



Review paper: The importance of consideration of collagen cross-links in computational models of collagen-based tissues

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ABSTRACT

Collagen as the main protein in Extra Cellular Matrix (ECM) is the main load-bearing component of fibrous tissues. Nanostructure and architecture of collagen fibrils play an important role in mechanical behavior of these tissues. Extensive experimental and theoretical studies have so far been performed to capture these properties, but none of the current models realistically represent the complexity of network mechanics because still less is known about the collagen's inner structure and its effect on the mechanical properties of tissues. The goal of this review article is to emphasize the significance of cross-links in computational modeling of different collagen-based tissues, and to reveal the need for continuum models to consider cross-links properties to better reflect the mechanical behavior observed in experiments. In addition, this study outlines the limitations of current investigations and provides potential suggestions for the future work.

1. Introduction

Collagen plays an important role in many biological tissues, including tendon, cartilage, bone, teeth, arterial walls and cornea (Fratzl, 2008; Buehler, 2008). It has a hierarchical structure consisting of collagen fibrils, which at nanoscale, are composed of an array of tropocollagens (i.e. collagen molecules). Tropocollagens are approximately 300 nm long and 1.5 nm in diameter, and are axially offset from each other by their D-spacing of about 67 nm. They pack laterally in a quarter-staggered array to form microfibrils as shown in Fig. 1a (Quan and Sone, 2013). Single collagen fibril made of collagen microfibrils has the diameter of 80–100 nm and the length of multiple microns. Tropocollagen is made up of three polypeptide strands: a triple helix with two α_1 chains and one α_2 chain (Ottani et al., 2001; Fratzl and Weinkamer, 2007). There are some covalent cross-links among two chains of a single triple helix, and a variable amount of covalent cross-linking between tropocollagens (Fig. 1b), forming the different types of collagen in different mature tissues and contributing to the transmission of forces in both healthy and aged tissues (Andriots et al., 2018; Parvizi and Kim, 2010).

The maturation of collagen occurs via multi-step intracellular and extracellular processes including the formation of several covalent cross-links to stabilize their structure and to provide biochemical properties to

tissues (Gaar et al., 2020). Cross-linking in the collagen molecule may be categorized into two functionally distinct groups. First, intramolecular cross-linking, where two chains within the same molecule are covalently linked. Second, intermolecular cross-linking which forms covalent bridges between chains in different collagen molecules (Light and Bailey, 1982). It is suggested that the intramolecular cross-link is not a separate entity but only an intermediate of an intermolecular cross-link (Kang and Gross, 1970). The mechanical stability of collagen fibrils is mainly dependent on the formation of covalent intermolecular cross-links (Kang and Gross, 1970).

Intermolecular cross-links are categorized into enzymatic and non-enzymatic cross-links, which are developed differently, and have been proposed to have different mechanical effects. Enzymatic cross-links are formed during the development of collagen fibrils, and they connect tropocollagen molecules at their ends and stabilize collagen molecules of the structure contributing to the mechanical resistance of a fibril under tension. The biosynthesis of enzymatic cross-links involves the covalent cross-linking of specific active sites (non-helical ends) in collagen molecules and begins with the enzymatic oxidation of lysine or hydroxylysine residues into aldehydes to form immature covalent cross-links. Enzymatic crosslinks are firstly formed between telopeptide and helical residues to produce immature (divalent) crosslinks connecting the end of the tropocollagen molecule to the nearest neighbor from an

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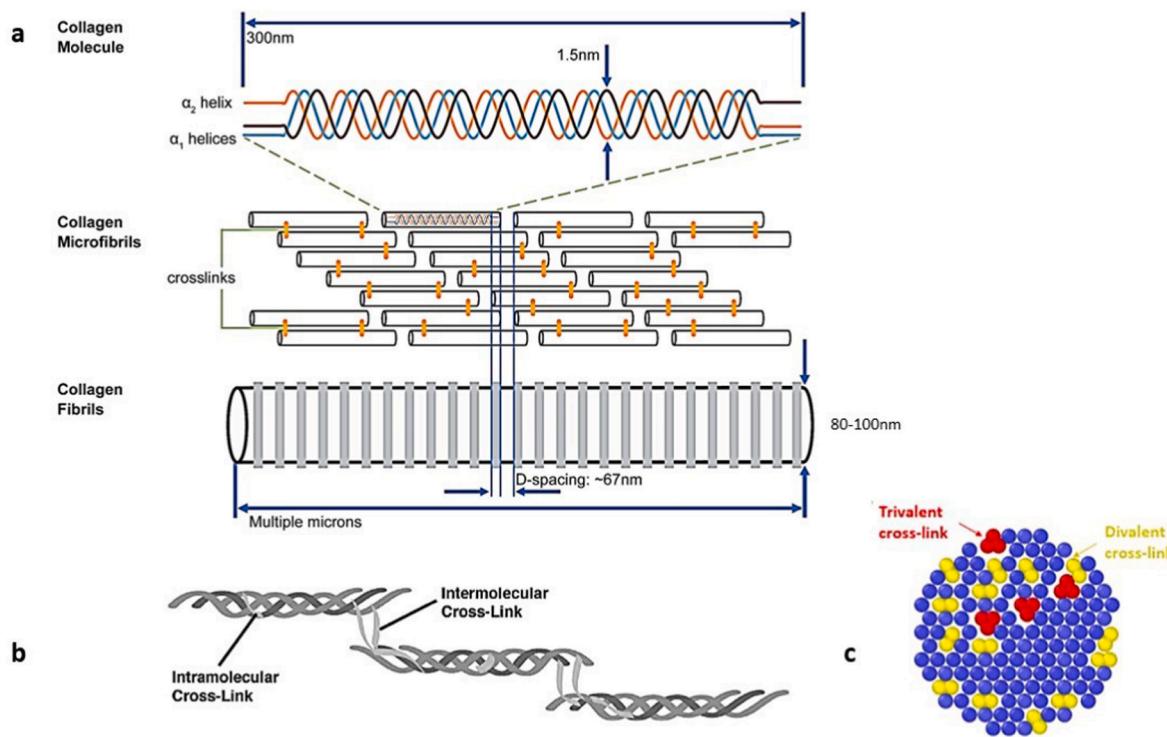


Fig. 1. a) Schematic of hierarchical structure of collagen fibrils. Triple helical collagen molecules are assembled in a quarter-staggered array to form microfibrils, which are connected by cross-links. Microscopically, collagen fibrils display a pattern of stripes formed by a sequence of more dense (dark grey) and less dense (light grey) fibrillar regions (adapted from Canelon and Wallace (2016)). b) Schematic of intramolecular and intermolecular cross-links (adapted from Diegelmann (2001)). c) Illustration of cross section of collagen microfibrils indicating divalent and trivalent cross-links (adapted from Khatib et al. (2021)).

adjacent molecule (Fig. 1c). This immature crosslink may then rejoin to another telopeptide residue producing a mature (trivalent) crosslink, which connects the end of the molecule to the two nearest neighbors from adjacent molecules (Khatib et al., 2021) (Fig. 1c). The cross-link maturation from covalent into multivalent (divalent and trivalent) is performed via poorly understood mechanisms (Gaar et al., 2020; Bailey et al., 1974). Formation of enzymatic cross-links is a beneficial process of collagen maturation (Eekhoff et al., 2018). These cross-links stiffen the fibrils by resisting intermolecular sliding. Therefore, greater enzymatic cross-linking leads to less compliant and stronger tissue as a result of stiffer fibrils, while, poorly cross-linked collagen fibrils show a weaker tensile strength and a dissipative deformation character (Eekhoff et al., 2018; Siegmund et al., 2008; Yoshida et al., 2014; Gaar et al., 2020).

On the other hand, non-enzymatic cross-links are not produced during development and they can attach at any point along the length of a tropocollagen molecule. The adventitious reaction of specific lysine residues on collagen with sugars happens naturally during ageing and results in the formation of non-enzymatic cross-links (Brennan, 1989). The non-enzymatic unselective glycation of lysine residues via the Maillard¹ reaction results in the formation of Advanced Glycation End products (AGEs), which are associated with the decreasing mechanical strength of collagen fibers (Paul and Bailey, 1996). The amount of AGEs formed on a protein increases with time until it is degraded, which allows large amounts of AGEs to accumulate due to the extensive half-life of collagen. AGEs have also been associated with the development of diabetes, where plasma glucose levels are elevated allowing extensive AGE formation (Brownlee, 2000). These cross-links are only expected to be present in significant quantities during aging (Siegmund et al., 2008;

Snedeker and Gautieri, 2014). Increased non-enzymatic cross-linking over time is generally considered unfavorable to normal function of fibril, and decreases microscale strain attenuation and the viscous response of tissue (Eekhoff et al., 2018). To study the detailed biosynthesis of these two types of collagen cross-links and their distinct effects on the mechanical properties across different length scales, the potential readers are referred to the reviews of Gaar et al. (2020), Eekhoff et al. (2018), Garnero (2012), and Zieman and Kass (2004).

The formation of intermolecular covalent cross-links in collagen is important in enhancing the mechanical stability of the collagenous tissues (Buehler and Yung, 2009; Fratzl and Weinkamer, 2007). Collagen fibrils are strengthened through covalent intermolecular cross-links formed enzymatically during the assembly between the collagen molecules. This processing results in collagen materials, which are mechanically very strong in tension, at all hierarchical levels. The hierarchical buildup from nm-scale fibrils to μm -fibers, fiber-bundles, and mm-scale fascicles, is perhaps best observed in tendon and ligament (Jawad and Brown, 2011).

There are several studies describing the effects of collagen cross-links density on the macroscopic mechanical response of different tissues such as tendons (Eekhoff et al., 2018; Marturano et al., 2013), articular cartilage (Chen et al., 2017), cervical tissue (Yoshida et al. 2014, 2019), arterial tissue (Holzapfel and Ogden, 2020b; Sáez et al. 2014, 2016), connective tissues of knee joint (Eleswarapu et al., 2011). These studies exclusively report that cross-linking of collagen fibers has a substantial impact on the tissue mechanics. However, the excessive presence of intermolecular cross-link (glycation reactions) over-stiffens the fibers, as found in the elderly, and alters normal cell-matrix interactions leading to obvious signs of ageing such as skin wrinkling, cartilage impairment or bone brittleness (Bailey, 2001). This highlights the role of cross-links in the alteration of material properties also in diseases which might lead to breakdown of key material components of the structure (Buehler and Yung, 2009). The increased cross-link density results in

¹ Maillard reaction generates several reactive intermediates that then undergo several oxidation reactions, or non-oxidative rearrangements, resulting in the formation of many cross-links between protein molecules Avery and Bailey (2006).

stronger but more brittle collagen fibrils as shown by coarse-grained computational simulations (Buehler, 2008), which investigated the influence of the cross-link density on the strength and the failure mechanism of a two-dimensional bead-spring representation of a collagen microfibril (Buehler, 2006). This altered mechanical response could have important effects on tissue viability (Buehler, 2008).

There are a number of objectives for this review article: i) it provides an overview of different cross-linking mechanisms typical of collagenous tissues; ii) it highlights the role of collagen cross-linking in mechanics of several fibrous tissues; iii) it summarizes some important experimental investigations giving insight into the role of and nature of cross-linking; iv) the important computational simulations taking cross-links properties into account are highlighted; v) the paper identifies the limitations of current methods and models and provides ideas for the future investigations. The coupling of the experimental observations with the numerical models draws special attention since the computational simulations are observed as a means to decrease the extent of expensive and time-consuming experiments, but also represent a possibility to predict processes and effects at different length and time scales that are often impractical or technically not yet accessible to discover through experimental facilities alone.

In this review, the role of cross-links in mechanics of collagenous tissues such as tendon and ligament, cartilage, cervix, collagen gels and arterial tissues are unraveled in Sections 2 to 6, respectively. Section 7 gives an overview of the computational models of collagenous tissue categorized into atomic/molecular models and continuum-based constitutive modeling. The paper then summarizes the current limitations and future perspective in experiments and computational models in Section 8, and finally provides the conclusion in Section 9.

2. Collagen cross-links in tendon and ligament

Tendons and ligaments are predominantly composed of collagen, which is arranged in a hierarchical manner parallel to the long axis of the tissue. As an example, the hierarchical structure of tendons is given in Fig. 2 showing that tendon is made of parallel fascicles containing collagen fibers. Furthermore, collagen fibers consist of fibrils, which are assemblies of parallel molecules (Fang and Lake, 2016). Tendons and ligaments are normally grouped into a single category sharing similar properties such as being flexible cords of fibrous tissue that join bone to either muscle (tendon) or bone (ligaments) (Provenzano and Vanderby, 2006), being both likely to rupture with only limited capacity to heal (Leong et al., 2020). In both tissues, collagen Type I accounts for about 80% of the dry weight and their deformation mechanisms are largely

governed by their hierarchical structure, which is mainly composed of collagenous tissue (Benjamin et al., 2008). For movement of the musculoskeletal system, appropriately functioning tendons and ligaments are vital (Benjamin et al., 2008; Asahara et al., 2017). Therefore, any changes in their mechanical properties may have important clinical implications (Gregory et al., 2021; Asahara et al., 2017).

