

# The Importance of Collagen Fibers in Vertebrate Biology

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## ABSTRACT

Collagen fibers form the basic structural components of extracellular matrix (ECM) of vertebrates that serve to: (1) store elastic energy during muscular deformation, (2) transmit stored energy into joint movement, and (3) transfer excess energy from the joint back to the attached muscles for dissipation. They also act as mechanotransducers by transferring stress borne by the musculoskeleton to the attached cells in order to either up - or down - regulate tissue metabolism as a result of changes in mechanical loading. Finally, they prevent premature mechanical failure of tissues by limiting deformation of most ECMs and organs.

## INTRODUCTION

Collagen fibers are the major constituents of tissues termed extracellular matrices (ECMs). ECMs make up the structural components of surface and internal linings found in the human body, connections in musculoskeletal and oral tissues, walls of conduits and holding structures of the cardiovascular system and gastrointestinal tract. They also compose the parenchyma or structural support for all of the organs [1, 2]. The primary roles for collagen fibers in tissues found in vertebrates are to prevent premature mechanical failure and to help store, transmit and dissipate energy imparted either by the musculoskeleton or as a result of externally applied forces. Collagen fibers are required for effective locomotion, and for tissue regeneration and repair through mechanochemical transduction processes [2-18]. The above mechanical functions are required for vertebrates to achieve locomotion and move efficiently. Vertebrates must be able to develop muscular forces, store elastic energy, and transfer this energy to the attached joints for locomotion to occur. In addition, energy remaining after movement is achieved must be transferred from the joints back to the muscles where it can be dissipated as heat [2, 19].

During the normal gait cycle in vertebrates, potential energy is stored as strain energy in tendons that are stretched after impact with the ground; elastic recoil primarily by these tendons converts most of the stored energy into kinetic energy [20, 21]. Decreased

energy storage and dissipation is associated with normal aging and with wear and tear diseases involving collagen fibers in ECMs [7, 8, 14, 15]. Energy storage, transmission and dissipation in ECMs are intimately associated with molecular changes to collagen molecules, fibrils, and fibers [2].

The purpose of this paper is to discuss how the structure and mechanical properties of collagen fibers play a role in energy storage, transmission and dissipation in vertebrate ECMs, based on studies conducted in the laboratories of the author during the last 30 years.

## STRUCTURE OF COLLAGEN FIBERS

The basic structural unit of collagen fibers is the collagen molecule in the form of a triple helix. The ability of collagen fibers to store and transmit energy in ECMs is due to the rod-like structure of the fibrous collagens (types I, II and III) and their assembly into crosslinked networks of staggered molecules [2, 12]. Collagen fibers found in most tissues are mixtures of different collagens but there usually are one or two predominant collagen types. Tendons are largely composed of type I collagen, while skin and blood vessels contain primarily types I and III, and cartilage contains predominantly type II collagen [2]. All of the fiber forming-collagens are found in cells in a rod-like form with large end components, termed procollagen (*Figure 1*). The end components, termed the propeptides, are cleaved with enzymes once procollagen molecules are assembled into fibrils within larger diameter fibers.

Collagen triple helices are packed into a “quarter-staggered” packing pattern that results in nearest neighbor molecules being staggered longitudinally by about 22% of their molecular lengths with a space or hole between the head of one molecule and the tail of the next (*Figure 2*). Five collagen molecules are packed laterally into a quarter-staggered unit that is in turn longitudinally packed into microfibrils that are believed to be continuous and run the length of a tissue [2]. Collagen microfibrils are laterally packed into fibrils and fibers in most tissues [2].

