ADVANCED-GLYCATION-ENDPRODUCTS CROSS-LINKING IN COLLAGEN FIBRILS – A NANOSCALE STUDY ON TISSUE

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Dedicated to those who are always around.

1989 – ...

...and to infinite jest...
ABSTRACT

Advanced Glycation End-products (AGEs) accumulate in the body of diabetic patients due to high glucose levels. Increased contents of AGEs have been found to correlate with impaired mechanical behavior of tissue. However, the exact mechanisms of how AGEs accumulation affects tissue mechanics remains largely unknown. Specifically, the cross-linking types of AGEs might be responsible for changing tissue mechanics since they are located between the tropocollagen molecules within the collagen fibril. Collagen fibrils are the main building constituent of tissue and their mechanical behavior determines tissue mechanics. By changing fibrillar mechanics in various ways, cross-links are considered to be a key component at the nano-scale of tissue. The underlying mechanisms causing alterations in collagen fibril mechanical behavior and how this is linked to tissue malfunction are still not well understood.

This thesis aims to examine the effect of various parameters, e.g. cross-link density and type, that are suspected to influence collagen fibril mechanics and to provide a fundamental understanding of cross-linking on the collagen fibril scale of tissue. We use coarse-grained molecular dynamics models to perform destructive tensile tests on collagen fibrils to investigate the influence of cross-linking on fibril mechanics in various configurations. We evaluate the influence of enzymatic and AGEs cross-linking on the deformation and failure mechanisms of the fibril. In an additional step, we insert mineral particles into the respective locations of the collagen fibrils, accounting for nano-scale behavior of hard tissue. While enzymatic cross-linking does not substantially contribute to changes in deformation behavior, AGEs cause fibril stiffening and a change in the deformation behavior: At large AGEs contents, the force is rather transferred through AGEs to the tropocollagen molecules than through sliding between these molecules. This finally leads to an abrupt failure of the bonds within the tropocollagen molecules and provides the causal link between increased AGEs content and inhibited intra-fibrillar sliding, increased stiffness, and abrupt fibril fracture.

Having revealed this general relation, we focus on different types of AGEs, characterized by their mechanical properties i.e. stiffness and fracture length, and how their different quantities influence the fibril’s mechanical behavior. We demonstrate that AGEs loading energy capacity and density are the unique factors for fibril mechanical behavior assessment. Density and loading energy capacity determine the failure mechanism – whether energy is rather dissipated through friction by sliding between the tropocollagen molecules or absorbed by stretching of the tropocollagen molecules, causing stiffening and finally brittle failure of the fibril.
Finally, we investigate the influence of AGEs cross-linking at different mineral content levels. We show that AGEs only influence the mechanical behavior of the mineralized collagen fibril at low mineral contents. When the mineral is dominating the mechanical response at high mineral contents, AGEs cross-links do not influence the deformation or failure behavior. It is generally agreed upon that the mineral phase in the collagen fibril of bone increases the strength of collagen, but how this interferes with AGEs cross-linking had not been investigated.

The findings of this research work provide a fundamental understanding of the mechanisms responsible for altered tissue behavior on the collagen fibril level caused by cross-linking, specifically AGEs cross-linking.

tem Gleiten der Tropokollagene, erhöhter Steifigkeit und schlagartigem Bruch der Fibrille.

Nachdem wir die grundlegende Wirkung von Cross-links beleuchtet haben, liegt der Fokus anschließend auf den unterschiedlichen Arten von AGEs-Cross-links und wie sich deren Konzentration auf das mechanische Verhalten der Fibrille auswirkt. Verschiedene AGEs-Typen werden in unserer Studie charakterisiert durch ihre mechanischen Eigenschaften, in diesem Fall Steifigkeit und Bruchlänge. Wir zeigen, dass neben der Anzahl der AGEs die sogenannte "Loading Energy Capacity" (Belastbarkeit) der entscheidende Faktor ist, um die Veränderungen im Verformungsmechanismus zu abzuschätzen. Sowohl die AGEs-Konzentration, als auch die Belastbarekeit bestimmen den Versagensmechanismus - ob Energie durch das Aneinandergleiten der Tropokollagene abgeleitet oder von den Tropokollagenen absorbiert wird, was zunächst zu einer Versteifung der Fibrille und schliesslich zu einem spröden Bruchverhalten führt.


Try not to become a (wo)man of success but rather try to become a (wo)man of value.
— AS EINSTEIN DIDN’T SAY

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Table A.1 Parameters used in coarse-grained molecular dynamics mesoscale model of collagen fibrils with varying AGEs types and densities.

ACRONYMS

AGEs Advanced Glycation End-products
MD Molecular Dynamics
CGMD Coarse-grained Molecular Dynamics
AFM Atomic-Force-Microscopy
SAXS Synchrotron Small-Angle X-ray Scattering
T2DM Type 2 Diabetes Mellitus
MEMS Micro-Electromechanical System
BMD Bone Mineral Density
TC Tropocollagen
BS Bruck syndrome
HAP Hydroxyapatite
ECLs Enzymatic cross-links

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Part I

INTRODUCTION & OVERVIEW

In this first part of the thesis, the reader can get an overview of the research topic within the extensive field of collagen tissue research. The question of why we specifically focus on collagen and its cross-links at the nano-scale level of tissue is answered. And why simulations can help to understand mechanisms behind changes and impairment of mechanical properties where common experimental techniques are still not able to give a complete answer.
Aging and the unhealthy lifestyle of poor exercise in combination with immoderate food intake have triggered the emergence of numerous diseases in many societies. Type 2 Diabetes Mellitus (T2DM) is one of them and has become a major concern in healthcare systems around the globe. The prevalence of diabetic patients was 425 million people worldwide in 2017 and is expected to rise to 629 million by 2045 due to the increase in unhealthy behavior leading to obesity within the population [56]. In Switzerland, currently about six percent of the population suffer from T2DM [186]. T2DM is the non-genetic form of diabetes, in which the blood glucose level is not regulated anymore due to reduced absorption of insulin caused by resistance of cells. The increased glucose level in the body leads to many health deteriorating effects. Amongst the most well-known are cardiovascular diseases like heart attack and stroke, diabetic foot, kidney damage, and blindness, but also other body functions are affected.

Numerous negative consequences associated with T2DM are related to impaired behavior of collagenous tissue within the body. For example, structural and functional abnormalities in the Achilles tendon of diabetic individuals are assumed to cause plantar forefoot ulcers by increased stiffness and changing of foot mechanics [1, 20, 76]. In bone, the Bone Mineral Density (BMD) is generally used as the standard measure for estimating tissue quality and fracture risk in terms of osteoporosis and osteopenia, but paradoxically, it is not applicable for T2DM patients [120]: studies have shown that, despite an increased BMD in T2DM patients, the fracture risk is also increased, while in non-diabetic bone a decrease in BMD is the decisive criteria for diagnosing osteoporosis [24, 41, 194]. This leads to the conclusion that the bone quality of diabetic patients is impaired, but mechanisms responsible for this reduced material performance could not be fully revealed to date. Potential causes include hyperinsulinemia, reduced serum levels of insulin growth factor 1 (IGF-1), hypercalciuria, renal failure, microangiopathy, inflammation and deposition of AGEs in collagen [91, 92, 120, 165, 173]. The latter are found in different forms in collagenous tissue at the nano-scale level, where they are suspected to influence the mechanical behavior and deformation mechanism of the collagen fibril, the main building constituent of tissue. While it has been demonstrated that an increased content of AGEs in various tissues correlates with impaired or altered
tissue behavior and negative effects, e.g., bone brittleness [5, 61, 107, 139, 162, 176, 190, 211, 213], the underlying mechanisms remain unclear.

Bone fracture is one of the major factors responsible for morbidity, mortality, and reduction in quality of life in aging societies [93, 94, 185] and therefore a burden for public healthcare systems concerning the impact in costs and care utilization [26, 36, 62, 146]. In combination with the worldwide exploding prevalence of diabetes due to obesity, lack of exercise, and aging, not only the Western healthcare systems will face severe challenges dealing with its negative effects. Therefore, targeted provision, medication, and treatment for T2DM patients is of great importance. Revealing the mechanisms causing the deterioration in tissue behavior is a key ingredient for establishing appropriate measures for adjusting treatment for increasing bone strength of T2DM patients.
SCOPE AND STRUCTURE OF THIS RESEARCH PROJECT

2.1 MOTIVATION AND PROBLEM STATEMENT

Collagenous tissues in general and bone in particular comprise a hierarchical structure, where changes in material composition and properties at each scale can influence the macroscopic mechanical behavior. AGEs cross-links accumulate in the collagen fibril at the nano level due to aging and increased glycation levels in the body, and are suspected to change the mechanical properties of the fibrils. Since the collagen fibril is the basic building unit of tissue, changes in its mechanics are believed to result in altered tissue behavior on the macro scale. However, it is difficult or impossible to obtain insights to these smallest scale levels in the laboratory, and very little is known about AGEs parameters i.e. types, properties, location, numbers and their influence on tissue mechanical behavior. This is where *in-silico* modeling provides researchers with a tool to overcome experimental limitations as a more viable way of accessing the nano scale. We built numerical models of the collagen fibril to explore various aspects of fibril mechanics, energy dissipation, and deformation, and to answer the fundamental question of how so far not quantified parameters influence collagen fibril on the small scale and tissue on the larger scale.

The aim of this thesis is to provide a fundamental understanding of collagen fibril mechanics in the presence of AGEs cross-links. This study is focusing on the mechanics of the collagen fibril – the main building constituent of bone and other tissues – to reveal the causes of altered collagen fibril behavior correlating with increased AGEs content [5]. The work provides a profound understanding of how changes in AGEs numbers and type influence collagen fibril mechanics and uncovers the origins of altered tissue mechanical behavior like increased bone brittleness.

The first objective is to explore the influence of cross-linking and the density of cross-linking on the mechanical behavior of the collagen fibril in general. Apart from non-enzymatic AGEs cross-links, enzymatic cross-links are naturally present in collagen fibrils. We address not only the number of AGEs, but also the additional contribution of enzymatic cross-links on the force transmission within the collagen fibril and their influence on energy dissipation and failure mechanism.

After investigating the influence of general cross-linking, including enzymatic and non-enzymatic cross-links, and the number of cross-links in particular, we focus on the type and variety of AGEs in the next step: AGEs appear in many different
chemical configurations and apart from structure, different mechanical properties are one of the main attributes determining their type. By varying mechanical parameters of AGEs cross-links, we account for different types and reveal the key mechanical property for the classification of AGEs influence on collagen fibril mechanics.

Up to this point, we concentrated on collagen fibrils in soft tissue, which only consist of collagen molecules. The collagen fibrils in bone, classified as hard tissue, have one specific characteristic: unlike collagen fibrils in soft tissue, they are mineralized, meaning that the growth of so-called hydroxapatite crystals is nucleated within the collagen fibril between collagen molecules. Therefore, in our third study, we investigate the influence of AGEs cross-linking on the mineralized collagen fibril i.e. the collagen fibril in bone. We aim to reveal the impact of cross-linking on the nano-scale structure to establish the link between increased AGEs content and bone brittleness due to changes in mechanical behavior and energy dissipation mechanism within the mineralized collagen fibril.

2.2 STRUCTURE OF THE THESIS

This thesis is structured in a cumulative format and organized in several parts and chapters, which can be summarized as follows:

PART I provides introductory information on the research project for a more profound understanding of the topic.

CHAPTER 1 gives a short general introduction to the background of the research topic and its societal impact.

CHAPTER 2 contains the motivation and problem statement, explaining the contribution of this research work. Further, it provides a short overview of the structure of the thesis.

CHAPTER 3 provides a comprehensive introduction to collagen and its structural composition and to bone as an example of collageneous tissue.

CHAPTER 4 contains the state of the art with an extensive literature review covering the main findings, in both, experimental research and computational modelling, in the field of collagen fibril mechanics and influence of AGEs on nano-scale mechanics of tissue.

CHAPTER 5 describes the numerical techniques utilized to carry out the simulations, addressing the objectives outlined in Sec. 2.1. As a result, a description of the
fundamental principles behind Molecular Dynamics Modelling and coarse-graining is given.

PART II presents the results and outcomes of the research project by providing the publications.

CHAPTER 6 provides a study on the influence of cross-linking (enzymatic and non-enzymatic) on the mechanical behavior of the collagen fibril. The effects of both cross-link types on the deformation and failure behavior are investigated and we show whether and how different cross-link types change the deformation mechanism within the collagen fibril. The results presented in this chapter provide a direct link between increased AGEs content, inhibited intrafibrilar molecular sliding, increased stiffness, and abrupt fibril fracture.

CHAPTER 7 contains a study on the effect of different AGEs types, i.e. cross-link mechanical parameters, and AGEs densities on mechanical properties and fracture behavior of the collagen fibril. We investigate how these parameters alter fibril deformation mechanisms and reveal that the AGEs loading energy capacity is, aside from their density in the fibril, the unique factor determining the effect of different types of AGEs on the mechanical behavior of collagen fibrils.

CHAPTER 8 concentrates on the mineralized collagen fibril in hard tissue like bone: The influence of mineral content and AGEs density on the fibril’s deformation and fracture behavior are investigated. We reveal the influence of these two factors on altered fibril mechanics and why this is important in bone and diabetes research.

PART III provides a summary of the findings of this work and an outlook concerning future research topics in the field.

CHAPTER 9 offers a comprehensive summary of the main findings, outcomes, and contributions arising from the current study. Furthermore, this chapter points out the main conclusions that contribute to our understanding of collagen fibril mechanics and the influence of AGEs cross-linking on fibril mechanical behavior, energy dissipation and failure mechanisms. Finally, it focuses on critically evaluating the conducted research and assessing the key findings, contextualizing their significance.