To understand the influence of collagen and cross-links on the deformation mechanics of collagen fibrils, a series of experiments by pulling individual collagen fibrils with changing density of cross-links was carried out, which allowed us to observe the difference in mechanical behavior (Fratzl, 2003, 2008; Abreu et al., 2008; Rigozzi et al., 2009; Al Makhzoomi et al., 2021). To better understand this, the three different regions of the stress-strain curve of rat tail tendon associated to deformations at different structural scales (Fig. 3a) and strain-rate dependence of the ratio of fibril elongation to tendon elongation (Fig. 3c) will be discussed. The rat tail tendon was tested at a strain-rate in which the strain of the fibril (ϵ_F) was approximately 40% of the total strain of the tendon (ϵ_T) in the linear region of the stress-strain curve (Fratzl and Weinkamer, 2007). As shown in Fig. 3a, in the “toe” region, a very small stress is enough to lengthen the tendon, related to the removal of a macroscopic crimp of the fibrils (Diamant et al., 1972). In the heel region, a significant increase of the stiffness happens with increasing the strain. Here, disordered molecular kinks in the gap region of collagen fibrils are straightened (Misof et al., 1997). However, once all the kinks are straightened, another mechanism of deformation must operate to explain the linear dependence of stress and strain in the third region. It is assumed that the stretching of the collagen triple-helices and the cross-links between the helices, implying a side-by-side gliding of neighboring molecules, likely leads to structural changes at the scale of the collagen fibrils (Fratzl and Weinkamer, 2007). The main conclusions derived from this graph where simultaneous tensile testing and synchrotron X-ray diffraction characterization (Folkard et al., 1987; Folkard et al., 1987) are employed are as follows:

- The strain of collagen fibrils is always considerably less than half of the total strain of the tendon (Fratzl et al., 1998), indicating large deformation must occur outside the collagen fibrils, presumably in the proteoglycan-rich matrix (Cribb and Scott, 1995), which mediates deformation by shearing between fibrils (Fig. 3b).
- The ratio between the extension of the fibrils and of the tendon increases with the strain rate (Puxkandl et al., 2002) in normal collagen (Fig. 3c), showing that most of the viscous deformation is due to the viscosity of the proteoglycan matrix. At adequately large

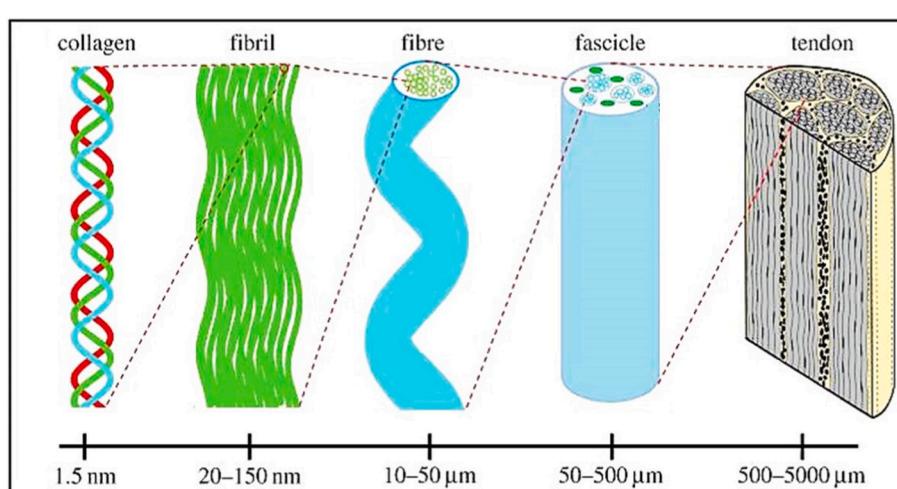


Fig. 2. Schematic of hierarchical structure of tendons. Tendon is made of a number of parallel fascicles containing collagen fibers. Collagen fibers consist of fibrils, which are assemblies of parallel molecules (adapted from Fang and Lake (2016)).

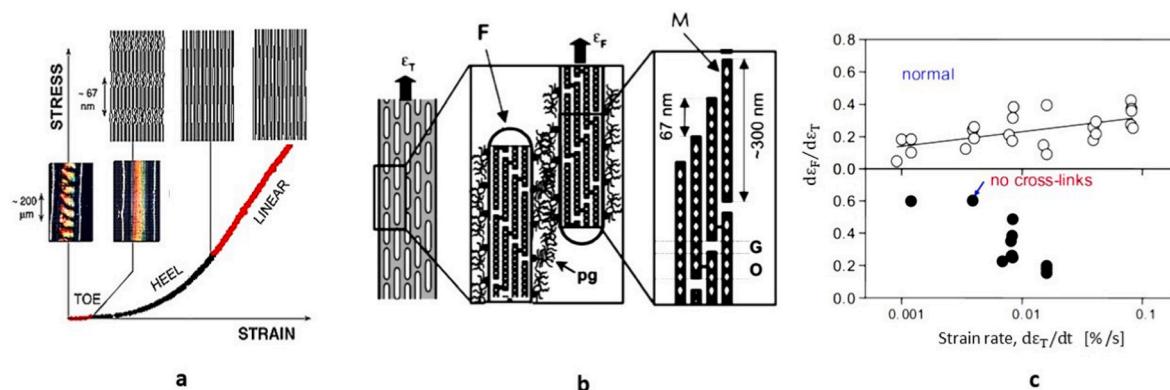


Fig. 3. a) Tensile behavior of the typical collagen fibril structure from rat tail tendon (adapted from Fratzl and Weinkamer (2007)). b) A composite of collagen fibrils (tendon fascicle) in a proteoglycan (pg)-rich matrix is subjected to ε_T . The strain transmitted to the fibrils (F) is denoted by ε_F . Triple helical collagen molecules (M) are packed within fibrils with gap (G) and overlap (O) zones (adapted from Fratzl (2003)). c) Strain – rate dependence of the ratio of fibril elongation $d\varepsilon_F$ to tendon elongation $d\varepsilon_T$ (adapted from Puxkandl et al. (2002)).

strains, the mature collagen fibrils can be considered as mostly elastic.

- In collagen with low content of cross-links, the ratio between the extension of the fibrils and of the tendon decreases with the strain rate (Puxkandl et al., 2002) as shown in Fig. 3c. Therefore, a plateau in the load/extension curve appears which indicates creep behavior. Consequently, further slippage of fibrils may result from the absence of covalent cross-linking between molecules in the fibrils, which specifies the major role of collagen cross-links in determining the fibrils' stiffness.

Due to the hierarchical structure of these tissues, it is important to investigate mechanical properties across length scales (molecular, fibril and tissue levels) and to widely understand how structural and compositional changes affect mechanics. To provide a broad insight to the potential readers, the review article by Fang and Lake (2016) and the book chapter by Marino and Vairo (2021) broadly frame mathematical and computational multiscale models that have been used to understand tendon and ligament mechanics at different hierarchical level and to reveal a number of mechanisms that transfer the strain from macro-to microscale (Marino et al., 2018; Hoegen et al., 2019; Hamdia et al., 2019; Fang and Lake, 2016, 2017). As highlighted in Eekhoff et al. (2018), both enzymatic and non-enzymatic collagen cross-links significantly influence the multiscale mechanical properties of tendon and therefore are worthy of further research. Recently, Moghaddam et al. (2023) showed experimentally and computationally that cross-linked collagen fibrils, i.e., the collagen fibrils that can interact with one another via strain transfer mechanisms, resist microscale longitudinal compressive forces and showed, via two-dimensional (2D) FEM simulation, the underlying deformation mechanisms that were responsible for this observation. The simulation demonstrated the prevention of buckling by cross-link-mediated interactions of collagen fibrils by showing that single fibril buckles in compression, but a group of several mutually engaged fibrils resists buckling and compressive forces.

It has been shown that at nanoscale, an elevated concentration of cross-links affects the mechanical behavior of tendon by perhaps locking adjacent helices together and would inhibit intermolecular sliding (Lee and Veres, 2019). Therefore, the chemistry, location and quantity of cross-links are believed to contribute to the tissue-specific differences observed in mechanical and biological properties of these tissues (Hudson et al., 2021). Understanding their specific molecular characteristics is further important for pathological purposes and improving the design of clinical treatment approaches (Hudson et al., 2021). As an example, molecular slippage which is likely required for the discrete plasticity failure mechanism observed in tendon (discrete plasticity is a characteristic nanoscale damage motif at the collagen fibril level in

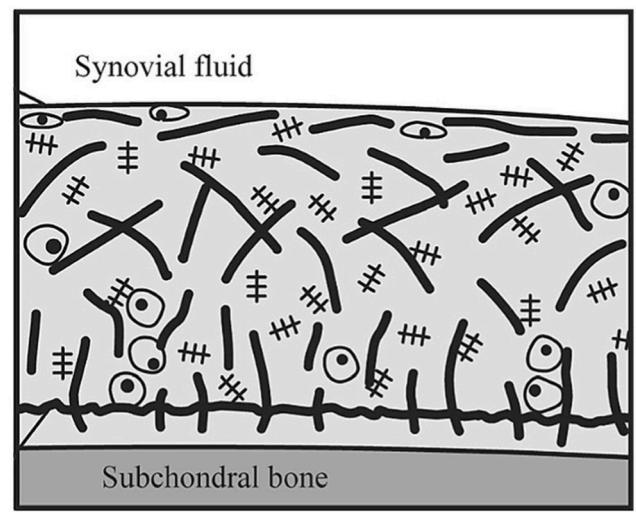
tendons caused by overload-induced kinking along the length of collagen fibrils leading to rupture) is correlated to cross-link density (Lee and Veres, 2019). Further improvement in modeling of microstructure can then help personalized treatments.

3. Collagen cross-links in cartilage

Cartilage is a dense loadbearing connective tissue that provides low friction articulation and facilitates the load transmission to the underlying subchondral bone. As shown in Fig. 4, cartilage is mainly comprised of collagen, proteoglycans and special types of cells called articular chondrocytes (Buckwalter et al., 2005). The mechanical properties of cartilage are highly complex and zonal (Nachtsheim et al., 2019).

Better understanding of the mechanics of the collagen meshwork in cartilage and the implications of its properties and connectivity are required in osteoarthritis pathogenesis, diagnostics and design of regenerative strategies. Experiments have been showing the role of collagen cross-links and their density (Chen et al., 2017) in enhancing cartilage integration (Athens et al., 2013; Ahsan et al., 2005) and articular cartilage repair (Masahiko et al., 2012; Wu et al., 2022).

Over decades, computational models have increasingly been



| Collagen **⊕** Proteoglycans **○** Chondrocyte

Fig. 4. Microstructure of cartilage containing collagen, proteoglycans and chondrocytes (adapted from Zhu (2018)).

developed to investigate tissue-level cartilage mechanics using complex constitutive models (Wilson et al., 2006, 2007; Ateshian et al., 2009; Pierce et al., 2013; Pierce, 2021; Deneweth et al., 2013). For instance, the relation between permeability and tissue composition of articular cartilage was investigated using a viscoelastic constitutive relation within a fibril-reinforced model to predict the equilibrium and transient response of articular cartilage during compression, indentation and swelling tests (Wilson et al., 2006). Ateshian et al. (2009) applied continuous fiber angular distributions to model the solid matrix of cartilage and predicted experimental observations of the tissue's equilibrium response to mechanical and osmotic loading. However, such models did not include details from individual fibril but rather considered the impact of average fibril orientations.

Although theoretical mechanics of upscaling (Davit et al., 2013), where material properties at the sub-continuum scale are incorporated into the constitutive relation of continuum models with controlled accuracy are advanced, these approaches are generally not controllable for collagen networks (Buehler, 2006). Therefore, presently it is not possible to assess the impact of individual cross-links in a continuum model. For this reason, Chen et al. (2017) adopted a bottom-up approach based on a spring-node model of cross-linked collagen and represented collagen fibrils individually. In this study, due to a lack of available data, enzymatic and AGE related cross-links were not probed. Instead, the cross-links referred to inter-fibril connectivity were modeled with a linear force-strain relation assuming small deformation. The impact of cross-link stiffness and density on the mechanical response in particular fibril alignment as a symbol of cartilage pathology was then investigated (Brown et al., 2012; Chen et al., 2017). They showed that although cross-link stiffness, within the parameter range considered, had no significant effect, the number of cross-links in each structure consistently altered the structure (Chen et al., 2017).

From pathological viewpoint, since degeneration at the fibril level can lead to severe injuries that will cause significant cartilage degradation (Faisal et al., 2019), a multiscale constitutive model of cartilage was created to clarify the effect of two reasonable fibril degradation mechanisms on the aggregate tissue: tropocollagen cross-link failure and a generalized surface degradation (Faisal et al., 2019). After cross-link failure and surface degradation, the yield stress was shown to be different between aggregate tissue and intact cartilage. In addition, the tissue-level aggregate behaviors were also observed to be different from the fibrillar behaviors in the molecular dynamics simulations (Faisal et al., 2019). Accordingly, understanding of the aggregate stress-strain behavior of cartilage tissue and its fundamental biomechanical factors are vital to develop therapeutic interventions for cartilage pathologies. Although multiscale modeling show promising results to define the mechanical microenvironment experienced by and within single cells regulating biological activity at the molecular level, currently no multiphase, multiscale models relevant to cartilage mechanics and mechanobiology exist due to poorly understood required knowledge (Wang et al., 2019). Further improvement in modeling of microstructure can help patient-oriented treatments and soft-tissue replacements in tissue engineering.

