Chapter 14
The Other Connective Tissue: Echinoderm Ligaments and Membranes as Decellularized Bioscaffold for Tissue Engineering

Kheng Lim Goh and Yos Morsi

Abstract This chapter examines the sea urchin ligament as a potential decellularized bioscaffold by discussing the significance of collagen fibrils, which are highly-paralleled slender structures embedded in the hydrated proteoglycan-rich (PG) extracellular matrix (ECM) of tendons, for reinforcing the soft connective tissue. The discussion is presented in two parts as follows. Part one examines the role of collagen fibrils for providing structural support for the tissue, in the context of the structure of the collagen fibril, and mechanics of stress transfer in the tissue. Part two will review the potential clinical applications of the decellularized bioscaffold, related to tissue implants for repair and regeneration.

Keywords Collagen fibrils · Taper · Extra-cellular matrix · Fibre composites

14.1 Introduction

Soft connective tissues, such as tendon, ligament and muscle, are biological examples of fibre reinforced composites. They share similar biomechanical functions. For instance, tendon, which connects bone to muscle, transmit the force from muscle to the bone during locomotion; ligament, which connects bone to bone, transmits force from one bone to the other [1]. The extracellular matrix (ECM) of these tissues also share similar structural features, comprising a blend of hydrated macromolecular assemblies of proteins and polysaccharides, collageneous fibrils, elastin fibres [2].

Collagen fibrils are the key components underpinning the reinforcement to the tissue, which is analogous to fibre reinforcement of a composite material [3–5].

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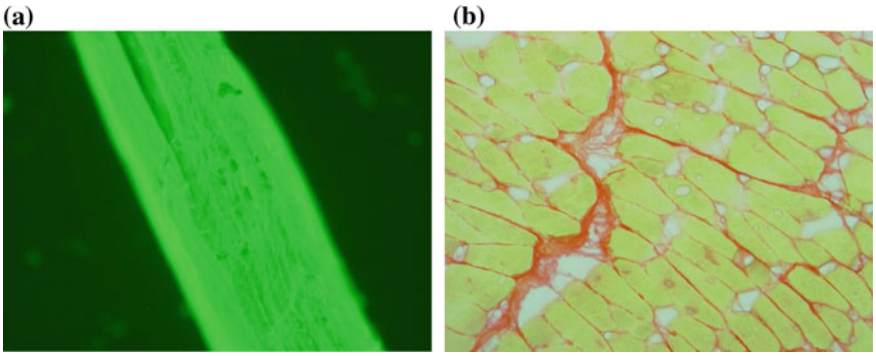


Fig. 14.1 Histology of extracellular matrix in connective tissues from the C57BL6 mouse. **a** Longitudinal section of a tail tendon, **b** cross section of the gastrocnemius muscle tissues

Figure 14.1a shows an image of a tendon under an optical microscope. Within the tendon are numerous lines depicting bundles of highly paralleled collagen fibrils which can only be seen under an electron microscope. When the tendon is loaded in tension, a high proportion of the fibrils will be recruited for force transmission. The direction of the force generated is along the axis of the tendon. Similar highly paralleled collagen fibrils are found in the ligament. In other connective tissues, e.g. muscles (Fig. 14.1b), the collagen fibrils may be less aligned; for muscles, apart from the muscle cells (i.e. fibre-like cells), the tissue also contain collagen fibrils (indicated by the region of red stain) which appears as thin mesh enclosing each muscle fibre-like cell (green in colour).

The proportion of the fibrils that can be easily recruited to carry the load from muscle to bone (in the case of tendons) or bone to bone (in the case of ligaments) depends on the orientation of the fibrils. These descriptions of the collagen fibrils and other ECM components apply to a great many soft connective tissues, including those of sea animals such as echinoderms, which is the subject of our discussion in this chapter.

With regards to the strategy for repairing damaged soft connective tissue in reconstructive surgical procedures, one important consideration is that the repair site must possess a structure that can support load [6]. From a mechanical engineering perspective, this means that such a repair material (known as the scaffold) would have to possess the desired structural and materials properties. In addition to this criterion are a range of other considerations, namely biocompatibility [7], such as immunological compatibility. It must be emphasized that given that there is still a lot of unknowns in the connective tissues, one would expect that the motivation to design and fabricate the material from scratch, such as the much-touted 3D printing approach to tissue engineering, would seem far-fetch. To this end, how can we acquire such a material for assisting tissue repair? One way is to manufacture biomaterials derived from extracts of biological tissues, such as chitosan from the exoskeleton of crus-

taceans [8, 9]. Another way is to harvest like-tissue from another species, followed by decellurizing the tissue to obtain the ECM [6].

There are a number of excellent tissue candidates that one can choose—namely porcine, bovine even human dermal tissues—that are now commercially available for obtaining the ECM for use as scaffold [6]. ECM-based biological scaffold materials from these intact mammalian tissues are applied in in tissue engineering as reported in preclinical studies and in clinical applications [10]. From the perspective of sustainable use of materials, interests in other sources are also being researched. Researchers are turning to marine sources such as echinoderms. These sea creatures are also able to provide collagen for producing ECM scaffold. The strategy is to separate the unwanted from the wanted (favourite edible) parts during harvesting. For instance, one may collect unwanted sea urchin soft connective tissues after the edible parts are removed [11–13].