CHAPTER 10 provides a broader perspective on the influence of cross-linking in the field of tissue mechanics and gives recommendations for future research.
APPENDIX includes further materials and results that contribute to a comprehensive understanding of the research approach and methodology.
Collagen type I is the main building component of mammalian tissue and responsible for stress carriage, energy storage, and force transmission [58, 86]. It provides bone with its fracture resistance and can transmit forces from muscles to bones in tendons and ligaments [195, 217]. In bone, it comprises about 95% of the collagen content and 80% of the total proteins [129, 195]. Collagen type I is the most prominent type of the over 50 different collagens found in human tissue [95, 150]. Its basic building unit are three left-handed polypeptide helices, two genetically identical $\alpha_1$ chains, and one distinct $\alpha_2$ chain, forming a right-handed triple helical structure (see Fig. 3.1c&d) [54]. The triple helix is called Tropocollagen (TC) molecule, obtaining its structure and cohesion due to the specific amino acid sequence Gly-X-Y the helices are built of (see Fig. 3.1d) [86]. The residues of glycine are directed towards the inside of the helix, forming the helix-core due to their small size and are stabilizing the TC structure. The residues of any amino acid X or Y are exposed to the outside of the helix and can interact with the environment. The TC molecules comprise a central helical region with a continuous repetition of the (Gly-X-Y)-sequence, flanked by comparatively small non-helical ends at C- and N-termini, called telopeptide ends with a size of about 20 amino acid residues (see Fig. 3.1b). A TC molecule is approximately 300 nm in length and 1.5 nm in thickness [59, 169].

In collagen type I, which is part of the fibrillar collagen family, the TC molecules are arranged in collagen fibrils, which display a specific repeating banding pattern [87]. This pattern is derived from the longitudinal arrangement of molecules within the fibril, where the TC molecules are staggered by 5 multiples of the so-called D-period of 64-67 nm, leading to gap- and overlap zones within the collagen fibril (Hodge-Petruska-Model, see Fig. 3.1a) [47, 137, 205]. While the arrangement in the longitudinal direction with the banding pattern is well understood, the organization in the cross-sectional area, meaning the lateral direction, has not been revealed so far. The TC molecules are packed in an axial structure which appears to be variable and might be dependent on function and tissue. The thickness of collagen fibrils varies between 50 and a few hundred nano-meters [86]. The diameter and length of the collagen fibril tend to be different depending on anatomical location [205].

The formation of collagen – also called fibrillogenesis – is a complex process consisting of numerous intra- and extracellular steps that influence the final struc-
ture and mechanical properties of the fibril. First, so-called procollagen molecules, soluble precursors of TC molecules, are created within the cell. After the transfer to the extracellular matrix, collagen fibrils are assembled by enzymes and finally stabilized using enzymatic covalent cross-linking at the telopeptide ends (see Fig. 3.1a) [86].

Fibrillar collagen is remarkably versatile in function and part of many different types of tissue, where mechanical behavior is tuned via the arrangement of collagen fibrils within the (hierarchical) structure of the tissue. The mechanical properties of the collagen fibril are derived from the structural organization of the TC-molecules and fine-tuning of structural properties, such as fibril size regulation, dispersion of crystallinity, and interfibrillar connectivity. Further, the mechanical behavior of collagen fibrils is highly prone to be influenced by one specific factor: collagen cross-linking [205].

3.2 COLLAGEN CROSS-LINKING

After the assembly of the collagen fibril into its specific staggering pattern, post-translational processes stabilize the fibrillar structure mechanically: cross-links are formed between and within the TC molecules (inter- and intramolecular). They are known to control strength and stiffness of the collagen fibril and their density is influenced by age, tissue turnover, and disease [176, 210]. Further, changes in the prevalence of cross-linking have been shown to alter mechanical properties, but the exact mechanisms underlying these behaviors are not fully understood. Generally, two groups of collagen cross-links are distinguished, depending on their way of synthesis: enzymatic and non-enzymatic cross-links.

3.2.1 Enzymatic cross-links

The stabilization process of the collagen fibril as the final step of fibrillogenesis is mainly initiated by enzymes of the lysil oxidase family [110, 116]. Therefore, these stabilizing cross-links are also called enzymatic cross-links. They are built at the telopeptide ends of the TC molecules (see Fig. 3.1a), where lysine or hydroxylysine residues in the N- and C-terminal telopeptides are converted to peptidyl aldehydes, which spontaneously condense and form various forms of intra- and intermolecular cross-links. After the formation of immature divalent cross-links, they react to mature trivalent cross-links. Apart from the respective enzymes, cross-linking depends on the presence of specific amino acid sequences and the quarternary structural arrangement in the fibril [86].

Strength and toughness of collagen result from the presence of enzymatic cross-links. Various diseases cause impaired tissue behavior as a result of abnormal or absent enzymatic cross-linking, for example in the bone of patients suffering from
Figure 3.1: Schematic overview of the structural organization of collagen type I from fibril to polypeptide structure: (a) Hodge-Petruska-Model: Collagen fibril is built in a staggered manner and comprises 5 gap and overlap zones per D-period. (b) TC molecule with a length of 300 nm and thickness of 1.5 nm: the main helical region in the central region and the small telopeptide ends. (c) TC molecules are formed of a right-handed triple helix built from three left-handed helices. (d) Collagen-specific repeating amino acid sequence in the helical region: Gly-X-Y responsible for packing and structure of the triple helix. (e) Cross-section of TC molecules: Triple helix consists of two genetically identical α1 chains and one distinct α2 chain.
the Bruck syndrome (BS) [71, 72]. While in healthy bone, enzymatic cross-linking contributes to bone strength, resistance to microdamage, and crack propagation, the brittleness of BS bone is increased due to unstable cross-linking (lack of trivalent cross-linking) caused by the mutation of genetic sequences encoding the lysyl hydroxylase enzymes responsible for cross-link formation. Apart from genetic diseases disturbing or inhibiting enzymatic cross-link formation and changing mechanical properties of the tissues’ major building component, any distortion of the collagen/cross-link relation seems to influence the mechanical behavior of tissue [5, 42, 170]. Therefore, also increased non-enzymatic cross-link formation is in focus of current research.

3.2.2 Advanced Glycation End-products (AGEs) – the non-enzymatic cross-links

Advanced Glycation End-products (AGEs), which are the focus of this thesis, are formed during non-enzymatic reaction in the helical regions of the TC molecules (see Fig. 3.1a). Their name is derived from their way of synthesis: glycation, a process that starts with the Maillard reaction and is generally followed by several oxidation reactions. Lysine residues of the TC molecules and either glucose or ribose form AGEs [118, 148, 156]. The rate of AGEs accumulation is dependent on tissue turnover and system glycose levels [13, 14, 21, 192]. Due to increased sugar presence in hyperglycemic systems, collagen in the tissue of diabetic patients is prone to accumulation of AGEs. It is commonly assumed that collagen tissue of elderly with a longer half-life and that of diabetic patients is specifically affected by AGEs accumulation since increased AGEs content has been shown to correlate with tissue dysfunction [147]. Apart from impaired microstructure and tissue behavior in collageneous tissues like tendon [106, 107] and the cornea [162], bone brittleness and fracture are associated with increased content of AGEs [25, 206]. Further, at the collagen fibril level, changes in the deformation behavior have been observed [5, 8, 52, 70, 177]. However, the mechanisms behind these alterations remain poorly understood.

Two groups of AGEs can be distinguished, characterized by their function: AGEs can be cross-linking (acting as an intermolecular connection between two TC molecules) or non-cross-linking (bonding to a single TC molecule). Collagen properties are affected by both but in different ways. Non-cross-linking AGEs are presumed to cause changes in protein function and tissue metabolism while cross-linking AGEs are suspected to be responsible for the changes in the mechanical behavior at the collagen fibril level, which may cause alterations at tissue level. This mechanism is of great interest with respect to increase of bone brittleness [5, 12, 83, 135, 171, 216]. Different structural configurations of cross-linking AGEs result in different types with different mechanical properties. Among the molecules that have been identified so far are glucosepane, pentosidine, GOLD (glyoxal-lysine dimer), MOLD
(methylglyoxal-lysine dimer), crossline and vesperlysine [125, 128, 157, 159, 177, 196, 207]. The occurrence of different AGEs is dependent on tissue type and location, but their quantification is still missing. The most prominent AGE cross-link found in human tissue is glucosepane and it is also the most investigated one. It is to current knowledge the most abundant one, reaching levels of 2000 pmol mol$^{-1}$ in collagen tissue of 90 years old patients and of 4500 pmol mol$^{-1}$ in diabetic patients, meaning there is one AGE every 5 molecules in aged collagen and one AGE every 2 molecules in diabetic collagen, which is about 1000 times higher than the contents of other cross-link types [166]. The location of different types of AGEs is also unclear. So far, only computational studies could give insights and provide potential binding sites of AGEs within the collagen fibril, based on the relative distance of lysine arginine residues [37, 64].

Complete information on cross-link quantity, type, and location in specific tissues is still missing. Hence, it is not possible to link cross-link properties and density to the mechanical behavior of the collagen fibril on the nano-level and further to altering tissue mechanics. Establishing this connection would be necessary for the quantitative prediction of acceptable AGEs content per type for healthy collagen fibril and tissue mechanics.

### 3.3 Bone – A Highly Specialized Load Carrier

Human tissue has complex functions and its reaction to loading conditions is highly specified. This specification is achieved by a sophisticated build-up on different scale levels (see Fig. 3.2), with mineralized collagen fibrils as the main building component. Bone is responsible for the principal support of the body and is one of the most complex tissues. As a metabolically active organ, it is constantly remodeled by bone cells at the nano-scale [85]. In three distinctive phases, the bone matrix is first resorbed by osteoclasts, then mononuclear cells appear on the bone surface and finally, osteoblast replace removed bone [78]. During this process, material architecture is continuously adapted to meet changing loading conditions and mechanical needs. The duration of turn-over is dependent on bone type, location, and age of the individual.

Bone is a hard tissue, meaning that apart from the organic matrix (95% collagen type I arranged in collagen fibrils and 5% proteoglycans and other non-collagenous proteins), it also consists of inorganic mineral, often referred to as Hydroxyapatite (HAP) crystals [187]. Compared to the "ideal" form of HAP, bone mineral is less regular and exhibits inclusions and modifications in its mineral structure [22, 123]. The complex process of collagen mineralization has not been revealed so far and the form and location of the mineral are still not well understood. HAP was believed to be located in the gap regions of the collagen fibril, but mineral volume measurements have shown that the space in these regions is insufficient.
Gap zones only comprise 12 volume % of the fibrils, while minerals constitute about 45 volume % of bone, meaning that about 73 volume % of the mineral must be located outside the gap zones [112]. Therefore, mineral is suspected to either extend interfibrillar into the overlap region or into the extrafibrillar space, but opinions are varying in this context. While some studies claim that mineral platelets are located in the extrafibrillar space surrounding the collagen fibril [105, 112, 175, 184], others state it extends to the overlap regions of the fibril [11, 130, 172]. The implication of the respective arrangement on the mechanical behavior of the mineralized collagen fibrils on the nano-scale and further on tissue at the macro-scale has not been evaluated to this point. Still, several computational studies have been conducted at the collagen fibril level, showing that mineral is responsible for limiting TC molecule sliding at large deformations, increasing ultimate tensile strength via a load transfer to the collagen [43, 170, 181].

The mineralized collagen fibril as a universal building component is the lowest level in bone hierarchy (see Fig. 3.2h&g). Collagen fibril bundles form collagen fibers (see Fig. 3.2g), which are then arranged into lamella structures (see Fig. 3.2f). These lamellae are either organized in a plywood structure around a vessel or cavity to form osteons or build bone substance themselves, depending on location [113]: Two types of bone are distinguished with respect to morphology and location – trabecular (or cancellous) bone and cortical bone (see Fig. 3.2b,c,d,e). Their structural properties are different on the respective scales, following their adaption to functional needs and loading [187].

Cortical bone (see Fig. 3.2d,e) is known as the load-bearing shell, providing bone with strength and protection. It comprises approximately 80% of the skeletal tissue mass, with a high matrix mass per unit volume and only about 10% porosity [202]. Trabecular bone (see Fig. 3.2c) is located in the epiphyses of long bones, where it is responsible for load transmission to the cortical shell. Only in vertebral bodies it serves as the load-bearing bone tissue. Trabeculae form a loosely organized porous scaffold. Their organization and structure are optimized for load transfer by a dynamic feedback loop where bone cells act as load sensors reacting to mechanical stimuli such as bending and compression and subsequently trigger bone remodeling. According to Wolff’s law, bone substance is arranged in a way that trabecular bone structure guarantees the best static support, i.e., building trabeculae where increased stresses require support [19, 133, 152] (see Fig. 3.2b).

The mechanical properties of bone vary widely across bone type, anatomic location, aging, and disease and are highly dependent on the mechanical properties of its building components and their structural arrangements. Due to the fact that the structural set-up has not yet been fully revealed and distinct knowledge about composition and bone architecture is still missing further research has to be conducted. Especially at the small scales, computational modeling provides a valuable tool for estimating the influence of various parameters.
Figure 3.2: Overview of the structural built-up of trabecular and cortical bone: (a) Macrostructure: Whole bone level (b) Cross-section of bone with (c) Trabecular Bone responsible for force transmission and (d) Cortical Bone as the main load carrier building the shell. The major building unit of cortical bone are osteons (e), which are formed from lamella structures surrounding a cavity containing blood vessels. From the micro-scale level, trabecular and cortical bone show the same structural built-up: (f) Lamellae consisting of (g) collagen fibers, which are bundles of collagen fibrils. The mineralized collagen fibril (h) is the basic building unit of bone, displaying its specific banding pattern of TC molecules (i) with gap and overlap zones, where mineral is located mostly in the gap regions.
Tissue research in general is a very broad field and due to the variety of tissue and the differences in hierarchical scales, there are still many open questions to answer. Apart from composition, the mechanical and metabolic behavior of normal healthy tissue, diseased tissue and the mechanisms behind tissue malfunction are of great interest. In this research project, the focus is put on the nano-scale of diabetic tissue in general and that of diabetic bone specifically, namely on collagen fibrils and their mineralized relatives, showing an increased level of AGEs cross-links. In the following, we give an overview of the current status of research in collagen fibril mechanics, specifically focusing on the influence of cross-links. The mechanics of collagen fibrils are influenced by various parameters, which are also tuning their functions dependent on their physical position and adapted in the respective tissue. However, the exact mechanism behind this tuning of mechanical functionalities has not been revealed and is a matter of ongoing research. Further, other factors changed by diseases, e.g. in diabetic tissue, are of great interest: increased AGEs cross-linking, mechanisms at work changing collagen fibril mechanical behavior, and how they interfere with other parameters, these points open another chapter of questions to be answered.