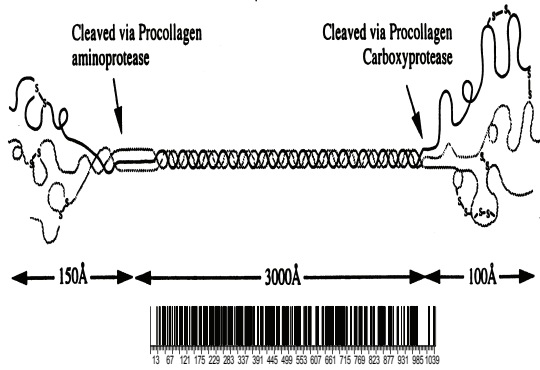


FIGURE 1. Diagram illustrating the structure of procollagen, the biosynthetic precursor of the collagen molecule [2].

Procollagen molecules are formed within the cell and the large propeptides are extracellularly cleaved during self-assembly into crosslinked collagen fibers. Collagen molecules are triple helical rods about 300 nm in length. The flexibility profile is shown below the diagram of the collagen triple helix and the 300 nm (3000Angstroms) line that represents the triple helical portion. The dark vertical lines represent rigid regions and the light areas depict the flexible domains of the collagen triple helix at the bottom of the figure.

In this packing pattern five collagen molecules are staggered by about 22% of the molecular length of 300 nm with respect to their nearest neighbors. A space or hole 0.6 D in length (D is between 64 and 67 nm) is left between neighboring molecules. The collagen molecule is 4.4 D long where D is the stagger between neighboring collagen molecules. The distance D consists of an overlap zone of 0.4D and a hole region of 0.6D as is shown by the vertical dotted lines that are superimposed on the microfibril in the diagram. The overlap and hole regions that make up the D repeat consist of 13 rigid and 12 flexible domains and are depicted by the rectangles and springs shown, respectively. These domains were identified based on flexibility analyses conducted on the triple helix [12] and a comparison with the collagen banding pattern [4]. The 12 flexible regions are identical to the 12 bands denoted c2 through c3 that are seen as dark vertical lines across the collagen fibril when collagen is stained with heavy metals and viewed in the electron microscope. The twelve flexible regions are believed to be stretched when collagen fibrils are mechanically deformed.

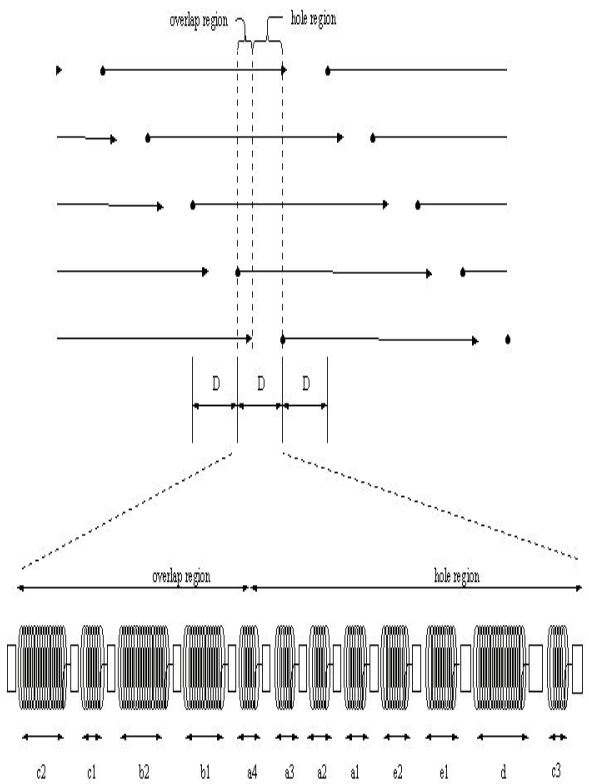


FIGURE 2. The top portion of this figure illustrates the structure of a five-membered microfibrillar unit that is believed to be the repeat unit found in collagen fibrils and fibers.