The focus of this chapter is on the highly paralleled collagen fibrils in ECM of ligaments from the echinoderms. Our argument is developed from the ECM biology and stress transfer mechanisms for tendons (next section). Here, we present an examination of the nature of collagen fibrils, and the laying down of collagen fibrils within the proteoglycan-rich hydrated ECM. This is followed by an exploration of the structure-function implications in relation to a framework for the mechanisms of stress transfer, focussing on how taper in collagen fibrils, through the inter-play between key proteoglycans and collagen macromolecules, influence the tissue to take up stress, as the tissue is subjected to an increasing applied load until it ruptures apart. Then we reviewed how manufacturing methods may influence the structural and mechanical properties of the scaffolds and also highlighted the implications on tissue functionality during in vivo degradation and remodeling of ECM scaffolds [10]. In Sect. 14.3, we discussed the recent clinical applications of collageneous bioscaffold from the echinoderm sources.

14.2 Basis of Structural Support in Soft Connective Tissue

14.2.1 Overview

Echinoderms such as the sea urchin of the Paracentrotus lividus species is an economically important human food source [12]. This species may also be regarded as a potential source of collageneous connective tissue for use as scaffold materials in biomedical engineering. The tissues of particular interest are the compass depressor ligament and peristomial membrane, which are by-products of the food industry [12]. The normal practice is that these tissues are discarded together with the body parts, after the gonad is removed [12]. This section is intended to discuss the fundamentals relating to structure and mechanical properties of collageneous connective tissues that have lend to our understanding of how the echinoderm connective tissue (e.g.

compass depressor ligament and peristomial membrane) can be processed into useful biomaterials for making bioscaffolds for tissue engineering.

14.2.2 Structure of Collagen Fibril

Collagen are protein molecules with a basic structure comprising three tightly bound helical polypeptide chains, and an overall shape which resembles a rod with an estimated length of 300 nm [14]. Each polypeptide chain is formed from Gly-X-Y repeats [14]. These molecules aggregate to form various types of structures for supporting physiological loads. The majority of the collagens can be classified according to the nature of their aggregated forms. As of now, there are more than 30 types of collagens and a thorough reveal of collagen and fibrils has been reported recently [15]. (For the interest of the reader, conventionally, to reference the variety of collagen types, the vertebrate collagens are given a Roman numeral, e.g. type I collagen.)

With regards to the different collagen types, the majority of collagen found in fibrils are types I, II, III; the minor collagens are types V and XI [14]. Thus, we find type I collagen predominates in tendons; type II collagen predominates in cartilage [14]. Two or more types of collagen could predominate in certain tissues, e.g. type I and III collagen in the vascular system. Type III related collagen fibrils can be found as with small diameter in embryonic and vascular tissues [14]. Some collagens, namely types IX, XII and XIV, are bonded to fibril forming collagens specifically at the fibril surface. Type IX collagen is found on the surface of the type II collagen fibre; it could be covalently bound to type II in an anti-parallel manner. While not much is known about the mechanical function of type IX, it could possess good thermal stability [16].

The collagen molecules are axially configured, in a so-called ‘quarter-staggered’ (over-lapping) arrangement, and laterally dispersed in a radial pattern, within the collagen fibril [17–20]. The meridional and equatorial intensities of the x-ray diffraction pattern respectively contain important information about the axial and lateral arrangements of collagen molecules in fibrils.

In fact, analysis of the x-ray diffraction patterns has enable the revelation of the anisotropic property of fibrils from the Bragg peaks in the meridional and equatorial regions. From the meridional regions, while one may infer about the ‘D repeats’, characterised by light-dark bands (period = 67 nm) observable in electron micrographs, from the regular-spaced Bragg peaks (Fig. 14.2a), these peaks also yield information about the axial staggering of collagen molecules. Any change in the position of these peaks may implicate changes in the long-range axial crystallinity of the semi-crystalline structure of the fibril [21]; this property has been used to determine the deformation at molecular level when the fibril is deforming under tension [22].

The characteristic triplet of diffraction peaks occurs in the equatorial region and is observable at short camera length [18]; Fig. 14.2b shows a single blob because the pattern was acquired at a long camera length of 10.2 m (which corresponds to