4.1 Experimental Studies

The mechanical behavior and functions of collagen fibrils are dependent on various parameters, for example on molecular structure [6, 29, 34, 60], post-translational modifications [6, 48] and intrafibrillar cross-linking [29, 176, 177].

The two big groups of experimental testing of collagen fibrils in-vitro for the assessment of their elastic behavior are nanoindentation and nanotensile tests [10], but also other methods e.g. Atomic-Force-Microscopy (AFM) bending tests [81] have been used to extract mechanical properties.

Nanoindentation tests using AFM [9, 82, 204] are very time-efficient for testing collagen fibrils for their linear elastic behavior and therefore commonly used in material science. The indentation modulus of collagen fibril reported varies from 1.25MPa to 25GPa and is highly dependent on environmental conditions, meaning the hydration level (dry or hydrated) of the fibril [9, 73]. These force-indentation tests use a sharp AFM tip apex at the end of a bending cantilever beam. The indentations are then performed over the region of interest in a way, that the
resolution of indentations provides at least one force indentation test at the crest of the fibril. From the reaction of the cantilever, the force-indentation data is measured and the mechanical data of the fibril is then extracted via different theories and models based on contact mechanics [10].

Nanotensile testing has been conducted with Micro-Electromechanical System (MEMS) [49] or AFM [189] on isolated collagen fibrils. AFM [144, 176, 211] as well as MEMS [108, 167, 168] nanotensile testing approaches were both adopted to perform dynamic destructive tensile testing of the fibrils to obtain further mechanical properties, also in the non-elastic range. Nowadays, custom instruments for tensile testing have been developed to increase the throughput, force and strain resolution compared to AFM tensile testing [126]. An additional but indirect method for measuring collagen fibril deformation is using Synchrotron Small-Angle X-ray Scattering (SAXS) during tensile testing of full bone samples, which allows for simultaneous, real-time measurements of the strain carried within collagen fibrils as compared to the bulk tissue strain [5]. Tensile testing has shown that collagen fibrils display a very specific stress-strain curve [60, 176], with three different phases I, II and III, associated with untangling of the molecules (I), molecules sliding (II) and molecules stretching (III). In general, mechanical properties of collagen fibrils are highly dependent on their environment and hydration state: the indentation modulus in aqueous solution is reduced by three orders of magnitude compared to air-dried fibrils [6, 7, 74], while dehydration with ethanol [6, 74] or poly-ethylene glycol [7] increases the indentation modulus.

Important to mention in the context of collagen fibril testing is that the fibrils used in in-vitro collagen tensile tests in order to investigate their mechanical properties are mainly obtained from tendon. These collagen fibrils are often referred to as collagen type I, but collagen fibrils are heterotypic, meaning that they consist of a mixture of collagen type I, III and V [10, 33, 100]. Tendon fibrils are used because their extraction is the most simple one compared to other tissue types, but also other tissues have been used as source for collagen fibril extraction, showing that collagen structure and mechanics are dependent on tissue type [136].

Another factor influencing mechanical properties is different types of cross-linking. Enzymatic cross-links and their maturation are known to be responsible for stabilization of the fibril structure and associated with improved mechanical properties [17], but data obtained from mechanical testing is contradictory. One study comparing tensile tests on collagen fibrils from different tendons with different enzymatic cross-link densities due to age [15, 176] shows that differences concerning cross-link maturity occur mainly at high strain levels [176], where fibrils with higher rates of mature cross-links displayed a significant rise in stress and stiffness, while stress of fibrils with mainly immature cross-links only reached a plateau level. A different study claims that neither age nor type of the tissue the collagen fibrils were extracted from, had significant effect on fibril mechanics, sug-
gesting that variations in enzymatic cross-link density are likely to play a minor role after initial tissue formation [177]. We are specifically interested in diabetic bone, where increased fracture risk is associated with the prevalence of AGEs, changing nano-mechanical behavior at the collagen fibril level [3, 5, 89, 155, 179, 180, 190]. To our knowledge, it has not been possible so far to isolate mineralized collagen fibrils from bone for mechanical testing.

A quantification of different AGEs types in different tissues is currently still missing. The most commonly used method is using indirect measurement of AGEs content via fluorescence of assays [5, 55, 119], since several products formed during glycation are fluorescent [117].

### 4.2 Computational Studies

On the sub-nano and nano-scale level, in-silico modelling provides a tool for getting insights into the mechanics within the collagen fibril, where common in-vitro experimental techniques lack resolution. Especially in the case of collagen fibril mechanics, where intrafibrillar cross-links are present, numerical simulations are currently the only way to investigate the influence of their respective levels on collagen fibril mechanics and deformations mechanisms. The mechanical reactions of TC molecules have been investigated in several computational studies, using full-scale molecular dynamics simulations in order to reveal mechanical properties under different loading conditions, e.g. tension, compression, shear, and bending [28, 63, 65, 66, 69]. Also additionally added enzymatic cross-links and their influence on the mechanical properties were investigated [104]. Gautieri, Buehler, and Redaelli [63] found that the uncoiling mechanism of the tripe-helical structure depends on the strain rate and that shear between two tropocollagen molecules is highly dependent on shear rate. Further, they speculate that H-bonds are controlling the intermolecular shear strength, showing that the presence or lack of water molecules is crucial for collagen fibril mechanics [66]. Intermolecular cross-links are also located at this scale between the TC molecules and especially the location of AGEs cross-links is a question of ongoing research. A computational study on the level of collagen molecules proposes eight potential sites for glucospane intermolecular cross-linking along the TC molecule [64], while a thermodynamic study only revealed five potential binding sites [37].

At the nano-scale, collagen microfibril mechanics from full-scale atomistic models were compared to nanotensile laboratory tests with good agreement [67, 68], where structural water has been found to act as a lubricant between TC molecules in forced axial stretching and as a glue forced axial sliding [214]. This is again indicating that water molecules and H-bonding, i.e. hydration, are playing an important role in collagen deformation [68].
For collagen fibrils in the laboratory, the specific stress-strain curve [176] is described by a three-phase behavior (phase I, II and II). Computational simulations can reveal the mechanism at molecule level responsible for this behavior and also how cross-linking is responsible for changing it [42]. When it comes to testing of full collagen fibrils with larger diameters, full-scale simulations reach their limits because of computational cost. This is where Coarse-grained Molecular Dynamics (CGMD) play an important role (see 5.1). The parameters used in coarse-grained modelling are usually extracted from full atomistic simulations. Based on studies of Buehler [30] on cross-linking using coarse-grained molecular dynamics, Depalle et al. [42] investigated how enzymatic cross-links and their properties influence the collagen fibril behavior. They state that collagen fibrils including enzymatic cross-linking show five instead of three [176] different forms of deformation mechanics: (1) alignment of TC molecules, (2) uncoiling, (3) uncoiling and sliding, (4) stretching of the bonds within the TC molecule backbone and (5) sliding and bond breaking. Still, the collagen fibrils tested only revealed this specific behavior for fibril tensile testing when doubling the enzymatic cross-links tensile stiffness.

In the laboratory, it has not been possible to test mineralized collagen fibrils originating from bone, but in-silico experiments provide researches with a tool to overcome this limitation. Several studies performed full-scale Molecular Dynamics (MD) simulations [114, 124] to reveal molecular mechanics of mineralized collagen fibril and how energy is dissipated, suggesting that the mineral phase bears up to four times the stress of the collagen fibrils, whereas the collagen is predominantly responsible for the deformation response of the fibril. Coarse grained modelling of the mineralized collagen fibrils could show that the collagen fibril in bone reaches its strength and toughness by multiplying its sources of energy dissipation and deformations mechanisms in five deformation phases: (1) molecular uncoiling, (2) molecular stretching, (3) mineral/collagen sliding, (4) molecular slippage, and (5) crystal dissociation. Mineral provides the fibril with up to 10 times increased strength and up to 35 times increased toughness compared to collagen fibrils without mineral [43]. The increase in strength has been shown to be caused by reduced intermolecular sliding and increased stretching of the TC molecules due to mineral [181]. A study on the influence of enzymatic and non-enzymatic cross-linking on a very reduced model of mineralized collagen fibrils using the finite element method [170] could show that specifically non-enzymatic cross-linking changes the deformation behavior of the fibril by inhibiting sliding of the TC molecules and transferring load to the mineral, increasing modulus but decreasing post-yield strain. This is suspected to be the origin of the reduced toughness of bone. Other studies also found that general cross-linking leads to increased brittleness of the mineralized collagen fibril [43, 181]. How different amounts and types of non-enzymatic cross-links could influence mechanics of the fibril is, however, still unknown.
SUMMARY The nanomechanical behavior of tissue in general and of bone in particular at the collagen fibril level is still not fully revealed and is a field of intensive research. Because of the reduced accessibility of this scale level to imaging techniques, computational modeling provides a valuable contribution. Cross-linking, specifically non-enzymatic cross-linking, has been demonstrated to cause deterioration in tissue mechanical behavior, but the origins on the fibril level could not be revealed so far. A comprehensive investigation on AGEs cross-links concerning identification, localization and quantification in different tissue types is still missing. Since computational modelling allows varying these parameters, it is possible to investigate their influence on deformation mechanisms within the collagen fibril in-silico and reveal the origins of impaired tissue mechanical behavior.
METHODOLOGY

In this research work, destructive tensile tests on collagen fibrils are performed \textit{in-silico}. We use Coarse-grained Molecular Dynamics (CGMD) simulations, a specific type of Molecular Dynamics (MD), for investigating the dynamic behavior of the fibril during these tests. The following chapter provides an overview of the applied modeling technique and implementation methods.

5.1 GEOMETRY IMPLEMENTATION USING COARSE-GRAINED MOLECULAR DYNAMICS

In coarse-graining, particles within discrete simulations do not represent one atom, but several, while the definition of the interactions between these particles represents the behavior of the system overall \cite{161} (see Fig. 5.1 a&b). For a closer description of interactions applied in this work, please refer to 5.2.1.

Coarse-grained Molecular Dynamics (CGMD) is a useful method were full-scale atomistic simulations (i.e. every particle represents one atom) are limited by computational expenses. This makes it possible to reach length scales of several micrometers and time scales of microseconds, to obtain insights on the next higher scale by linking atomistic-scale studies with the mesoscopic level, e.g. TC molecules with collagen fibrils \cite{27}.

In our collagen fibril model, the TC molecule is represented by a chain of particles, where the bonds between these particles represent the mechanical behavior of the TC molecule in atomistic simulations (see Fig. 5.1 b, following \cite{42, 43}). The particle chains have a length of 300 nm and a diameter of about 1.5 nm, represented by the dispersive parameter $\sigma$. Collagen particles were placed equidistantly along the TC molecule axis, where the equilibrium distance accounts for particle diameter (for exact description and parameters, please refer to Sec. 6.2.1, 7.2.1, 8.2.1, 8.2.4 and A.3). The collagen fibril is implemented as a bundle of these TC molecule (see Fig. 5.1c&d) with the characteristic 5-staggering pattern following the Hodge-Petruska Model (compare Fig. 3.1a and Fig. 5.1c. The D-period including one gap and overlap zone has a length of 67 nm. Cross-links are added between the TC molecules (see Fig. 5.1b): enzymatic cross-links are located at the respective ends of the TC molecules and \textit{AGE}s cross-links are randomly placed between the non-helical central part in every TC molecule respectively, since the location of \textit{AGE}s is still a question of ongoing research. The content of \textit{AGE}s is defined as cross-links per TC molecule $\left(\frac{\text{AGE}}{\text{TC}}\right)$. In mineralized collagen fibrils, where the mineral phase is
Figure 5.1: Schematic overview of the principle of coarse-graining: (a) The mechanical behavior of the TC molecules in full atomistic scale is represented by the forces acting between larger particles that are arranged in a string and replace the TC molecule. (b) Collagen molecules represented by the grey particles, mineral (HAP) molecules by the red particles (present only in the mineralized collagen fibrils of bone) located in the gap region of the collagen fibril. Bonds modeled between collagen particles within the TC molecules and as cross-links between the TC molecules. (c) Schematic drawing of the fibril geometry: strings of beads representing TC molecules are arranged in characteristic 5-staggering pattern with one gap and overlap zone per D-period. (d) Visualization of mineralized collagen fibrils before tensile testing.
characteristic for hard-tissue like bone (compared to soft-tissue like tendon), the mineral particles are inserted in the gap zones of the fibril, using two different approaches: (1) mineral is “grown-in” from the sides of the gap and (2) mineral growth is nucleated from the center of the gap. The mineral content is measured in length % of the gap length.

5.2 A General Introduction to Molecular Dynamics Simulations

Molecular Dynamics (MD) is a discrete modeling technique calculating the time-dependent behavior of a system of particles based on pre-defined interactions and showing their dynamic evolution. The trajectory is calculated via the integration of Newton’s equation of motion, where the forces result from interaction potentials acting between the particles [161].

