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Collagen in Human Tissues: Structure, Function, and Biomedical Implications from a Tissue Engineering Perspective

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Abstract The extracellular matrix is a complex biological structure encoded with various proteins, among which the collagen family is the most significant and abundant of all, contributing 30–35% of the whole-body protein. "Collagen" is a generic term for proteins that forms a triple-helical structure with three polypeptide chains, and around 29 types of collagen have been identified up to now. Although most of the members of the collagen family form such supramolecular structures, extensive diversity exists between each type of collagen. The diversity is not only based on the molecular assembly and supramolecular structures of collagen types but is also observed within its tissue distribution, function, and pathology. Collagens possess complex hierarchical structures and are present in various forms such as collagen fibrils (1.5–3.5 nm wide), collagen fibers (50–70 nm wide), and collagen bundles (150–250 nm wide), with distinct properties characteristic of each tissue providing elasticity to skin, softness of the cartilage, stiffness of the bone and tendon, transparency of the cornea, opaqueness of the sclera, etc.

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There exists an exclusive relation between the structural features of collagen in human tissues (such as the collagen composition, collagen fibril length and diameter, collagen distribution, and collagen fiber orientation) and its tissue-specific mechanical properties. In bone, a transverse collagen fiber orientation prevails in regions of higher compressive stress whereas longitudinally oriented collagen fibers correlate to higher tensile stress. The immense versatility of collagen compels a thorough understanding of the collagen types and this review discusses the major types of collagen found in different human tissues, highlighting their tissue-specific uniqueness based on their structure and mechanical function. The changes in collagen during a specific tissue damage or injury are discussed further, focusing on the many tissue engineering applications for which collagen scaffolds are currently being applied.

Keywords Cardiac • Collagen type I • FACIT • Fibrillar • Skin

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1 Introduction

The extracellular matrix (ECM), by definition, is the organic matter that is found between the cells in plants and animals [1, 2]. The ECM is a relatively stable structural material that lies under the epithelia and surrounds the connective tissue cells and provides a stable framework for multicellular organisms under gravity and physical loading, whereby it maintains the integrity of tissues and enables physiological functioning. Over the years, there has been a gradual change in our understanding of the ECM as a static "connective tissue" that binds everything together to one of the ECM as a dynamic biomaterial that performs multiple functions such as providing strength and elasticity, activating growth factors during development and

controlling their availability, cell–surface receptor interactions, etc. In addition, ECM is essential for morphogenesis and assists in the regeneration of multicellular organisms and tissues. The integrin receptors on the cell surface along with the ECM can be pictured as intricate nanodevices that allow cells to physically organize their three-dimensional (3D) environment and to sense and respond to various types of mechanical stress [3]. Although the composition of ECM is a complex alloy of variable members of diverse protein families such as the collagens, proteoglycans, glycosaminoglycans (GAGs), and elastin, its main function is to support the tissue with specific mechanical and biochemical properties. For example, the collagens are a source of strength to the tissues; elastin and proteoglycans provide matrix resiliency; and other structural glycoproteins aid in inducing tissue cohesiveness. The most plentiful proteins in the ECM are the collagen family of proteins, and collagens form the fundamental organic matrix of the bone, skin, arteries, ligaments, cartilage, and most of the ECM in general. They are responsible for the stability of tissues and organs, helping them to withstand stretching. Contributing ~30% of the total protein mass in mammals, the collagenous proteins are a broad class of molecules found with extreme heterogeneity. Though there is still uncertainty in classifying a protein under the collagen family on the basis of a single criterion, the trademark and common structural feature of collagens is the presence of three polypeptide chains, each containing one or more regions characterized by a repeating amino acid motif, allowing the formation of a triple helical structure.

Modern research on collagen at the molecular level began in the 1950s when Hall et al. [4] and Schmitt et al. [5] characterized the collagen molecule under the electron microscope and Ramachandran and Kartha [6] and Rich and Crick [7] developed models to explain the triple helical structure of collagen. To date, 29 types of collagen have been identified in the collagen superfamily and these 29 types of collagen are discriminated by considerable complexity and diversity in their structure, their splice variants and the presence of additional non-helical domains, their assembly, and their function [8]. Based on the domain structure homology, the collagen types are classified and assigned with Roman numerals according to the chronological order of their discovery. Collagens can be homotrimetric (identical alpha chains) or heterotrimetric (genetically distinct alpha chains) and the distinct alpha chains within the same collagen type are denoted by Arabic numerals based on their order of discovery. All these collagens arrange themselves into a variety of supramolecular structures including fibrils, microfibrils and network-like structures that are responsible for the structure and function of the ECM. The mechanical properties of the ECM are mostly a consequence of the collagen fiber architecture and kinematics. The interesting fact is that one type of collagen can predominate in tissues that have extremely diverse mechanical properties, asserting that collagen suprastructures are a major determinant of tissue-specific architecture and function. The versatility of collagens is due to their complex hierarchical structure, leading to a great variety of properties. Similarly to the ECM, collagens were initially thought of as a group of proteins that provide structural support on the basis of their characteristic molecular architecture, thereby contributing to the extracellular scaffolding and preserving the shape and mechanical properties of a specific tissue. With advanced characterization in the last two decades, there has

been a tremendous change in the viewpoint and now it is widely known that the functions of the collagen family are not confined to a structural role but that they also perform myriad other additional functional roles. Collagens have several developmental and physiological functions and they are involved either directly or indirectly in cell attachment and differentiation, as chemotactic agents, as antigens in immunopathological processes, and as a defective component in certain pathological conditions [9]. Collagen mainly contains glycine and proline. Collagen is predominantly synthesized by fibroblastic-like cells (chondrocytes, osteocytes, and tenocytes), though epithelial cells also synthesize smaller amounts of collagen. Collagens inherently possess high tensile strength, which is due to the intermolecular covalent crosslinks that are reducible in the early stages of fibril development but become stable as the tissue matures. The creep resistance property of collagen-rich tissues is based on the small-diameter collagen fibrils, whereas tissues that encounter high stress levels contain a higher percentage of large-diameter collagen fibrils. The ability of a tissue to sustain an applied load depends on the amount of collagen content per unit mass of the tissue and hence tissues with greater tensile strengths are those with higher collagen content.

Various disorders are associated with abnormalities in collagen caused by mutations in genes coding for collagen α chains or abnormal folding of collagen molecules. Such structural changes in collagen are related to a variety of diseases of the bone, ligament, or even blood vessels. For example, degradation, disturbed metabolism, and molecular defects of collagen can be related to conditions such as chondrodysplasias (type II collagen), osteogenesis imperfect/brittle bone disease (type I collagen), Alport syndrome (type IV collagen), Ehlers–Danlos syndrome (type IV collagen), osteoporosis (type I collagen), osteoarthritis (type II collagen), atopic dermatitis (type III collagen), etc. [10, 11]. It is therefore essential to understand the molecular structure and assembly of collagen in relation to the mechanical properties of specific tissues together with their sources, type, and distributions to have a deeper knowledge on the influence of collagen on disease origin, prevention, and regeneration. Also, collagens play a major role in wound healing and tissue repair by contributing to the entrapment, storage, and delivery of growth factors and cytokines, thereby establishing them as the first choice of natural polymeric material for effective tissue regeneration and drug delivery. The many applications of collagen in tissue engineering include construction of artificial skin substitutes, bone graft substitutes, dental implants, corneal implants, artificial vascular grafts, and regeneration of nerve, skin, cartilage, etc. which are discussed in Sect. 4.

2 Collagen

2.1 Types of Collagen

The collagen family, as we know, is complex and marked by huge diversity in molecular assembly and supramolecular structure, tissue distribution, and function. Collagens are classified into different groups on the basis of their structural

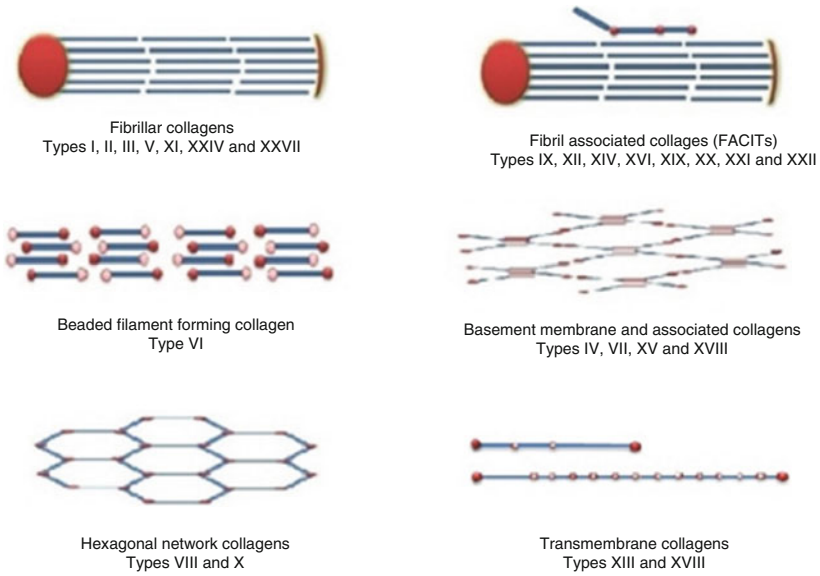

Fig. 1 Supramolecular structures of collagen

organization and sequence homologies, and further diversity occurs in the collagen family due to the presence of several molecular isoforms for the same collagen type. In humans, the types of collagen present can be classified into different subfamilies such as fibrillar collagens (types I, II, III, V, XI, XXIV, and XXVII), fibril-associated and related collagens (types IX, XII, XIV, XVI, XIX, XX, XXI, and XXII), beaded filament-forming collagen (type VI), basement membrane and associated collagens (types IV, VII, XV, and XVIII), transmembrane collagens (types XIII, XVII, XXIII, and XXV), and hexagonal network collagens (types VIII and X) [12–16]. In other words, grouping of the collagen family can also be based on their supramolecular assemblies, as represented in Fig. 1.