4. Collagen cross-links in cervix

Cervix is a muscular, tunnel-like tissue located in the lower part of uterus. Cervical tissue comprises collagen fibers, elastic fibers, various proteoglycans, hyaluronic acid, matricellular proteins and a population of cervical fibroblasts and smooth muscle cells (Hao et al., 2018). Cervix is known to be highly collagenous, and collagen Types I and III as the most abundant proteins (34–77% dry weight) in the extracellular matrix (ECM) act as the structural support for the tissue (Yoshida et al., 2014). The alignment and concentration of collagen within the human cervix was shown to be variable across patients and across anatomic locations (Hao et al., 2018).

During the gestation, several changes happen gradually in the

structure of the cervical tissue, an increase in the hydration, decrease in elasticity and disorganization of collagen cross-links can be observed (Peralta et al., 2015). The collagen structure disorganization during cervical ripening is complex: collagen fibers (fibrils) become thicker (Akins et al., 2011) and wavier (Feltovich et al., 2012) as the gestation progresses, while pores between collagen fibers (fibrils) become larger and separated as shown in Fig. 5 (Peralta et al., 2015; Hao et al., 2018). Accumulation of poorly cross-linked collagen results in dispersion of collagen fibrils, leading to a loss of tissue integrity and increased tissue compliance (Myers et al., 2008). Cervical remodeling requires progressive alterations and turnover in the ECM composition and microstructure that result in reorganization of collagen fibril structure with a gradual loss of tensile strength and the alteration in the mechanical integrity of the cervix during pregnancy results in a successful delivery (Akins et al., 2011). Cervical collagens are recognized to remodel extensively with progressing gestation leading to a soft cervix at term (Mead et al., 2018; Colon-Caraballo et al., 2022; Myers et al., 2008; Moghaddam et al., 2022; Torres et al., 2019).

Several experimental studies highlighted that the reduction of stiffness during gestation is directed by the collagen structure through a reduction of cross-linking between fibers as well as the distinctive rearrangement of the fibers from being aligned to curling (Torres et al., 2019; Timmons et al., 2014; Feltovich et al., 2012; Akins et al., 2011; Yoshida et al., 2014). The factors modulating collagen structure during early mouse pregnancy such as expression of proteins involved in processing of procollagen, assembly of collagen fibrils, cross-link formation, and deposition of collagen in the ECM were experimentally evaluated by Akins et al. (2011), where they showed that the mature (older) cross-links decreased significantly with pregnancy and cervical remodeling of mouse. Yoshida et al. (2014) also reviewed the current understanding of cervical remodeling during pregnancy in rodent models and showed that during the process of pregnancy in a mouse model, mature cross-linked collagens are hypothesized to be replaced with immature less cross-linked collagens to facilitate cervical softening and ripening. Timmons et al. (2014) also found that tensile mechanical response of the cervix changed considerably from a stiff structure into a soft structure as pregnancy progressed, which is attributed to cross-linking alteration during pregnancy. In a review performed by Yoshida et al. (2019), the collective contribution of multiple mechanical studies on rodents' cervical tissue was highlighted, which gives evidence that cervical softening coincides with known cross-link changes throughout the pregnancy.

Peralta et al. (2015) proposed a multiscale model, which consists of a finite difference time domain (FDTD) algorithm, constitutive mixture theory and a collagen morphology model that quantitatively explains the relationship between micro scale and tissue scale mechanical properties. They claimed that FDTD simulation technique is advantageous since no physical approximations are made. Consequently, the FDTD simulations can be used both to show the interaction between shear waves and the hierarchical structure of connective tissues, and to study the mechanical properties of soft tissues. In their model, cervix mechanics was shown to be more sensible to the alterations in the microstructure (cross-links) than to the ECM composition (Peralta et al., 2015).

Recently, an extensive combined experimental-theoretical model of both human and mouse cervical tissues was developed capturing the tension-compression asymmetric material responses and the remodeling characteristics of the tissue (Shi et al., 2022). In this 3D constitutive model, according to the previously published cervical material models (Shi et al., 2019; Lee et al., 2022; Myers et al., 2010), the cervix was modeled as a composite material with a collagen network embedded within an isotropic, compressible matrix. Directionality, dispersion and cross-linking of collagen fiber were also included in the model. In this model, the collagen molecules are assumed to comply with the freely-jointed chain model, and any kind of interactions among monomers were neglected. A similar drastic softening pattern was captured in

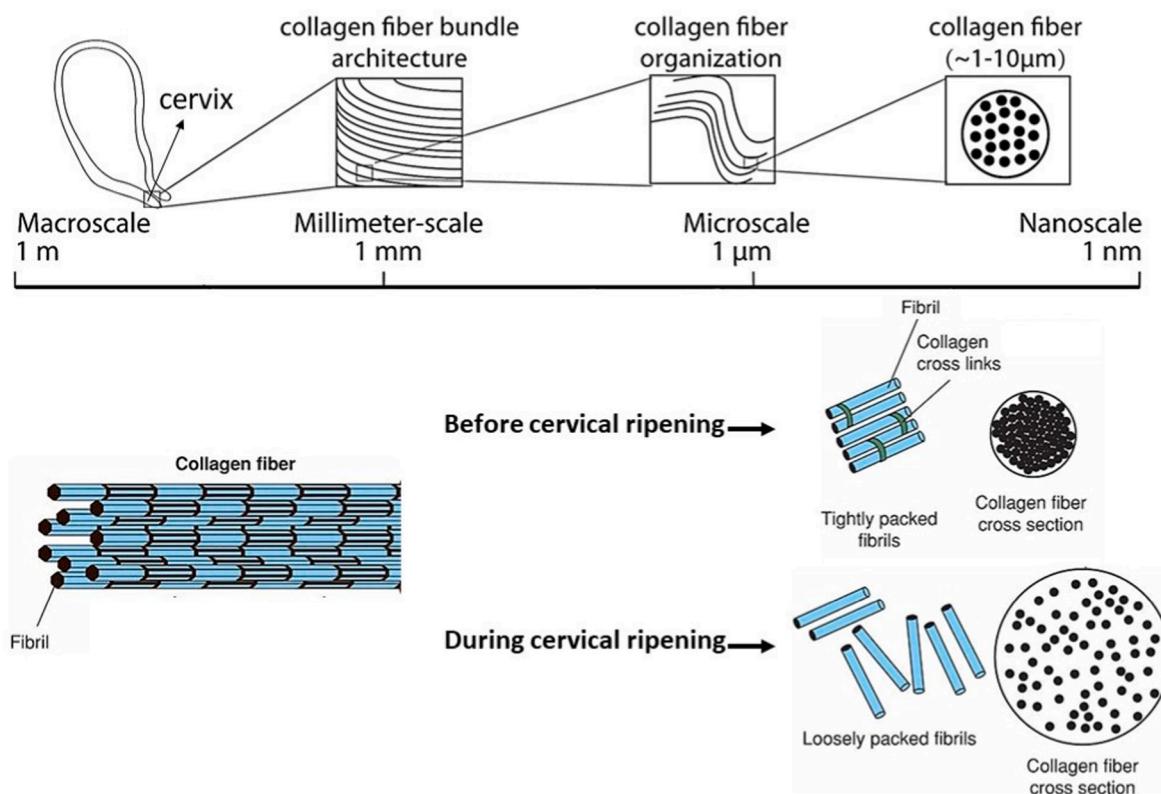


Fig. 5. Collagen organization in cervical tissue. Before cervical ripening, fibril size is uniform, and fibrils are well packed. During cervical ripening, fibril size is less uniform, and spacing between collagen fibrils and fibers is increased and disorganized (adapted from Hao et al. (2018) and reproduced from www.obgynkey.com/physiology-of-labor).

both human and mouse tissues due to an increase in extensibility of pregnant cervix, decrease in collagen cross-linking and ground substance compressibility. Since mouse and human cervices share similar ECM components (Myers et al., 2015) and show similar pattern during pregnancy, mouse cervix is used as an acceptable model when human cervix is difficult to obtain.

The dominant orientation of collagen fibrils was reported to be region-specific in cervix, perpendicular to the cervical canals in the outer zone and septum and parallel to the cervical canals in the inner zone (Moghaddam et al., 2022). Moghaddam et al. (2023) showed experimentally and computationally that the changes in the cross-linked collagen fibril orientation affected the indentation modulus of the inner cervical zone. The indentation modulus had a strong and positive correlation with the orientation of collagen fibrils, agreeing with observations in tendon, despite structural and functional differences. These results highlight the importance of shear between collagen fibrils and shear-regulating mechanisms in the elastic response and help to understand the underlying physical mechanisms that control the mechanical properties of tissue during disease or remodeling.

All studies mentioned above emphasize the important role of cross-links on the mechanical behavior of cervix during gestation. However, still abnormal cervical remodeling remains a significant clinical dilemma in obstetrics (Iams and Berghella, 2010; Colon-Caraballo et al., 2022; Vink and Feltovich, 2016). Therefore, increasing the capacity to capture the microstructure of collagen including cross-links in computational models of cervix could be less expensive compared to the experiments and would provide more flexibility to assess how the alteration in cross-links can affect the mechanics.

5. Cross-links in collagen gels

A network of polymer filaments embedded on an aqueous substrate

forms hydrogels and their mechanical properties are mainly defined by the filament network architecture and properties. To increase properties of native collagen, such as stiffness or strain stiffening, these networks can be modified by adding cross-linking agents that alter the network architecture (Valero et al., 2018). Collagen Type I, a major component of the ECM of connective tissues, was cross-linked under different conditions to provide collagen gels for different tissue engineering applications (Parenteau-Bareil et al., 2010). Collagen Type I is widely used as a three-dimensional scaffold for cell cultures capable of providing optimal environments in the form of physical and chemical cues (Lin and Gu, 2015). The structural properties of collagen gel provide the basis of cell-scaffold interactions and were considered in many scaffold designs and tissue engineering (Vader et al., 2009; Wallace and Rosenblatt, 2003; Xu et al., 2011; Chuang et al., 2018). Microstructure modulates the macroscopic properties of cross-linked fiber networks.

There have been different methods to improve the mechanical properties of collagen scaffolds including physical cross-linking (Chen et al., 2023; Anderson et al., 2022) through exposure to a radiation sources such as UV light (Romppainen et al., 2007) and chemical cross-linking (Nair et al., 2020). The central aim of chemical cross-linking of collagen is to improve the mechanical properties and stability of the final processed collagen product. Therefore, the selection of an appropriate cross-linker based on the properties required by the application of interest is necessary. For detailed information about the effect of cross-linking methods on various physical, chemical and biological properties in collagen-based constructs for collagen Type I, the readers are referred to the review of Nair et al. (2020).

Since collagen is often used in biomedical applications to replicate the biochemical environment experienced in vivo, amine-based cross-linkers are often selected to mimic the lysine-based cross-links which are naturally present in collagen (Mouw et al., 2014). Glutaraldehyde (GA) is one of the most common chemical amine-based cross-linking reagents

for collagen, which helps to keep many of the viscoelastic properties of collagen fibrillary network, and it reacts relatively fast (Olde Damink et al., 1995; Cheung et al., 1985). Collagen gels cross-linked with GA have already been studied for heart valve (Sung et al., 1999; Lopez-Moya et al., 2018; Liu et al., 2022b), corneal tissue engineering scaffolds (Duan and Sheardown, 2006; Doillon et al., 2003; Lai, 2012; Mahdavi et al., 2020), ocular surfaces (Geggel et al., 1985) and collagen membranes (Charulatha and Rajaram, 2003). Addition of GA will induce covalent bonds between collagen fibrils from aldehyde-amino reactions as well as from aldol condensation (Jayakrishnan and Jameela, 1996), which results in a more tightly cross-linked network. In addition, GA can lead to intramolecular cross-links formed between two α -chains by aldol condensation. Although GA is widely used, it has potential toxic effect and prevents its application for *in vivo* studies. Recently emerging cross-linking methods include the use of genipin (GP), a compound extracted from the fruit of the *Gardenia Jasminoides* that has been shown to effectively cross-link cellular and acellular biological tissues as well as hydrogels and hydrogel composites (Sung et al. 2000, 2003). GP was also found to be significantly less cytotoxic than GA (Sundararaghavan et al., 2008).

There have been several experimental studies to investigate the relationship between the mechanical properties of collagen gel and the quality of cross-linked fiber structure including fiber dimensions, fiber strength, collagen concentration, pH, and etc. as well as how to improve the mechanical properties of collagen hydrogels (Vader et al., 2009; Engler et al., 2006; Lin and Gu, 2015; Xu et al., 2011; Sander et al., 2009; Valero et al., 2018; Tamura et al., 2022; Lotz et al., 2017; Princen et al., 2023). All these studies correlated the microscopic cross-link properties with the macroscopic gel mechanics. For example, Motte and Kaufman (2013) have shown that Type I collagen gel stiffness and failure stress increased with collagen concentration, pH, or temperature during polymerization.