Given a closed system of particles, the definition of the interaction of the particles is based on the function returning the potential energy function of this system. The potential energy is the sum of all force field terms

\[ V(x) = \sum V(\{x_i \mid i = 1, 2, \ldots, N\}) \]  \hspace{1cm} (5.1)

where \( V \) is the potential energy, \( x \) is the 3N-dimensional coordinate vector and \( x_i \) are the 3d coordinates of \( N \) particles of the system [88]. This definition of interactions is the so-called force field, essentially the “heart” of every MD simulation (see Fig. 5.5). In other words, the force field is defined as the potential energy \( V(x) \) of the system in the given configuration \( x \). For performing MD simulations, the corresponding forces on each particle \( F_i(x) \) are required. This force is the negative derivative of the potential energy

\[ F_i(x) = -\nabla V(x) = -\frac{\partial V(x)}{\partial x_i} \]  \hspace{1cm} (5.2)

and the forces are typically calculated simultaneously with the potential energy during the evaluation of a system. Interactions between particles are distinguished between bonded and non-bonded. Since the total potential energy \( V \) of a system is the sum over all force field terms (equation 5.1), it is defined as

\[ V_{\text{total}} = V_{\text{bonded}} + V_{\text{non-bonded}} = \sum_{\text{bond}} \Phi_{\text{bond}} + \sum_{\text{non-bonded}} \Phi_{\text{non-bonded}} \]  \hspace{1cm} (5.3)

with \( V_{\text{bonded}} \) as the potential energy calculated from the sum of all bonded interaction \( \sum_{\text{bond}} \Phi_{\text{bond}} \) and \( V_{\text{non-bonded}} \) as the potential energy calculated from the sum of non-bonded interaction \( \sum_{\text{non-bonded}} \Phi_{\text{non-bonded}} \).

The force \( F_i \) acting per particle is also a sum over force field terms

\[ F_i(x) = \sum_{\text{bond}} F_{i,\text{bond}} + \sum_{\text{non-bonded}} F_{i,\text{non-bonded}} \]  \hspace{1cm} (5.4)
where bond interactions can be referred to as covalent bonds, resulting in forces between particles due to bond stretching or bond-angle bending and non-bonded interactions are caused by electrostatic interactions or van der Waals forces.

Knowing the forces acting on each particle, the dynamical trajectory is calculated via Newton’s equation of motion

$$F = ma \quad (5.5)$$

where forces $F$ result from interaction potentials acting between the particles and masses $m$ are given. The particle accelerations $a$ are calculated, leading to new velocities and positions (see Fig. 5.5).

### 5.2.1 Force field terms

The forces acting on each particle can be calculated from equation 5.2. For example, if $r$ is the distance between two respective particles, the force between these particles is defined as the negative derivative of the potential energy

$$F(r) = -\frac{\partial \Phi(r)}{\partial r}, \quad (5.6)$$

where $F$ is the force, $r$ is the distance and $\Phi$ is the potential energy. In a 3-dimensional system, this results into

$$F(r) = -\nabla V(r). \quad (5.7)$$

Bond stretching between two particles within the TC molecules and as cross-links is described via harmonic oscillators

$$\Phi_{\text{bond}}(r) = \frac{1}{2}k(r - r_0)^2, \quad (5.8)$$

where $r$ is the distance between two particles, $r_0$ is the equilibrium distance and $k$ is the bond stiffness.

In this study, we use specific trilinear bonds (see Fig. 5.2), describing the force between two particles, derived from the potential energy (see Eq. 5.2), as

$$F_{\text{bond}}(r) = \begin{cases} 
-k_0(r - r_0) & \text{if } r < r_1 \\
-k_1(r - r_0) & \text{if } r_1 \leq r < r_{\text{break}} \\
z k_1(r - r_0) & \text{if } r_{\text{break}} \leq r < r_{\text{break}} + a \\
0 & \text{if } r \geq r_{\text{break}} + a,
\end{cases} \quad (5.9)$$

where $F_{\text{bond}}$ is the force acting between the two particles that are connected with a bond, $k_0$ and $k_1$ are the respective spring constants of the bond deformation and
5.2 A General Introduction to Molecular Dynamics Simulations

\[ \Phi_{\text{bond}}(r) = \frac{1}{2}k(r - r_0)^2 \]

(a) \[ F(r_0) = 0 \]

(b) \[ F(r) = -k^{(0)}_r(r - r_0) \quad \text{if } r < r_1 \]
\[ F(r) = -k^{(1)}_r(r - r_0) \quad \text{if } r_1 \leq r < r_{\text{break}} \]

(c) \[ F(r) = z \cdot k^{(1)}_r(r - r_0) \quad \text{if } r_{\text{break}} \leq r < r_{\text{break}} + \alpha \]
\[ F(r) = 0 \quad \text{if } r \geq r_{\text{break}} + \alpha \]

Figure 5.2: Bond interaction definition due to covalent bonding between two particles within TC molecules collagen and between TC molecules as cross-links (AGEs and enzymatic cross-links). (a) Equilibrium distance \( r_0 \) with no forces between the bonded particles. (b) Two different bond stiffnesses account for stiffening of the bond after the distance between the particles reaches \( r_1 \). (c) Bond breaking with regularization factor \( z \) for computational stability.

\[ \Phi_{\text{angle}}(\phi) = \frac{1}{2}k_{\theta}(\phi - \phi_0)^2 \]

(a) \[ F_{\text{angle}}(\phi_0) = 0 \]

(b) \[ F_{\text{angle}}(\phi) = -k_{\theta}(\phi - \phi_0) \]

Figure 5.3: Bond angle bending accounts for tetrahedral interactions between three particles, force and potential energy are dependent on the angle \( \phi \).
Figure 5.4: Non-bonded interactions are described via a soft-core Lennard-Jones potential; with a common Lennard-Jones potential, when particles get closer than the equilibrium distance, they experience strong repulsion: These high forces are damped by applying a soft-core. When the distance between two particles is larger than the equilibrium distance, particles attract each other due to van der Waals forces.

\( r_0 \) is the equilibrium distance between the two bond particles. \( z \) accounts for regularization after bond breakage to avoid discontinuities and provide computational stability, with \( a \) defined as \( a = z (r_{\text{break}} - r_1) \).

Bond angle bending (see Fig. 5.3) is represented in a similar way via

\[
\Phi_{\text{angle}}(\phi) = \frac{1}{2} k_B (\phi - \phi_0)^2
\]

(5.10)

where \( \phi \) is the angle between three particles, \( \phi_0 \) is the equilibrium angle and \( k_B \) is the angle stiffness. This results in inter-particle forces defined as

\[
F_{\text{angle}}(\phi) = -k_B (\phi - \phi_i) \cdot \phi,
\]

(5.11)

derived from the potential energy via (5.2).

In general, non-bonded interaction between particles occur due to electrostatic forces or van-der-Waals forces, but only the latter occur in our system. In the collagen fibril models, van-der-Waals forces are calculated between collagen molecules, and if mineral is introduced in the case of mineralized collagen fibrils, between collagen and mineral and between two mineral particles. The common method to calculate the potential \( \Phi_{\text{LJ}} \) of van-der-Waals forces between all atom pairs closer than a specific cut-off is a Lennard-Jones potential, e.g.

\[
\Phi_{\text{LJ}}(r) = 4\varepsilon \left[ \left( \frac{\sigma}{r} \right)^{12} - \left( \frac{\sigma}{r} \right)^6 \right]
\]

(5.12)
where $\varepsilon$ is the well-depth of the potential function and $\sigma$ is the particle distance at which the potential energy is 0. For computational stability, we introduced a Lennard-Jones potential with a soft-core (see Fig. 5.4), where forces between particles are calculated following

$$F_{\text{non-bond}}(r) = \begin{cases} F_{\text{LJ}}(r) & \text{if } r \geq \lambda \sigma_{\text{LJ}} \\ F_{\text{LJ}}(\lambda \sigma_{\text{LJ}}) & \text{if } r < \lambda \sigma_{\text{LJ}} \end{cases}$$

(5.13)

where

$$F_{\text{LJ}}(r) = \frac{1}{r} \left[ 48 \varepsilon_{\text{LJ}} \left( \frac{\sigma_{\text{LJ}}}{r} \right)^{12} - 24 \varepsilon_{\text{LJ}} \left( \frac{\sigma_{\text{LJ}}}{r} \right)^{6} \right].$$

(5.14)

with $\lambda$ as the parameter to adjust the critical force associated to the soft core.

Since the force field describes the particle interaction via the potential energy function, force field definition, i.e. the definition of particle interactions, is a crucial step when it comes to using MD. The specific parameter choice for our models is described in the respective chapters (see 6.2, 7.2, 8.2).

### 5.2.2 Molecular Dynamics Numerical Algorithm

The algorithm in MD is based on updating schemes of the system after every timestep $\Delta t$ by integrating the classical equations of motion (equation 5.5). From a given set of particles, using their coordinates, masses, velocities and the volume of the system, via the potential energy function, the forces acting on every particle are calculated. From these forces, via calculating accelerations with newtons equation of motion (equation 5.5), new velocities and coordinates at the time $t + \Delta t$ can be calculated. In classical MD simulations the integrator used it the Leap-frog integration, where velocities are updated in $t + \frac{1}{2} \Delta t$ and coordinates in $t + \Delta t$, subsequently (see Fig. 5.5).
Figure 5.5: Working principle of MD algorithm: From the input configuration at time $t$ forces are calculated and via integrating classical equations of motion, new velocities and coordinates are calculated using the Leap-Frog algorithm.
Part II

RESEARCH RESULTS – PUBLICATIONS

In the second part, the outcomes of the research project are presented in form of publications. One chapter is assigned per publication. The structure follows a logical order, where cross-linking and the influence of different cross-link contents in collagen are investigated in the first chapter, followed by a parameter study varying AGEs cross-link mechanical properties i.e. types and finally in the last chapter, we evaluate the influence of AGEs cross-linking on the mineralized collagen fibril in bone.
THE INFLUENCE OF AGES AND ENZYMATIC CROSS-LINKING DENSITY ON THE MECHANICAL PROPERTIES OF COLLAGEN FIBRILS

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KEY FINDINGS:

• High contents of AGEs cause stiffening of the collagen fibril in higher strain ranges.

• AGEs cross-links dominate force transfer between tropocollagen molecules.

• Load transfer to tropocollagen molecules is causing stiffening of the fibril.

• Reduced sliding and stiffening lead to less energy dissipation in the tissue.

• Strengthening and stiffening of the collagen fibril may cause impaired tissue behavior.

AUTHOR CONTRIBUTIONS: Conceptualization, all authors; methodology, all authors; model implementation, J.K.; validation, J.K.; formal analysis, J.K.; investigation, all authors; resources, D.S.K., C.A.; data analysis, J.K.; writing–original draft preparation, J.K.; writing–review and editing, all authors; visualization, J.K.; supervision, D.S.K., C.A.; project administration, D.S.K.

1 This is a post-print of Kamml et al. [97], differing from the published paper only in terms of layout and formatting.
ABSTRACT

Collagen, one of the main building blocks for various tissues, derives its mechanical properties directly from its structure of cross-linked tropocollagen molecules. The cross-links are considered to be a key component of collagen fibrils as they can change the fibrillar behavior in various ways. For instance, enzymatic cross-links (ECLs), one particular type of cross-links, are known for stabilizing the structure of the fibril and improving material properties, while cross-linking Advanced Glycation End-products (AGEs) have been shown to accumulate and impair the mechanical properties of collagenous tissues. However, the reasons for whether and how a given type of cross-link improves or impairs the material properties remain unknown, and the exact relationship between the cross-link properties and density, and the fibrillar behavior is still not well understood. Here, we use coarse-grained steered molecular models to evaluate the effect of AGEs and ECLs cross-links content on the deformation and failure properties of collagen fibrils. Our simulations show that the collagen fibrils stiffen at high strain levels when the AGEs content exceeds a critical value. In addition, the strength of the fibril increases with AGEs accumulation. By analyzing the forces within the different types of cross-links (AGEs and ECLs) as well as their failure, we demonstrate that a change of deformation mechanism is at the origin of these observations. A high AGEs content reinforces force transfer through AGEs cross-links rather than through friction between sliding tropocollagen molecules, which leads to failure by breaking of bonds within the tropocollagen molecules. We show that this failure mechanism, which is associated with lower energy dissipation, results in more abrupt failure of the collagen fibril. Our results provide a direct and causal link between increased AGEs content, inhibited intra-fibrillar sliding, increased stiffness, and abrupt fibril fracture. Therefore, they explain the mechanical origin of bone brittleness as commonly observed in elderly and diabetic populations. Our findings contribute to a better understanding of the mechanisms underlying impaired tissue behaviour due to elevated AGEs content and could enable targeted measures regarding the reduction of specific collagen cross-linking levels.

6.1 INTRODUCTION

Aging and diabetes impair mechanical properties and healing capacities in human tissues [38, 57, 75, 121]. However, precise mechanisms explaining such behaviour at different tissue scales remain still unknown. Tissue mechanical properties are conferred by its complex hierarchical structure. The most important structural protein maintaining the mechanical function and structural integrity of most tissues is collagen [59]. Amongst different forms arising from the collagen family, collagen type I is the most abundant in the human body, occurring in tendon, bone, skin, cornea, lung, and vasculature [86]. Its main building components are polypeptide chains called tropocollagen (TC) molecules that self-assemble into staggered fibrils with a characteristic periodic banding pattern (see Fig. 6.1c). Post-translational
enzyme-driven modifications stabilize the fibrillar structure via cross-linking between TC molecules with Enzymatic cross-links (ECLs). With aging and other factors like diabetes so-called Advanced Glycation End-products (AGEs) are formed via a non-enzymatic glycation process, called the Maillard reaction. Some of these AGEs molecules occur as cross-links connecting individual TC molecules within the collagen fibrils. It has been observed that an increased density of such AGEs cross-links alters the mechanical properties of the collagen fibrils on the nano-scale [40, 52, 70]. However, whether or how this increased cross-linking within the collagen fibril (intrafibrillar) and the precise nano-scale mechanisms are responsible for impaired tissue properties has not been revealed so far.