Figure 2 shows the suprafibrillar architectures formed by the collagen fibril bundles in mature tendon, ligament, human dentin, and connective stroma of the small intestine as seen using scanning electron microscopy (SEM) [17]. In addition to all these types, there are many other collagen-like proteins that are not called "collagen," most of which are soluble proteins, such as adiponectin CTRP9, macrophage scavenger receptor, surfactant proteins, hibernation proteins, and C1q. Table 1 summarizes the distinctive features of the major classes of the collagen family and describes their associated functions.

2.1.1 Fibrillar Collagens

The fibrillar collagens are the most profusely found collagens in human tissues and, as a consequence, they were the first members of the collagen family to be discovered. The fibril-forming or fibrillar collagen subfamily includes collagens I,

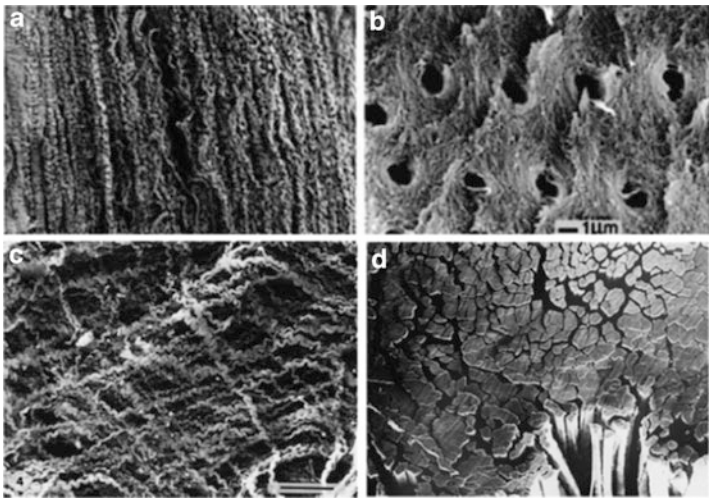

Fig. 2 Suprafibrillar architectures of collagen shown by SEM: (**a**) mature tendon and ligament, (**b**) human dentin, (**c**) connective stroma of small intestine, and (**d**) transected tendon (reprinted with permission from [17])

II, III, V, XI, XXIV, and XXVII. From the biomechanical point of view, the fibrillar collagens are considered to be the most significant type as they constitute the main functional support material of almost every connective tissue in human body. The basic building blocks of a collagen-rich tissue are the collagen fibrils, which measure approximately 50 to a few hundred nanometers in thickness. The arrangement of fibrils includes different 3D patterns such as parallel bundles (in tendons and ligaments), orthogonal lattices (in the cornea), and concentric weaves (in bone). The fibrils are synthesized and secreted by fibroblasts but how this process is orchestrated and controlled, particularly during embryonic development, regeneration, and tissue repair, is poorly understood. With recent characterization techniques such as electron microscopy and X-ray diffraction [18–23], an attempt has been made to comprehend the collagen organizations in fibrils and it was found that the longitudinal packing has a high degree of order, forming banding patterns with a periodicity of $D = 64$–67 nm, as observed under the electron microscope. The same characteristic feature was identified in the X-ray diffraction studies, with the length of the fibrillar collagen molecules measured as 300 nm. The D-periodicity varies from 64 nm in skin and cartilage, to 67 nm in tendons, ligaments, bones, and several other tissues, which evidently constitutes the major structural difference in collagen fibrils in different tissues. The characteristic feature of classic fibrillar collagens is that they aggregate themselves into highly ordered fibrils with a typical quarter-staggered fibril array and most of the collagen fibrils are made up of different fibrillar collagen types and are referred to as heterotypic fibrils. Also, the presence of different collagen types within a single fibril is a structural

Table 1 Distinctive structural features of the major collagen classes and their associated functions

Collagen type	Distinctive structural feature	Associated function
Fibrillar collagens	300 nm uninterrupted triple-helical structure; aggregate themselves into highly ordered fibrils with a typical quarter-staggered fibril array	Influence the mechanical properties; tissue-specific features based on fibril arrangement
FACIT collagens	Collagenous domains interrupted by non-helical domains and trimeric molecules with alternatively spliced domains	Modulators of surface properties; control interactions between each fibril and the cell–matrix environment
Multiplexins	Homotrimers with strong sequence and structural homologies in the C-terminal; multiple triple helical domains	Maintain the structural integrity of basement membrane
Network forming collagens	Molecule of about 400 nm long that forms hexagonal networks	Supporting structures for cells and tissues
Transmembrane collagens	Homotrimer with N-terminal intracellular domain, single transmembrane stretch, and a large extracellular domain (characteristic of type II orientation)	Function as cell surface receptors and extracellular matrix components

prerequisite in many tissues. The collagen fibrils provide the key to scaffolding structures in the body from the nanoscopic to macroscopic length scales.

Type I collagen is the most extensively occurring collagen (up to 90% of all the collagen types) found in bone, tendon, skin, ligament, cornea, lung, vasculature, and in many other interstitial tissues except hyaline cartilage and brain vitreous body. Type II collagen is the predominant component of hyaline cartilage and is also found in vitreous body, corneal epithelium, notochord, nucleus pulposus of intervertebral discs, and embryonic epithelial–mesenchymal transitions. Type III collagen is present in relatively elastic tissues such as embryonic skin, lung, and blood vessels and is widely distributed in collagen I-containing tissues with the exception of bone. It is also an important component in reticular fibers in the interstitial tissues of the lungs, liver, spleen, vessels, etc. Type V collagen is found in association with type I collagen in minor quantities in the bone matrix, liver, lungs, and placenta and in particularly high amounts in cornea. Type XI collagen is found in minor quantities in the articular cartilage in association with collagen II. These classic fibrillar collagens have a long uninterrupted triple helical domain [24] and they can associate into homotrimers (collagens II and III), heterotrimers (collagen XI) or both (collagens I and V) depending on tissue specificity. Since these collagen fibrils are present almost everywhere in human tissues, they require different collagen gene products that lead to qualitatively different fibrillar forms, resulting in variation in properties. Though all collagen fibril structures contains the 300 nm triple-helical structure, some variations occur in the size and complexity of the telopeptides (the ends of the molecule) and the

interactions between the fibril-forming collagens within a fibril influence the mechanical properties. Collagen fibrils present in the ECM of connective tissues such as tendon, skin, and bone help them tolerate tensile forces and are crucial for skeletogenesis.

2.1.2 FACIT and FACIT-Like Collagens

FACIT (fibril-associated collagens with interrupted triple helices) collagens are non-fibrillar collagens as they do not form fibrils by themselves, but they are associated with the surface of collagen fibrils and some have transient interaction with fibrils during development. The first discovered protein of this family was the type IX collagen which was found to interact with type II collagen fibrils [25] and is a vital component of the cartilage collagen fibrils, along with type II and type XI collagens. Following this was the discovery of collagen types XII and XIV, which are present in tissues containing either type I or type II collagen [26, 27]. At present, the FACIT subfamily has become a large group with eight different members and forms a heterogeneous family of molecules. The FACIT collagens have their most important part at the fibril surface where the specificity of interactions can be conferred by the presence of many non-helical sequences of FACIT collagens. The FACITs are not only involved in modulating the surface properties but are also involved in packing of the fibril-forming collagens during fibril assembly. For example, collagen IX is a prototype FACIT collagen with interruptions in the triple helix and is an example of this class of molecule in which the globular structural features disrupt the internal structure of a collagen fibril. Electron micrographs show that the type IX collagen protrudes from the surface of the fibrils and establishes itself as modulator of surface properties [28]. Collagen IX is the most studied FACIT collagen [29–32] and is covalently linked to the surface of cartilage collagen fibrils of type II; the type IX collagen does not self-assemble into fibrils, and collagens XII and XIV are associated with collagen fibrils of types I and II. Evidence for the association of FACITs with banded fibrillar collagen in tendon was previously studied by Keene et al. [33]. The FACIT domain, i.e., a relatively short C-terminal triple-helical stretch flanked by a cysteine-containing motif found between the triple helix and C-terminal non-helical region, is a common feature of the FACIT collagens. The FACIT domain of type IX collagen may be incorporated into the gap between the consecutive fibrillar collagen molecules in cartilage fibrils [34]. Collagens IX and XII have GAG chains covalently attached to them, and alternative splicing of collagens IX, XII, and XIV expresses short and long variants that might facilitate different molecular mechanisms for modulating properties of the fibrils in tissues such as the cartilage, skin, and tendon [35–37]. Type XIV collagen is also a fibril diameter regulator in the early stages of fibrillogenesis [38]. Evidence suggests that, in addition to a fibril-associated form, type XII collagen possess aggregation properties involving interactions with basement membrane components, evidently signifying that many collagens can be involved in more than one kind of supramolecular assembly [39–43].