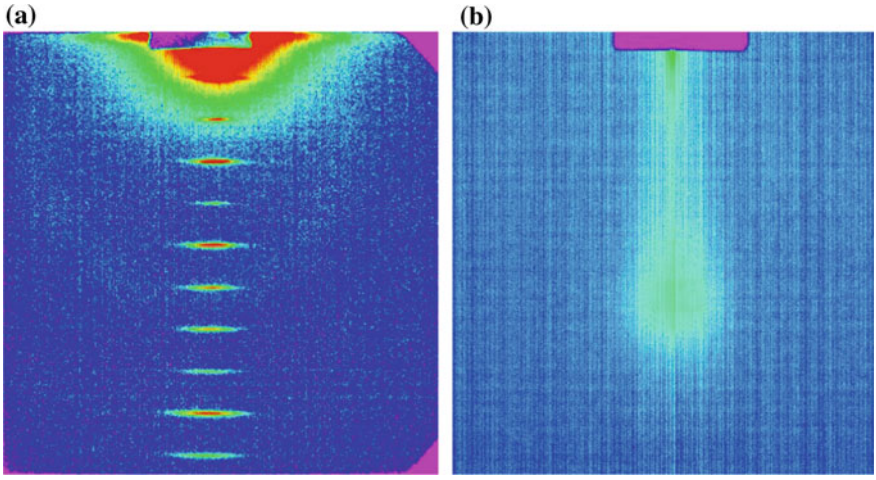
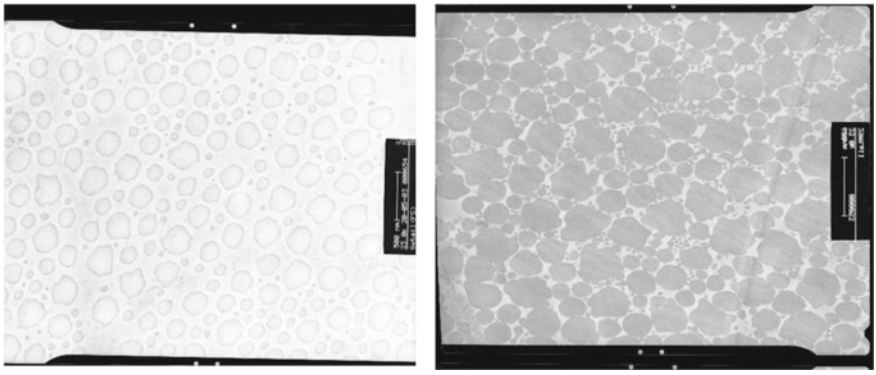


Fig. 14.2 X-ray patterns of collagen fibrils from tail tendons of C57BL6 mice. **a** The meridional peaks as revealed from small-angle x-ray scattering patterns. **b** The diffuse peak as shown in wide-angle x-ray diffraction patterns. These results were obtained using synchrotron x-rays (Beam-line 2.1, at Daresbury Laboratories, UK)

small angle scattering). It is possible to evaluate these peaks (Fig. 14.2b) using a model of the gap and overlap regions for the three-dimensional axial staggering of collagen molecules [17, 19] to derive important information about the packing of the collagen molecules in the radial direction. Such a model could help identify the precise changes in the radial packing influencing the Poisson ratio of these fibrils as demonstrated very recently in ageing studies by Haverkamp and co-workers [23].

While small angle x-ray scattering has been proposed as a viable method to study collagen fibril sizes [24], many studies prefer to rely on electron microscopy. Indeed, electron micrographs of the transverse section of connective tissues such as tendons have been used to study the sizes and dispersion of collagen fibrils in the PG-rich hydrated ECM (Fig. 14.3a). What is found commonly is that the cross section of fibrils is near-circular and non-uniform in sizes in young tissues [2, 25–27].

The fibril size effects on the mechanical properties of the tissue has been a subject for debate until 2012 when Goh and co-worker established a detailed analysis of the connection of the different size distribution of collagen fibrils to the resilience and fracture toughness of the tendons [26]. The origin of these sizes is a subject for speculation: the small fibril size may be associated with short fibrils, and may have been formed shortly while the large fibril size may be the result of further fibril self-assembly after extrusion from the fibroblast, as well as fusion of two or more fibrils post fibrilogenesis [2, 25–27]. On the other hand, if we consider that these are tapered fibrils, then the different sizes could be attributed to the sectioning of fibrils at different points [27]. Additionally, there are difficulties arising from the serial sectioning of fibrils: (1) there is limited information concerning whether the fibrils seen so far possess lengths that span the entire tissue, (2) is the small size fibril



(a) 11 WEEKS OLD **(b) 29 MONTHS OLD**

Fig. 14.3 Cross-sections of tail tendons in young (11 weeks old) and old (29 months old) C57BL6 mice. Images (unpublished) were provided by David F. Holmes. Similar patterns may be found elsewhere in an earlier publication [25]

cross-section a part of a longer fibril with larger fibril diameter at the fibril centre but not observable by this method [27].

Figure 14.3b shows the increasing irregularity of the morphology of the fibril cross section with age; no appreciable increase with age was observed for the packing of fibrils. Quite a while back, it is believed that decorin proteoglycans are capable of regulating the size and morphology of collagen fibrils [28]. There is a high similarity in the irregularity observed in tissues from older animals with those from knock-out mice deficient in the gene for decorin [28].

It has been proposed that the irregularity is due to fibril tip to shaft fusion or fibril shaft to shaft fusion [29] but whether this could be caused by a decrease in the decorin density on the fibrils remains to be tested. Decorin is a member of the family of small leucine repeats PGs (SLRPs) that includes fibromodulin and lumican [30]. Decorin binds to procollagen [31] and evidence of surface-bound decorin proteoglycans at the ends of collagen fibrils led to speculations that decorin might have an important role in the fibrillogenesis process because the collagen may be able to accommodate the proteoglycan during fibrillogenesis [29].

Although some investigators have shown that there are no changes in the mechanical properties with the removal of the decorin proteoglycans [32], Soslowsky and co-workers have recently shown that decorin contribute to tendon's response to load based on studies using decorin knockout mice [33]. Decorin-null tendons revealed significant changes in the mechanical properties across different age groups, accompanied by location-dependent collagen fibre re-alignment changes, suggesting a site-specific role for these molecules in loading [33]. That decorin proteoglycans could result in reduced magnitudes in the mechanical parameters as they are associated with the interfibrillar matrix is still an ongoing topic for debate.