The two types of cross-links are known to present different characteristics. ECLs are located at the end of the TC molecules (Fig. 6.1d-e) and their quantity controls strength and stiffness in collagen [28, 42, 176]. These cross-links are formed via an enzymatic reaction mediated by lysil oxidase mainly at the ends of the TC molecules (see Fig. 6.1d). Initially, immature divalent cross-links will be formed, which are then reacting to form mature trivalent cross-links [18]. While throughout the early life the concentration of immature and mature cross-links reduces and increases, respectively, the cumulative ECLs content stabilizes after the age of 20 years at approximately 1.2 ECLs per TC molecule [50, 159]. AGEs, the second type of cross-links, are non-enzymatic since they are formed during glycation of proteins in the helical regions between TCs. They are predominantly formed in hyperglycemic systems due to the increased presence of sugar [138]. All pathways of their formation reaction have not fully been revealed, as multiple reactions can be involved [16], leading to different molecular structures. Several types of AGEs have been found as intermolecular cross-links [14], linking the TC molecules within the collagen fibril. AGEs accumulate with age, due to the long half-life of collagen, e.g., the number of pentosidine in bone was found to grow by a factor of 5 from age 10 up to 80 years [159]. In tissue of diabetic individuals, the process of AGEs formation is accelerated due to augmented blood glucose levels. By far the most abundant AGEs cross-link is glucosepane, which reaches levels about 1000 times higher than the contents of other cross-link types. Specifically, one glucosepane cross-link in every five TC molecules was found in tissue of 90 years old patients, whereas the AGE content reaches levels of one cross-link every two molecules in diabetic individuals [166]. Eight potential binding sites for glucosepane’s interfibrillar cross-linking have been proposed by Gautieri et al. [64], but it has not been possible to experimentally quantify the exact amount, type and distribution of all types of AGEs present in the fibril.

Despite limited knowledge about the type and quantity of AGEs in collagen fibrils, it is known that their presence is a major cause of dysfunction in mature collagenous tissues in elderly patients, which is accelerated in diabetic subjects because of higher levels of glucose leading to increased AGEs formation [18, 164].
Collagen tissue with a longer half-life is specifically affected by AGEs formation and their increased occurrence correlates with inferior material properties, e.g., with an increased fracture risk in bone \cite{14, 98}. Several experimental studies have investigated the relation between cross-link density and mechanical behaviour of collagenous tissue \cite{5, 176, 211}. Nevertheless, the underlying mechanisms of how cross-links and specifically AGEs influence fibrils at the nanoscale could not be revealed so far due to limitations of imaging techniques and the complexity of collagen.

In-silico modeling provides a tool to overcome these experimental limitations and presents an opportunity to reveal the biomechanical effects of enzymatic and non-enzymatic intrafibrillar cross-linking on the mechanical properties of collagen fibrils at the small scale and the tissue at the larger scale. For studying the structure and mechanical behavior of TC molecules and AGEs cross-links in collagen fibrils, full atomistic simulations could be used \cite{28, 64, 127, 188}. For instance, atomistic simulations have been used to describe the mechanical properties of collagen depending on TC molecule length, amount of enzymatic cross-links and mineralization \cite{29, 30}, and to investigate the effect of AGEs cross-links on the structure of collagen fibrils \cite{127}. However, full-scale simulations are computationally far too expensive to study multi-molecule fibril mechanics in full three dimensions through a full tensile testing procedure. This is where coarse-grained modeling can overcome the limitations of computational cost by representing the mechanical properties of the TC molecules using data from full-atomistic simulation results.

In this paper, we build a 3D coarse-grained model of the collagen fibril using steered molecular dynamics, where the mechanical response of TC molecules and cross-links are derived from reactive molecular dynamics simulations with atomistic resolution. With our simulations, we investigate the influence of cross-link density between TC molecules on stiffness, strength, and toughness (represented by work to failure) of collagen fibrils with a particular focus on AGEs increase on top of normal enzymatic cross-links. We show that higher densities of AGEs cause fibrilar stiffening (when fibrilar strain reaches a critical value of $\varepsilon_0 \approx 0.15$) and affect the failure mechanisms by causing the breaking of the TC molecules which results in abrupt failure of the fibril.

6.2 MATERIAL AND METHODS

We build a coarse-grained model of a collagen fibril including enzymatic and AGEs cross-links at various densities. Both types of cross-links are considered because AGEs mostly occur in aged or diabetic tissue where enzymatic cross-links are naturally present, since they have mostly been formed during growth and adult development. We simulate a destructive tensile test of collagen fibrils and investigate various quantities related to their deformation, failure, and energy
dissipation and how these depend on various cross-link densities. Our model is a coarse-grained molecular model where TC molecules forming the collagen fibril are represented by particles arranged in a chain and interacting according to multi-body potentials [28, 29, 42]. This approach allows us to reach length scales of hundreds of nanometers and time scales of microseconds, which would not be possible in full-scale atomistic modeling due to high computational costs.

6.2.1 Geometry of the collagen fibril

Collagen fibrils are bundles of TC molecules that are aligned in the longitudinal direction in the collagen-specific five-staggered pattern with gap and overlap zones (see Fig. 6.1c). In-vivo, TC is formed of three polypeptide chains (see Fig. 6.1a) twisted into a right-handed triple-helical structure with a diameter of about 1.5 nm and a length of 300 nm [23]. Three different domains are distinguished in the TC molecule: the central triple helical domain and the two non-helical telopeptide ends called C- and N-terminal (see Fig. 6.1a). The central domain is by far the largest, comprising of about 95 percent of the total length of the molecule. In fibrillar collagen, the TC molecules self-assemble into staggered fibrils with a characteristic repeating banding pattern. The periodicity of the pattern is $D = 67$ nm, where TC molecules are staggered with respect to their neighbors by multiples of $D$ (see Fig. 6.1c). Collagen fibrils have a thickness of about 50 to a few hundred nanometers [86].

We build one representative region of a collagen fibril showing five gap and overlap zones as shown in Fig. 6.1e. First, the geometry of the single TC molecules was obtained from the Protein Data Bank entry 3HR2, the atomistic model of a collagen type I microfibril based on X-ray crystallography [134]. We represent several amino acids of one TC molecule by a single particle and the mechanical properties of the entire molecule by a chain of these particles (see Fig. 6.1a-b). The coordinates of the particles in the longitudinal direction are obtained by using spline-fitting along the TC molecule structure and then aligning the particles equidistantly along this spline. The distance between the particles is chosen as the equilibrium distance $r_0 = 14.0$ Å of the inter-particle potential, approximating the diameter of the TC molecule [42]. This results in 218 particles per TC molecule. The angles between the two outer particles of each particle triplet along the chain are serving as equilibrium angles $\phi_i$ in the coarse-grained model. After the cross-section of collagen fibrils is built by meshing a sphere with a diameter of $d = 20.2$ nm with a triangular mesh, 155 TC molecules are replicated and aligned along the longitudinal axis of the fibril, resulting in a cylindrical shape. In the longitudinal direction the geometry is implemented with five gap and overlap zones of the TC molecules, where the measure of the gap $0.6 \cdot D$ and the overlaps $0.4 \cdot D$ accordingly. Finally, the collagen fibril was extended with 40 additional particles at the end of
each TC molecule in order to ensure smooth force transmission by constraining and strengthening the fibril where external forces are applied.

6.2.2 Insertion of enzymatic cross-links

Enzymatic cross-links occur as immature divalent cross-links or mature trivalent cross-links (see Fig. 6.1d-g) between the telopeptide ends of the TC molecules and the helical domain of adjacent molecules in collagen fibrils [158]. They are covalent bonds between lysine or hydroxylysine residues. We model divalent and trivalent cross-links by linking two and three different TC molecules, respectively [42]. We vary the content of enzymatic cross-links between 0, 25, 50, 75 and 100 percent, where 100 percent corresponds to two enzymatic cross-links per TC molecule (2 mol/mol), i.e. one at each end. For enzymatic cross-link contents of less than 100 percent, we distribute them randomly amongst all the telopeptide ends of the collagen fibril such that no telopeptide end has more than one cross-link. The molecule to link to is chosen to be the one closest to the respective telopeptide end. The process of enzymatic cross-link insertion is done after equilibration of the collagen fibril model, as discussed in more detail in Sec. 6.2.5.

6.2.3 Insertion of AGEs cross-links

The density of AGEs cross-links in vivo depends on the tissue type, where tissue with low turnover and higher age generally shows a higher amount of cross-links [178]. Very little is known about the exact location of AGEs in collagen. AGEs are formed in vivo via non-enzymatic reactions. AGEs cross-links have several different molecular compositions and emerge between TC molecules in the helical regions, depending on the sugars and amino acids involved in their formation and their relative distance. In general, a sugar moiety is added between two proteins involving lysine-to-lysine or lysine-to-arginine residues of two TC molecules leading to covalent bonding [14, 138]. Due to its frequency of occurrence, we use glucosepane as a representative for AGEs cross-links in our simulations: it is the most prominent AGE, reaching levels of 2000 pmol/mol in collagen tissue of 90 years old patients and of ~ 4500 pmol/mol in diabetic patients [166]. Hence, there is one AGE every 5 molecules in aged collagen and one AGE every 2 molecules in diabetic collagen.

In our model, cross-links are inserted randomly between two neighboring TC molecules. Telopeptide ends of the TC molecules (consisting of four particles in our model) are excluded as potential binding sites. Random cross-link density is measured as the number of AGEs cross-links per TC molecule. The potential binding sites between particles of every TC molecule are extracted and then cross-links are randomly created. If the density is less than 1 cross-link per TC molecule, TC molecules where cross-links are created are randomly chosen and then cross-links
Figure 6.1: Schematic overview of creation of the coarse-grained model of collagen fibrils. (a) TC molecule with telopeptide ends and helical main region: consists of 3 α-polypeptide helices twisted into each other. (b) Full-scale atomistic TC molecule is represented by a string of particles (coarse-grained model) - the forces between the particles represent the mechanical properties of the full-scale molecule. (c) Typical 5-staggering arrangement pattern of TC molecules within the collagen fibril with gap and overlap zones, leading the characteristic banding pattern. (d) The coarse-grained model parameters represent the mechanical properties of TC and the inter-molecular cross-links (AGEs and enzymatic cross-links). (e) Schematic representation of the configuration of the model as a representative part of the collagen fibril with 5 gap and overlap zones. Particles at the ends are rigidly constrained for applying force during displacement-controlled tensile tests using steered molecular dynamics. (f) Different types of bonds inserted in the model: Particles are linked via collagen covalent bonds within each TC molecule; ECLs as intermolecular covalent bonds (dark blue: divalent, light blue: trivalent) at the ends to the TC molecules, AGEs cross-links randomly placed between two TC molecules (red). (g) Cross sections with cross-links shown schematically. (h) Steered molecular dynamics tensile tests on collagen fibril model.
Table 6.1: Parameters used in coarse-grained molecular dynamics mesoscale model of collagen fibrils [42]

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<thead>
<tr>
<th>Components</th>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen molecules</td>
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</tr>
<tr>
<td></td>
<td>Critical hyperelastic distance ($r_1$, Å)</td>
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<td></td>
<td>Bond breaking distance ($r_{\text{break}}$, Å)</td>
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<td>Equilibrium angle ($\phi_0$, degree)</td>
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<td>Soft core parameter ($\lambda$, -)</td>
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<th>Components</th>
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<td>Particles at ends of TC molecules</td>
<td>same parameters as collagen molecules except:</td>
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<td>8.00</td>
</tr>
</tbody>
</table>

| Mass of each mesoscale particle, atomic mass units | 1358.7 |
are randomly created at potential binding sites. We use densities of 0, 0.50, 1, 2, 5, 10, and 40 AGEs cross-links per TC molecule, which goes beyond the above-mentioned measured values, since the exact numbers of different AGEs cross-links have not been quantified so far.

6.2.4 Parameterization of the force field for coarse-grained modeling

We now will summarize the various particle interactions applied in our coarse-grained model. We consider the total energy of the modeled system as follows

\[ E_{\text{total}} = E_{\text{bond}} + E_{\text{angle}} + E_{\text{inter}} \]

\[ = \sum_{\text{bond}} \Phi_{\text{bond}}(r) + \sum_{\text{angle}} \Phi_{\text{angle}}(\phi) + \sum_{\text{inter}} \Phi_{\text{inter}}(r) \]  

(6.1)

where \( E_{\text{bond}} \) is the bond energy due to stretching, \( E_{\text{angle}} \) the three-body interactions energy due to bending, and \( E_{\text{inter}} \) the pairwise interaction energy due to molecular interactions such as Van-der-Waals forces.

The force between two particles linked via a bond is given by

\[ F_{\text{bond}}(r) = -\frac{\partial \Phi_{\text{bond}}(r)}{\partial r}, \]  

(6.2)

where \( \Phi_{\text{bond}} \) is the bond potential and \( r \) is the radial distance. We model the nonlinear deformation behaviour of a single collagen molecule in tensile tests as reported previously \([31, 32]\) with a tri-linear relation (more details provided in Appendix A.1), as follows

\[
F_{\text{bond}}(r) = \begin{cases} 
-k_0(r - r_0) & \text{if } r < r_1 \\
-k_1(r - r_0) & \text{if } r_1 \leq r < r_{\text{break}} \\
-z \cdot k_1(r - r_0) & \text{if } r_{\text{break}} \leq r < r_{\text{break}} + a \\
0 & \text{if } r \geq r_{\text{break}} + a 
\end{cases}
\]  

(6.3)

where \( r_0 \) is the equilibrium distance between two particles linked by a bond, \( k_0 \) and \( k_1 \) are spring constants of the deformation, and \( a \) is defined as \( a = z \cdot (r_{\text{break}} - r_1) \).