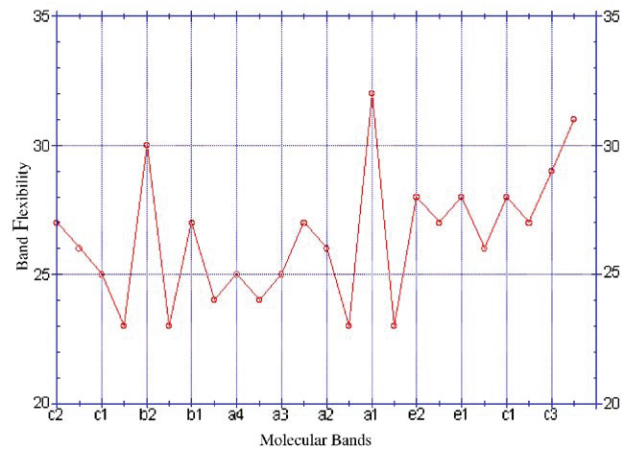


FIGURE 3. A plot of theoretical band flexibility as a function of axial position along a positive staining pattern for the type I collagen fibril.

Collagen fibril flexibility was estimated from the area under the conformational map for different dipeptides in a subfibril containing five quarter staggered collagen molecules. The plot of flexibility (number of

conformations, a unitless variable) vs. band number shown indicates that in general the flexibility is greater at the band center, while it decreases between the bands [4].

When the sequence of type I, II and III collagens are analyzed to evaluate the flexibility of dipeptide sequences that make up the backbone of the collagen triple helix, it is observed that the flexibility of the backbone is periodic. Domains of the molecule are flexible and alternate with rigid domains that are rich in proline and hydroxyproline [4, 12]. The flexible domains coincide with the 12 bands (c2 through c3 in *Figures 2 and 3*) that are the finger print of the collagen repeat unit (termed D period) when collagen fibrils are stained with heavy metals and viewed in the electron microscope. Electron microscopic images confirm the presence of numerous bends and points of flexibility in the collagen triple helix [2]; however, it was not until recently that the significance of these regions as energy storage elements became clear [4, 12]. The flexible domains are poor in the imino acids, proline and hydroxyproline, and appear to be deformed when external mechanical forces are applied to collagen fibrils based on free energy calculations made on molecular models of a stretched collagen microfibril [22, 23].

Energy storage during stretching appears to be associated with conformational changes in the collagen triple helix caused by repulsion between sets of like charges based on energy minimization calculations using molecular modeling [23] as discussed further below. In contrast, energy dissipation appears to be associated with the viscous sliding of collagen fibrils by each other during tensile deformation [3-6, 10]. Thus, the long collagen fibrils and fibers in tendon maximize energy storage and transmission while minimizing energy dissipation during locomotion [3-6]. In the same manner, energy storage and transmission are putatively maximized in bone; however, this is not the case for all ECMs. In skin, the fraction of energy dissipated can approach 50% during tensile deformation while that in tendon is only about 10% to 20% [4]. Skin protects inner organs from mechanical injury by energy dissipation during stretching [24].

#### **PACKING OF COLLAGEN MOLECULES IN COLLAGEN FIBRILS**

It is believed that the structure of collagen molecules in collagen fibrils is similar in many tissues based on the similarity between the D repeat found for tendon fibrils when compared to the D period found in other tissues. However, there is some evidence to support

differences in collagen structure in cardiovascular tissue where the fibrils may have an additional level of twist [2]. With this difference in mind, the structure of collagen fibrils in tendon has been characterized extensively from x-ray diffraction and electron microscopy and serves as a basis for analysis of structure-property relationships for collagenous tissues. Beyond the observation that collagen molecules are packed into a “quarter-staggered” packing pattern, the literature suggests that a 4 nm microfibrillar repeat unit exists within cross sections of collagen fibrils in vivo and in vitro and that collagen fibrils may be packed into rows of molecules with different lateral organizations [24-27].