It is an attractive theory because of the following findings: (1) Optical tweezer mechanics study of decorin-decorin interaction shows that the interaction may be disrupted at a force of order of 10 pN [34]. The low force suggests that the interaction is not contributed by covalent bonding. As ECM occupies the bulk of the tissue, the non-covalent, and presumably reversible, forces between these proteoglycans could be an important contributory factor to the structural and mechanical integrity of the connective tissues; (2) Evidence of decorin proteoglycans on the surface of collagen fibrils [35, 36]. The proteoglycan can in turn modulate fibril size during fibrillogenesis [30]. It then follows that absence of these molecules could have a profound effect on the fibril size; transgenic mice which are null for decorin show abnormal collagen fibril diameters [30].

ECM is a hierarchical architecture [2, 27]. At the length scale of the fibril, the fibril is made up of microfibrils [19]. At a larger length scale, the collagen fibrils bundle up to form collagen fibres. At the next higher length scale, the collagen fibres bundle to form fascicles.

14.2.3 Mechanism of Reinforcement

When connective tissues, such as tendon and ligaments, are subjected to an increasing tensile load the mechanism of stress up-take in the tissue varies throughout the loading process until the tissue ruptures [2, 4, 27].

How the tissue takes up load finds an analogy to how fibre reinforced composites can withstand loads that tend to pull them apart [5]. In this case, the collagen fibrils are responsible for reinforcing the weak PG-dominated hydrated interfibrillar matrix [4, 37]. Collagen fibrils are long and slender structures that taper (somewhat paraboloidal) to each end, as observed under a transmission electron microscope [38, 39].

It has been shown using atomic force microscopy that collagen molecules possess a rupture force of the order of 1000 pN [40]. Taking the energy to rupture to be equal to the displacement at rupture multiply to the force to rupture, one finds that the energy to rupture is of order of 1×10^{-4} pNm. Clearly the mechanical properties of collagen fibrils are remarkable but how does the fibril provide reinforcement to the ECM? The biomechanical properties of the tissue, namely fracture strength and modulus of elasticity, may be said to depend on the collagen fibril volume fraction V_f and the material properties of the fibrils, according to the rule of mixtures for fracture strength and modulus of elasticity that are often applied for studying engineering fibre composites [5]. The fibril volume fraction is a parameter which provide an indication of the number of fibrils present in ECM but could also accounts for the inter-fibrillar distance which we will see in the following section. However, these do not help explain how collagen fibrils provide reinforcement to the ECM.

Over the last ten years, using concepts applied to engineering discontinuous fibre reinforced composites [3, 41–45], Goh and co-workers have laid down the key foundations for the reinforcement mechanisms that relate to how collagen fibrils take up

stress, at different stages of the loading process [2, 4, 27, 37, 46]. These mechanisms are the elastic stress transfer, plastic stress transfer, and rupture [2, 4, 27, 46]. This section will discuss the key concepts underpinning these mechanisms that have helped shaped our insights for collagen fibril reinforcing connective tissue.

We begin with the concept of elastic stress transfer mechanism, which regulates the initial loading stage [4]. Here, the proteoglycan-rich matrix deforms elastically. At the interface between the collagen fibril and the proteoglycan-rich matrix, the interactions of decorin proteoglycans on the fibril with macromolecules in the PG matrix and with decorin PGs on adjacent fibrils generate interfacial shear stresses which place the fibre in tension as it stretches elastically.

If the fibril were paraboloidal in shape at the ends [38], the stress would be a minimum at the fibril centre and increases and peaks near the ends of the fibril. If the fibril were uniform cylindrical in shape, the stress would peak at the centre of the fibril and decreases non-linearly to zero at the fibril end [4]. Concentrating stresses near the fibril end makes good sense because should a portion near the end breaks, the effectiveness of the bulk of this fibril for reinforcement would not be appreciably compromised. This would not be the case should stresses be concentrated at the fibril centre; the fibril would suffer a significant lost in length should the fibril break at the fibre centre.

Unfortunately, while it is certainly more advantageous for a fibril to be paraboloidal in shape at its ends than uniform cylindrical, the question of whether the fibril is uniform cylindrical or paraboloidal in shape is still debatable because the only people who have witnessed the latter shape are Holmes and co-workers. Another point to note here is the parameter known as the relative stiffness of the fibril to the proteoglycan-rich matrix, E_f/E_m. In all the four cases shown here in Fig. 14.4, it is observed that high E_f/E_m corresponds to high stress uptake in the fibril; on the other hand, low E_f/E_m corresponds to low stress uptake in the fibril. What this mean is that while high E_f/E_m bestows the fibril with the capacity to take up high stress, providing good stress transfer from the proteoglycan-rich matrix to the fibril, under an increasing applied load, this mean the stress in the fibril will increase and if it eventually reaches the fracture stress of the fibril, the fibril breaks. On the other hand, low E_f/E_m reduces the capacity of the fibril to take up high stress; this means that the stress transfer mechanism is not as effective as when the E_f/E_m is high. Thus, when E_f/E_m is low, a part of the load is taken up in the proteoglycan-rich matrix and the matrix will readily disrupt at a lower applied load as compared to the case when the E_f/E_m is high.