The chosen tri-linearity accounts for breaking of the bond, which occurs at \( r_{\text{break}} \).

The breaking is regularized with the \( z \)-factor to avoid any discontinuities and provide computational stability. The \( z \)-factor is chosen small enough, such that its finite value does not affect the simulation results.

Forces arising from angle bending between triplets of particles are defined as

\[ F_{\text{angle}}(\phi) = -k_B(\phi - \phi_i) \cdot \phi, \]  

(6.4)
with $\phi_i$ being the varying equilibrium angles obtained from the initial coarse grained geometry (see Sec. 6.2.1), and $k_B$ being the bending stiffness of the molecule \cite{29, 32, 42}.

The non-bonded interactions of particles are modeled with the Lennard-Jones potential with a soft core, which is given by

$$ F_{\text{non-bond}}(r) = \begin{cases} F_{\text{LJ}}(r) & \text{if } r \geq \lambda \sigma_{\text{LJ}} \\ F_{\text{LJ}}(\lambda \sigma_{\text{LJ}}) & \text{if } r < \lambda \sigma_{\text{LJ}} \end{cases} \quad (6.5) $$

where

$$ F_{\text{LJ}}(r) = \frac{1}{r} \left[ 48 \epsilon_{\text{LJ}} \left( \frac{\sigma_{\text{LJ}}}{r} \right)^{12} - 24 \epsilon_{\text{LJ}} \left( \frac{\sigma_{\text{LJ}}}{r} \right)^{6} \right]. \quad (6.6) $$

In these equations, $\epsilon_{\text{LJ}}$ is the well depth between two particles, $\sigma_{\text{LJ}}$ is the distance at which the intermolecular potential between the two particles is zero and $\lambda$ is the parameter to adjust the critical force associated to the soft core.

The parameters for the coarse-grained model of the interactions in the TC molecules as well as for enzymatic cross-links are taken from full-scale simulations in literature and adjusted to our approach \cite{28, 42}. For the AGEs cross-links, we conducted steered molecular full-scale tensile test simulation and a literature study and extracted parameters for the observed mechanical behavior (details provided in Appendix A.1). All interaction parameters used in the simulations are displayed in table 6.1.

### 6.2.5 Simulations

All coarse-grained molecular dynamics simulations on single collagen fibrils were performed in LAMMPS \cite{141}. Ten particles at the end of the system are constrained to a rigid body similar to a clamp (see Fig. 6.1e,h) and kept at a stable constant distance apart from each other. In this configuration and without any cross-links (yet), a first equilibration is performed, where, first, an energy minimization using steepest descent is performed followed by a conjugate gradient energy minimization. This sets the system to an energetically favorable configuration. Further, the system is equilibrated for 80 ns using an NVT ensemble at a temperature of 300 K with a time step of $\Delta t = 10$ fs. After equilibrium is reached, enzymatic and AGEs cross-links are inserted following the procedure described in Sec. 6.2.2 and 6.2.3. At this point, another equilibration is performed with the inserted cross-links for 10 ns.

Next, the tensile tests are performed in the NVT ensemble, where the rigid bodies at the end of the system are moved apart from each other along the longitudinal axis of the fibril with a constant velocity of 0.0001 Å/fs ($= 10$ m/s) with a time step of $\Delta t = 1$ fs, using a steered-molecular dynamics approach. The applied strain rate
is, for computational reasons, considerably faster than commonly used values in experiments. We verified that slower values do not lead to qualitative differences in our simulation results. We use the force calculated between the two groups of atoms being moved apart during the tensile tests to calculate the engineering stress, where the area of the cross-section of the fibril is calculated during the construction of the fibril geometry.

Figure 6.2: Stress-strain curves of tensile tests until rupture of a representative collagen fibril with different AGEs cross-link densities. Simulations were performed with 0 enzymatic cross-links per TC molecule.

6.3 RESULTS

6.3.1 Fibrilar stress-strain response dependent on AGEs cross-link density

In a first series of simulations, we vary the AGEs cross-link content and investigate its effect on the mechanical properties of the collagen fibril. Our results show that the modeled collagen fibrils present a linear deformation behavior at low strains, i.e. $\varepsilon < \varepsilon_0 \approx 0.15$, where $\varepsilon_0$ defines the limit of the linear elastic regime.(see Fig. 6.2). This behavior is qualitatively and quantitatively independent of the AGEs density.
The effect of the AGEs content starts to appear for strains beyond this limit, where fibrils with lower cross-link densities (e.g., 0-1 AGEs/TC) show softening mechanisms. The fibril stiffness reduces continuously, transitioning into a reduction of stresses, which indicates the presence of localization in terms of reduction in cross-sectional area, and eventually leading to total failure of the fibril without any load-carrying capacity (Fig. 6.2). The failure process appears to be overall smooth (no sudden stress drops) and occurs at relatively low stress and strain levels. Fibrils with higher AGEs densities present a very different mechanical behavior (e.g., 10-40 AGEs/TC in Fig. 6.2). At \( \varepsilon > \varepsilon_0 \), they present a stiffening, which leads quickly to considerably higher stresses for relatively moderate strain levels. Moreover, failure of these fibrils appears more abrupt, where stresses drop fast and without any prior softening that would indicate an impeding fracture (note sharp peak in stress-strain curves for 40 AGEs/TC in Fig. 6.2).

These differences in the collagen fibril behavior translate into various mechanical properties that are affected by the AGEs density, and which may result in some of the observed impairments at the tissue level. First, we observe that the peak stress \( \sigma_{\text{peak}} \) increases with a larger AGEs cross-link content \( N_{\text{AGE}} \) (see Fig. 6.3a, b, and Appendix A.2). This observation is consistent across all modeled fibrils and for any enzymatic cross-link content, as will be discussed in Sec 6.3.2. Second, the work to failure \( W_f = \int \sigma \, d\varepsilon \) is also increasing with increasing \( N_{\text{AGE}} \) (see Fig. 6.3c, d, and Appendix A.2). We observe that small variations in these mechanical fibril properties (see Fig. 6.3) occur due to individual cross-link arrangements in each simulation. The discussed overall trends are, however, strong and consistent across all simulations.

The increases in \( \sigma_{\text{peak}} \) and \( W_f \) are directly associated with the observed stiffening of the fibril at \( \varepsilon > \varepsilon_0 \). To evaluate the contribution of the stiffened regime, we compute the elastic-to-peak stress difference given by \( \Delta \sigma = \sigma_{\text{peak}} - \sigma_0 \), where \( \sigma_0 = \sigma(\varepsilon_0) \) is the stress indicating the start of the stiffened regime. We observe that \( \Delta \sigma \approx 0 \) for fibrils with low \( N_{\text{AGE}} \) (see Fig. 6.3e, f) confirming the absence of this stiffening regime, whereas a higher \( N_{\text{AGE}} \) leads to an increasing \( \Delta \sigma \).

6.3.2 Influence of enzymatic cross-linking

In physiological conditions, AGEs cross-links are built in the process of aging or due to increased glycation levels in diabetic patients’ tissue. Therefore, enzymatic cross-links will always be present when AGEs occur but their content may vary. Since the influence of these enzymatic cross-links cannot be neglected, we investigate their effect with a series of simulations by varying their content and type, i.e. divalent or trivalent.

Overall, our results show that trivalent enzymatic cross-links have a larger influence than divalent on key mechanical properties of the collagen fibril (compare
Figure 6.3: Summary of key mechanical properties of collagen fibrils with varying AGEs and enzymatic cross-link content. (a,b) peak stress, (c,d) work to failure and (e,f) $\Delta \sigma$ of collagen fibril with varying AGEs content where $\Delta \sigma \geq 0$ by definition and (a,c,e) divalent or (b,d,f) trivalent enzymatic cross-links. Results for $N_{AGE} = 0$ not shown but approximately equivalent to $N_{AGE} = 0.5$ CLs/TC. Lines are drawn as guides for the readers’ eyes.
top with bottom in Fig. 6.3). We see that the development of increasing peak stress and increasing work to failure is the same for increasing enzymatic cross-link content independent of cross-link type. Specifically, the contribution of ECLs on the peak stress remains relatively constant across various AGEs cross-link densities $N_{AGE}$, while their influence on the work to failure decreases slightly with increasing $N_{AGE}$.

Particularly important is the effect of enzymatic cross-links on the actuation of the stiffened regime. First, we note that enzymatic cross-links alone cannot cause the stiffened regime, as can be seen by $\Delta \sigma \approx 0$ for $N_{AGE} = 0.5$ CLs/TC (see Fig. 6.3c). However, at low contents of AGEs cross-links, enzymatic cross-links, and in particular trivalent ones, may cause a stiffening of the collagen fibril that would not occur without them (see $N_{AGE} = 1 - 5$ CLs/TC in Fig. 6.3e). In fibrils with high $N_{AGE}$, we observe that enzymatic cross-links increase further the stiffened regime by increasing the peak strength, but they do not appear to cause any qualitative differences in the behavior of the fibril.

6.3.3 Local force distribution in collagen fibril

The observed differences in the global mechanical properties of the collagen fibril are the results of changes in how the force is transmitted through the fibril. Hence, we analyze the force distribution within the TC molecules and the different types of cross-links. Our model shows that the average force within the TC molecules $\bar{F}_{TC}$ follows the stress-strain curves on the global fibril level (compare Fig. 6.4a with Fig. 6.2). The same stiffening effect at higher strains for fibrils with $N_{AGE} \geq 5$ CLs/TC is observed, where $\bar{F}_{TC}$ increases after a global strain reaches the critical value of $\epsilon_0 \approx 0.15$. This shows that the higher loads are mainly carried by the collagen molecules. It also suggests, as can be expected, that the TC molecules are not causing the changes in global behavior but are simply the carrying element in the system.

The average forces in the enzymatic cross-links $\bar{F}_{ECL}$ presents a different behavior. Their maximum values appear to be independent of the cross-link density (see Fig. 6.4b). This shows that failure of ECLs is not the governing factor of fibrillar failure under the investigated conditions. Furthermore, we note that the ECLs are activated at somewhat higher global strain values for fibrils with higher $N_{AGE}$ (see shift to higher strain values in Fig. 6.4b). For the linear range $\epsilon < \epsilon_0$, where global stress-strain relation is independent of $N_{AGE}$, this shows that the force transmission from on TC molecule to another happens through a different part of the fibril, i.e. the AGEs.

The most prominent differences appear in the average forces in AGEs cross-links $\bar{F}_{AGE}$ (see Fig. 6.4c). Larger $N_{AGE}$ lead to a lower $\bar{F}_{AGE}$ at the same global strain level, with $\bar{F}_{AGE}$ at 40 CLs/TC being less than half of that at 0.5 CLs/TC. These
results provide an explanation for the observed stiffening behavior of the collagen fibril. At low $N_{AGE}$, forces transmission between TC molecules within the fibril at $\varepsilon > \varepsilon_0$ happens to some degree through the AGEs and the ECLs but mostly through direct interactions between TC molecules (e.g., friction). Even an increase by a factor 4 from 0.5 CLs/TC to 2 CLs/TC does not cause any significant changes to $F_{AGE}$. Consequently, global fibrillar deformation in this strain range and at these $N_{AGE}$ becomes increasingly dominated by intra-fibrillar sliding. This is confirmed by decreasing local strain of the TC molecules at increasing global fibrillar strain (see Fig. 6.5).

At $N_{AGE} > 2$, however, the lower $F_{AGE}$ indicates that the AGEs cross-link become the primary medium for force transmission between TC molecules (same global force is distributed over more cross-links). As a consequence, sliding between TC is mostly oppressed (confirmed by Fig. 6.5) and global deformation is the direct result of the deformation of the TC molecule. Since these molecules are the stiffest part of the collagen fibril (see Fig. A.1), this transfer of deformation from sliding between TC molecules to deformation of the TC molecules explains the observed stiffening of the collagen fibril.

6.3.4 Local origins of collagen fibril failure

Modifications to the deformation mechanisms of collagen fibrils incurred by changes in $N_{AGE}$ are likely causing also alteration to their failure mechanisms. These changes manifest themselves in the timing and quantity of failed AGEs and ECLs cross-links, as well as the breaking of the TC molecules.

First, we note that in the elastic regime $\varepsilon < \varepsilon_0$, the deformation is mostly driven by stretching of the TC molecules (see $\Delta \varepsilon > 0$ Fig. 6.5b). Consequently, with only very few exceptions, no cross-links are broken in this regime (note almost zero broken AGEs and ECLs even for $\varepsilon = 0.25 > \varepsilon_0$ in Fig. 6.6&6.7). As the deformation mechanism transforms into a process dominated by intermolecular sliding, the breaking of cross-links slowly starts to appear. This is expected as sliding strains the cross-links. The failure of cross-links then reduces the resistance against intermolecular sliding, which causes sliding to become increasingly dominant throughout this process (note how $\Delta \sigma$ grows negatively in Fig. 6.5b).

Our model shows that AGEs are the first cross-links failing in collagen fibrils with relatively low $N_{AGE}$, independent of the ECLs content (see Figs. 6.6&6.7). Overall, 40 – 60% of the AGEs cross-links break until failure of the collagen fibril. The breaking of ECLs occurs at higher strain levels but eventually reach a similar overall failure ratio of 40%. Most importantly, these collagen fibrils with low $N_{AGE}$ do not show any breaking of TC molecules. Hence, it is the ECLs and AGEs cross-links that govern the overall failure of collagen fibrils with low $N_{AGE}$, which confirms our observations from Sec. 6.3.3 that their failure mechanism is sliding-dominated.
Collagen fibrils with high $N_{AGE}$, however, we observe a different mechanisms. As discussed in Sec. 6.3.3, the large number of $AGEs$ cross-links act as sliding inhibitors, and fibrillar deformation is dominated by the deformation of the TC molecules. Consequently, less cross-links fail during the deformation of the fibril (see Figs. 6.6&6.7). However, the TC molecules break at high fibrilar strain levels (see Figs. 6.6d&6.7d). Since the TC molecules are considerably stronger than the cross-links (see Fig. A.1) and fail abruptly, the global failure mechanism of the collagen fibril also becomes more abrupt, as we have observed in Fig. 6.2.