#### **ARRANGEMENT OF COLLAGEN FIBERS IN DIFFERENT ECMs**

To a first approximation the mechanical properties of ECMs reflect the behavior of the collagen triple helix and its arrangement in 3D. However, nature has given ECMs a variety of differing macroscopic properties by altering the orientation and arrangements of the fibers and other non-collagenous components in three dimensions. Other factors contributing to dictating the mechanical properties of collagenous tissues found in ECMs include the relationship between collagen fibers and other tissue components such as elastic fibers, proteoglycans, smooth muscle, and the extent of crosslinking [2]. In addition, tissues containing layers, such as the aorta, must be considered as composites of elements with different orientations. In the case of tissues which are rich in type I collagen such as tendon, the elastic and viscous behaviors reflect the behavior of the collagen fibers. Therefore mechanical studies on tendon give us information concerning the molecular basis of elastic (energy storage) and viscous (energy dissipative) behaviors [2].

#### **MOLECULAR BASIS OF ENERGY STORAGE AND DISSIPATION BY COLLAGEN**

When tendons are subjected to tension, stress is developed by stretching collagen fibril bundles which in turn cause stretching of collagen fibrils and molecules [3, 4]. Increases in the D period are reported to exceed increases in the h spacing (rise per amino acid residue) for stretched rat tail tendon [28]; for every 10% increase in the macroscopic strain of tendon, the h spacing, e.g., the axial displacement per amino acid residue, in tendon increases only about 1% [3, 4, 28]. Therefore, 90% of the strain applied to tendon causes fibrillar and molecular slippage while only 10% of the macroscopic strain causes direct stretching of the collagen triple helix.

A molecular modeling program has been used to calculate the change in free energy arising from a 1-3% increase in the h spacing of type I collagen triple helices packed into five-membered units [22, 23]. The resulting free energy calculations were compared to the change in energy under the molecular stress versus strain curve for tendon [22, 23]. From these considerations it was concluded that changes in free energy during stretching collagen fibers were proportional to the energy changes under a macroscopic stress-strain curve [22, 23]. It was further determined, that regions without imino acids appear to “open up” or uncoil when stress is applied to a collagen fiber and the flexible regions can serve as sites of energy storage in the triple helix [5]. Peterlini et al. [29] have predicted that the flexible regions can form folds in the collagen triple helix, the folds may unfold when stress is applied and energy is stored. Molecular modeling results suggest that stretching increases steric energy of the triple helix that is attributable to van der Waals and electrostatic interactions between amino acids that are charged [22, 23].

An estimate of the elastic modulus of the collagen triple helix can be obtained from the slope of the stress-strain curve corrected for the actual molecular strain and collagen content. This assumes that tendon tissue is tested in tension after all the viscous relaxation has occurred. The actual measurement is made by allowing the stretched tissue to relax at constant length until the stress does not decrease any further [3, 4,] (*Figure 4*). This relaxation process may take a matter of several hours [4-6, 9, 10, 13, 30]. The stress at a particular strain at equilibrium, that is, when the stress no longer decreases with time, is the elastic or time-independent stress [3-5, 24]. This is the stress used to estimate the elastic modulus of the collagen molecule from stress-strain curves corrected for the molecular strain and collagen content [3-5, 24]. The difference between the initial stress at a fixed strain and the equilibrium stress is the stress dissipated as heat [3-5, 24].

The viscoelastic properties of ECMs have been obtained by constructing incremental stress-strain curves for a variety of tissues including tendon [2]. Such incremental stress-strain curves are derived for tendon and other ECMs by stretching the tissue in a series of strain increments and then allowing the stress to relax to an equilibrium value at each strain increment before another strain increment is added (*Figure 4*) [3-5]. By subtracting the elastic stress (equilibrium stress value) from the initial or total stress value, the viscous stress is obtained (stress that is converted into heat). By plotting the equilibrium

stress versus strain and the total stress minus equilibrium stress versus strain we get elastic and viscous stress-strain curves [3-5]. *Figure 5* shows the initial (total), equilibrium (elastic) and dissipated (viscous) stress-strain curves for tendon. All of these curves are approximately linear once the planar waveform known as crimp is removed in tendon.

