-3288. DOI: 10.1016/j.biomaterials.2008.04.028

[62] Younesi M, Islam A, Kishore V, Anderson JM, Akkus O. Tenogenic induction of human MSCs by anisotropically aligned collagen biotextiles. Advanced Functional Materials. 2014;**24**:5762-5770. DOI: 10.1002/adfm.201400828

[63] Gurkan UA, Cheng X, Kishore V, Uquillas JA, Akkus O. Comparison of morphology, orientation, and migration of tendon derived fibroblasts and bone marrow stromal cells on electrochemically aligned collagen constructs. Journal of Biomedical Materials Research. Part A. 2010;**94**:1070-1079. DOI: 10.1002/jbm.a.32783

[64] Abu-Rub MT, Billiar KL, Van Es MH, Knight A, Rodriguez BJ, Zeugolis DI, et al. Nano-textured self-assembled aligned collagen hydrogels promote directional neurite guidance and overcome inhibition by myelin associated glycoprotein. Soft Matter. 2011;**7**:2770-2781. DOI: 10.1039/C0SM01062F

[65] Kishore V, Bullock W, Sun X, Van Dyke WS, Akkus O. Tenogenic differentiation of human MSCs induced by the topography of electrochemically aligned collagen threads. Biomaterials. 2012;**33**:2137-2144. DOI: 10.1016/j.biomaterials.2011.11.066

[66] Chen Z, Liu X, You J, Song Y, Tomaskovic-Crook E, Sutton G, et al. Biomimetic corneal stroma using electro-compacted collagen. Acta Biomaterialia. 2020;**113**:360-371. DOI: 10.1016/j.actbio.2020.07.004

[67] Learn GD, McClellan PE, Knapik DM, Cumsky JL, Webster-Wood V, Anderson JM, et al. Woven collagen biotextiles enable mechanically functional rotator cuff tendon regeneration during repair of segmental tendon defects in vivo. Journal of Biomedical Materials Research. Part B, Applied Biomaterials. 2019;**107**:1864-1876. DOI: 10.1002/jbm.b.34279

[68] Kato YP, Christiansen DL, Hahn RA, Shieh SJ, Goldstein JD, Silver FH. Mechanical properties of collagen fibres: A comparison of reconstituted and rat tail tendon fibres. Biomaterials. 1989;**10**:38-42. DOI: 10.1016/0142-9612(89)90007-0

[69] Kew SJ, Gwynne JH, Enea D, Brookes R, Rushton N, Best SM, et al. Synthetic collagen fascicles for the regeneration of tendon tissue. Acta Biomaterialia. 2012;**8**:3723-3731. DOI: 10.1016/j.actbio.2012.06.018

[70] Dunn MG, Avasarala PN, Zawadsky JP. Optimization of extruded collagen fibers for ACL reconstruction. Journal of Biomedical Materials Research. 1993;**27**:1545-1552. DOI: 10.1002/jbm.820271211

[71] Cavallaro JF, Kemp PD, Kraus KH. Collagen fabrics as biomaterials. Biotechnology and Bioengineering. 1994;**43**(8):781-791. DOI: 10.1002/bit.260430813

[72] Siriwardane ML, DeRosa K, Collins G, Pfister BJ. Controlled formation of cross-linked collagen fibers for neural tissue engineering applications. Biofabrication. 2014;**6**:015012. DOI: 10.1088/1758-5082/6/1/015012

[73] Zeugolis DI, Paul RG, Attenburrow G. Post-self-assembly experimentation on extruded collagen fibres for tissue engineering applications. Acta Biomaterialia. 2008;**4**:1646-1656. DOI: 10.1016/j.actbio.2008.05.015

[74] Pins GD, Christiansen DL, Patel R, Silver FH. Self-assembly of collagen fibers. Influence of fibrillar alignment and decorin on mechanical properties. Biophysical Journal. 1997;**73**:2164-2172. DOI: 10.1016/S0006-3495(97)78247-X

[75] Nerger BA, Brun PT, Nelson CM. Microextrusion printing cell-laden networks of type I collagen with patterned fiber alignment and geometry. Soft Matter. 2019;**15**:5728-5738. DOI: 10.1039/c8sm02605j

[76] Lai ES, Anderson CM, Fuller GG. Designing a tubular matrix of oriented collagen fibrils for tissue engineering. Acta Biomaterialia. 2011;**7**:2448-2456. DOI: 10.1016/j.actbio.2011.03.012

[77] Caves JM, Kumar VA, Wen J, Cui W, Martinez A, Apkarian R, et al. Fibrillogenesis in continuously spun synthetic collagen fiber. Journal of Biomedical Materials Research. Part B, Applied Biomaterials. 2010;**93**:24-38. DOI: 10.1002/jbm.b.31555

[78] Malladi S, Miranda-Nieves D, Leng L, Grainger SJ, Tarabanis C, Nesmith AP, et al. Continuous formation of ultrathin, strong collagen sheets with tunable anisotropy and compaction. ACS Biomaterials Science & Engineering. 2020;**6**:4236-4246. DOI: 10.1021/acsbiomaterials.0c00321

[79] Lai ES, Huang NF, Cooke JP, Fuller GG. Aligned nanofibrillar collagen regulates endothelial organization and migration. Regenerative Medicine. 2012;**7**:649-661. DOI: 10.2217/rme.12.48

[80] Ziolko FJ. Method of Making a Tubular Collagen Casing. Somerville,

NJ, USA: Devro Inc.; 1979. Patent No: US4138503.