Between the stages regulated by the elastic and plastic stress transfer mechanisms, there is an intermediate stage in which the fibril undergoes one or more of the three modes of stress transfer, namely Mode α, β, and χ. Mode α is said to occur when the deforming proteoglycan-rich matrix yields and turns plastic adjacent to the fibril-matrix interface. In the case of uniform cylindrical fibrils, stress concentrates in the proteoglycan-rich matrix around the end and this may lead to matrix yielding. For tapered fibrils the lower stress concentrations in the proteoglycan-rich matrix around the ends may make them less susceptible to Mode α. Mode χ is said to occur when a crack at the debonded fibril end propagates into the proteoglycan-rich matrix but

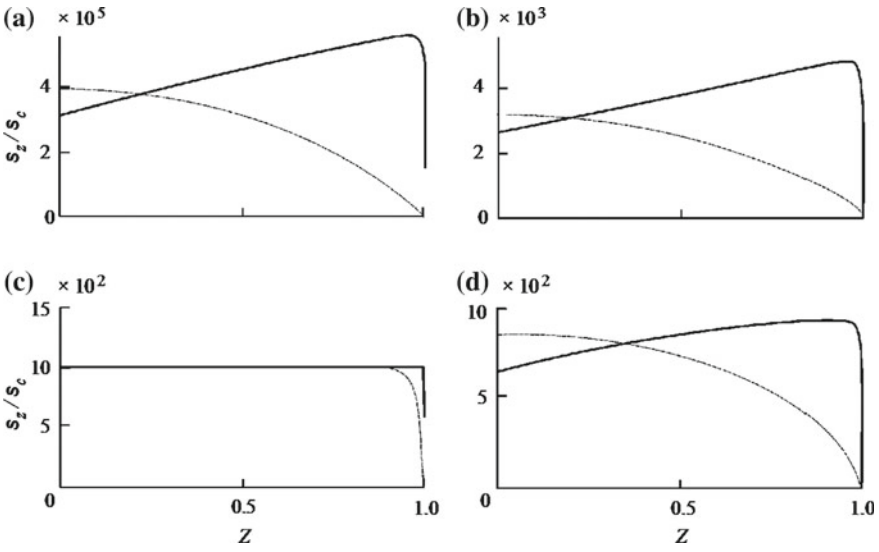


Fig. 14.4 Distributions of normal stresses, σ_z, versus distance along the collagen fibril axis, Z, during elastic stress transfer. **a** High E_f/E_g and high q. **b** High E_f/E_g and low q. **c** Low E_f/E_g and high q. **d** Low *Ef/Eg* and low q [4]

not along the fibril-matrix interface. There is some similarity between Mode χ and the engineering concept of mode I crack, i.e. parting of two surfaces; in this case both concepts suggest that stress transfer will not occur across the crack planes and subsequently reduces the effectiveness of stress transfer between the proteoglycan-rich matrix and fibril.

Mode β is said to occur when a crack initiates in the interface at the debonded fibril end and propagates along the interface. Frictional stress transfer occurs as the deforming proteoglycan-rich matrix slides over the fibril surface. The rate of debonding is related to the relative stiffness of the fibril to the proteoglycan-rich matrix, E_f/E_m; high E_f/E_m means that the fibril can take up higher stress but that also mean that if the applied load increases to a high level, this translate to a high stress in the fibril which could exceed the fracture stress of the fibril and hence induces fracture. In other words, the greater the elastic mismatch between the fibril and proteoglycan-rich matrix, the higher the rate of fibril fracture. On the other hand, low E_f/E_m means that the fibril has a lower stress uptake capacity; as less stress can be transferred to the fibril, the rest is bore by the proteoglycan-rich matrix and this will readily induce failure in the matrix at a lower applied load.

The failure may appear in the form of cracks or debonding; in the case of the latter, shear-sliding action begins when the interfacial shear stress overcomes the frictional stress and a cohesive sliding resistance. When sliding occurs between the fibril and the surrounding matrix, the value of the interfacial shear stress is constant throughout the interface. There is some similarity between Mode β and the engineering concept

of mode II crack, i.e. shear failure; in both cases, it suggests that stress transfer via friction at the crack surfaces. A modified Rice and Tracey micro-void nucleation, growth and coalescence model has been used to predict the crack propagation in a finite-element analysis, leading to insights concerning how a proteoglycan-rich matrix crack is formed and how the crack propagates in the presence of voids in the region ahead of the crack which nucleate, grow and coalesce due to the presence of high stress at the fibril corner. A critical fracture strain may be defined as a function of void size and the level of the stress magnitude around the region; when the stress at a point in the proteoglycan-rich matrix was greater than this critical fracture strain, this results in a crack.

After the intermediate stage, as the applied load increase, the reinforcement of the ECM by the collagen fibrils is regulated by the plastic stress transfer mechanism. This mechanism involves the disruption of the interfacial bonds between the collagen fibrils and the PG-rich matrix. The disruption cuts across the entire interface, initiating at the fibril end, propagating towards the fibril centre and ends at the fibril centre. The proteoglycan-rich matrix now deforms plastically and shear-slides over the fibril surface. The plastic stress transfer mechanism is essentially a simultaneous occurrence of mode β and α at the interface, and in the adjoining proteoglycan-rich matrix region, respectively.