Many computational models have been developed to deeply understand biopolymer hydrogels, and to evaluate the mechanical properties of hydrogels under various loading modes, which can be categorized into continuous and discrete approaches. Among the continuum approaches, the worm-like chain (WLC) type models have been used to simulate biopolymer behavior (Palmer and Boyce, 2008; Kuhl et al., 2005; Arruda and Boyce, 1993). Even though these models represent actin networks, they could be adapted to other biopolymers such as collagen with similar behavior. Others have proposed continuous approaches different from WLC (Holzapfel et al., 2014; Castro et al., 2016). Discrete models have been widely used because of their ability to reproduce network geometry which simulate the biopolymers filamentous structures using various lattice methods e.g. (Rens et al., 2016; Lee et al., 2014; Kwansa et al., 2016). Molecular dynamics has been also used to model collagen cross-link to investigate their mechanical properties (Depalle et al., 2015; Buehler and Wong, 2007).

Examples of computational models of collagen gels that provide a theoretical framework to understand relationships between the cross-link structure and tissue-level mechanical properties are also available (Xu et al., 2011; Chandran et al., 2012; Lin and Gu, 2015). An image-based multiscale model was developed using Galerkin finite element method based on the theory of volume averaging that captured the anisotropy and heterogeneity of a cell-compacted collagen gel subjected to an off axis hold mechanical test and biaxial extension (Sander et al., 2009). Cross-links were constructed to allow fibers to rotate but not bend or slide past each other. Their model predicted that tensile and compressive fiber forces were produced to accommodate macroscopic displacements. The nonlinear material response captured in the model arises mainly from the properties of the network, where fibers accommodate the macroscopic stretch through rotations at the cross-links before stretching along the fiber axis.

Xu et al. (2011) used a previously established anisotropic hyperelastic constitutive model (Gasser et al., 2006) in combination with experiments to study the structure and function of type I GP- cross-linked

collagen gel with the effects of cross-linking and spatial fiber alignment. The tangent modulus, the storage, and loss modulus of collagen scaffolds were shown to increase with GP concentration. Therefore, cross-linking of the scaffolds was shown to tune the overall scaffold's stiffness with no influence on the presence of anisotropy in the collagen matrix (Xu et al., 2011).

Lin and Gu (2015) developed a model with randomly distributed 3D cross-linked collagen fiber network using the random seed algorithm to predict tensile behavior under various cross-link stiffness and density. The Type I collagen gel in this model was represented by a representative volume element (RVE) with a fiber volume fraction of 0.073%. The cross-links were generated between nodes when their distance is less than or equal to a certain value, referred to as cross-link threshold. Various cross-link thresholds and cross-link stiffness showed that the increased cross-link density has higher impact on the gel stiffening than the cross-link stiffness. Both collagen fibers and cross-links were modeled as linear elastic materials; however, the fiber network exhibited obvious strain stiffening. The network stiffness was increased with strain, and its magnitude is much less than the stiffness of either fibers or cross-links due to low fiber volume fraction, which could be visualized by continuous fiber alignments. This result is consistent with the experimental study by Vader et al. (2009) and the theoretical hypothesis by Onck et al. (2005) that strain stiffening in polymer gel was governed by the fiber rearrangement.

A continuous computational model based on the WLC has been also used to reproduce the mechanical behavior of the collagen gels (also networks made of different biopolymers) and estimate the parameters of the biopolymer networks without more sophisticated methods, such as image processing or network reconstruction (Valero et al., 2018). This WLC model accurately predicted the strain stiffening and the strain that initiates strain softening, although it cannot simulate softening when collagen concentration is increased.

The investigations mentioned above clearly demonstrate that physical and chemical modification can alter mechanical properties of collagen gels to tune for different tissues demands. The ability to control the mechanical properties and degradation of collagen hydrogels using different cross-linking mechanisms is a great tool to optimize systems for use in different tissue engineering purposes (Sarrigiannidis et al., 2021; Skopinska-Wisniewska et al., 2021). These results could be further used to guide the design of scaffold with tunable material properties.

6. Collagen cross-links in arterial tissue

Arteries are made of four main components; collagen, elastin, smooth muscle cells (SMC), and water and they have a clear composite-like structure (Sáez et al., 2014; Iaizzo, 2010). The arterial wall consists of ECM and several types of cells organized into different layers: intima, media and adventitia (Fig. 6) (Taki et al., 2016; Chow et al., 2014; Iaizzo, 2010). SMC mostly found in media react mechanically to external stimuli by contracting or dilating the vessel. Elastin, which is distensible and has a low tensile strength, determines low-strain properties and functions primarily as an elastic reservoir. It distributes stress evenly throughout the wall and onto collagen fibers (Wolinsky and Glagov, 1967), while collagen has a low contribution at low strains due to its wavy nature and it protects the wall from overstretching and contributes mainly to the wall's properties at higher strains (Roach and Burton, 1957; Chow et al., 2014; Sugita and Matsumoto, 2017). Collagen, the main load-bearing component of the ECM, exhibits high non-linear behavior bearing the major part of the load transmitted through the tissue (Sáez et al., 2014).

The intima layer is mainly occupied with endothelial cells (ECs), which synthesize proteins, such as collagen IV and laminin, to create basal lamina. Its main function is to transmit signals, which control vascular tone, and has a minimal contribution to the artery's mechanics. The media as the thickest layer is composed of multiple lamellar units (LU) (Fig. 6), which consists of elastin encompassing SMC and collagen

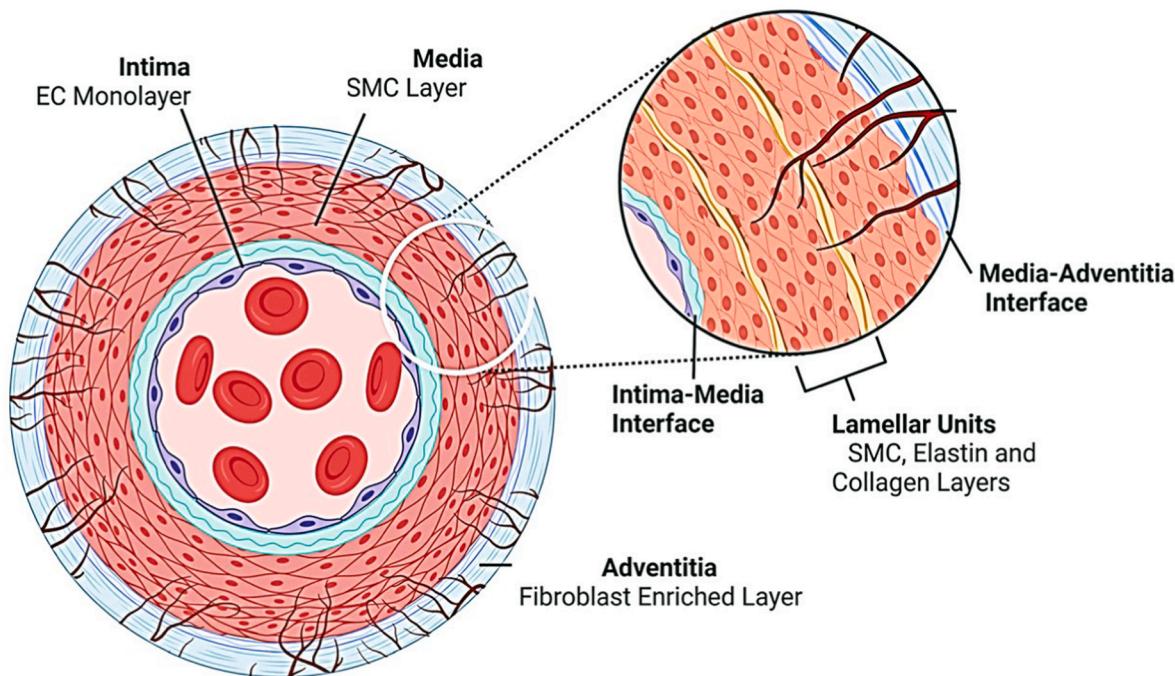


Fig. 6. Typical cross-section of an artery showing intima, media, and adventitia layers. The intima is the inner layer consisting of an endothelial cells (ECs), the media is concentrically layered with lamellar units (LUs) of smooth muscle cells (SMCs) confined by elastin and collagen fibers. The outer layer is the adventitia, which is an ECM and fibroblast-rich layer (adapted from [Bax et al. \(2022\)](#)).

fiber network. The media serves as the primary load bearing component ([Mozafari et al., 2019](#)) because the interconnecting lamellar network is designed to transfer stress throughout the vessel wall. Bundles of collagen between the lamellar layers show no definite overall arrangement at low pressure but become circumferentially aligned as pressure increases ([Wolinsky and Glagov, 1964](#)). With additional increase in the wall strain, there is little further change in radius as additional collagen fibers are recruited, accounting for the nonlinear nature of vascular elasticity ([Wolinsky and Glagov, 1964](#)). The adventitia anchors the vessel to the surrounding tissue. It mainly contains fibroblast that is mainly responsible for the synthesis of collagen and a collagen-rich ECM ([Chow et al., 2014](#)). Adventitial fibroblasts respond to a variety of chemical and mechanical cues. For example, hypertensive environments result in increased fibroblast proliferation and collagens I and III synthesis. Adventitia provides mechanical strength to prevent vessel over-expansion and bears over half of the load at abnormal pressure due to the high relative collagen content ([O'Connell et al., 2008](#)). Multi-photon image analysis of biaxially stretched aortic tissues has shown that the medial collagen is being recruited throughout the stretching process while the adventitial collagen shows a delay in the fiber recruitment and starts to be recruited at a later stage of stretching ([Chow et al., 2014](#)).

Collagen and elastin fibers are not individually placed along the arteries, they are connected via cross-links, and therefore the 3D hierarchical arrangement of the collagen fibers and cross-links is crucial to understand mechanical behavior of the arterial tissue ([Humphrey, 2013](#)). Elastin cross-linking similar to collagen cross-linking (as explained in Section 1) is also involved in the stabilization of fibrils via intra- and intermolecular cross-links and the strength of the of ECM connective tissue, i.e., the aorta ([Gaar et al., 2020; Hornstra et al., 2003](#)). Lysyl oxidase promotes cross-link formation in embryonic fibrils of elastin by conversion of lysine and hydroxylysine side-chain residues to aldehydes ([Reiser et al., 1992](#)).

The density of cross-links in collagenous tissue has a stiffening effect on the mechanical response of the tissue. Aging and diabetes have also been associated with increasing the density of non-enzymatic cross-links leading to increasing the stiffness and brittleness of the tissue ([Brüel and](#)

[Oxlund, 1996; Zieman and Kass, 2004; Snedeker and Gautieri, 2014; Wang et al., 2023; Briiel and Oxlund, 1996](#)). Excessive collagen cross-linking has been observed in patients with hypertension, atherosclerosis, and diabetes ([Safar et al., 2000](#)) and in dilated carotid arteries ([Nuthakki et al., 2004](#)). Interestingly, a reduction of elastin cross-linking in abdominal aortic aneurysms has been reported ([Gandhi et al., 1994](#)), which is consistent with the role of inhibition of elastin cross-linking in enlargement remodeling. Collagen and elastin cross-linking can participate in the healing process, leading to constrictive remodeling after balloon injury; inhibition of collagen and elastin cross-linking resulted in disorganization of collagen fibers and a significant reduction of constrictive remodeling and restenosis ([Brasselet et al., 2005](#)).

Microstructure of ECM can vary by some diseases, and collagen cross-link disruption has been observed in arteries. Therefore, appropriate cross-linking can prevent severe consequences in some arterial diseases and reduce the failure rate in treatment. As an example, a weak venous wall is one of the major reasons of vein graft failure after coronary artery bypass grafting. Adventitial glutaraldehyde cross-linked collagen in human saphenous veins was shown to reinforce venous wall by increasing stiffness and decreasing extensibility and alleviated the endothelial damage under high-pressure distension ([Liu et al., 2022a](#)). Adventitial collagen cross-linking also remarkably reduced intimal hyperplasia in a rabbit arteriovenous graft model ([Liu et al., 2020](#)). The mechanobiology and modeling of aneurysms as an arterial disease have been studied e.g. ([Vorp, 2007; Humphrey and Holzapfel, 2012](#)). Currently, the common treatment for large abdominal aortic aneurysm (AAA) is the repair surgery, and no therapeutic option is available now for the small AAA. [Chirila and Suzuki \(2022\)](#) found that the mechanical stiffness of blood vessel walls was enhanced by the adventitial collagen cross-linking through a photochemical process promoted by ultraviolet-A (UV-A) radiation. The stabilization of adventitia may prevent the rupture of the aneurysm when this method is applied to an aneurysmal dilated wall region. With further investigation, this method can become a therapeutic approach for arresting the progression of AAA. Protein photo cross-linking has also been demonstrated to stiffen and strengthen tissues, decrease inflammatory responses and

facilitate tissue bioengineering. The review by [Redmond and Kochevar \(2019\)](#) summarized research on the potential applications of photo-sensitized cross-linking of tissue proteins in surgery such as reattaching blood vessels and strengthening vein.