[81] Hoogenkamp HR, Bakker GJ, Wolf L, Suurs P, Dunnewind B, Barbut S, et al. Directing collagen fibers using counter-rotating cone extrusion. Acta Biomaterialia. 2015;**12**:113-121. DOI: 10.1016/j.actbio.2014.10.012

[82] Yang S, Shi X, Li X, Wang J, Wang Y, Luo Y. Oriented collagen fiber membranes formed through counter-rotating extrusion and their application in tendon regeneration. Biomaterials. 2019;**207**:61-75. DOI: 10.1016/j.biomaterials.2019.03.041

[83] Yunoki S, Sugimoto K, Ohyabu Y, Ida H, Hiraoka Y. Accurate and precise viscosity measurements of gelatin solutions using a rotational rheometer. Food Science and Technology Research. 2019;**25**:217-226. DOI: 10.3136/fstr.25.217

[84] Saeidi N, Sander EA, Ruberti JW. Dynamic shear-influenced collagen self-assembly. Biomaterials. 2009;**30**:6581-6592. DOI: 10.1016/j.biomaterials.2009.07.070

[85] Saeidi N, Sander EA, Zareian R, Ruberti JW. Production of highly aligned collagen lamellae by combining shear force and thin film confinement. Acta Biomaterialia. 2011;**7**:2437-2447. DOI: 10.1016/j.actbio.2011.02.038

[86] Lanfer B, Freudenberg U, Zimmermann R, Stamov D, Körber V, Werner C. Aligned fibrillar collagen matrices obtained by shear flow deposition. Biomaterials. 2008;**29**(28): 3888-3895. DOI: 10.1016/j.biomaterials.2008.06.016

[87] Lanfer B, Seib FP, Freudenberg U, Stamov D, Bley T, Bornhäuser M, et al. The growth and differentiation of mesenchymal stem and progenitor cells cultured on aligned collagen matrices. Biomaterials. 2009;**30**:5950-5958. DOI: 10.1016/j.biomaterials.2009.07.039

[88] Lanfer B, Hermann A, Kirsch M, Freudenberg U, Reuner U, Werner C, et al. Directed growth of adult human white matter stem cell-derived neurons on aligned fibrillar collagen. Tissue Engineering. Part A. 2010;**16**(4):1103-1113. DOI: 10.1089/ten.TEA.2009.0282

[89] Giraud-Guille MM, Besseau L, Martin R. Liquid crystalline assemblies of collagen in bone and in vitro systems. Journal of Biomechanics. 2003;**36**:1571-1579. DOI: 10.1016/s0021-9290(03)00134-9

[90] Paten JA, Siadat SM, Susilo ME, Ismail EN, Stoner JL, Rothstein JP, et al. Flow-induced crystallization of collagen: A potentially critical mechanism in early tissue formation. ACS Nano. 2016;**10**:5027-5040. DOI: 10.1021/acsnano.5b07756

[91] Yunoki S, Hatayama H, Ebisawa M, Kondo E, Yasuda K. A novel fabrication method to create a thick collagen bundle composed of uniaxially aligned fibrils: An essential technology for the development of artificial tendon/ligament matrices. Journal of Biomedical Materials Research. Part A. 2015;**103**:3054-3065. DOI: 10.1002/jbm.a.35440

[92] Yunoki S, Hatayama H, Ebisawa M, Kondo E, Yasuda K. A novel method for continuous formation of cord-like collagen gels to fabricate durable fibers in which collagen fibrils are longitudinally aligned. Journal of Biomedical Materials Research. Part B, Applied Biomaterials. 2019;**107**:1011-1023. DOI: 10.1002/jbm.b.34194

[93] Schulze-Tanzil G, Al-Sadi O, Ertel W, Lohan A. Decellularized tendon

extracellular matrix-a valuable approach for tendon reconstruction? Cell. 2012;**1**:1010-1028. DOI: 10.3390/cells1041010

[94] Li Y, Zhu T, Wang L, Jiang J, Xie G, Huangfu X, et al. Tissue-engineered decellularized allografts for anterior cruciate ligament reconstruction. ACS Biomaterials Science & Engineering. 2020;**6**:5700-5710. DOI: 10.1021/acsbiomaterials.0c00269