6.4 Discussion

6.4.1 Limitations of numerical model

Our model is a simplification of a collagen fibril, which is a complex biological system. Hence, there are various underlying assumptions that may need further investigation in the future. For instance, we modeled one specific type of $AGEs$ cross-links, namely glucosepane, which is known to be the most abundant one in collagenous tissues [166]. Other $AGEs$ cross-links have not been taken into account because most of them have not been quantified in tissue so far. Furthermore, the locations of $AGEs$ cross-linking remain largely unknown, which is why we considered a random $AGEs$ distribution along the TC molecule to approximate the amino-acids-based process responsible for cross-link position selection [64]. Apart from $AGEs$, also enzymatic cross-links display a higher configurational variety than displayed in our study. For example, divalent and trivalent cross-links are not mutually exclusive and co-exist, which was not considered in our model.

The range of $AGEs$ content in our model exceeds considerably their occurrence as observed in measurements with current technology [166]. While this is true for glucosepane alone, the observed mechanical behavior is likely still to occur for two reasons. First, other non-glucosepane $AGEs$ are also present in collagen fibrils but have been neglected here, and second, the exact mechanical properties of $AGEs$ still remain unknown, and, hence, stiffer and stronger $AGEs$ are likely to cause the stiffening of the fibril at lower $AGEs$ content.

When it comes to collagenous tissues, there is great variability in structure and composition. Collagen type I is not the only collagen responsible for the structural properties. Further, the turn-over, i.e. the physiological rebuilding of tissue is important for the accumulation of $AGEs$ since they are only built during the process of aging or with enhanced exposure increased glycation levels [192]. Depending on the glycation level and exposure, the $AGEs$ density might also vary across the cross-section of the fibril and influence the mechanical properties additionally. Types of $AGEs$ might also vary depending on the tissue type [51]. We do not account
Figure 6.4: Force distribution among bond types within a collagen fibril. Average force in (a) TC molecule bonds, (b) enzymatic cross-links, and (c) AGEs. Fibril with 25 percent ECLs.
Figure 6.5: Deformation mechanisms in collagen fibril with an ECL content of 75 percent. (a) Local strains in TC molecules $\varepsilon_{TC}$ at global fibrillar strain $\varepsilon$. (b) The difference between average strain in TC bonds and global strain $\Delta \varepsilon = \varepsilon_{TC} - \varepsilon$ as function of global fibrillar strain.
Figure 6.6: Failure of cross-links in collagen fibril with 25 percent ECL. (a) Stress-strain curve of collagen fibril. (b) Percentage of broken AGEs cross-links. (c) Percentage of broken enzymatic cross-links. (d) Percentage of broken bonds in TC molecules.
Figure 6.7: Failure of cross-links in collagen fibril with 75 percent ECL. (a) Stress-strain curve of collagen fibril. (b) Percentage of broken AGEs cross-links. (c) Percentage of broken enzymatic cross-links. (d) Percentage of broken bonds in TC molecules.
for these biological variabilities in our model, so the collagen fibril is a generic representative.

Direct and quantitative comparison between our numerical results and experimental measurements on collagen fibrils \([49, 167, 177]\) are restricted by uncertainties in the measurements of fibril geometry caused by experimental procedures. Differences may occur due to the definition of the numerical force field deduced from full-scale simulations \([42]\), which relies on strong assumptions at the atomistic scale and might overestimate the strength of the collagen bonds. However, the absolute value of the force fields is not expected to affect the qualitative behavior of the collagen fibril.

Despite all of these limitations, the proposed model captures the relevant mechanical properties of the TC molecule and the various cross-links and provides a valuable qualitative description of the deformation and failure mechanisms inside collagen fibrils. Finally, we note that our model is limited to the scale of collagen fibril and thus we can only analyze the effect of AGEs density on the fibril. Intramolecular AGEs, which are present within the TC molecules or bonded to these, may also reduce the strength of the TC molecules and therefore affect the tissue properties (e.g., strength, stiffness). This AGEs contribution is beyond the scope of the present work and is left for future models.

### 6.4.2 Physiological implications of results

Collagen fibrils are a main building component of numerous tissues and their behavior directly affects the macroscopic mechanical properties of the tissue. For example in bone, researchers found that an increased glycation level with increased AGEs density is associated with increased brittleness and increased likelihood of fracture \([3, 4, 89, 155, 179, 180, 190]\). Fracture in the case of bone tissue is fundamentally different from fracture of collagen fibrils, despite using the same terminology. In bone, it refers to micro-cracking of the extracellular matrix, whereas in collagen fibrils, fracture simply results from the breaking of molecular bonds and sliding of the TC molecules. It has been commonly argued that an increased amount of AGEs causes brittleness of bone by inducing a stiffening of the collagen fibril and by preventing fibrillar sliding, which is assumed to be the natural energy dissipation mechanism \([3, 5, 216]\). Our results support this perspective of the cause for bone brittleness. In fact, our model provides a clear causal link between increased cross-linking within the collagen fibril and the occurrence of stiffening and strengthening of the fibril. The simulations demonstrate that both of these modifications of the fibrillar deformation mechanisms are caused by inhibited fibrillar sliding between TC molecules due to increased AGEs content. This suggests that energy dissipation is reduced because only little friction occurs, and, consequently the tissue appears to be more brittle. Hence, our results demonstrate that increased fibrillar cross-linking
with AGEs is directly responsible for bone brittleness. It is important to note that this brittleness occurs despite an increase in the work to failure, which is not an appropriate descriptor for fracture toughness. In contrast, our results clearly show that fibrils with higher AGEs content fail in a considerably more abrupt manner (see the post-peak slope in Fig. 6.2).

Aside from more brittle behavior of the collagen fibril, there are additional mechanisms contributing to an increased brittleness of bone. For instance, collagen might not be the origin of failure in bone with high AGEs cross-linking due to its increased strength. Instead, fracture initiation might occur in the mineral component that surrounds the collagen, due to load transfer to the mineral, as shown by Siegmund, Allen, and Burr [170] in their 2D FEM model. As minerals are known to be brittle, this would inherently increase the overall brittleness of the bone. To evaluate these contributions to impaired material properties of bone, future models should target a scale that combines collagen fibrils and minerals.

It is important to note that since these hypotheses for the origin of brittleness in bone with high AGEs content are based purely on numerical simulations, it would be important to provide experimental confirmation. For this, it would be advisable to mechanically test individual collagen fibrils with different cross-link densities. Such experiments would also provide data to better calibrate the numerical model and address some of the limitations mentioned above.

Aside from bone, the mechanical behavior of collagen also affects other tissues. For instance, tendons show increased failure stress and strain with increased AGEs content as well as an increased peak modulus on the fascicle and on the fibril level [177], which is also observed in our study (see Fig. 6.2). Studies on tendons [70, 107] further suggest that AGEs reduce tissue elasticity by limiting fiber-fiber and fibril-fibril sliding, which leads to increased fiber and fibril stretching, as also confirmed by our numerical observations. While this results in a loss of the post yield plastic behavior, the elastic modulus is not affected by increased AGEs contents. However, a recent study by Zellers et al. [213] argues that the change in mechanical behavior of tendon is rather caused by collagen disorganization than AGEs content, since no statistically significant relationship was observed between AGEs and mechanical parameters. This shows that further studies are needed at different scales to determine the origin of changes in mechanical parameters in tendon tissue with increased AGEs content.

Other tissues that appear to be affected by AGEs in their mechanical behavior include the following. In intervertebral discs, increased levels of AGEs are associated with functional changes such as increased stiffness, increased torque range and torque failure [103, 201]. Further, cartilage shows altered tensile properties, where an induction of AGEs leads to increased stiffness and strength and additionally to a decrease in failure length causing more severe brittleness [35, 193]. At the nano scale, however, increases in stiffness are caused by cartilage network embrittlement
All in all, there are many open questions regarding the root of impaired mechanical behavior in collageneous tissues, and despite its many limitations, our numerical results provide a fundamental insight into potential nano-scale origins of various tissue-related observations.

We performed in-silico tensile tests on collagen fibrils using a coarse-grained molecular simulations of a representative part of the fibril. We analyzed the effect of increased amounts of AGEs non-enzymatic cross-links on the mechanical properties of the collagen fibril. We found that higher AGEs cross-link densities cause increased strength and work to failure of the fibril. Furthermore, we discovered that the collagen fibril presents an increased stiffness for large strains at AGEs cross-link densities that exceed some critical level. We showed that this is valid for fibrils with varying contents of enzymatic cross-links. We demonstrated that the stiffening is the result of the AGEs acting as the main medium for force transfer between the TC molecules, which inhibits intrafibrillar sliding and causes the macroscopic deformation being dominated by the deformation of the (stiffer) TC molecules. Consequently, energy dissipation, which in fibrils with low cross-linking occurs mostly through friction as TC molecules slide against each other, is considerably reduced in collagen fibrils with dense AGEs cross-linking. Hence, our model results provide direct and causal evidence for the link between high AGEs cross-link content and impaired mechanical material properties (e.g., increased brittleness) in collageneous tissue, as commonly observed in aged and diabetic bone.

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ADVANCED GLYCATION END-PRODUCTS: HOW CROSS-LINKING PROPERTIES AFFECT THE COLLAGEN FIBRIL BEHAVIOR

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KEY FINDINGS:

• Higher bond breaking distance and stiffness cause collagen stiffening.
• The stiffening of the fibril is caused by reduced sliding between tropocollagens.
• Decreased sliding leads to less energy dissipation.
• When inter-molecular sliding is reduced, stretching of the tropocollagens increases.
• More stretching leads to energy absorption in the tropocollagens and brittle failure.

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1 This is a post-print of Kamml et al. [97], differing from the published paper only in terms of layout and formatting.
Advanced Glycation End-products (AGEs) are known to be a major cause of impaired tissue material properties. In collagen fibrils, which constitute a major building component of human tissue, these AGEs appear as fibrillar cross-links. It has been shown that when AGEs accumulate in collagen fibrils, a process often caused by diabetes and aging, the mechanical properties of the collagen fibril are altered. However, current knowledge about the mechanical properties of different types of AGEs, and their quantity in collagen fibrils is limited owing to the scarcity of available experimental data. Consequently, the precise relationship between the nano-scale cross-link properties, which differ from type to type, their density in collagen fibrils, and the mechanical properties of the collagen fibrils at larger scales remains poorly understood. In our study, we use coarse-grained molecular dynamics simulations and perform destructive tensile tests on collagen fibrils to evaluate the effect of different cross-link densities and their mechanical properties on collagen fibril deformation and fracture behavior. We observe that the collagen fibril stiffens at high strain levels when either the AGEs density or the loading energy capacity of AGEs are increased. Based on our results, we demonstrate that this stiffening is caused by a mechanism that favors energy absorption via stretching rather than inter-molecular sliding. Hence, in these cross-linked collagen fibrils, the absorbed energy is stored rather than dissipated through friction, resulting in brittle fracture upon fibrillar failure. Further, by varying multiple AGEs nano-scale parameters, we show that the AGEs loading energy capacity is, aside from their density in the fibril, the unique factor determining the effect of different types of AGEs on the mechanical behavior of collagen fibrils. Our results show that knowing AGEs properties is crucial for a better understanding of the nano-scale origin of impaired tissue behavior. We further suggest that future experimental investigations should focus on the quantification of the loading energy capacity of AGEs as a key property for their influence on collagen fibrils.

7.1 Introduction

The accumulation of Advanced Glycation End-products (AGEs) in the body is a major concern for elderly individuals and patients with diabetes [139]. It has been demonstrated that AGEs are responsible for a large number of negative effects, such as chronic kidney diseases or an increased risk of macrovascular and microvascular complications [45, 80, 145]. The presence of AGEs is also associated with impaired material properties in collagenous tissues, such as tendons, vertebrae, bones, or the cornea [61, 107, 162, 191]. For example, in experimental studies on bone, an increased AGEs content has been observed to be correlated with an increased fracture risk and bone brittleness at the tissue level [3, 89, 155, 179, 180, 190], and changes to the collagen fibril deformation mechanisms at the nano-scale [5]. In tendon, it has been demonstrated that the presence of AGEs altered stress relaxation behavior, failure stress and yield behavior of the tissue [107]. Furthermore, AGEs are
also suspected to cause alterations to the mechanical behavior of all types of tissue by changing hydration [8] and reducing tissue viscoelasticity through changes at the nano-scale [5, 70, 107, 211]. Based on these findings, it is commonly assumed that AGEs are an important source of reduced functionality in elderly and diabetic patients because these groups show increased AGEs content in their tissues [159]. However, the precise mechanisms by which AGEs affect tissue behavior and impair body function remain unknown.

Collagen fibrils, which constitute a fundamental component of tissue at the nanoscale, are highly susceptible to the accumulation of AGEs. Among the various types of collagen, collagen type I is the most abundant in the human body. Its fibrils exhibit a distinctive banding pattern, which is the result of the five-staggered assembly of tropocollagen (TC) molecules, each comprising three polypeptide helices (see Fig. 7.1a,b). The non-enzymatic glycation process, known as the Maillard reaction, leads to the formation of AGEs at lysine residues of the TC molecules in the presence of sugars. Previous studies have demonstrated that the accumulation of AGEs alters the mechanical properties of collagen fibrils [8, 70, 97]. However, the specific mechanisms caused by these changes within the fibril structure remain poorly understood. Since the macroscale behavior of the tissue is directly influenced by the behavior of collagen fibrils, understanding the effects of AGEs at the nanoscale is crucial for uncovering alterations in tissue behavior caused by AGEs accumulation.