Computational simulations have been implemented for many decades to investigate the mechanical behavior of arteries. The arterial wall has been modeled as a single layer ([Kiousis et al., 2009](#)), two or three layers ([Holzapfel and Ogden, 2010](#)). The applied constitutive relations to the arterial wall have been formulated by hyperelastic material with orthotropic, transverse isotropic, and isotropic behavior e.g. ([Holzapfel et al., 2000; Taber and Humphrey, 2001; Kural et al., 2012](#)). These models predicted the macroscopic mechanical properties of the artery, but highly heterogeneous microstructure of the arterial layers requires additional consideration. Micromechanical modeling has then been developed to include the geometries and properties of the individual constituents and better describe the mechanics of the tissue. Capturing the hyperelastic responses of tissues with multiple families of collagen fibers ([deBotton and Oren, 2013](#)) or explaining the interaction between collagen and non-fibrillar matrix ([Lake et al., 2012](#)) are some examples of micromechanical modeling.

As observed, pathology of many vascular diseases has been related to structurally disordered ECM and its altered interaction with other arterial constituents. Mechanical characteristics of intact and diseased vascular tissues are important, and require robust constitutive descriptions to capture the vessel wall's anisotropic and non-linear properties. Specifically, the complex 3D network of collagen and the mechanics of its cross-links has a governing effect on arterial properties at higher stress levels.

7. Computational models of cross-link in fibrous tissue

The macroscopic properties of collagen-based tissues have broadly been studied ([Sander et al., 2009; Mononen et al., 2011; Svensson et al., 2021; Barrett et al., 2021; Karimi et al., 2017; Krasny et al., 2018; Li et al., 2017](#)). However, less is known about the microstructure of tropocollagen as their basic building blocks and the relationship between collagen molecule and tissue properties. Therefore, the models, which take the microstructural features of collagen molecule such as cross-link into account, are required to facilitate comprehension of the mechanics and failure mechanisms of the tissues.

7.1. Atomistic-molecular models

To develop a fundamental and measurable understanding of collagen mechanics, theoretical models at the atomistic scales (considering atomistic and chemical interactions during deformation) are essential. This represents an alternative strategy capable of predicting the properties of collagen tissue in a bottom-up approach.

7.1.1. Atomistic models

Fully atomistic modeling of stretching single molecules of tropocollagen was performed by [Buehler and Wong \(2007\)](#) using classical force field CHARMM, implemented in the molecular dynamics (MD) program NAMD, capable of describing different regimes of elastic and permanent deformation (transition from entropic to energetic elasticity). They showed a dominance of entropic elasticity for small deformation, while a transition to energetic elasticity at larger deformation, which is characterized by breaking of hydrogen bonds, followed by deformation of covalent bonds in the protein backbone, leading to molecular fracture. Previously, it was shown that entropy determines the network elasticity at small strains, while enthalpy plays a larger role at larger strains ([Storm et al., 2005](#)). Enthalpic effects have also been proposed as sources of nonlinear bond elasticity, which in turn causes stiffening ([Rosenblatt et al., 2006](#)). The assembly of single tropocollagen molecules into fibrils was also shown to significantly decrease their bending flexibility, leading to decreased contributions of entropic effects during

deformation ([Buehler and Wong, 2007](#)).

A two-dimensional atomistic model of the large-strain deformation regime of collagen fibrils ([Buehler, 2008](#)) was used to investigate the effect of cross-link density, which further linked properties of individual molecules with the overall mechanical response using a fiber-reinforced composite constitutive model. The effect of cross-links was modeled by modification of the adhesion strength at the terminals of collagen molecules in the Lennard-Jones potential to account for the stronger interaction between molecules. This study showed that greater cross-linking led to increased fibril tensile strength that exhibit an increasingly brittle deformation character, while cross-link deficient collagen fibrils show a highly dissipative behavior with large yield regimes ([Buehler, 2008](#)). These results did not however replicate the three-phase response of human fibrils tested experimentally ([Svensson et al., 2013](#)). This observation resulted in the development of a 3D coarse-grained model of a collagen fibril with divalent or trivalent cross-links based on fully atomistic simulations ([Tang et al., 2009](#)). Similar to the previous models, increased density of either type of cross-link is related to a greater ultimate tensile stress of the fibril and an increased maximum strain. In addition, the model also successfully replicated the experimental results, incorporating divalent or trivalent cross-links bearing similarity to the mechanical responses of fibrils from a rat tail tendon and human patellar tendon, respectively. Increased cross-link density was shown to resist sliding between individual molecules in the model, which resulted in an increase in molecular strain relative to fibril strain ([Tang et al., 2009](#)).

[Uzel and Buehler \(2011\)](#) developed a simple and fully atomistic model of an entire overlap region of two collagen molecules in collagen Type I based on large-scale molecular dynamics simulation in explicit water solvent to investigate the influence of only enzymatic cross-links on the deformation mechanism and strength of the structure under different loading scenarios. The protein was subjected to stretching via the steered molecular dynamics (SMD) simulation protocol ([Lu et al., 1998](#)), where the center of mass of a collection of chosen atoms is pulled via a spring along the direction of the molecular axis, while the center of mass of another group of atoms was kept fixed via a stiffer spring ([Uzel and Buehler, 2011](#)). In this work, the effect of cross-links was found to be relatively minor for small deformations, in agreement with the previous results ([Buehler, 2008](#)). However, this effect was not negligible for large deformations, that is the presence of cross-links causes significant strengthening ([Kwansa et al., 2016; Uzel and Buehler, 2011](#)). Their findings also verified that cross-links contribute to the mechanical stability of collagenous tissues by enhancing the adhesive strength between collagen molecules at a molecular scale.

[Uzel and Buehler \(2011\)](#) additionally proposed a simple rheological model to reproduce the results of their molecular dynamics simulation, i.e. the elastic, slippage and delayed elastic regimes. The deformation mechanism of the structure was shown to be greatly affected by the presence of the cross-link at larger deformation levels. The presence of a cross-link leads to a greater strength during deformation since complete intermolecular slip is prevented. On the contrary, the lack or absence of a cross-link (e.g. in diseases that prevents the cross-link to form) causes the onset of intermolecular sliding during deformation leading to an overall weaker structure, and could lead to a catastrophic failure as reported before ([Gautieri et al., 2009](#)).

7.1.2. Discrete fiber network models

Biological materials are invariably heterogeneous at small scales ([Tseng and Wirtz, 2001](#)). Therefore, specific network architecture, such as the number of filaments and the number of cross-links per filament, plays an important role in governing elasticity ([Lindström et al., 2010; Kasza et al., 2009](#)). Discrete fiber network (DFN) models have been developed to better understand fiber-level kinematics, and structural inhomogeneity within the network ([Yu and Zhang, 2022](#)). In addition, the constitutive models often rely on the affine deformation assumption. Although there have been theoretical models to trace the nonlinear elasticity of single filaments by assuming affine network deformation e.

g. (Storm et al., 2005), some two dimensional (Head et al. 2003a, 2003b; Wilhelm and Frey, 2003) and three dimensional (Huisman et al., 2007) networks show evidence of nonaffine behavior. The degree of network affinity has been shown to be a function of strain (Onck et al., 2005) as well as characteristic length scales of the network, such as the average distance between cross-links, fiber length and persistence length (Head et al., 2003a).

Therefore, considerable efforts in developing DFN models have been made to describe and understand the cross-link structural effects on mechanical behavior of the structure. One of the preliminary efforts is the application of a 2D anisotropic random network of rigid rods to investigate the elasticity of stiff networks developed by Wilhelm and Frey (2003). Here, the building blocks of the model are homogeneous and randomly distributed elastic rods of equal length, connected by a cross-link with zero extensibility at intersections, where the cross-links either fix the relative orientation of the rods (stiff cross-links) or allow free rotation (free hinges). The elastic response of a rod between two neighboring cross-links is characterized by length dependent force constants for elongation and bending. In addition to a critical rigidity percolation region and to a homogeneously elastic regime for high density rods which are dominated by the compressive modulus of the individual filaments, Wilhelm and Frey (2003) found a novel intermediate scaling regime, where the elasticity is dominated by bending stiffness of the filaments. It has been argued that an alternative mechanism involving network rearrangement and a transition from bending-to stretch-dominated deformation can cause nonlinearity even without the nonlinear force-extension relation of single chains (Onck et al., 2005).

The review of Picu (2011) presents the different parameters of DFN structure such as cross-linking, fiber density, and fiber orientation distribution in determining the mechanical properties. DFNs are usually created by randomly placing fibers in a given domain with the network structure depending on the generating method. Based on the DFN generating method, fiber coordination (number of fibers intersect at one cross-link) can be different, which is important for the stability of the network (Picu, 2011). A two-dimensional DFN model of arterial elastic fiber network was generated from multiphoton images using Otsu's thresholding method, which predicted the stress-strain behavior of the elastin network and the cross-linking was shown to affect the local fiber mechanics and kinematics. Mechanical behavior of the collagen gel was also captured by DFN models using Voronoi tessellation generation method (Lake et al., 2012; Nachtrab et al., 2011). There are DFNs available, which are built by randomly depositing fibers of equal length into a given domain (Wilhelm and Frey, 2003; Stylianopoulos and Barocas, 2007). DFNs created by Delaunay triangulation of a set of randomly distributed points were used to model fibrous microstructures and electrospun fibrous scaffolds (D'Amore et al., 2010).

In three-dimensional DFN models, cross-links were modeled as short fibers that can rotate and extend (Žagar et al., 2015; Stylianopoulos and Barocas, 2007) when two fibers are closer than a threshold distance, or within the interpenetrating fibers (Dirrenberger et al., 2014). Whereas, in two-dimensional DFN models the inter-fiber cross-linking is treated as either pin joints (Mauri et al., 2016; Bircher et al., 2017; Yu and Zhang, 2022; Thunes et al., 2016) or weld joints (Onck et al., 2005; Abhilash et al., 2014; Yu and Zhang, 2022). Yu and Zhang (2022) assumed the cross-links to have a rotational stiffness that varies from zero (pin joint) to infinity (weld joint) while the cross-link density changed from being fully to partially cross-linked. Pin joints were shown to be suitable to describe cross-links in either flexible polymer networks or networks of rigid filaments with flexible cross-linkers (Pritchard et al., 2014), while weld joints have been used to model rigid cross-link (Gardel et al., 2004). Yu and Zhang (2022) showed that the pin and weld joints do not seem to have clear effect on the arterial network stress-strain behavior.

In DFN models, both the properties of fibers and cross-links contribute to the nonlinear elasticity of fibrous networks (Kasza et al., 2009; Žagar et al., 2015; Pritchard et al., 2014). In addition, the density

of cross-links can also significantly impact the mechanical behavior of biological tissue (Shahsavari and Picu, 2012, 2013a; Yu and Zhang, 2022; Žagar et al., 2015). DFN models can be incorporated into a multiscale models and help to modify the constitutive governing equations to further provide microstructural understandings on the tissue macroscopic mechanical properties (Shah et al., 2014; Korenczuk et al., 2019; Yu and Zhang, 2022) and failure of tissues (Witzenburg et al., 2017; Yu et al., 2020).

7.1.3. Worm-like chains (WLC) models

Another type of models are worm-like chains (WLC) and glassy WLC models, where sticky interactions between biopolymers allow exponential stretching of the relaxation spectrum (Kroy and Glaser, 2007; Kroy, 2008). Kurniawan et al. (2012) developed a 3D model of realistic, cross-linked semiflexible network to study the nonlinear network response (strain stiffening and the effect of fiber volume-occupancy) from small to large strains through the structural parameters of network influence (network connectivity and fiber entanglements), that fully govern the nonlinear behavior. The fibers in the network, which are represented as an array of beads of diameter d were modeled by modifying the standard bead model typically used to represent coarse grained polymer and WLCs (Fig. 7). The model includes intra- and interfiber interactions that furthermore include two subclasses. Thus, stretching and bending belong to intrafiber interactions, whereas cross-link and entanglement are classified as interfiber effects. All of these interactions are characterized by potentials of the harmonic type as shown in Fig. 7. Here, the stretching potential U_s depends on the distance between beads r and the bead's diameter d , bending potential U_b depends on the angle between the neighboring beads θ , cross-link potential U_{cl} relies on the difference between the distance r and the cross-link length r_0 , and entanglement potential U_i relies on the difference between the diameter d and distance r under the condition that $r \leq d$. Furthermore, k_s, k_b are stretching and bending constant, k_{cl} is the cross-link compliance and k_i determines the "softness" or the degree of penetrability of the fibers. The results reported by Kurniawan et al. (2012) using this model revealed distinct deformation mechanisms at different length scales. For the strains lower than critical strains (the critical strain corresponding to the onset of nonlinearity, and the instantaneous large-strain modulus), the overall deformation is dominated by short-scale fiber mechanics, and the network stiffness is governed by steric interaction at short length scales and by cross-links at larger length scales. However, once the network is sufficiently strained, reorganization causes the effects of local heterogeneity to diminish and be replaced by a more homogeneous response. Biological networks may therefore take advantage of the heterogeneity at small strains to accommodate various functions, while retaining the ability to accurately control large-strain responses through active cross-linkers for the benefit of network integrity.