Goh and co-workers have evaluated a finite element model consisting of an elastic fibre in tension under the application of a constant shear stress on its surface to study plastic stress transfer [41]. If the fibril were uniform cylindrical in shape, the axial stress peaks at the centre of the fibril and decreases linearly to zero at the fibril end (Fig. 14.5). If the fibril were paraboloidal in shape at the ends, the axial stress also peaks at the fibril but decreases non-linearly to zero at the fibril end (Fig. 14.5).

During the process of deformation, stress transfer from the proteoglycan-rich matrix to a fibril may cause the fibre to break at fibril centre where the stress reaches the fracture stress. This results in fibril fragments. Further fragmentation process may

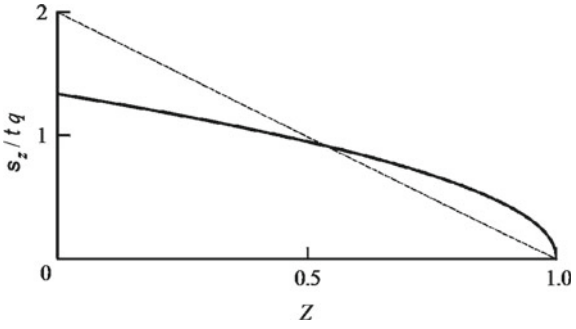


Fig. 14.5 Distributions of normal stresses, σ_z, versus distance along the collagen fibril axis, Z, during plastic stress transfer. Dark bold line refers to paraboloidal shape; light thin line refers to uniform cylindrical shape. Symbols τ and q denote fibril-matrix interfacial shear stress and fibril aspect ratio (a parameter for the slenderness of the fibril)

occur, but this would eventually stop when the fragments are too short. This leads to the idea of a critical length parameter for the collagen fibril; this is the length of the fibril below which the stress uptake into the fibril would not be sufficient to reach the fracture stress and the fibril would not fracture. Although one could estimate the length of collagen fibrils in tissues, e.g. on the basis of the number of fibrils ends observed in a cross section [47], no attempts has been made to measure the critical length of collagen fibril experimentally.

Finally, beyond plastic stress transfer, at higher applied loads, the tissue begins to fail by a variety of mechanisms: debonding at the fibril proteoglycan-rich matrix interface, fibril fragmentation, cracking in the proteoglycan-rich matrix and fibril pull-out. The order and importance of these events is immaterial, but it is important to note that the variety of failures have been well-observed in scanning electron micrographs in other studies such as thermal effects and ultraviolet irradiation effects [48, 49]. Fibril pull-out may occur when fibrils are drawn out from the crack faces within proteoglycan-rich matrix. This may be initiated by the combination of Modes β and χ. If Mode β did not occur, the proteoglycan-rich matrix crack arising from Mode χ may propagate to neighbouring fibrils; these neighbouring fibrils become responsible for bridging cracks in the proteoglycan-rich matrix. In both cases, fibril pull-out may occur when the fibril is unable to bridge the crack. The ability of a fibril to bridge a proteoglycan-rich matrix crack depends on the interface, the fibril strength and fibril modulus. If bonding is present at the interface, then the trigger for interfacial failure depends on the yield stress related to the interfacial shear; if debonding has occurred, then the degree of pull-out depends on friction as a result of sliding. There are two possible outcomes of fibril pull-out while bridging a proteoglycan-rich matrix crack. The first is that if the crack to propagate were to be deflected by neighbouring fibrils, the crack could propagate along the interface instead (Mode β). The second outcome is that the fibril could fracture when the applied load increases so that attempts to bridge the crack results in high stress uptake in the fibril beyond the level of the fibril fracture stress.

14.3 How Processing Bioscaffolds Affects the Scaffold Structural/Mechanical Integrity

14.3.1 Overview

The approach to treat the connective tissue of the echinoderms to derive the ECM scaffold material involves physical/mechanical and biochemical processes. The processing steps typically involve decellularization, hydration, dehydration, powdering and gelation, with disinfection during the process (Fig. 14.6). Each of these steps may affect the structural/mechanical integrity of the bioscaffold (as well as the type of host response) that which it is intended for. Here we shall focus on the first three steps to illuminate the effects on the mechanical integrity of the scaffold.