The FACIT-like collagens are found in the basement membrane junctions between tissues and share common features with the FACITs, but vary in their structure and function. This class includes collagen types XVI, XIX, XXI, and XXII [44–47]. Type XXII collagen is found in the myotendinous junction of basement membrane and also in association with cartilage fibrils [48]. Collagen type XVI is produced as a homotrimer and is enhanced during cell growth arrest and in fibrotic diseases. It is reported that collagen XVI takes part in stabilizing fibroblasts in dermal matrices [49, 50]. Collagen XVI is widely distributed in embryonic and adult tissues [51] and its supramolecular organization is tissue-specific. For example, collagen XVI is present in papillary dermis as a component of specialized fibrillin-1-containing microfibrils instead of banded collagen fibrils, whereas in territorial cartilage it occurs in a discrete population of thin, weakly banded collagen fibrils also containing collagens II and XI [52]. Type XIX collagen is reported to be present in the endothelial, neuronal, mesenchymal, and most epithelial basement membrane zones of all tissues [53].

2.1.3 Network-Forming Collagens

Collagen types IV, VI, VIII, and X make linear and lateral associations to form open networks rather than fibrils and they come under the network-forming collagen subfamily. Though present in smaller quantities than other collagen types, the networking collagens perform a variety of functions. Collagen networks act as supporting structures for cells and tissues, serve as selective molecular filters and barriers, function as anchorage for neighboring cells, and contain and protect the developing embryos [54]. Type IV collagen is the most important member of this class and is the main component of the basement membrane. It is composed of three 400 nm long polypeptide chains but the triple-helical domains of the polypeptide chains are often interrupted and it is shown that the flexibility of the interruptions is necessary to allow for the formation of a network rather than a fiber [55]. In the basement membrane, type IV collagen exists mainly in three different forms and this collagen forms a stable 3D basement membrane network via three types of interactions. Detailed explanation of the self-assembly, molecular architecture, and supramolecular organization of the type IV collagen in the basement membrane was discussed by Yurchenco and colleagues [56–58]. Type VI collagen occurs mainly as a heterotrimer and is a rod-like molecule about 105 nm long. Collagen VI molecules assemble into the so-called beaded filaments with a periodicity of 110 nm. Other forms of this collagen include beaded microfibrils, hexagonal networks, and broad-banded structures. Collagen VI has a ubiquitous distribution throughout the connective tissues where it forms an extensive microfibrillar network, linking cells and many matrix components, and is also important in preserving the integrity of tissues such as blood vessels, lung, and skin [59]. Types VIII and X collagens are called short-chain collagens [60–62] and collagen X is present in specific tissues such as the hypertrophic zone of cartilage during endochondral ossification. Type VIII and type X collagen help in the formation of a hexagonal

supramolecular network in the Descemet's membrane in cornea [63] and in calcifying cartilage [64], respectively.

Type VII collagens form anchoring fibrils and possess the longest triple-helical region among vertebrate collagens, measuring about 420 nm in length. This collagen is present underlying the basement membrane at the dermal–epidermal junction. Being a major component of the anchoring fibrils, type VII collagen combines with the banded collagen fibrils of the dermis and connect the epidermis to the dermis [65].

2.1.4 Multiplexins

Type XV and type XVIII collagens are homotrimers that include various collagenous domains and together they are called as multiplexins (multiple triple helices with interruptions). These two collagens occur in the endothelial and epithelial basement membrane zones of a wide variety of tissues [66]. Type XV collagen functions as a structural component to stabilize skeletal muscle cells and microvessels [67] and is also hypothesized to be a tumor suppressor [68]. Collagen XVIII is a heparan sulfate proteoglycan and it is the first reported collagen with heparin sulfate side-chains [69]. This collagen is reported to be present in the perivascular basement membrane zones. The many functions of the type XVIII collagen include maintaining the structural integrity of basement membranes, epithelial branching morphogenesis in the lung and kidney [70], and a major role in determining the retinal structure as well as the closure of the neural tube.

2.1.5 Transmembrane Collagens

Transmembrane collagens include specialized collagenous proteins that function concomitantly as cell surface receptors and perform structural and regulatory roles. The transmembrane collagen family includes types XIII, XVII, XXIII, XXV, and gliomedin, which are inserted in the plasma membrane in a type II orientation. The transmembrane collagens are widely expressed and participate in cell adhesion, epithelial–mesenchymal interactions during morphogenesis, neuromuscular signaling, and host defense against microbial agents. The biology and pathology of collagenous transmembrane proteins was explained in detail by Franzke et al. [71]. Collagen XVII is the characteristic and one of the best studied members of the transmembrane collagen family [72]. Also, collagen XVII was the first collagen type found to constitutively shed from the cell surface, yielding a shorter soluble form of the molecule [73, 74]. Collagen XVII is a keratinocyte surface protein in the epidermis and is a structural component of the hemidesmosomes, which mediate adhesion of epidermal keratinocytes and some other epithelial cells to the underlying basement membranes. Mutations in collagen XVII lead to diminished epidermal adhesion and skin blistering in response to minimal shearing forces. Collagen XIII occurs in many tissue junctions and cell–matrix interaction sites in epithelial, mesenchymal, and neural tissues, e.g., the myotendinous junction in skeletal

muscle and at cell–cell interaction sites such as the intercalated discs in the heart or adherence junctions in the epidermis. It is a component of focal adhesions in cultured fibroblasts [75]. During late fetal development, type XIII collagen can be observed in many tissues, including the cartilage, bone, skeletal muscle, lung, intestine, and skin. Type XIII collagen expression was observed in the central and peripheral nervous systems of the developing mouse fetus in mild-gestation [76]. Collagen XXV serves as a part of the cell adhesion machinery since it shares structural features with collagen XIII. It is described as brain-specific, named as CLAC-P (collagen-like Alzheimer amyloid plaque component precursor) [77] and is exclusively expressed by the neurons. The arrangements of the amino acids in collagen XXIII have close similarities to those in collagen XIII and XXV. It is observed in placenta, kidney, skin, and neural cells. Also, collagen XXIII induces adhesion and spreading of keratinocytes and its expression is restricted to basal keratinocytes, with uniform distribution on the cell surface of those cells [78].

3 Collagen in Human Tissues: A Mechanical Perspective

Every tissue is well adapted to its respective mechanical function and has an intrinsic tissue-specific structure–property relation. In general, naturally occurring tissues that are designed to carry mechanical loads are made of composites that are present in the form of fibers. Collagen fibrils form the principal building block for collagen-rich tissues in the human body. The thickness of the fibrils is in the range of 50 to a few hundred nanometers and the dominant mechanical properties of the tissues depend greatly on the fibrillar collagen structure. Collagen present in the human tissues is assembled in a complex hierarchical way into macroscopic structures and the methodological developments of the last decade have led to determination of the collagen morphology, which has enabled the understanding of biomechanical tissue properties of collagen-rich tissues. In this section, the major types of collagen and their structure and function in different tissues are discussed.

3.1 Bone

Bone is a stiff skeletal material and it is the most mineralized tissue of the human body, comprising primarily type I collagen fibrils reinforced with carbonated apatite crystals of a few nanometers in thickness. The organic material of bone consists of 85–90% of type I collagen [79] by mass and a series of non-collagenous lipids and proteins. Although bone collagen is predominantly type I, it also contains very small amounts of type III and type V collagen. Osteoblasts deposit the triple-helical collagen molecules into the ECM and the 300 nm long and 1.5 nm thick collagen molecules build fibrils by a self-assembling process [80]. Adjacent molecules are staggered in their long axis by 67 nm, generating a characteristic pattern of gap zones of 35 nm length and overlap zones of 32 nm length within the