Benetatos et al. (2012) calculated the effect of random cross-links on the force-extension relation of an array of parallel-aligned anisotropic network of WLCs, which have been aligned along a preferred axis. The cross-link was modeled as a harmonic spring, confining the transverse motions of the chains at the midpoint, while leaving their orientation free to fluctuate. The effect of the cross-links is purely entropic in the strong stretching limit, independent of the bending rigidity of the chains. Cross-links enhance the differential stretching stiffness of the bundle. For hard cross-links, the contribution of cross-link to the force-extension relation was inversely proportional to the force. The dependence of force-extension relation on the cross-link density, close to the gelation transition, is the same as that of the shear modulus. The qualitative behavior is captured by a toy model of two chains with a single cross-link in the middle.

Benetatos et al. (2014) have studied two reversibly cross-linked, semiflexible filaments under tension and demonstrated that the two filaments are always in a (weakly) bound state. Within mean-field theory, there is a discontinuous phase transition as a function of applied

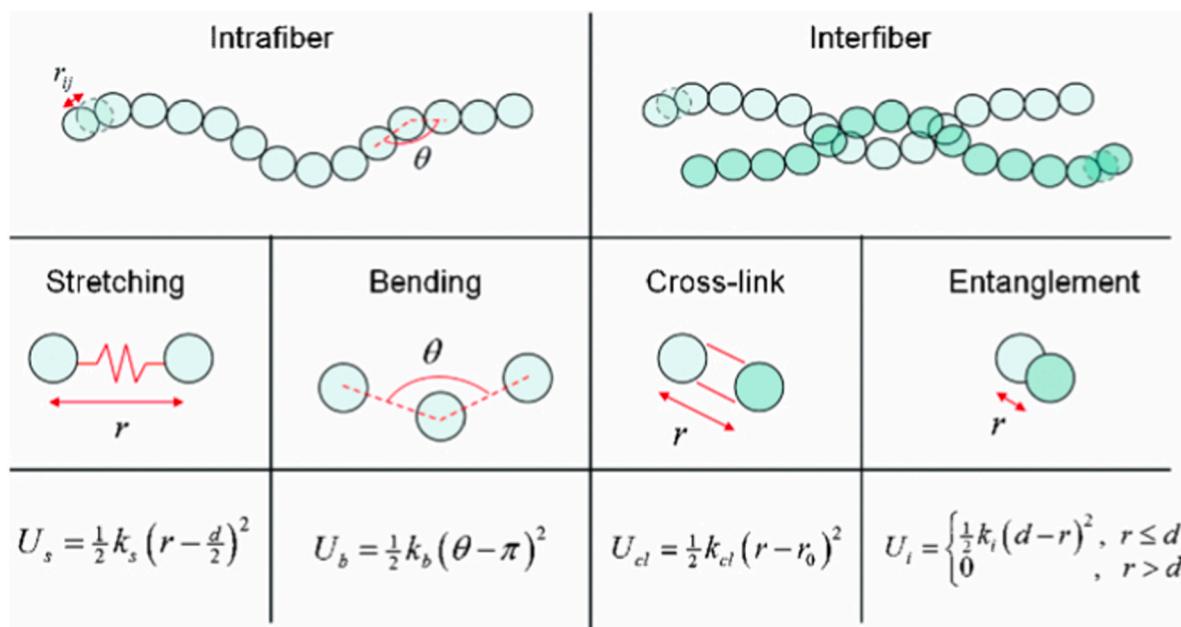


Fig. 7. Summary of standard bead model and interfiber and intrafiber interactions together with corresponding potentials (adapted from [Kurniawan et al. \(2012\)](#)).

stretching force from a weakly bound state at small force to a strongly bound state at large force. The critical force as a function of either cross-link strength or chemical potential displays a scaling, which can be derived from a model of a single polymer with a confining potential. The analysis performed by [Benetatos et al. \(2014\)](#) neglects entanglement effects and twist. Their results can be generalized to (1 + 2) dimensions, since in the weakly-bending approximation, the two transverse directions decouple.

In the aforementioned studies, a cross-link is defined as an additional bond either between two parts of the same polymer or between two different polymers. Microscopically, this means that cross-links are modeled to form between two monomers. This situation holds true in many materials, whereas there are also other important classes of cross-links that connect more than two monomers ([Degtyar et al., 2014](#); [Reinecke et al., 2016](#); [Harrington et al., 2009](#)). Therefore, a few studies exist that investigate the influence of the coordination of cross-links (coordination of cross-links is referred to the number of monomers involved in one cross-link) on the mechanical behavior ([Depalle et al., 2015, 2018](#); [Shabbir et al., 2019](#); [Shabbir and Hartmann, 2017](#)). Here, di- and trivalent cross-links in collagen are investigated.

The 3D molecular computational model of [Shabbir et al. \(2019\)](#) studied the tensile deformation behavior of parallel fibers connected by two- and three-fold coordinated cross-links using coarse-grained approach as a continuation to their previous work ([Shabbir and Hartmann, 2017](#)), where the linear polymer is described using a bead-spring model (Fig. 8). Each bead is modeled as a covalently bound hard sphere, and several sticky beads are introduced to model cross-links capable of forming reversible cross-links. Fig. 8 illustrates a two-dimensional schematic, but the model is fully three-dimensional so that beads can move in all three directions of space. In their study, higher coordination of the cross-links has been shown to have a positive effect on the strength of the overall fibrous system because a three-fold coordinated system prevents the premature rupture of the covalent backbone frequently observed in classical two-fold coordinated cross-links ([Nabavi and Hartmann, 2016](#)). Therefore, controlling the coordination of cross-links can be potentially used to modify the mechanical properties of polymeric structures.

Very recently, [Kamml et al. \(2023\)](#) built a 3D coarse-grained model of the collagen fibril considering both enzymatic and AGEs cross-links using steered molecular dynamics, where the mechanical response of

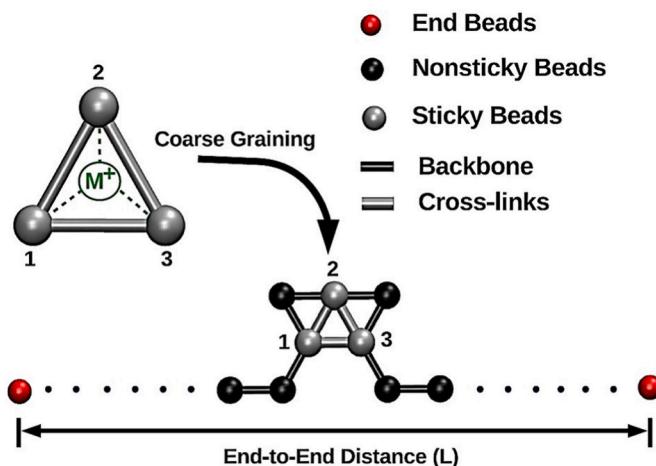


Fig. 8. Bead-spring model, where the polymer is defined as a chain of covalently bound hard spheres. Sticky sites can form cross-links via coordination with metal ions as shown in the upper left (adapted from [Shabbir et al. \(2019\)](#)).

tropocollagen molecules and cross-links are derived from reactive molecular dynamics simulations. They showed that higher density of AGEs cause fibrilar stiffening and strengthens force transfer through AGEs cross-links rather than through friction between sliding tropocollagen molecules, which results in the breaking the bond of the tropocollagen molecules (associated with lower energy dissipation) leading to an abrupt failure of the fibril. Therefore, these results provide a direct relation between increased AGEs content, inhibited intra-fibrillar sliding, increased stiffness, and abrupt fibril fracture. This study can explain the mechanical origin of bone brittleness observed in aging, which contributes to a better understanding of the mechanisms underlying impaired tissue behavior due to elevated AGEs content and could enable targeted measures regarding the reduction of specific collagen cross-linking levels.

The insight gained from fundamental molecular models of cross-linking in biopolymers is vital to understand collagenous materials, and the information can be used for the continuum modeling of fibrous tissues. It could also help in advancing the development of materials for

applications in regenerative medicine and scaffold design (Harley et al., 2007).

7.2. Constitutive models of fibrous tissues

Many constitutive models, either phenomenological or structural, have been proposed to describe the mechanical properties of fibrous tissues like large elastic arteries. The constitutive modeling of collagenous tissues relies on definition of strain energy function (SEF) for collagen fibers embedded within an isotropic matrix associated with elastin (Carpenter et al., 2020; Ibrahimbegovic, 2009; YC Fung, 1967; Gasser, T., 2019). Readers who require a detailed description of the constitutive theory for highly anisotropic solids and nonlinear elasticity are referred to continuum theory of the mechanics of fiber-reinforced composites by Spencer (1984), Holzapfel (2002) and Ibrahimbegovic (2009).

7.2.1. Phenomenological versus structural models

In phenomenological arterial models, collagen is commonly modeled by exponential-type expressions. The phenomenological approach with polynomial, exponential or logarithmic function (Humphrey, 1995; Takamizawa and Hayashi, 1987) captured hyperelastic behavior, but did not provide information about arterial material and structure and, sometimes, the fitted parameters do not have physical meaning or do not match with experimental observations (Sáez et al., 2014). Phenomenological SEFs are purely based on mechanical input information; however, structural SEFs integrate microstructural histological data, and are able to link the macroscopic loading to potential cellular responses.

Structural SEFs used for arterial wall are based on fiber-reinforced composite concepts, which assume (families) of fibers to be straight and parallel-aligned (Sokolis, 2010; Holzapfel et al., 2000), straight and orientation-dispersed (Gasser et al., 2006), undulated and parallel-aligned (Zulliger et al., 2004a), or undulated and orientation-dispersed (Martufi and Gasser, 2011). Extensive effort has been dedicated to develop and numerically implement structural SEFs, nonetheless, only very few studies consistently validated structural SEFs, i.e. by treating histological and mechanical input information strictly separately (Gasser et al., 2012; Polzer et al., 2015; Wan et al., 2012). As observed, there have been studies to develop constitutive models of the arterial wall by taking experimentally measured structural information into account (Zulliger et al., 2004a; Holzapfel et al., 2000). However, in these structure-inspired constitutive models, appropriate microstructural information was usually not considered (Holzapfe and Weiszäcker, 1998; Holzapfel et al., 2000; Kao et al., 2011). Therefore, incorporation of microstructural characterization of vessel wall components including cross-links can help to achieve a more realistic constitutive modeling.

To include the inter-fiber interactions in constitutive models, Nerurkar et al. (2011) added shear interactions between two opposing fiber families to the SEF to model the intervertebral disc. Interactions between collagen fibers and myofibers was considered in a constitutive model for passive right ventricular myocardium (Avazmohammadi et al., 2017). Various SEF can be defined for arterial walls (Holzapfel et al., 2000), upon which different properties of collagen cross-linking can then be built and incorporated into basic models. These properties of collagen cross-linking include orientation of the fibers (Sáez et al., 2016; Linka et al., 2016; Holzapfel and Ogden, 2020b; Wang et al. 2016, 2023), gradual recruitment of fibers in the model (Mattson et al., 2019; Wang et al., 2016), 3D description of arterial tissue including cross-links content and stiffness and fiber orientation (Holzapfel and Ogden, 2020b; Tian et al., 2016; Yu and Zhang, 2022), and considering the effect of fiber and cross-link dispersion on the tissue mechanics (Teichtmeister and Holzapfel, 2022).

7.2.2. Incorporation of cross-links in constitutive modeling

A precise investigation of stress states is necessary in order to

investigate the contribution of collagen and other constituents in load bearing properties of the tissue. For hyperelastic materials, stress tensor is determined as the derivative of the strain energy function (Ψ). In fibrous tissues, the collagen fibers are embedded within an isotropic matrix. The contribution of each phase to the SEF is defined based on their volume fraction. The isotropic contribution to the energy (Ψ_{iso}) is found using the neo-Hookean formulation. According to the standard reinforcing model, anisotropic collagen fibers' contribution to SEF (Ψ_f) has the form:

$$\Psi_f(I_4) = k(\rho)(I_4 - 1)^2 / 2, \quad (1)$$

where $k(\rho)$ is the cross-link-dependent fiber stiffness, and $I_4 = \mathbf{M} \mathbf{C} \mathbf{M}$ is the fourth invariant of the right Cauchy-Green tensor $\mathbf{C} = \mathbf{F}^T \mathbf{F}$ where \mathbf{F} is the deformation gradient and \mathbf{M} is the fiber direction. Note that some authors only consider the deviatoric part of deformation in the definition of Ψ_f . The derivative k should be positive to reflect the increasing stiffness with increasing density of cross-links (Holzapfel and Ogden, 2020b). Alternatively, an exponential function can be used for Ψ_f since based on experimental data of the fibrous tissues, this exponential function is shown to more realistically describe collagen fibers' contribution to SEF (Holzapfel et al., 2000; Holzapfel and Ogden, 2020b; YC Fung, 1967; Sáez et al., 2016; Gasser et al., 2006; Wang et al., 2023):

$$\Psi_f(I_4) = \frac{k_1(\rho)}{2k_2} \{ \exp[k_2(I_4 - 1)^2] - 1 \}, \quad (2)$$

where $k_1 > 0$ is a parameter with the dimension of stress, while $k_2 > 0$ is a dimensionless parameter which measures the exponential behavior of the response. Eq. (1) and Eq. (2) do not still include the cross-link properties. Therefore, Sáez et al. (2014) formulated a continuum-based approach by extending Helmholtz SEF with an isotropic contribution of the cross-links connecting parallel fibers in carotid arteries to address structural features. The modification proposed by Sáez et al. (2014) introduces the cross-links attachment to the main fibers and these cross-links confer a non-negligible stiffness in the transversal direction of the fibers, which now overcame the limitations observed in the previous models. For the contribution of collagen fiber including cross-links properties to the stored energy (Ψ_{fc}), they employed Eq. (2) and accounted for the cross-links using a parameter ($\alpha \in [0, 0.5]$) which provided a weighting between the isotropic and the anisotropic response (Eq. (3)):

$$\begin{aligned} \Psi_{fc}(I_1, I_4) = & \sum_{i=1}^N (1-\alpha) \Psi_f^i + \alpha \left[\frac{k_1}{2k_2} \{ \exp[k_2(I_1 - 3)^2] - 1 \} - \Psi_f^i \right] = \sum_{i=1}^N (1-2\alpha) \Psi_f^i \\ & + \alpha \frac{k_1}{2k_2} \{ \exp[k_2(I_1 - 3)^2] - 1 \}. \end{aligned} \quad (3)$$

Here, $I_1 = \text{tr } \mathbf{C}$ is the first isotropic invariant of the right Cauchy-Green tensor \mathbf{C} , N is the number of collagen fiber and Ψ_f is the strain energy of a single collagen fiber using Eq. (2).