When two rod-like elements slide past each other in a solution containing small solvent molecules, one can calculate the element length, in this case the collagen fibril length, knowing the viscous stress and the fibril diameter [2-5]. Using this approach collagen fibril lengths calculated from the viscous stress for developing tendons in chicks [17] coincide well with the observed fibril lengths for the same tendons based on measurements made in the electron microscope reported by Birk and coworkers [31]. These observations suggest that energy dissipation in tendon and other ECMs occurs through the viscous sliding of fibrils and bundles of fibrils by each other during tensile deformation [15-18]. Energy dissipation, in tendon, is minimized by maximizing the collagen fibril diameter. This result would suggest why the collagen fibrils in tendon are much larger in diameter than those found in other ECMs [3-5, 9-12]. Small average collagen fibril diameter is associated with increased energy dissipation during ECM stretching; this occurs in tissues including skin and vessel wall [33].

#### **VISCOELASTIC BEHAVIOR OF TENDON**

Much of current understanding of the relationship between hierarchical structure and viscoelastic behavior of ECMs is based on studies of the mechanical properties of developing tendon [34-36]. The properties of developing tendon rapidly change just prior to the onset of locomotion. The maximum total stress that can be borne by a 14 day old embryonic chick leg extensor tendon is about 2MPa and increases to 60 MPa, 2 days after birth [34-36]. This rapid increase in tensile stress by tendon occurs without large changes in its hierarchical structure [34-36]. In this case, the collagen fibril length appears to be more important for energy storage than fibril diameter; but the two parameters are linked together since fibrils have been shown to grow in length by lateral fusion of fibril bundles [17, 31, 34].

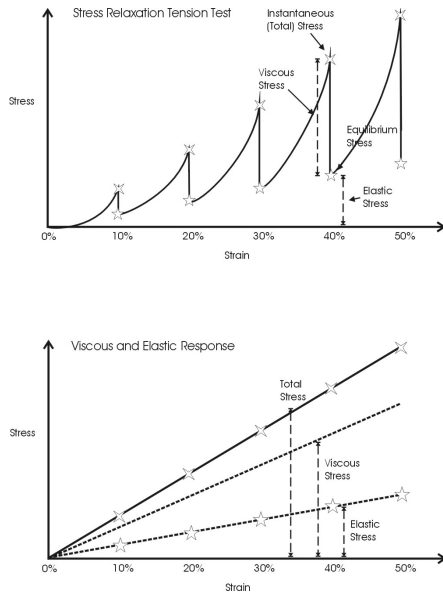


FIGURE 4. Incremental stress-strain curves for ECMs tested in tension.

(Top) A strain increment is applied to the ECM and the initial stress is measured. The strain increment varies from about 2% for tendon to about 10% for skin. The stress is allowed to relax at room temperature until an equilibrium value is reached. The process is repeated until the sample fails. (Bottom) Plots of all the initial (total) and equilibrium stresses are made versus strain as well as plot of the total minus equilibrium stress versus strain. The equilibrium stress versus strain curve is equivalent to the elastic stress-strain curve while the difference between the total and equilibrium stress is the viscous stress [32].

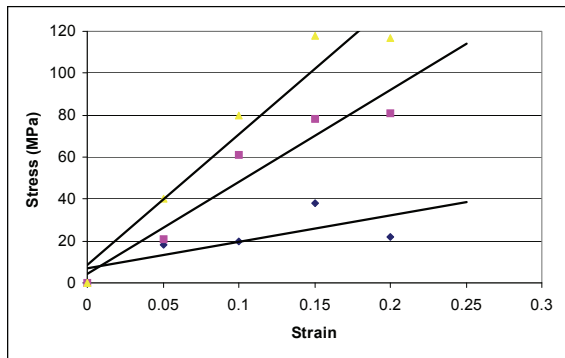


FIGURE 5. Total, elastic and viscous stress-strain curves for tendon.

This figure shows that the elastic stress-strain curve (squares) is approximately linear with strain is above the viscous curve (triangles). This diagram illustrates that more energy is stored during tensile deformation of tendon than is dissipated as heat during stretching [2].