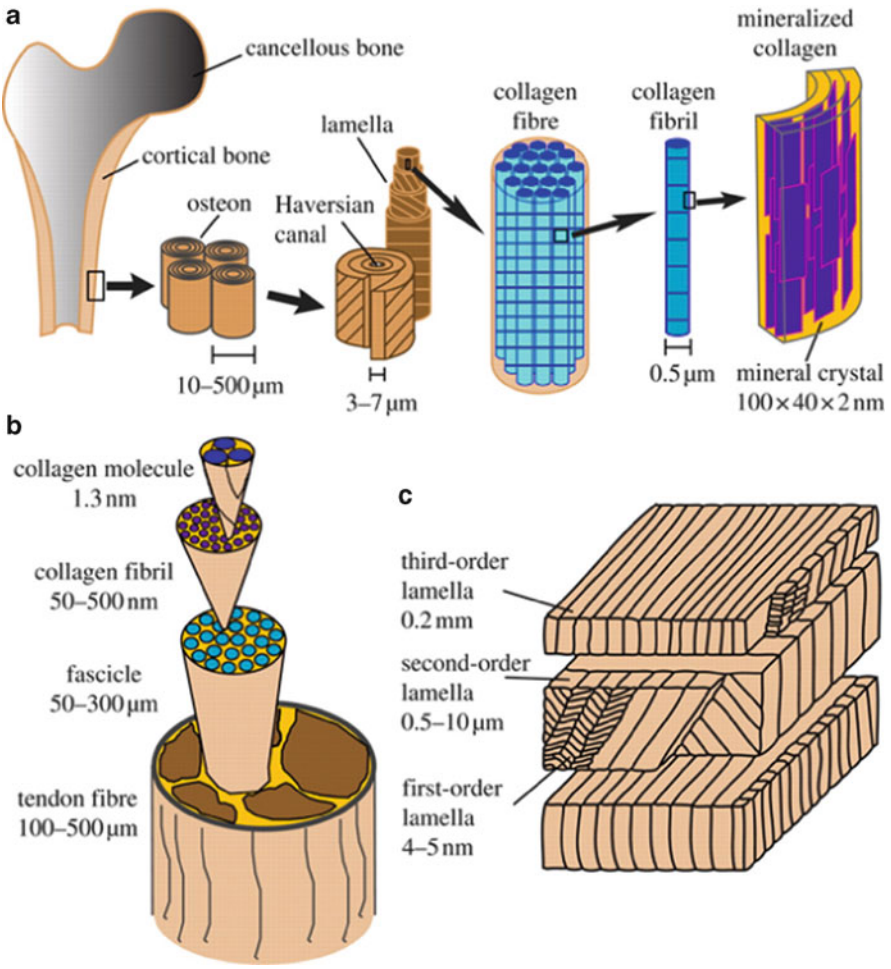

Fig. 3 Typical hierarchical structures of load-bearing biological materials: (**a**) bone, (**b**) mineralized tendon fibre, and (**c**) shell (reprinted with permission from [82])

fibril. The mineral crystals present in the bone either lie within the fibrils, disrupting them as they grow, or lie between the fibrils. Self-remodeling or regenerating occurs in bone, which makes it a mechanically anisotropic material both at the macroscopic and microscopic level. The bone tissue family includes the dentin, cementum, and mineralized tendon, all of which have different structural organization but share the same basic building blocks – mineralized collagen fibrils [81]. Bone has several levels of structural hierarchy above the molecular level and this has specific effects on the mechanics of bone. This scale dependence of mechanical properties is most probably a result of the hierarchical structure of bone, which comprises a number of structural elements on the millimeter to nanometer scale, as shown in Fig. 3 [82].

The collagen fibrils have a variety of arrangements distinctly associated with their function. In parallel lamellar bone, the fibrils are co-aligned whereas in woven bone, fibrils are randomly oriented or present in the form of small bundles [83]. Type I collagen present in the bone contributes towards the toughness (energy to fracture) of the tissue, mitigating the brittleness of mineral and also offering strength to the bone [84, 85]. Denaturing the collagen from the mineral phase leads to significant decrease in the modulus of elasticity, ultimate stress, and toughness [86, 87] while the denaturation of collagen triple-helical molecules may have a few effects on the viscoelasticity of bone [88]. Collagen provides bone with its tensile strength and forms a matrix for the deposition of mineral, which in turn confers rigidity to the bone. Initially, it was assumed that the stiffness of the bone is a consequence of the quantity of minerals present but recent results suggest that the properties of the collagen fibrils as well as the geometrical arrangement of the two components have a much larger influence than assumed. The modulus of elasticity of the bone corresponds to that of collagen [89] and collagen fiber orientation is considered to be a more important factor than porosity and mineralization in influencing the tensile strength of the bone [90]. Osteons containing longitudinal collagen fibers possess higher ultimate tensile strength and modulus of elasticity and lower percentage of elongation under tension than osteons with circumferentially oriented fibers. Transversely oriented collagen fibers are present in regions of compact bone experiencing higher compressive stress. The most unique feature of type I collagen fibrils in bone is the crosslinking chemistry and molecular packing structure, which provides the fibrillar matrices with a variety of properties such as tensile strength and viscoelasticity [91]. Collagen crosslinks are essential for bone to provide sufficient deflection capacity, bending strength, and stiffness. The non-collagenous acidic proteins present in the interfibrillar space form a supramolecular network that holds the collagen fibrils together and contributes to the fracture resilience of bone. The influence of collagen and other organic components on the mechanical properties of the bone can be assessed from the moduli and fracture strain of the bone constituents (mineral 135 GPa and 0.1%; collagen 1 GPa and 10%, respectively) and bone itself (10–25 GPa and 1–1.5%) [92]. The high modulus achieved by the collagen fibrils may be because the helices are physically confined between the mineral platelets. This shows that bone possess a unique combination of stiffness and fracture strain, making the bone stiff like mineral and also tough like collagen. Figure 4 shows the strain in collagen fibrils and mineral in human cortical bone as a function of applied strain in the tissue [93].

3.1.1 Relation of Collagen Content to Aging and Disease in Bone

Aging tends to influence the post-yield properties of whole bone, especially the toughness, which is particularly related to the reduction in strength of the collagen network. Hydroxyproline analysis showed a 50% decrease in total collagen concentration in cancellous bone with age, for both males and females. The study also revealed a steady reduction in mechanical properties of the cancellous bone with

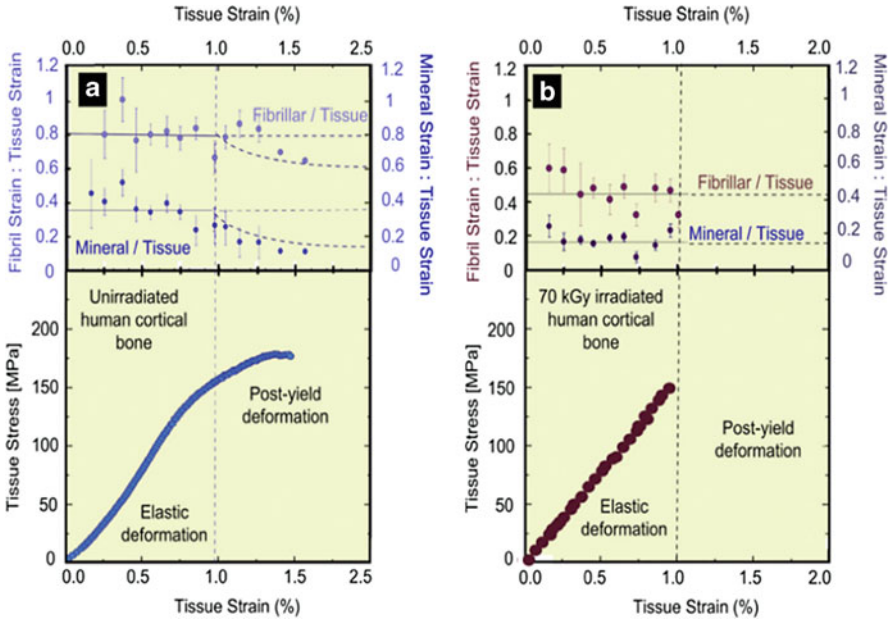

Fig. 4 Strain in collagen fibril, and mineral in bone as a function of applied strain in the tissue for human cortical bone in the (**a**) unirradiated and (**b**) 70 kGy-irradiated condition. The *upper graphs* show the ratios of fibril to tissue strain, $\varepsilon F/\varepsilon T$, and of mineral to tissue strain, $\varepsilon M/\varepsilon T$, for the (**a**) unirradiated and (**b**) 70 kGy-irradiated bone, averaged from $N = 20$ samples for each group. *Solid lines* represent the constant strain ratio expected before yield. *Dotted lines* represents where the ratio would vary if the fibril and mineral strain remained constant beyond the yield strain. The *lower graphs* show the corresponding stress–strain curves for the (**a**) unirradiated and (**b**) 70 kGy-irradiated human cortical bone (reprinted with permission from [93])

age, which is paralleled by the reduction in collagen content [94]. Studies using 30 human femora, measuring the mechanical properties of the demineralized bone and bone samples, showed that the mechanical integrity of the collagen network deteriorates with age. This was reflected as a 35% decrease in failure strength, 30% decrease in work to fracture, and a 10% decrease in strain to fracture [95]. Moreover, the porosity of bone was found to increase with age. However, it is important to note that neither bone porosity nor collagen integrity correlate significantly with the transverse fracture toughness of bone. The decrease in collagen crosslink concentration is found to decrease bone stiffness and its ability to absorb energy to fracture and such reductions occur especially with age and are very pronounced in osteoporosis. Collagen fibrils become narrower and apparently more disorganized with a reduced level of crosslinks, which is characteristics of osteoporosis [96]. The role of collagen in bone structure, the fibril diameter, and architecture in normal bone and during osteoporosis have been explained by a few researchers using thin section transmission electron microscopy (TEM) and computer-assisted analysis [96]. Osteogenesis imperfecta (brittle bone disease) is a heritable type I collagen disorder that results

in weak bones. Studies [97, 98] have shown that the weakness of the osteogenesis imperfecta bones is probably due to the de-arrangement of collagen molecules. However, what exactly happens in bone initially during its optimal property development (such as stiffness and toughness) and how the tremendous reduction in such properties during aging or bone diseases like osteoporosis occurs is still unclear. However, evidence collected so far indicates that the changes in bone, be they structural, mechanical, or biochemical, are correlated to the structure, concentration, and properties of collagen.