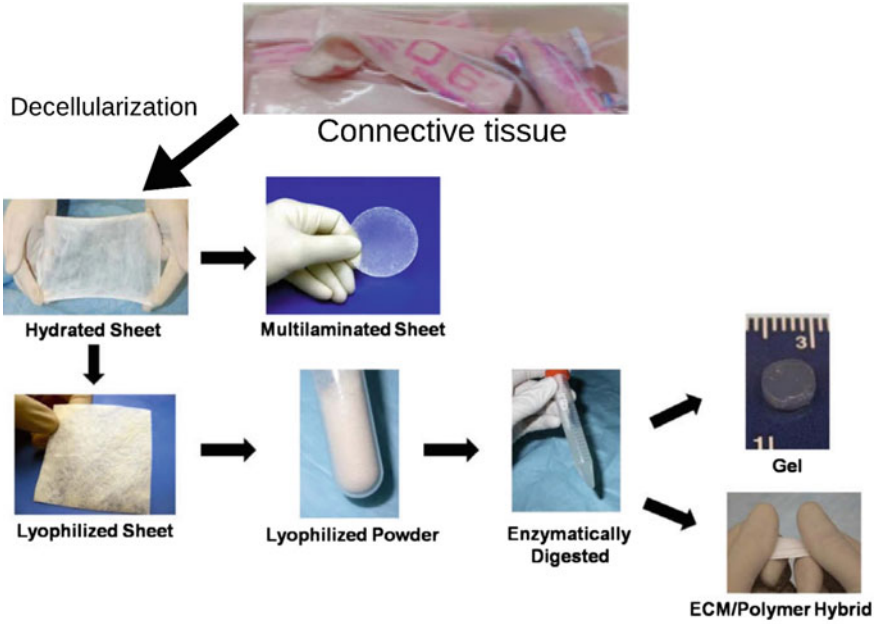


Fig. 14.6 Schematic of the processing of extracellular matrix (ECM) scaffolds from connective tissue. After the tissue is harvested, it undergoes decellularization to obtain a hydrated bioscaffold. Three-dimensional scaffold may be obtained by vacuum-pressing several such hydrated sheets to form a laminate scaffold. Lyophilization of the hydrated sheets, followed by a powdering process, may be implemented. The power may be enzymatically digested into a liquid; further processing by repolymerization or blending into a synthetic polymer then yields the ECM/polymer scaffold. Reprinted from [10], with permission from Elsevier

14.3.2 Decellularization

The aim of decellularization is to remove cellular material in the tissue so that what remains is the ECM [50]. This is important because cellular material may cause immunologic response in the host body; further discussion is found in the last paragraph of this section. Ideally, the method must not affect the ECM composition and structural/mechanical integrity (as well as biological activity). The strategy to remove cells is concerned about the removal of cell contents. To this end, one may target the breakup of the cell membrane by sonication, agitation and freezing/thawing [51]. As the membrane burst, cell contents are released but only further rinsing of the tissue may remove the cell remnants from the ECM [51].

Ideally, any decellularization process should not alter the structural/mechanical properties and biological properties of ECM [51]. In practice, this is not the case. One finds that fibrils are broken up during decellurization; the fibril-matrix interface and proteoglycan-rich matrix are disrupted. Detergents ae used in the decellularization process but while some detergents may be disruptive to ECM collagen in some

tissues, the same type may have minimal disruption to other tissues [51]. Additionally, glycosaminoglycans (GAGs), which holds water in ECM, may also be removed from the scaffold during decellularization [51]. Unfortunately, GAGs play an important role in regulating the viscoelasticity of the tissue and the absence of GAGs may result in diminution of the viscoelastic properties of the tissue [51]. Clearly it is important to develop effective methods for decellularization without drastically affecting the mechanical properties of the tissue [51].

In practice, decellularization methods may not be able to completely remove all cellular DNA-related materials [51]. Adverse immunologic response by allogeneic and xenogeneic recipients of the ECM scaffold material follows when antigenic epitopes found in the cell membranes and intracellular components of tissues and organs are not removed [51]. This is because xenogeneic and allogeneic cellular antigens are respectively recognized as foreign by the allogeneic and xenogeneic host [51]. The immunologic response may be inflammation or overt immune-mediated rejection [51]. Of note, it is worth noting that many molecules that are native to the extracellular matrix are well conserved across species and that they pose a lower risk of immunologic response in xenogeneic recipients [51].

14.3.3 Hydration

For the ECM scaffold it is important to maintain hydration within the scaffold in order to ensure that the structural/mechanical integrity are not altered [51]. Implementing hydration to recover the structural/mechanical integrity after subjecting to possible dehydration (during decellularization and sterilization) assumes that the dehydration/hydration is reversible, but this is not necessarily true [51]. When the ECM is dehydrated, the collagen fibrils are drawn closer to one another and this could fracture the fibril in the absence of a hydrated matrix which provides a buffer between the fibrils [52].

It has been pointed out that bioscaffolds that remain hydrated throughout the decellularization and sterilization process are better able to facilitate cellular attachment (and cell infiltration) than bioscaffolds that are subjected to a dehydration step followed by rehydration [51] but the key reason could be that the fibrils, being more intact in the former, allows for inter-twinning which lends to a more mechanically stable network of fibrils in the ECM. However, hydration comes with a price: it is said that the bioscaffold would leach off soluble growth factors (such as VEGF and b-FGF) present in the material during storage [51].

14.3.4 Dehydration

There are two advantages of having a dehydrated bioscaffold. First, dehydrated bioscaffolds are easier to handle (e.g. for vacuum pressing to create laminates) dur-

ing processing; second, dehydrated bioscaffolds can also minimised leaching/loss of growth factors during storage [51]. Dehydration can be carried out by lyophilization, followed by sterilization [51]. Lyophilization is a freeze drying (low temperature, low pressure) process by which the water is removed from the bioscaffold by sublimation.