As discussed above, the slope of the elastic stress-strain curve is proportional to the elastic modulus of the collagen molecule [5], while the viscous stress at a particular strain is a measure of the fibril length [5]. An estimate of the elastic modulus of the collagen molecule in tendon is obtained by dividing the slope of the elastic stress-strain curve by the collagen content and by the ratio of the molecular strain (change in h spacing) divided by the macroscopic strain (0.1). A value of between 7 to 9 GPa for the elastic modulus of the collagen molecule [3-5] is found for rat tail tendon (*Table I*). The value for collagen molecules in diseased tissues such as osteoarthritic cartilage is markedly reduced as shown in *Table I*. Collagen fibril lengths calculated from the viscous stress and hydrodynamic theory [2] range from about 20  $\mu\text{m}$  for developing tendon to in excess of 1 mm for adult tendons (*Table II*) [2].

TABLE I. Estimated elastic moduli for collagen based on elastic stress measurements for various ECMs [2].

Molecule	Elastic Modulus (GPa)
Type I	Self-assembled 6.51
"	Rat Tail Tendon 7.69
"	Turkey Tendon 4.20
"	Turkey Tendon (min) 7.22
Types I/III	skin 4.4
Type II	articular cartilage 7.0
"	OI cartilage 0.092
Elastin	Skin 0.040
"	Vessel Wall 0.01

OI=osteoarthritic min=mineralized

TABLE II. Estimated collagen fibril lengths based on mechanical measurements of viscous loss in different ECMs [2]

Tissue	Fibril Length (mm)
RTT	0.860
Self-Assembled	0.0373
Turkey Tendon	0.108
Turkey Tendon (min)	0.575
Human Skin	0.0548
Articular Cartilage	1.265
Osteoarthritic	0.164

RTT-rat tail tendon min=mineralized

## **VISCOELASTICITY OF SELF-ASSEMBLED TYPE I COLLAGEN FIBERS**

Additional information concerning the viscoelasticity of ECMs can be derived from understanding the behavior of model systems such as self-assembled type I collagen fibers derived from solubilized rat tail tendon collagen [1, 3, 11, 26]. The fibers are self-assembled under conditions that produce D-banded collagen fibrils similar to those seen in rat tail tendons [1, 26]. The purified type I collagen fibrils produced by self-assembly are much narrower, for example between about 20 and 40 nm in diameter, as compared to those in tendon which are as large as several 1  $\mu\text{m}$  [1, 26]. Incremental stress-strain curves for self-assembled purified type I collagen are linear for uncrosslinked collagen fibers [3-5]. However, unlike the incremental stress-strain curves for rat tail tendon, the viscous stress-strain curve for tendon is above the elastic stress-strain curve [3-5]. This result suggests that in the absence of crosslinks the ability of collagen fibers to store elastic energy is impaired; elastic energy storage appears increased by the formation of crosslinks [1, 3-5]. When the self-assembled collagen fibers are subsequently crosslinked by aging at room temperature, the elastic stress-strain curve is then above the viscous one [1]. On comparison of the slopes of the elastic stress-strain curves for tendon and self-assembled collagen fibrils, the slope of the elastic stress-strain curve for crosslinked self-assembled collagen fibrils is much closer to that of tendon than is the slope for uncrosslinked collagen fibers [3-5]. This result underscores the need for end-to-end crosslinks between collagen molecules in order to promote energy storage during stretching [3-5]. The viscoelastic behavior of tendon and self-assembled collagen fibers are very similar. The energy storage capability of tendon is attributable to direct stretching of the triple helix and energy dissipation occurs through the sliding of fibrils and bundles of fibrils during tensile stretching. However, the behavior of other ECMs is a bit more complicated because of the addition of additional components including elastic and smooth muscle fibers and differences in collagen fiber orientation [2].