3.2 Cartilage

Cartilage is a type of connective tissue with a tough, flexible matrix made primarily of collagen and other proteins. It is also described as a tissue with low friction and high capacity to bear load and serves the essential function of allowing movement of bones against each other. Cartilage functions to provide a wear-resistant surface, facilitating load support and transfer over decades of constant use. In adults, cartilage is present in the trachea, bronchi, nose, ears, larynx, and intervertebral discs. Three types of cartilage are distinguishably known: hyaline cartilage, elastic cartilage, and fibrocartilage [99]. Hyaline articular cartilage is glass-like in appearance and is the resilient layer of the connective tissue that coats the ends of bones in a joint. The ECM of hyaline cartilage comprises differentially distributed collagen fibrils and non-collagenous proteins to form an extensive network. The composition of the articular tissue keeps changing as the tissue develops such that mature articular cartilage contains mainly collagens (two-thirds by dry weight), proteoglycans (15–30%) and some other minor protein molecules and chondrocytes [100]. Water (70–80% by weight) and the synovial fluid are the primary fluid components of the articular cartilage. Different types of collagens are present in the cartilage, among which type II and type IX collagen form the main part of the fibrils and type XI collagen is present within and on the surface of the fibril. These three types of collagen are cartilage-specific [101]. The core fibrillar network in developing cartilage is a crosslinked copolymer of collagens II, IX, and XI. The minor collagens in the cartilage include types III, VI, X, XII, XIII, and XIV. Observation under light microscopy revealed different collagen fibril orientations in different zones of the cartilage [102]. The arrangement of collagen fibrils in the articular cartilage is such that deep-zone collagen fibrils are assembled perpendicular to the articular surface, curve away obliquely to form arches in the middle zone, and run parallel to the articular surface in the superficial zone. The material strength and biological properties of articular cartilage depend heavily on its uniquely and extensively crosslinked collagen network and characteristic fibrillar organization that varies with tissue depth and proximity to the cells. Each collagen type has a specific role in maintaining the structure of the cartilage. Type II collagen is a fibrillar molecule with 67-nm periodicity and it contributes towards tensile strength, enables the tissue to resist shearing forces, supports chondrocyte adhesion, and

induces differentiation of cells. Type IX collagen facilitates fibril interaction with the matrix proteoglycan molecules [101] and type XI contributes to the regulation of fibril size. Type X collagen organizes the collagen fibrils into a 3D hexagonal lattice [103]. Type VI collagen is mainly found at the periphery of the chondrocytes and helps to attach the cells to the matrix framework. The collagen fibrils form a random, loose network in the articular cartilage compared with collagen in most other connective tissues. The fibrils are thin in the surface zone and are parallel to the plane of the articular surface, with some degree of preferred orientation [104]. Collagen fibers tend to appear coarser and more banded going farther from the chondrocytes [103]. Type IX and type XI collagens are found in higher proportions in the thinnest fibrils and remodeling/maturation of the thin, newly formed collagen fibrils involves removal of types IX and XI collagens and addition of type II collagen [105, 106]. The tensile properties of cartilage are nonlinear due to the behavior of collagen fibers in the tissue. The collagen fibers align in the direction of loading and stretch as the tension increases, as a result of which the tissue exhibits higher stiffness at larger strains [107]. Collagen fibril orientation is a major factor in influencing the tensile strength and cartilage exhibits greatly increased stiffness with increasing deformation or strain parallel to the fibrils. The interactions between collagen and proteoglycan networks influence the viscoelastic and shear behavior of cartilage.

3.2.1 Relation of Collagen Content to Injury and Disease in Cartilage

Cartilage injuries are mainly due to injurious impact, repeated loading, torsional loading, joint malalignment, and foreign bodies in the joint and they are generally classified as osteochondral defects, chondral defects, and cartilage microfractures. Although cartilage is a metabolically active tissue, it has a limited capacity for intrinsic repair and even minor injuries may lead to progressive damage or joint degeneration in the case of articular cartilage [108, 109]. It is important to note that the chondrocytes can repair defects if the fibrillar collagen meshwork remains intact. The collagenous matrix is one main sources of destructive processes and the major aim of therapeutic cartilage repair is the development of an adequate collagen framework [110]. Overall, no collagen is lost during cartilage degeneration, but loosening of collagen network occurs as a major factor [111, 112]. During osteoarthritis, loosening of collagen network causes loss of proteoglycans, which results in mechanical overload and damage [113]. The collagen network of the osteoarthritic cartilage contains increased levels of degraded collagen molecules and the weakened collagen network leads to swelling and instantaneous deformation since it cannot counteract the swelling properties of proteoglycans or the rapid bulk movement of the proteoglycan–water gel. In osteoarthritis, the decrease in stiffness of the collagen is found to be largest in the surface region. The remodeling process generates hyaline-cartilage-like repair tissue, the ECM of which was shown to contain types II, IX, and XI collagens similar to those of the normal articular cartilage, however, the collagen fibrils in the repair cartilage are randomly

distributed and the cellular density and arrangement also differs [114, 115]. The diameter of the collagen fibrils in cartilage increases with aging and this leads to changes in the packing density of the molecules. Also, accumulation of non-enzymatic glycation products such as pentosidine can be related to an increase in collagen stiffening, making it more brittle and contributing to age-related decrease in the resistance of cartilage to fatigue and other damages.

3.3 Skin

Skin is the largest organ of the human body and plays a crucial role in a wide variety of functions such as protecting the organs from the environment and external insults, thereby preventing hazardous substances and bacteria from entering the body, controlling loss of water from the human body, fluid homeostasis, sensory direction, self-healing, protecting the cells from mechanical damage, etc. [116]. Human skin consists of two primary layers, a stratified, cellular epidermis and an underlying dermis of connective tissue, and a third layer, the hypodermis, consisting mainly of fat and a layer of loose connective tissue. Together, these three layers help in protecting the body from any mechanical damage such as injury. The epidermis is a thin and highly cellular layer that consists of keratinocytes and acts as a barrier to infection. The dermis contains a collagen-rich ECM, also comprising other proteins such as elastin and GAGs, and contributes to the bulk of the skin. The fibroblasts, which are the major cell type in dermis, play an important role in wound healing by producing remodeling enzymes such as collagenases. Immediately beneath the epidermis is the most superficial region of the dermis, known as the papillary dermis, which has finer collagen fibers and a higher density of fibroblasts than the deeper, reticular dermis. Hypodermis contains the adipose tissue, which is well vascularized and contributes to both the thermoregulatory and mechanical properties of the skin. Human skin has a unique combination of strength and elasticity, which is predominantly due to the dermal collagen network [116]. Dermal collagen is randomly organized and collagen bundles appear in a basket-weave-like pattern in the human skin [117]. The reason for the random structure, especially in parallel planes at the deeper levels, can be related to the functions of the skin such as enduring considerable shearing and pulling forces and it can be understood that the collagen structure is such that it resists these forces [118]. Human skin contains interstitial collagen types I, III, and V as well as the basement membrane collagen type IV [119–121]. The macromolecular organization and quantity of the collagen types varies in different layers of human skin. Type I and III are the major dermal fibrillar collagens and type IV is a basal lamina collagen present at the dermal–epidermal junction and around blood vessels and nerves, while type V collagen is found diffusely throughout the dermis and is concentrated in or near basement membranes [121–125]. Among the different types of collagen, 85–90% is type I, 8–11% is type III, and 2–4% is type V collagen in adult skin while during all gestational ages 70–75% is type I, 18–21% is

type III and 6–8% type V. It can be seen that the accumulation of dermal fibrillar collagen with age enhances the proportion of type I collagen [126]. In the early stages of gestation, the dermis contains fine, individual collagen fibrils over the surfaces of mesenchymal cells. The randomly assembled fibers of the skin permit considerable extension of the tissue until the fibers themselves are loaded. In skin, the collagen fibers are biaxially oriented and the elastic modulus of skin, which is about 4 GPa, is similar to the elastic modulus of normal type I collagen. At low strains, the elastic and viscous stresses are almost the same as the viscoelasticity of skin and this is mainly due to the stretching of elastic fibers that surround the collagen fibers. But, at higher strains, the collagen fibers bear the loads and store energy, which prevents premature mechanical failure of skin, and also the elastic stress is much greater than viscous stress in this case.

3.3.1 Relation of Collagen Content to Aging, Wound Repair, and Healing of Skin

With increasing age, the collagen matrix in the ECM and the size of the collagen fibril diameter increase and a greater number of collagen fibrils associate to form fiber bundles. Aging is associated with a decrease in collagen turnover due to a decrease in the number of fibroblasts and their collagen synthesis [127]. Skin collagen decreases linearly by about 1% per year throughout adult life. During the aging process, the elastic properties of collagen fibers change by biochemical transformations such as crosslink formation, fragmentation, etc. Progressive skin thinning occurs with age and this corresponds to changes in the microarchitecture of the dermis following biochemical modifications of the collagen fibers. The elasticity of skin decreases and the stiffness of skin increases with increase in age. Collagen has a close connection to wound repair since the ultimate result of most repair is the formation of scar tissue, which is composed of collagen fibers. The first step in healing is the formation of a clot, which involves adhesion of circulating blood platelets to collagen. Aggregation of platelets is caused by collagen-induced release of adenosine diphosphate, which acts as a hemostatic plug at the site of injury. The final stages of healing involve the production, maturation, and degradation of collagen fibrils, which form a scar tissue of appreciable tensile and breaking strength [128]. The collagen morphology of scar tissue is significantly different to normal skin because the scar tissue consists of smaller bundles aligned in a parallel fashion to the epidermis [117]. Schilling [129] reported that the collagen fibers of scars align themselves in a direction parallel to that of tension or stress forces. In normal human skin, type I collagen exceeds type III by a ratio of 4:1 but during wound healing this ratio decreases to 2:1 due to an early increase in the deposition of type III collagen, which is because the fibroblasts transform into myofibroblasts and deposit mainly type III collagen [130, 131]. The preliminary deposition of collagen is followed by a slow process of remodeling, and wound healing is associated with a more regular alignment of collagen than that present in normal tissue [128, 132, 133].