The structural constitutive model developed by Sáez et al. (2016) was based on individually measured collagen orientation densities using three histological sections across the wall, which captured the biaxial and uniaxial properties of the common carotid artery. The anisotropic contribution of the collagen fibers reinforcement is defined as:

$$\Psi_{fc} = \frac{1}{4\pi} \int (\rho + \alpha \hat{\rho}) \Psi_f dA, \quad (4)$$

where ρ is the Bingham orientation density function (ODF) (Bingham, 1974), Ψ_f is the SEF of the collagen fiber using Eq. (2), $\hat{\rho} = [\max(\rho) - \rho]/\max(\rho)$ is an effective orientation density that accounts for cross-links between the main collagen fibers, and α represents the relative amount of cross-links with $\alpha = 0$ and $\alpha = 1$ indicating no cross-links and fully cross-linked collagen, respectively. Eq. (4) is

numerically integrated over the unit sphere. Since the model required coupling amongst thick collagen fibers, an effective orientation density that accounts for cross-links between the main collagen fibers was proposed. This work was limited by approximation of the arterial wall's multilayered structure by a one-layered homogeneous model without residual strains in the unloaded configuration. In addition, the use of limited number of sections to identify the collagen organization questioned potential gradual changes previously reported for the porcine aorta (Polzer et al., 2015). A 3D continuum arterial constitutive model with the ability of differentiating the mechanical consequences of changes in collagen content distinct from cross-linking was also proposed (Tian et al., 2016). An eight-chain orthotropic element model to characterize collagen mechanical behavior and an isotropic neo-Hookean model of elastin were combined. In this model, a single chain in the eight-chain element denotes a single tropocollagen molecule section between two adjacent cross-links (Fig. 9). In the model, eight identical chains make up an element, where a single chain is made of multiple subunits with fixed length denoting the repeating amino acid motif on a tropocollagen molecule. A single chain in the eight-chain model represents the section of tropocollagen molecule between neighboring cross-links. The length of each chain depends on the number of subunits, which in turn depends on the density of cross-links. The SEF of collagen fibers inspired from Bischoff et al. (2002) considering incompressibility and absence of shear deformation as a consequence of the sum of the entropy of all tropocollagen molecules and the repulsion between them has the form:

$$\Psi_f = \Psi_0 + \frac{nk\Theta}{4} N \sum_{i=1}^4 \left[\frac{\beta_p^{(i)}}{N} \beta_p^{(i)} + \ln \frac{\beta_p^{(i)}}{\sin h \beta_p^{(i)}} \right] - \frac{nk\Theta}{4} \frac{\beta_p}{\sqrt{N}} \times \ln \left(\lambda_r^{C_r^2} \lambda_\theta^{C_\theta^2} \lambda_z^{C_z^2} \right), \quad (5)$$

where Ψ_0 is a constant related to the non-zero entropy of the undeformed chains in the model, λ_r , λ_θ , and λ_z are the stretches in the radial,

circumferential, and longitudinal respectively, n is the chain density per unit volume (note that there are eight chains per unit element), N represents the number of subunits (the number of repeating amino acid motifs) per chain, k is Boltzmann's constant, Θ is the absolute temperature, $\beta_p^{(i)}$ is the normalized deformed length of the chains in the unit element where the superscript i denotes the chain number ($i = 1, \dots, 8$), β_p is the inverse Langevin function, and C_r , C_θ , and C_z are normalized dimensions along the material axes r , θ , z , respectively (Tian et al., 2016).

Here, the section of the tropocollagen molecule between two neighboring cross-links was assumed to have the same length (i.e., the same N in the model). The advantage of using Eq. (5) is the ability to investigate the separate effects of collagen content and collagen cross-linking and to differentiate the mechanical consequences of changes in collagen content distinct from cross-linking. Collagen content and cross-linking were confirmed to contribute differently to arterial mechanics as a function of pressure, depending on collagen fiber engagement. Both collagen content and cross-linking are important to understand arterial stiffening due to pulmonary hypertension (Wang and Chesler, 2012; Wang et al., 2013). It is important to note that, the eight-chain model is used to characterize rubber-like materials, which is unsuitable for fibrous tissues. Their work is significant in a way that previous studies did not uncouple collagen content from cross-linking, which is an important aspect in arterial mechanics (Tian et al., 2016).

The amount of cross-links was so far controlled by a scalar parameter included in the anisotropic energy function. To transit from a fiber-related energy function towards a cross-links related energy function, Holzapfel and Ogden (2020b) investigated the overall mechanical response of fibrous tissue accounting for cross-link stiffness, cross-link orientation and cross-link density by developing an invariant-based SEF to account for isotropy, collagen fibers (using Eq. (2)), cross-links (Eq. (6)) as well as the interaction of collagen fibers and cross-links

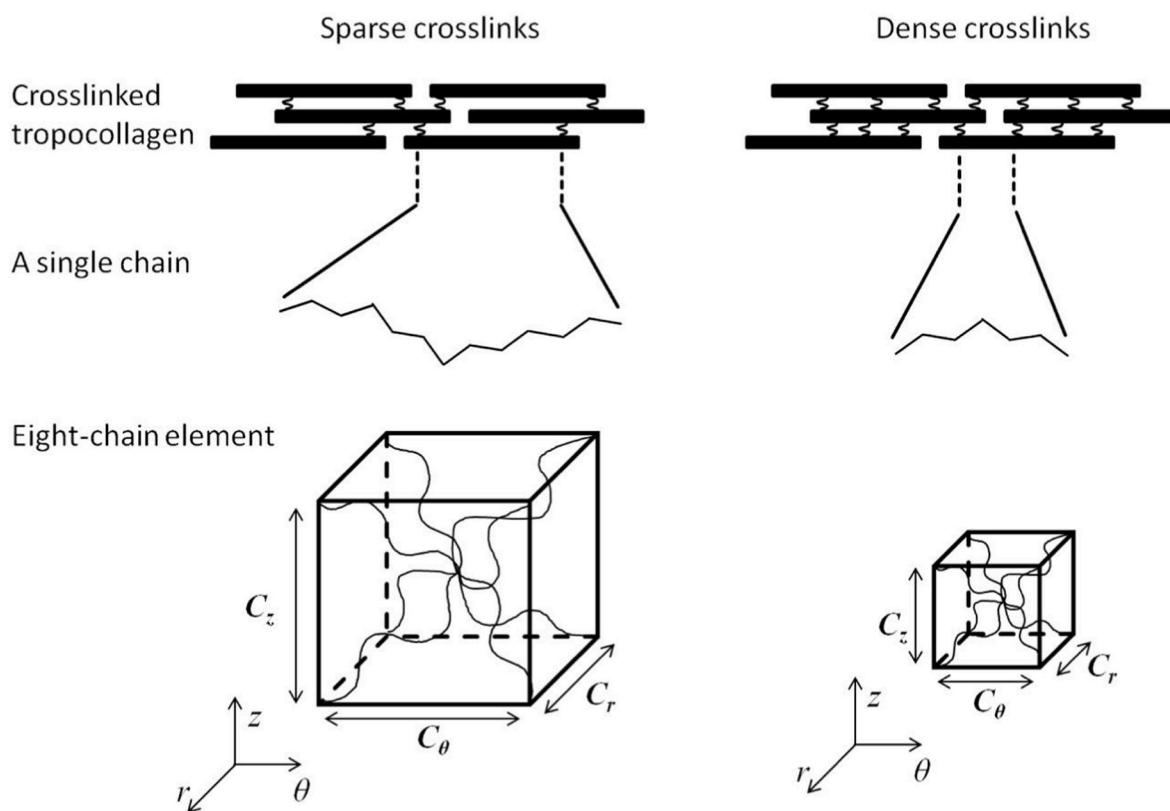


Fig. 9. Schematic of sparsely and densely cross-linked tropocollagen models. The element has normalized dimensions $C_r \times C_\theta \times C_z$ along the material axes r , θ , z respectively (adapted from Tian et al. (2016)).

(Eq. (7)). Since limited data are available on mechanical behavior of cross-links, Holzapfel and Ogden (2020b) suggested to assume quadratic reinforcing form for the energy stored in cross-links (Ψ_c) as

$$\Psi_c = \frac{1}{2}v(I - 1)^2, \quad (6)$$

where v is a positive parameter measuring the strength of the cross-links, and is referred to as the cross-link parameter with the dimension of stress, the invariant I is the square of the stretch in each of the cross-link directions, and calculated as $I = \lambda^2 \cos^2 \alpha_0 + \lambda^{-1} \sin^2 \alpha_0$, where α_0 defines orientation of families of cross-links relative to the collagen fiber direction (Fig. 10), and λ is the stretch ratio of uniaxial deformation.

Similar to Eq. (6), the interaction energy between the collagen fibers and the cross-links (Ψ_{fc}) is represented as

$$\Psi_{fc} = \frac{1}{2}\kappa(I_8^+ - \cos \alpha_0)^2 = \frac{1}{2}\kappa(I_8^- + \cos \alpha_0)^2, \quad (7)$$

where κ is a positive stress-like parameter that measures the strength of the interaction between the fibers and the cross-links, and $I_8^\pm = \pm \lambda^2 \cos \alpha_0$ (I_8^\pm is defined as the coupling between the collagen fiber and cross-link directions) (Holzapfel and Ogden, 2020b; Wang et al., 2023). A more general 3D model with an arbitrary fiber direction and with two symmetrically disposed families of cross-links was also proposed in the same study by Holzapfel and Ogden (2020b) to demonstrate how cross-link stiffness and relative orientation of the cross-links and the fibers affect the uniaxial response. To extend the model described above, Holzapfel and Ogden (2020a) formulated collagen fiber damage with intact cross-links in the framework of pseudo-elasticity (Ogden and Roxburgh, 1999).

The limitation of the above-mentioned continuum-based models is the assumption of parallel fibers that are connected by cross-links, while collagen fibers are distributed differently in fibrous tissues, for example, they are nearly parallel in tendons, but dispersed in arterial walls (Fratzl, 2008). Therefore, Teichtmeister and Holzapfel (2022) proposed a planar continuum model of collagenous tissues including the stiffness and relative orientation of the cross-links and the fiber-cross-link interaction, considering dispersed fibers connected by randomly distributed cross-links. They investigated the influence of different cross-link configurations on the stress response, in particular simple shear and the sign of the normal stress perpendicular to the shear planes, which is referred to as the Poynting effect (Janmey et al., 2007; Conti and MacKintosh, 2009; Destrade et al., 2015). The presence of cross-links caused stiffening of the tissue, and demonstrated a significant dependence of the stress response on the orientation distribution of fibers and cross-links (Teichtmeister and Holzapfel, 2022). Their investigation of the Poynting effect happening in a circular hollow cylinder under pure torsion was again emphasized the pronounced role of cross-links properties on the

collagenous soft tissue mechanics. This work may provide a profounder insight into the microstructural mechanisms of semi-flexible biopolymer gels that, in simple shear, are in charge of the tendency of the top and bottom faces to move towards each other (Teichtmeister and Holzapfel, 2022). It should be noted that the constitutive models mentioned above refer to the fibers, not the fibrils, which is the focus of the atomistic models. In the future, the constitutive models based on the insight gained by the atomistic models could more realistically represent the tissue mechanics at the fibril level.

Table 1 briefly summarizes the main features of available molecular and constitutive models of cross-link mentioned in this paper.

It is important to note that the SEF of the constitutive models mentioned here considered the cross-links at the fiber level. This emphasizes the importance of molecular level models investigating the role of collagen cross-links on the mechanical behavior at the fibril level. The insight gained from them can be incorporated to modify the fiber-level constitutive models.