While lyophilization can be used to preserve the ECM of biological graft tissues from degradation during storage, but at the expense of modifying the collagen fibril ultrastructure [51]. In particular, as the thickness of the bioscaffolds may be decreased following lyophilization (typically by 30%) because the water phase is removed, this results in a collagen ultrastructure that features a more tightly packed fibrils. Additionally, as the proteoglycan-rich matrix is now greatly reduced in volume fraction because the water phase is removed, this means that there is a significant disruption to the interaction between proteoglycans and proteoglycan and collagen. These changes may be irreversible when water is reintroduced: (1) the more compact fibril packing will not make it easy for the bioscaffold to recover the original water phase, and (2) the disruption of the interaction between the proteoglycans and collagen at the fibril-matrix interface means that the effectiveness of fibril stress uptake is reduced as it can only occur via the shear-sliding action, regulated by either Mode β or plastic stress transfer mechanisms [2, 27].

Vacuum pressing process is intended to produce a 3D enhanced-anisotropic structure by tightly stacking layers of thin bioscaffolds. This is analogous to the stacking of layers of unidirectional continuous carbon fibres (where fibres in consequentive layers are in different directions) to form a carbon fibre reinforced polymer composite (CFRP) for structural application. This is also analogous to the anisotropic collagen ultrastructure in skin dermis [53]. In all cases, the laminate results in enhanced mechanical properties, such as higher strength and stiffness as compared to the individual layer. More importantly, it is intended to mimic the structural/mechanical environment of the specific tissue in which the implant would be introduced for, e.g. tissue repair [51]. However, such a vacuum-pressed bioscaffold can also exhibits reduced extensibility, as compared to the individual layer [52]. Thus it is important to develop an optimal configuration and method of processing of an ECM scaffold for the intended clinical application.

14.4 Potential Clinical Applications

So far, the commercially available and FDA-approved biological scaffold materials are made from human skin, human fascia lata, human dermis, cadaveric human dermis, porcine small intestinal submucosa, horse pericardium, fetal bovine skin, cadaveric fascia lata, in dry or hydrated forms [10]. While there are several reports highlighting the possible use of echinoderm connective tissues, such as sea urchin ligaments and membranes, as biomaterials for constructing bioscaffolds for tissue engineering and other biomedical applications, these marine-related bioscaffolds have not appear in the market yet. This section is intended to assess some of the possibilities proposed by the researchers, in the context of mechanical viability.

The most commonly suggested area of application for the echinoderm connective tissue is for tissue repair. The processing of bioscaffolds from connective tissue has been discussed in Sect. 14.3. One group has already embarked on a long-term study (known as the MIMESIS project) to develop and commercialise collagen films made from sea urchin connective tissues [54]. Collagen extraction was performed according to the protocol of Matsumura [55] with additional modifications established by the group [12, 56]. They have claimed that their method could also extract intact collagen fibrils; their aim is to be able to process a bioscaffold with the appropriate hierarchical architecture as that found in the host tissue where the bioscaffold would be implanted. In the report, they have also carried out test on their bioscaffold using mammalian cells [11]. More specifically, it has been suggested that the decellularized material may be used to treat connective tissue pathologies characterized by alteration of the viscoelastic properties [57]. This could be implemented by implanting the treated decellularized material into the pathological site. The mechanical properties of the implanted material would have to be of similar viscoelastic properties as that of the host tissue. To be able to address this effectively, the implanted material would have to be treated to an appropriate level of hydration over a given duration. Recall that if the hydration is not adequately achieved, this would result in a high E_f/E_m which is fine if the fibrils in the ECM can undergo elastic stress transfer as a high E_f/E_m means that the fibrils are able to take up high stress during elastic loading via the shear-lag action. But if the inadequate hydration results in poor interaction between the proteoglycan-rich matrix and the fibrils, when the implanted material is loaded, the fibrils would only be able to undergo plastic stress transfer, which is not ideal as stress transfer via the shear-sliding action is not as effective as via the shear-lag action.

The mutability of the echinoderm connective tissues has been well-studied and it has been suggested that this unique property can be exploited to develop collagen barrier-membranes for Guided Tissue Regeneration (GTR) [13]. Of note, one can now find commercially available membranes for GTR or soluble/reassembled (fibrillar) bovine collagen substrates but alternative sources from marine animals have yet to be commercialised. Like the bovine sources, use of the tissue from sea urchins is sensible in terms of eco-sustainability as this addresses the recycling tissues from food wastes. Ferrario and co-workers have used the mutable connective tissues (MCTs) from different echinoderm models (sea urchin, starfish and sea cucumber) to produce echinoderm-derived collagen membranes (EDCMs). These EDCMs could result in similar rate of cell (e.g. fibroblasts) proliferation and cell morphology as those from commercially available bovine collagen substrates.

The mutability of the MCT has inspired Trotter and co-workers [56] to propose a ‘hybrid’ biomaterial inspired by the MCT. Such a hybrid biomaterial could compose of synthetic interfibrillar matrix and collagen fibrils of sea cucumber dermis origins [56]. Clinically speaking, MCT-like biomaterials could be useful for tissue engineering of soft tissues, i.e. for application in regenerating tissues in a dynamic environment [58]. This concept is still a novelty but deserves further consideration in the near future.

Finally, the echinoderm connective tissue that exploits the destiffening property could have useful application in the cosmetic industry [57]. Cosmetically speaking, the decellularized ECM could provide anti-aging therapy for destiffening the aged skin.

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