3.4 *Myocardium*

The myocardium is the thickest contractile middle layer of the heart wall and is composed of cardiac muscle cells that form the bulk of the heart. The myocardium consists of muscle fibers and blood vessels, which are connected and interspersed by a network of connective tissue. The uniqueness of heart lies in its dynamic functionality, which requires sophisticated tissue architecture with specialized cellular and extracellular components. Every excitation and contraction cycle of the cardiac muscle involves a number of mechanical events and recent studies have shown more significant association of the ECM in all aspects of the electromechanically active myocardium than previously believed [134–137]. Collagen networks contribute chiefly to the cardiac ECM and they play a vital role in the myocardial structure and function. Collagen provides strength and stiffness to the myocardium and establishes a structural framework for the myocytes and provides myocyte-to-myocyte connections (collagen struts) that are vital in adhering the cells. Five collagen isoforms are present in the myocardium: types I, III, IV, V, and VI. The most abundant forms of collagen are type I and type III collagens, contributing 80% and 12%, respectively, which also constitute the bulk scar tissue following myocardial infarction (MI) [138, 139]. Type I and type III collagen molecules form aggregate struts of varying thickness and are widely distributed between myocytes and among muscle fibers [140, 141] whereas type IV collagen molecules arrange themselves to form end-to-end aggregates characterized by a fishnet appearance in the basement membrane of cardiac myocytes and fibroblasts [56, 142]. Type VI collagen is present as fine filaments in the myocardium oriented perpendicularly opposite to other collagen fibers [143]. Collagen types present in the myocardium are relatively insoluble and are characterized by abundant inter- and intramolecular covalent crosslinkage [144]. Also, a hierarchy of decreasing tensile strength exists among cardiac collagen (type I > type III > type VI and fibronectin > basement membranes) so that changes induced by contraction and relaxation can be effectively distributed throughout the heart [139]. Przyklenk et al. [145] found that the stiffness and tensile strength of the myocardium correlated directly with collagen content and, as such, a collagen-rich matrix is crucial in maintaining the integrity of the cardiac muscle.

3.4.1 Relation of Collagen Content to Infarction and Aging of Myocardium

MI is a condition that occurs when there is a blockage in one or more of the blood vessels supplying blood to the heart. A series of pathological processes follows MI, which includes an initial inflammatory response, loss of cardiomyocytes, infarct expansion, degradation of the myocardial ECM, etc. After MI, the collagen network is damaged and this weakened collagen network leads to wall thinning and ventricular dilation. Infarct expansion is closely associated with the impairment of the collagen network of the heart, which is due to apparent loss of collagen struts.

This loss provides the additional damage essential for significant infarct expansion. When collagen has been deposited in the infarct to form the scar tissue, the infarcted myocardium becomes capable of resisting further expansion to a certain extent, suggesting that the damaged collagen network is vulnerable but normal collagen is able to withstand expansion [146]. Therefore, efficient and rapid deposition of type I collagen is fundamental for healing since the collagen matrix provides a mechanically strong network, minimizes infarct expansion, and resists maladaptive remodeling [147]. It is suggested that in spite of the deleterious consequences of degrading collagen fibrils, cleavage of type I collagen is essential for effective fibrotic healing after MI [148]. Also, with increase in age, there is an apparent increase in type I collagen content, which may be due to loss of myocytes leading to accumulation of collagen in the left ventricular wall. With age, the type I collagen fibers tend to increase in number, thickness, and diameter and the increased myocardial collagen content results in a decrease in the ventricular elasticity [149]. In general, aging and myocardial disease are associated with enhanced deposition of myocardial collagen types I and type III, with the former being more predominant [150].

4 Biomedical Implications of Collagen as Scaffold Material

Collagen is pliable for different processing techniques and can be fabricated into a wide variety of forms such as films, strips, sheets, sponges, beads, discs, etc. for biomedical applications. Detailed imaging analysis of the structure of collagen at each stage of the manufacturing process reveal that it maintains its original triple-helical structure throughout and does not become denatured. Collagen is highly dynamic and undergoes constant remodeling for proper physiological functions and performs myriad functions in the human body. Collagen plays a dominant role in maintaining the biological and structural integrity of tissues and imparts tensile strength to the tissues. From the biophysical aspect, collagen aides in transmitting forces, dissipating energy, preventing premature mechanical failure, and providing biological signals to adjacent cells to regulate functional responses [151]. In addition, mimicking the ECM using naturally occurring proteins will provide better infiltration of cells into the scaffold and also provide sufficient nutrient supply. Collagen also helps in the formation of tissues and organs and has a distinct mode of interaction in the body compared with other natural and synthetic polymers. The collagen structure offers the cells a suitable biological environment for embryologic development, organogenesis, cell growth, and wound repair [152]. The repair and restoration of function and structural integrity of disrupted tissues after an injury largely depends on the deposition and production of collagen. Scarce collagen deposition will make the wound weak and may cause rupture. On the other hand, loss of anatomical structure and function and fibrosis will occur due to excessive deposition of collagen at the wound site [153]. All these properties have contributed to the extensive use of collagen in coatings for clinically used hernia meshes.

Collagen hernia meshes derived from human dermis are also being developed to improve the biological properties of the implant.

Collagenous scaffolds have found extensive usage in various tissue engineering (TE)-based applications due to properties such as excellent biodegradability, weak antigenicity, and superior biocompatibility. Collagen scaffolds produced as gels or sponges lack mechanical and structural stability and certain crosslinking techniques using glutaraldehyde vapors, formaldehyde, and epoxy compounds have been attempted in an effort to improve mechanical properties. Glutaraldehyde-treated type I collagen scaffolds were found to match the tensile strength of several commercially available wound care products such as Beschitin and Resolute LT [154]. However, this approach causes an increased risk of cytotoxicity and calcification when used in vivo. Barnes et al. [155] developed a technique for the crosslinking of electrospun collagen using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in ethanol, which imparted desirable mechanical properties, prevented any cytotoxic effects, and also maintained the nanofibrous structure. Dong et al. [156] experimented with electrospinning of type I collagen using buffer/ethanol and crosslinking using EDC and proved that the triple-helical structure of collagen can be preserved. In another experiment, the morphology, cell growth, adhesion, and osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (MSCs) on EDC crosslinked electrospun collagen fibrous mats was assessed [157]. Collagen is known to have different effects on tissue restoration depending on the type, structure, degree of crosslinking, and chemical treatment. The effect of glutaraldehyde crosslinking on mineralized polyanionic collagen (PAC) membranes was analyzed by conducting a histological evaluation of the tissue response and by assessing the biodegradation of subcutaneous membrane implants in rats [158]. The inflammatory responses to membranes without crosslinking were found to be higher, which was seen from the reduced leukocyte count observed in the histological analyses of the glutaraldehyde-crosslinked collagen membranes. The healing period was found to be longer in membranes without crosslinking as the space occupied by the membrane and multinucleated cells/leukocytes was slowly replaced by connective tissue, bone, and cementum. Another finding from this study was that the crosslinked collagen membranes had the added advantage of supporting healing, even when prematurely exposed to the environment, which can be explained by the fact that the crosslinked collagen membranes have the capacity to withstand bacterial collagenolytic degradation while facilitating soft tissue healing over the exposed membranes [159]. Angele et al. [160] assessed the differences between the properties of bovine- and equine-based collagen as TE scaffolds and showed that the results obtained for scaffolds based on a certain collagen species may not be transferable to scaffolds based on another, because of the differing physico-chemical properties. Table 2 give details of the mechanical properties (tensile strength, compressive strength, and Young's modulus) of different tissues in the human body and indicates the type of collagen found abundantly in that tissue. Things are more complicated than they look as far as collagen is

Table 2 Mechanical properties and type of collagen present in different human tissues

Tissue	Tensile strength (MPa)	Compressive strength (MPa)	Young's modulus (MPa)	Collagen type
Bone				
Cancellous	2–20	4–12	0.02×10^3 to 0.5×10^3	Collagen I
Cortical	60–160	130–180	3×10^3 to 30×10^3	Collagen I
Cartilage	3.7–10.5	–	0.70–15.3	Collagen II
Myocardium	3×10^{-3} to 15×10^{-3}	–	0.02–0.5	Collagens I and III

concerned and therefore it is very important to get the right type and quantity of collagen for a suitable tissue, thereby making it appropriate for a particular application such as bone, cartilage, skin, or heart tissue engineering.