8. Limitations and future work in computational modeling of cross-link in fibrous tissue

The present work reviews the models incorporating cross-links and their influence on the behavior of fibrous tissues, which is already observed by many experimental studies (see Sections 1–6). However, the limitations associated with the cross-link properties from experimental and modeling point of view have to be discussed. Any attempt to address the limitations can tremendously improve the existing constitutive models and will take a step towards future work and enabling new treatment targets.

Limitations from the experimental viewpoint: it is important to note that animal models remain popular in clinical hypothesis testing, where specifically the pig carotid artery has a central role in arterial studies (Sáez et al., 2016). Moreover, the use of animal tissue allow us to collect a large sample size for acceptable data analysis. However, apart from the ethical issues related to this, translation from animal samples to human tissue is still an open question. Since efforts in experimental and computational biomechanics are made to understand how human tissue would react to certain conditions, further research in biomechanical behavior of human collagen structure is certainly required to provide data for validation of the computational models (Holzapfel et al., 2007).

Usually the microscopic methods of detecting collagen identifies only thicker collagen fibers, but the collagenous tissue included also finer collagen fiber structures like vascular wall (O'Connell et al., 2008), which also could be mechanically relevant. Interestingly, these finer structures seem to cross-bridge thicker fibers in a mesh-like network without a clear preferred fiber orientation (Sáez et al., 2016). This highlights that currently available methods, in spite of valuable insight

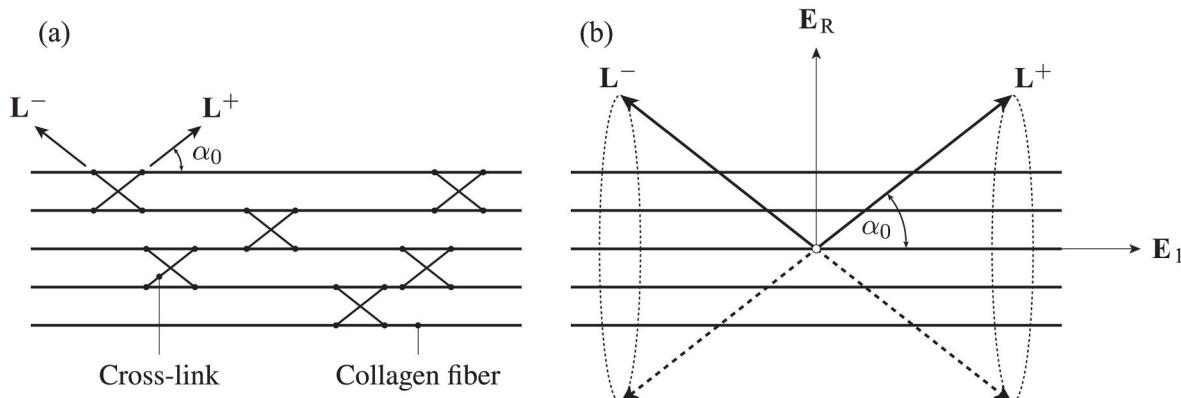


Fig. 10. a) Schematic of aligned collagen fibers in the direction E_1 with two families of interconnecting cross-links with directions L^+ and L^- making an angle α_0 with E_1 . b) Focus on a pair of cross-links indicating their rotational symmetry about E_1 with the radial vector E_R (adapted from Holzapfel and Ogden (2020b)).

Table 1

Summary of computational models available considering the cross-link properties.

Type of models	Authors	Main features
Atomistic-molecular models	Atomistic models Buehler and Wong (2007), Buehler (2008), Tang et al. (2009), Uzel and Buehler (2011)	- 2D and 3D atomistic model of cross-link considering the atomic structure of cross-links and their chemical interactions during deformation - Use of molecular dynamic simulation (MDS) - Showing the mechanism responsible for elasticity in small vs. large deformations - The effect of cross-link density on mechanical behavior - 2D and 3D networks - Definition of inter-fiber cross-linking as either pin joints or weld joints - Investigation of the cross-link density on mechanical behavior - Incorporation of DFN models into multiscale models - DFN generating method, fiber coordination, network stability - 2D and 3D WLC models - Standard bead model - Force-extension relation of an array of parallel-aligned network of WLC - Importance of consideration of cross-links' coordination - Enzymatic and AGEs cross-links
Discrete fiber network (DFN) models	Wilhelm and Frey (2003), Onck et al. (2005), Stylianopoulos and Barocas (2007), Kasza et al. (2009), Picu (2011), Nachtrab et al. (2011), Shahsavari and Picu (2012), Lake et al. (2012), Shahsavari and Picu (2013a), Shahsavari and Picu (2013b), Dirrenberger et al. (2014), Abhilash et al. (2014; Thunes et al.), Žagar et al. (2015), Thunes et al. (2016), Mauri et al. (2016), Bircher et al. (2017), Korenczuk et al. (2019), Yu et al. (2020), Yu and Zhang (2022)	
Worm-like chain (WLC) models	Kroy and Glaser (2007), Kroy (2008), Kurniawan et al. (2012), Benetatos et al. (2012, 2014), Depalle et al. (2015), Shabbir and Hartmann (2017), Depalle et al. (2018), Shabbir et al. (2019), Kamml et al. (2023)	
Continuum-based constitutive models	Constitutive models Nerurkar et al. (2011), Sáez et al. (2014, 2016), Wang et al. (2016), Linka et al. (2016), Tian et al. (2016) Avazmohammadi et al. (2017), Holzapfel and Ogden (2020b), Teichtmeister and Holzapfel (2022), Yu and Zhang (2022) Wang et al. (2023)	Definition of strain energy functions (SEFs) to consider different properties of collagen cross-linking such as • cross-link presence • cross-link density • cross-link stiffness • fiber orientation • considering the effect of fiber and cross-link dispersion on the tissue mechanics

provided, cannot still fully capture the microstructure of collagen cross-links. More advanced technologies that provide a clearer picture of the detailed microstructure might be needed in order to be capable of identifying both coarse and fine collagen fibers and their interaction and the role they play in the mechanical behavior of the tissues.

In arterial tissues, measurement of circumferential and longitudinal directions of collagen fiber orientation and different properties of collagen in these two directions will enable better quantification of anisotropy and will affect the model parameters. Collagen fiber distribution and alignment is tissue-as well as layer-specific in both healthy and diseased states. Therefore, more investigations are required to distinguish contribution of collagen structure in different layers to the mechanical behavior since it might be inappropriate to treat all layers of arterial wall tissue similarly (Holzapfel et al., 2007).

Due to the small accessibility to tissues, obtaining both collagen content and cross-linking from the same tissue is difficult (Tian et al., 2016). Thus, normalizing collagen content and cross-linking by wet weight in the model is not possible. The model that captured the probability of collagen engagement (Zulliger et al. 2004a, 2004b; Hill et al., 2012), fraction of collagen fibers cross-linked, and the separate effects of the collagen subtypes and cross-link types could better describe the mechanical properties.

Limitations from the modeling viewpoint: Computational modeling of collagenous tissues is aimed to quantify and predict phenomena that would otherwise be hard or impossible to evaluate using available experimental methods. While existing models provide insight into mechanical quantities such as strain and stress, the leading edge of the field has yet to connect these data to the known architectures of matrix molecules like collagen subtypes, proteoglycans, and glycoproteins and to regional variations in water content.

Most of the currently available atomistic models have only considered enzymatic cross-links. However, there are other types of cross-links in collagen fibrils, specifically non-enzymatic cross-links known as AGE cross-links, which are also believed to be involved in the alteration of the mechanical properties of collagenous tissues. The very recent work of Kamml et al. (2023) modeled only one specific type of AGEs cross-links

(glucosepane), but other AGEs cross-links exist that need to be considered. The only type of AGE cross-link that would likely affect the mechanical behavior of the fibrils is glucosepane, an arginine-lysine glycated cross-link whose concentration, in aging or diabetic tissue, could reach values sufficiently high not to be neglected (Gautieri et al., 2014). Little is known about the formation pathways and exact locations of these cross-links and that is why Kamml et al. (2023) considered a random AGEs distribution along the tropocollagen molecule to approximate the amino-acids-based process. Additionally, depending on the glycation level and exposure (Verzijl et al., 2000), the AGEs density might change across the cross-section of the fibril and affect the mechanical properties. Types of AGEs might also vary depending on the tissue type (Eyre et al., 2008), which also needs to be taken into account for more realistic modeling.

Variations of the water content is an important issue because the hydration can have high effects on the mechanical behavior of a collagen assembly at multiple levels (Grant et al., 2008). The behavior of a single pair of the collagen molecules equilibrated in a large explicit water box was studied (Uzel and Buehler, 2011), however, the hydration state was not changed. Therefore, the consideration of water content will help to have a better description of the mechanical behavior of collagen as well as the tissue. In addition, instead of modeling a single pair of molecules which have been mostly carried out, considering many cross-linked fibrils (with more than one cross-link in a single pair of molecule) can realistically describe their interactions because telopeptides can contain up to two covalent cross-links (Reiser et al., 1992).

The exact formation of inner- and inter-molecular cross-links in collagen fibrils is important to investigate. Cross-links can form at both N- and C-termini (Reiser et al., 1992), therefore, it would be valuable to model a fully atomistic 3D collagen fibril containing both N- and C-terminal cross-links. Since very little is known from experiments on the exact geometrical organization of the N-telopeptide, mostly the modeling focuses on C-termini e.g. (Uzel and Buehler, 2011), therefore, this is left to the future work.

Presently, the general molecular or constitutive models developed for cross-linked collagen are used for all collagenous tissues, however,

the distribution of collagen fibers is different in fibrous tissues and this has rarely been considered in the literature (Teichtmeister and Holzapfel, 2022). In addition, the cross-linking of collagen in tissues are different due to the differences in the hydroxylation of lysine at both telopeptide and helical cross-linking sites (Yamauchi and Sricholpech, 2012), which suggests that the different types of collagen cross-links are tissue specific rather than species specific (Gerriets et al., 1993). Therefore, further research is required to deeply address the effect of different fiber distribution and cross-link dispersion in different collagenous tissues on the mechanical response.

Collagen Type I has been mostly the focus of modeling but it is not the only collagen responsible for the structural properties. The role that the local variations in collagen types and molecular cross-linkers play in the stiffness and damage resistance of tissue is still an open issue. Therefore, taking other types of collagen into consideration in experiments and computational models is an important step in improving the understanding of the role of collagen type's structure and composition in mechanics of collagenous tissues.

9. Concluding remarks

This review paper provides a brief introduction to biophysics of enzymatic and non-enzymatic collagen cross-links as the building blocks of collagen in the extra cellular matrix of fibrous tissues. The importance of this topic is highlighted by providing an overview of experimental and modelling efforts capturing the presence of cross-links in maintaining the function of different collagenous tissues such as ligament and tendon, cartilage, cervix, collagen gel and arterial tissue. For each of these tissues, the paper provides data on the contribution of the cross-linking to the biological structure and to their healthy function. It also describes the most important experiments and computational techniques together with the crucial results and their clinical and pathological relevance.

The effects of cross-linking manifests over different length scales, which motivated the development of different numerical methods for their simulation. The fully atomistic models capture the effects at the nanoscale, whereas discrete fiber network models deal with the fiber level kinematics and worm-like chains models consider polymer chains as an array of single beads where different potentials regulate stretching, bending, cross-link and entanglement. Finally, constitutive models categorized as the phenomenological and structural simulate fibrous tissues following the principles of the continuum theory.

The investigation of cross-linking opens many perspectives toward the timely diagnosis and successful treatment of different diseases. As an example, molecular slippage, which is likely required for the discrete plasticity failure mechanism in tendon is correlated to the cross-link density. Cross-linking plays an important role in enhancing cartilage integration and articular cartilage repair. Furthermore, the abnormal cervical remodeling remains a significant clinical dilemma in obstetrics that might be explained by cross-linking effect. The cross-linking might also be the key for the EMC microstructure variation typical of some diseases as well as for the collagen cross-link disruption in arteries. Moreover, it is supposed to be the major reason of vein graft failure after coronary artery bypass grafting and has a crucial role in the mechanobiology and modeling of aneurysms. Not only diagnosis and treatment of diseases but also the tissue engineering profits from cross-linking effects. Here, the collagen Type I is a major component of collagen gels for different applications that performs remarkably with regard to the cell-scaffold interactions.

This review intends to demonstrate the substantial efforts, which have been made to take the properties of cross-links into consideration. However, it is true that these models still hold some limitations as mentioned in section 8, which need to be addressed. Considering the previous argumentation, it can be expected that the further investigation of cross-linking effects will have a significant contribution in diagnosis and treatment of diseases as well as in the tissue engineering. Moreover,

it opens possibilities to the most modern personalized treatments. The interplay of the experimental and computational methods represents an indispensable tool to achieve these goals. More advanced methods and models will certainly help to apply the lessons learned from structure-function of the fibrous tissue to the design of new engineering materials or methods.

Statements and declarations

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CRediT authorship contribution statement

Melika Mohammadkhah: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Sandra Klinge:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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