## **MECHANOCHEMICAL TRANSDUCTION BY COLLAGEN FIBERS**

Mechanochemical transduction is a process by which external mechanical loading to collagen fibers in ECMs increases cell division and tissue generation. It is thought to involve several different macromolecular components and processes. Among them, one process involves direct stretching of protein-cell surface integrin binding sites that are present on all eukaryotic cells (integrin-dependent

mechanisms). Such stretching involves direct collagen fibril-integrin interactions [2, 18]. Stress-induced conformational changes in ECM may alter integrin structure and lead to activation of several secondary messenger pathways within the cell. Activation of these pathways leads to altered regulation of genes that synthesize and catabolize extracellular matrix proteins as well as to alterations in cell division. A second process by which mechanochemical transduction occurs involves deformation of gap junctions containing calcium-sensitive stretch receptors [2, 18]. Once activated, these channels trigger secondary messenger activation through pathways similar to those involved in integrin-dependent activation and allow communication between cells with similar or different phenotypes. A third process by which mechanochemical transduction occurs is through the activation of ion channels in cellular membranes. Mechanical forces have been shown to alter cell membrane ion channel permeability associated with  $\text{Ca}^{+2}$  and other ion fluxes [18]. The application of mechanical forces to cells also leads to the activation of growth factor and hormone receptors, even in the absence of ligand binding, as recently reviewed [2, 18].

Mechanochemical transduction is an important process that results in changes in ECM hierarchical structure. Therefore, the relationship between external loading of collagen fibers and the ensuing changes in cell metabolism are important characteristics of the dynamic nature of extracellular matrices.

## **CONCLUSIONS**

Collagen fibers are involved in storage of elastic energy during muscular deformation, transmission of stored energy into joint movement, and in the transfer of excess energy from the joint back to the attached muscles for dissipation. These behaviors are a reflection of both the chemistry, through the amino acid sequence, and the physical structure of the collagen molecule, through its triple helical form. Collagen triple helices are flexible rods that have a repeat domain structure consisting of a flexible region followed by a rigid region that is repeated many times along the length of the molecule. Collagen triple helices are packed into a "quarter-staggered" packing pattern that results in nearest neighbor molecules being staggered longitudinally by about 22% of their molecular lengths with a space or hole between the head of one molecule and the tail of the next. The domain structure is conserved both at the microfibril level and at the fibrillar level resulting

in the ability of collagen fibrils to mimic the behavior at the molecular level.

The mechanical properties of ECMs reflect the behavior of the collagen triple helix and its arrangement in 3D. However, nature has given ECMs a variety of differing macroscopic properties by altering the orientation and arrangements of the fibers and other non-collagenous components in three dimensions. Other factors that contribute to dictating the mechanical properties of collagenous tissues found in ECMs include the relationship between collagen fibers and other tissue components such as elastic fibers, proteoglycans, smooth muscle, and the extent of crosslinking. The orientation of collagen fibers varies from tissue to tissue depending on the mechanical requirements of the tissue.

Viscoelasticity of collagen molecules is the result of both the flexible domains which behave as elastic storage elements during axial deformation as well as the viscous sliding of collagen molecules by each other in the fibril that contributes to the time-dependent behavior. Collagen viscoelasticity is important in resisting impact loads especially in the musculoskeleton. The elastic behavior varies from as high as about 90% of the total stress for tendon to as low as about 50% of the stress for skin depending on the collagen fiber orientation, rate of loading and the quantity of other tissue constituents. Collagen fibers prevent premature mechanical failure of all tissues by virtue of their elastic modulus that is as high as 7 to 8 GPa.

ECMs also act as mechanochemical transducers through integrin-dependent and integrin-independent processes. Direct collagen fibril-integrin interactions have been observed and lead to stimulation and/or modulation of protein synthesis and cell division. Stress-induced conformational changes in ECM putatively alter integrin structure and lead to activation of several secondary messenger pathways within the cell. Activation of these pathways leads to altered regulation of genes that synthesize and catabolize extracellular matrix proteins as well as lead to alterations in cell division. These data suggest that tissue metabolism is directly influenced by internal and external mechanical loading of collagen fibers in making mechanochemical transduction one of the most important properties of ECMs.

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