Taking into account all these features, electrospun collagen nanofibers present themselves as an appropriate material for tissue engineering of scaffolds. Fabricated and modified collagen nanofibers are becoming more common in bone, cartilage, skin, cardiovascular, and numerous other TE applications [161–164].

4.1 Collagen in Bone Tissue Engineering

Scaffolds for the repair of bone must provide mechanical support and simultaneously induce bone mineralization and 3D cell–cell interaction. Since type I collagen is the key constituent of bone, it is most obvious to use it for engineering this type of tissue. Liao et al. [165, 166] reported the fabrication of mineralized collagen matrix with or without growth factor and utilized it for the posterolateral lumbar spinal fusion in rabbit models. Biomimetic mineralization of collagen and its use as improved bone implants was also reported by Bradt et al. [167]. Electrospun type I collagen nanofibers of different nanosized diameters (50–200, 200–500, and 500–1,000 nm) were utilized for the osteogenic differentiation of human bone marrow-derived MSCs [168]. Researchers found that the cells on collagen nanofibers with higher fiber diameter (500–1,000 nm) showed significantly higher cell viability than the tissue culture polystyrene control and suggested its application for treatment of skeletal injuries. Modifying the structure and tailoring the desirable properties of type I collagen for targeted applications is also possible via chemical and surface modifications using relatively simple techniques. Alkaline treatment is one such example, wherein anionic collagen with enhanced piezoelectric properties can be obtained through hydrolysis of the carboxamide groups of asparagine and glutamine residues of collagen [169]. The resultant product has a sponge-like structure with heterogeneous pore size. This study characterized the morphological properties of anionic collagen matrices produced from bovine pericardium and the results indicated the formation of

bone within a scaffold of collagen with three different charge densities, obtained through the hydrolysis. The anionic collagen matrices were implanted in surgically created bone defects in rat tibias, where empty defects served as controls for assessment of their biocompatibility. Preliminary evaluation of the osteoconductivity of the matrices was carried out and the results reported low inflammatory response and bone formation within a short period of time.

4.2 Collagen in Cartilage Tissue Engineering

Collagen accounts for about two-thirds of the dry weight of adult articular cartilage, of which type II collagen is the principal molecular component (50–80%) though other collagen types such as III, VI, IX, X, XI, XII, and XIV are also found in the mature matrix [170]. The extensive crosslinking of the collagen and changes in fibrillar architecture gives strength to the tissue [27]. The near frictionless cartilage lines the articulating joints and performs functions such as energy absorption and load distribution to the bones. Compositional and structural degenerative changes of the articular cartilage modify chondrocyte function and lead to osteoarthritis, which ultimately results in the inefficiency of mature cells to restore the damaged cartilage [171]. The articular chondrocytes do not have the ability to restore the overall collagen structure if the tissue is injured or degenerated. In such cases, it is necessary to recreate both the function and morphology of the native articular cartilage using a bioengineered graft, and the most sensible choice of protein for this purpose will be type II collagen since it is the predominantly found material in the ECM of the native articular cartilage. Collagen has been under investigation for some time, with early in vivo studies being performed in animal models to evaluate healing after seeding chondrocytes. Chondrocytes seeded in type I and type II collagen scaffolds when implanted helped in the repair of chondral defects in a canine model. Type II collagen matrix showed a higher percentage of seeded chondrocytes with spherical rather than elongated and flattened morphology compared to type I collagen matrix and also greater GAG production by the cells. Type II collagen scaffold also showing higher filler amount and more intensive staining for type II collagen.[172]. However, it was found that the reparative tissue consisted primarily of unwanted fibroblasts. The importance of improving the mechanical properties of such scaffolds with regard to their applications was considered important.

Mathews et al. [173] experimented on the feasibility of electrospinning type II collagen and seeded chondrocytes on scaffolds for application in cartilage TE. The electrospun collagen fibers obtained from this experiment were around 110 nm, which were comparable to the 80 nm diameter of the collagen type II fibers found in the native tissue. These type II collagen fibers possessed structural integrity and had the ability to undergo manual manipulation after stabilization with glutaraldehyde vapor fixation. The seeded chondrocytes supported cell growth and infiltrated within the electrospun type II collagen nanofibers.

SEM analyses and histological studies confirmed the formation of confluent layers of cells on the external surface after 2 weeks and uniform distribution of the chondrocytes throughout the scaffold thickness with no overt changes in the structure of the matrix. The same researchers evaluated electrospun type II collagen nanofibers under three different conditions: uncrosslinked, crosslinked, and crosslinked/seeded [174]. Average fiber diameter and thickness of the uncrosslinked scaffolds were 496 nm and 0.20 ± 0.02 mm, respectively, and for the crosslinked scaffolds were 1.46 μm and 0.52 ± 0.07 mm, respectively. The increased thickness of the crosslinked scaffolds was attributed to the hydration of the scaffold, allowing the interstitial space between the fibers to expand. The type II collagen fibers crosslinked using glutaraldehyde were found to be stiffer, whereas differences between natural tissue and the uncrosslinked scaffolds were attributed to the lack of hydration. SEM studies of seeded, crosslinked type II collagen scaffolds revealed the adherence, proliferation, and infiltration of chondrocytes with formation of pseudopodia, responsible for cell attachment and communication within the type II collagen scaffold. In order to maintain the fiber morphology it was also necessary to prevent or minimize the shrinkage of the electrospun fibers and a new method of crosslinking electrospun type II collagen scaffolds was tested by Barnes et al. [155]. This group used EDC in nonaqueous solution to crosslink electrospun type II collagen fibrous matrices in a manner comparable to that of typical glutaraldehyde fixation protocols. Since collagen fibers electrospun from pure collagen disintegrate when placed in an aqueous solution, the crosslinking procedure involved the use of ethanol as the solvent for the crosslinking agent and as the proton donor for the EDC reaction instead of a buffered solution. The electrospun type II collagen nanofibers after crosslinking displayed similar behavior to that of the native tissue and it was observed that the stiffness of the collagen nanofibers did not inhibit chondrocyte mobility. The crosslinking treatment using EDC in ethanol provided sufficiently greater crosslinking and mechanical strength than the typical glutaraldehyde crosslinking. Pieper et al. [175] described the preparation of highly purified type II collagen from bovine tracheal cartilage and the development of porous EDC/*N*-hydroxysuccinimide (NHS)-crosslinked type II collagen matrices with and without attached chondroitin sulfate (CS). These collagen matrices were studied in vitro by culturing chondrocytes. EDC/NHS-crosslinked collagenous matrices showed porous morphology and maintained their structural integrity during culture. These researchers observed that the type II collagen matrices showed a distribution of chondrocytes throughout the matrix, which will strongly facilitate cartilage regeneration.

4.3 Collagen in Skin Tissue Engineering

Skin is the largest organ of the integumentary system and protects the underlying muscles, bones, ligaments, and internal organs by serving as a barrier against the

environment. The skin contains many types of collagens, type I and type III accounting for most of the dermal ECM. Appendages such as hair and glands are derived from the epidermis, but they project deep into the dermal layer. The integrity of this vital organ is threatened by severe damage caused by trauma or injury and in these cases it is necessary to recreate normal physiological skin function and homeostasis. Since the introduction of skin grafts by Reverdin in 1871 [176], several novel techniques for skin replacement have been developed such as cultured autologous and allogenic keratinocytes grafts, autologous or allogenic composites, acellular biological matrices, and cellular matrices including biological substances such as various types of collagen. These dermal matrices can be grafted onto deep wounds after early excision and can promote the reconstruction of a new dermis suitable to support the graft of autologous cultured epidermal sheets. One such commercially available graft that is well documented in the literature is the SkinTemp (Biocore, Topeka, KS). It is a type I bovine sponge-like collagen matrix that can provide a safe, readily available approach to secondary intention healing in patients where immediate reconstruction is contraindicated and who need a long-term biological dressing that stimulates wound healing [177, 178]. Similar collagen-based biomaterials were developed and designed for clinical and experimental purposes. The use of collagen gels (Apligraft), collagen sponges (Integra) and decellularized collagenous tissues (Alloderm) have all been approved by the US Food and Drug Administration and shown to improve wound healing in clinical applications [179–182]. A very notable advance in the field of wound healing is tissue-engineered skin and the goal of tissue engineers is to restore skin ontogenesis and regeneration. The characteristics of scar-free healing after incisional wounding include minimal inflammation and complete restoration of normal skin structure with normal collagen deposition and regularly distributed hair follicles, capillaries, and glands. Collagen fibrils are chemotactic and promote cellular proliferation, differentiation, and provide considerable strength. Venugopal and Ramakrishna [183] produced randomly oriented collagen nanofibers in the range of 200–250 nm and in a comparative study of human dermal fibroblasts proliferation on collagen blended PCL scaffolds showed that the collagen nanofibers improved (54% after 72 h) cell proliferation of fibroblasts. In another work, 3D collagen scaffolds having precisely controlled pore structures (pore sizes, 160 and 300 mm; >95% porosity) and successive layers of perpendicular collagen strands (300–320 mm in size) were fabricated by using an innovative cryogenic dispenser system. According to Hollister [184], a mechanical modulus ranging from 0.4 to 350 MPa should be adequate for soft tissue. Young's moduli of the scaffolds before and after crosslinking using EDC were documented as 4.9 ± 1.4 and 7.7 ± 2.2 MPa, respectively. Normal human keratinocytes and fibroblasts isolated from adult foreskins were cultivated and it was confirmed that the keratinocytes/fibroblasts were sufficiently proliferated. Hematoxylin staining and immunohistochemical studies showed that the proliferated keratinocytes migrated completely from the bottom to the surface of the porous collagen scaffolds and that fibroblasts were well dispersed within the scaffold, proving yet again that collagen fibers support skin tissue regeneration [185]. The hydroxyallysine crosslinking

levels of collagenous matrices obtained in vitro varied the susceptibility of the collagen matrices to matrix metalloproteinases, which is responsible for increased deposition of collagen leading to fibrosis. In vivo evidence on the importance of the type of crosslinking in determining the reversibility of the fibrotic process was found using the bleomycin-induced skin fibrosis mouse model. Analysis of the accumulated collagen in the fibrotic skin of bleomycin-treated mice did not reveal an increase in hydroxyallysine crosslinking levels. The authors proved their hypothesis by showing that the collagen accumulation resolved in time when the mice were no longer receiving bleomycin treatment, showing the reversibility of the fibrosis [186].

4.4 Collagen in Cardiovascular Tissue Engineering

Collagen is one of the most abundantly found proteins in the native artery and provides smooth muscle cells (SMCs) with greater wall strength and resistance to vessel rupture in a harsh in situ environment. According to the vascular physiology, the native artery comprises three distinct layers – intima, media, and adventia – in order to withstand the high flow rate, high pressure, and pulsatile nature of blood flow. The intimal layer is composed of a single layer of endothelial cells on a thin basal lamina and a sub-endothelial layer of collagen IV and elastin. The thick medial layer comprises several layers of SMCs in a matrix of collagen types I and III; elastin and proteoglycans are also present. The outermost advential layer is made up of fibroblasts and type I collagen [187]. In the heart tissue, the complex structural matrix of the ECM primarily consists of fibrillar collagen type I (80%) and type III (11%), and smaller amounts of type IV and type V collagens along with GAGs [162, 163]. The major requirements for regenerating elastic tissues such as blood vessels and smooth muscles include excellent mechanical integrity, porosity for tissue ingrowth, lack of thrombus or embolism formation, absence of fatigue, and resistance to wear and tear. Over the years, several attempts have been made to engineer the cardiovascular region, including classic in vitro TE approaches for myocardial TE using pre-formed 3D porous scaffolds or dense patches of synthetic/natural polymers to support the diseased region of the heart and the creation of a bioresorbable vascular graft that is eventually replaced by autologous tissue for cardiovascular TE. Wesolowski et al. [188, 189] first introduced the concept of a bioresorbable vascular prosthetic made up of a variety of Dacron yarns, collagen coatings, and collagen fibers. Collagen fibers were used in the prosthetic so that it promoted bioactivity and native tissue ingrowth and was subsequently degraded. Because type I collagen is the principal protein of the heart tissue, several authors experimented on the fabrication of type I collagen for cardiovascular TE [190]. Scaffolds of collagen were obtained by freeze-drying type I collagen and two different crosslinking methods (carbodiimide alone or a combination of carbodiimide and a diamine) were used to improve the stability of the obtained matrices. SMCs isolated from

human umbilical and saphenous veins were cultured for different periods of time and it was observed that no differences in cell attachment and proliferation were seen between scaffolds crosslinked using the two methods. A multilayer of cells was observed from the histological analyses and it was noted that the orientation of the cells resembled the orientation of native collagen and elastin fibers, and the cells were seen not only at the surface but also inside the scaffolds. Type I collagen was used for engineering a cardiac muscle construct, known as engineered heart tissue (EHT), with a combination of neonatal cardiomyocytes and an artificial ECM [191]. The applicability of EHT graft on the heart of syngeneic rats was assessed and it was discovered that the EHTs were heavily vascularized and retained a well-organized heart muscle structure 14 days after implantation. The contractile function of EHT grafts was preserved in vivo [192]. In order to enhance the in vivo performance of the EHT, certain modifications were implemented [193] and after 28 days, systolic wall thickening of the infarct region and electrical coupling to the native myocardium was observed. These results ascertain that EHT grafts fabricated from type I collagen and other ECM proteins (Matrigel) can be constructed in vitro, implanted in vivo, and can eventually support infarcted myocardial muscle. Kofidis et al. [194, 195] engineered a novel type of artificial myocardial tissue (AMT) by seeding neonatal rat cardiomyocytes with a commercially available, clinically approved bovine collagen-based 3D matrix (Tissue Fleece), which can be manufactured in various shapes to fit into infarction scars. Migration of myocardial cells into collagen-based scaffolds occurred and resulted in the development of artificial myocardium-like tissue with improved mechanical stability compared with that of the initial matrix material. Passive stretch curves and force measurements from the spontaneously beating construct were obtained. Elasticity was found to be similar to that of the native tissue and histological studies confirmed that cardiomyocytes seeded into the collagen scaffold maintained intrinsic activity and responded to external stimuli.

Recently, a new process has been devised to extract pure collagen of type I and III in fiber form using microbial treatment of bovine Achilles tendon [196]. Using this process, regularly ordered fibers of collagen possessing a rope-like structure were obtained and these collagen fibers were made non-immunogenic by the removal of certain terminal peptide chains. These non-cytotoxic collagen fibers retained the mechanical strength of the native tendon and their flexibility and compatibility assure them as potential fibers for cardiac TE. The collagen fibers showed shrinkage to half of their original length at 65°C, confirming that the fiber obtained is stable. In vivo biocompatibility and antigenicity studies showed that the cells adhered well on the collagen film and that collagen had been reabsorbed at the implanted site; a small circular patch of the implant was seen remaining after 14 days. Collagen is one of the first materials to be explored as an injectable for regeneration of the infarct myocardium. The feasibility of injecting commercially available collagen has been tested and favorable results such as improved thickness of the left ventricular wall, prevention of further infarct expansion, neovasculature formation, etc. have been reported [197].

5 Current Perspective on Collagens in Tissue Engineering

Collagen, which is currently being utilized for tissue engineering and other biomedical applications, is derived from animals such as cows and this might increase the possibility of contamination or generate harmful immune responses when the tissue-engineered scaffold is used in vivo. Such negative responses have raised the necessity to develop artificial collagens for practical applications. Koide et al. [198] developed collagen-like supramolecules by the self-assembly of a novel, trimeric peptide-based system. The obtained collagen-like peptides were found to be self-complementary trimers that formed a staggered arrangement. On the other hand, Kotch et al. [199] synthesized collagen fragments directly on a solid support by orthogonal deprotection of the cysteine residues. Atomic force microscopy and TEM studies revealed that the fragments self-assembled via intramolecular triple helix formation, resulting in fibrils that resembled natural collagen. Yamazaki et al. [200] reported the self-assembly of chemically synthesized peptides to form collagen-like gels. Hydrogels of collagen developed by spontaneous intramolecular triple helix formation upon cooling the peptide solutions. Though various attempts and approaches for producing synthetic collagens have been carried out during the past decade, none was fully successful in mirroring all levels of the structural assembly (peptide to triple helix to nanofibers or even hydrogels) of natural collagen. The latest approach carried out in this direction was to identify a method to produce synthetic collagen in the laboratory. O'Leary et al. [201] demonstrated the design and synthesis of a self-assembling peptide that forms a sticky-ended collagen-like triple helix. The authors made chains of amino acids that assembled, initially, into nanoscale fibrils, then into larger fibers and finally formed a hydrogel reflecting the multi-hierarchical self-organizational sequence of natural collagen. The long-term goal is to tailor synthetic collagen with tunable attributes that can be used as collagen substrates and templates without any immunological side-effects for tissue engineering applications.

6 Conclusion

The collagen family of proteins present in human tissues has always fascinated and intrigued researchers, with their diversity, versatility, and adaptability at each functional level leading to a variety of properties enabling delivery of a particular function. With 29 known types, the collagens serve as the basic building material present ubiquitously in almost every extracellular tissue with outstanding mechanical properties. Collagen, as the basic structural protein for most tissues in the human body, also plays a vital role in maintaining the biological and structural integrity of tissues. The supramolecular structures and the hierarchical organization of the collagen types, from the molecular level to the functional tissue, aids the intervention and interplay of a series of design features and also influences the normal growth and development of a specific tissue and contributes significantly in wound

repair/damage. Since the collagen family with its extensive network primarily regulates and defines most tissues, research involving collagen inspires tissue engineers. The ideal goal of tissue regeneration is achieved when the structural integrity and the remodeling process of native ECM is restored, in particular when reconstituting the delicate collagen networks under which normal physiologic regeneration occurs. Therefore, it is essential to develop novel biomaterials that match and duplicate both the structure and properties of the native collagen matrix, especially type I collagen fibrils. Although extensive research is being done using collagen-based materials, the work is still in its infancy. Advances in materials science engineering and cell biology will help in utilizing the full potential of collagens for the purpose of mimicking the nanoscaled structures and supramolecular assemblies, thereby taking tissue regeneration to the next level.

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