The correlation of AGEs density and altered collagen behavior has been shown in several studies [8, 52, 70, 107], but AGEs occur in different types and functions [98, 140, 163, 166]. Concerning their functions, we distinguish between cross-linking and non-cross-linking AGEs, where AGEs can either accumulate intramolecular, bonding to a single TC molecule, or as intermolecular bonds, cross-linking two or more TC molecules within the collagen fibril. Both non-crosslinking and crosslinking AGEs affect the collagen properties but in different ways. The non-crosslinking AGEs may cause changes in the tissue metabolism and protein function, whereas the cross-linking AGEs affect the mechanical behavior of the collagen fibril at the nanoscale, which may lead to changes at the tissue level. A computational study has shown that glucosepane, the most abundant AGE in tissue, may cause fibrillar stiffening [97], but otherwise, there is very limited information about their exact quantity and location, since common imaging techniques do not provide access to the nanoscale within the collagen fibril. Glucosepane is a lysine-arginine cross-link, which, based on molecular dynamic studies, has been shown to have 6 to 8 potential binding sites for cross-links along the TC molecules [37, 64]. Their positioning may not only influence mechanical properties of collagen, but have significant effects on tissue function and integrity due to their locations being key collagen biomolecular sites. Hence, it remains unknown whether AGEs cross-linking is causative to the observed impairment of mechanical properties in the tissue, as the exact relations
Several types of AGEs cross-links have been identified so far, e.g., glucosepane, pentosidine, GLUCOLD, crossline, vesperlysine, GOLD and MOLD [125, 128, 157, 159, 177, 196, 207]. Each of these AGEs presents a different structural configuration and, therefore, has different mechanical properties, e.g., strength and stiffness. While various experimental studies have shown the influence of AGEs content on the mechanical properties of collagenous tissues [5, 176, 211, 213], it is unclear which type of AGEs is responsible for these observations. Specifically, there is no complete information about the content of the various types of AGEs in these experiments, and hence, current studies cannot link the mechanical property of the collagen fibril to a specific AGE or a specific (mechanical) AGE property. In other words, knowledge about how AGEs cross-link properties translate to the fibril behavior remains missing, which prevents quantitative prediction of acceptable AGEs content per type for healthy collagen fibril mechanics.

In this paper, we study the link between the mechanical properties of AGEs cross-links at the nanoscale and the properties of the collagen fibril at the larger scale to evaluate how different types of AGEs cross-links influence the collagen behavior. We perform destructive tensile tests on a 3D coarse-grained steered molecular dynamics model of the collagen fibril with randomly inserted cross-linking in the helical regions of the TC molecules mimicking the contribution of AGEs. The properties of the AGEs cross-links, including stiffness and critical fracture length are varied systematically to account for differences in the structure of different AGEs cross-links. We quantify their effect on the mechanical properties of the collagen fibril at various cross-link densities with particular focus on fibril stiffness and strength, but most importantly, we show their influence on the deformation and fracture mechanism of the fibril on the molecule level.

7.2 MATERIAL AND METHODS

We build a 3D coarse grained model of a representative collagen fibril and perform tensile tests using steered molecular dynamics. AGEs cross-links are inserted randomly along the TC molecules at different densities and we vary cross-link parameters, namely stiffness and critical bond breaking length. The model is based on our previous study on the influence of cross-link density on the mechanical properties of the collagen fibril [97]. Our fibril geometry was implemented without enzymatic cross-links, since we previously showed that additional enzymatic cross-links do not influence or modify fibril mechanics in a crucial way [97]. In our approach, TC molecules are represented by particles arranged in a chain and we apply particle interactions according to multi-body potentials. The chains are placed in a staggered fashion to form a collagen fibril that is consistent with their
Figure 7.1: Schematic overview of model implementation and evaluation of deformation mechanisms. (a) Hodge-Petruska Model for collagen fibril: Displaying characteristic banding pattern with gap and overlap zones of TC molecules. (b) Coarse grained molecular dynamics model: The mechanical behavior of the TC molecules is represented by a string of particles mimicking the mechanical response that has been extracted from full scale simulations [42]. (c) Schematic representation of our implementation of a representative collagen fibril geometry: 5 gap and overlap zones; AGEs cross-links were randomly inserted between TC molecules (red) with different densities; fibril is strengthened at the ends (blue area) to guarantee smooth force transmission. (d) Definition of bond interactions (collagen bonds in TC molecules and AGEs cross-links): trilinear bond behavior, where the force depends on the distance \( r \) between two particles. The varied parameters in our simulations are \( k_1 \) and \( r_{break} \); the loading energy capacity of a single bond, e.g., \( W_{AGE} \) indicated by orange area. (e) Schematic representation of original configuration between two staggered TC molecules before tensile testing is started. (f) Sliding between TC molecules during tensile testing: AGEs cross-links rupture, the sliding of the TC molecules is responsible for energy dissipation in the collagen fibril, and the gap length \( g \) is increased by \( \Delta g \). (g) Stretching of the TC molecules during tensile testing: Load transmission to the TC molecules, AGEs withstand the force, eventually fracture of TC molecules.
physiological arrangement (see Fig. 7.1a-d) [28, 29, 42, 97]. For a consistent overview of the applied parameters in the coarse-grained model, please refer to Tab. 7.1 and Appendix A.3.

7.2.1 Geometry of the collagen fibril and insertion of cross-links

The geometry of the collagen fibril model is built to represent the biological configuration of collagen type I in tissue. Specifically, the TC molecules are arranged in a 5-staggering pattern with gap \((0.6 \cdot D)\) and overlap \((0.4 \cdot D)\) zones and a periodicity of \(D = 67 \text{ nm}\), also represented in Fig. 7.1a-d. One period with 5 gap- and overlap zones is used as a representative part of the collagen fibril (see Fig. 7.1c). The TC molecules are represented by chains of particles where the particle-interaction potential models the mechanical behavior of the molecules (see Fig. 7.1b). Each of these particle chains has a length of 300 \text{ nm}\) and a diameter of about 1.5 \text{ nm}\), represented by the dispersive parameter \(\sigma = 1.472 \text{ nm}\). The geometry of the single TC molecules was obtained from Protein Data Bank entry 3HR2, the atomistic model based on X-ray crystallography [134]. In total, 218 particles are placed equidistantly \((r_0 = 14.0 \text{ Å})\) along a spline which was fitted along the longitudinal direction of the TC molecule. In nature, collagen fibrils are bundles of TC molecules with a diameter between 20 and several hundred nanometers [86]. The modeled representative part of the collagen fibril has a diameter \(d = 20.2 \text{ nm}\) and includes 155 TC molecules aligned through the cross section along the longitudinal axis. At the ends of the collagen fibril specimen, where forces are applied, the TC molecules are extended with 40 additional particles with strengthened bonds to guarantee smooth force transition.

AGEs cross-links, which are at the focus of our study, are naturally built via glycation between the centrally located helical regions of the TC molecules. In our model, we insert AGEs cross-links randomly between the central 95\% of particles of two TC molecules, since the exact locations of AGEs in physiological collagen fibrils remains generally unknown. Exceptions are limited to studies of few individual types of AGEs (e.g., glucosepane) that determined their location [37, 64]. The insertion process consists of a random selection of particles in the respective parts of each TC molecules. Specifically, we make sure that the distance with a neighboring TC molecule is smaller than a certain threshold and that the particle is not already cross-linked. Further details about the precise insertion procedure of the cross-links are provided in Kamml et al. [97]. The fibril geometry used for all simulations shows a representative fibril mechanical behavior for all examined cross-link densities (see Appendix A.5). To explore the effects of AGEs cross-link density on the mechanical performance of the collagen fibril, and to account for their cumulative presence, we vary the inserted density. The exact number of AGEs in physiological collagen is unknown for most types of AGEs, except for glucosepane, the most
prominent AGE, which reaches levels of 2000 pmol/mol in collagen tissue of 90 years old patients, and \( \sim 4500 \) pmol/mol in diabetic patients. This corresponds to one AGE every 5 molecules and 2 molecules, respectively [166]. However, many other types of AGEs, which have not been quantified so far, co-exist in collagen fibrils, and, hence, the exact numbers of cumulative AGEs cross-links is unknown. Finally, we note that for a given cross-link density, we use the exact same cross-link location when we vary the mechanical properties of the AGEs cross-links to separate deterministic from statistical effects, and all collagen fibril models were visually controlled in order to avoid concentration batch effects.

### 7.2.2 AGEs cross-link parameters

Table 7.1: Parameters used in coarse-grained molecular dynamics mesoscale model of collagen fibrils. Reference values for glucosepane are indicated in grey.

<table>
<thead>
<tr>
<th>Components</th>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGEs Cross-links</td>
<td>Equilibrium particle distance ((r_0, \text{Å}))</td>
<td>18.52</td>
</tr>
<tr>
<td></td>
<td>Critical hyperelastic distance ((r_1, \text{Å}))</td>
<td>22.72</td>
</tr>
<tr>
<td></td>
<td>Bond breaking distance ((r_{break}, \text{Å}))</td>
<td>25 - 27 - 30 - 33 - 35 - 40 - 50 - 60</td>
</tr>
<tr>
<td></td>
<td>Glucosepane: Bond breaking distance ((r_{break}, \text{Å}))</td>
<td>31.72</td>
</tr>
<tr>
<td></td>
<td>Tensile stiffness parameter ((k_0, \text{kcal, mol}^{-1}\text{Å}^{-2}))</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Tensile stiffness parameter ((k_1, \text{kcal, mol}^{-1}\text{Å}^{-2}))</td>
<td>6 - 8 - 12 - 16 - 24</td>
</tr>
<tr>
<td></td>
<td>Glucosepane: Tensile stiffness parameter ((k_1, \text{kcal, mol}^{-1}\text{Å}^{-2}))</td>
<td>8.00</td>
</tr>
</tbody>
</table>

The total energy of the coarse-grained model is the sum of all pairwise particle interactions, as described by:

\[
E_{total} = E_{bond} + E_{angle} + E_{inter} = \sum_{bond} \Phi_{bond}(r) + \sum_{angle} \Phi_{angle}(\phi) + \sum_{inter} \Phi_{inter}(r) \tag{7.1}
\]

where \(E_{bond}\) is the bond energy due to stretching, \(E_{angle}\) the dihedral bond interactions energy due to bending (for further description, see Appendix A.3), and \(E_{inter}\) the pairwise interaction energy due to molecular interactions such as Van-der-Waals forces. Specifically, the bond energy represents the particle interactions though fixed interactions, as they occur within TC molecules, but also between them as cross-links. In our model, there are two different types of bonds, which contribute to the bond energy via

\[
\sum_{bond} \Phi_{bond}(r) = \sum_{collagen} \Phi_{collagen}(r) + \sum_{AGE} \Phi_{AGE}(r) \tag{7.2}
\]
where $\Phi_{\text{collagen}}$ is the bond potential between particles within any given TC molecule chain, and $\Phi_{\text{AGE}}$ is the bond potential of AGEs cross-links, linking particles between two adjacent TC molecules. The AGE cross-link force between two TC molecules is then given by

$$F_{\text{AGE}}(r) = -\frac{\partial \Phi_{\text{AGE}}(r)}{\partial r}, \quad (7.3)$$

where $r$ is the radial distance. In our model, AGEs forces are modeled as trilinear springs as follows

$$F_{\text{AGE}}(r) = \begin{cases} 
-k_0(r - r_0) & \text{if } r < r_1 \\
-k_1(r - r_0) & \text{if } r_1 \leq r < r_{\text{break}} \\
z \cdot k_1(r - r_0) & \text{if } r_{\text{break}} \leq r < r_{\text{break}} + a \\
0 & \text{if } r \geq r_{\text{break}} + a 
\end{cases} \quad (7.4)$$

where $r_0$ is the equilibrium distance between two linked particles, $k_0$ and $k_1$ are spring constants of the deformation, and $a$ is defined as $a = z \cdot (r_{\text{break}} - r_1)$. The chosen tri-linearity accounts for breaking of the bond, which occurs at $r_{\text{break}}$, and a gradual transition to its broken state via a regularization factor $z$ [97]. All other bond forces in our model follow the same trilinear behavior but with different parameters, as detailed in Appendix A.3.

We perform a parameter study and vary strength and stiffness of AGEs cross-links. As a reference, we use the approximated values of glucosepane obtained from full-scale simulations with a reactive force field [39, 97]. We then vary stiffness and bond breaking distance (the inter-particle distance at which AGEs cross-links break), since these two values define the loading energy capacity of AGEs cross-links, meaning how much force it can withstand while stretching. The full range of applied AGEs parameters are displayed in Tab. 7.1.

### 7.2.3 Simulations

The simulations of the destructive tensile tests are performed in LAMMPS [141]. The same procedure is applied as in our previous study [97] using steered-molecular dynamics at a constant velocity of 0.0001 Å/fs ($= 10m/s$) with a time step of $\Delta t = 1$ fs. We verified in our previous study that lower strain rates do not lead to qualitative differences in simulation results [97]. The two ends of the fibril are constrained and moved apart during the tensile tests, and the required force is calculated and converted into engineering stress.
Figure 7.2: Stress-strain curves of destructive tensile tests of representative collagen fibril with different AGEs cross-link densities and varying AGEs tensile stiffness $k_1$ and critical breaking length $r_{break}$. The effect of glucosepane corresponds approximately to (b).
7.3 Results

7.3.1 Deformation and strength behavior

In our simulations, we vary the AGES cross-links stiffness $k_1$ and the bond breaking distance $r_{\text{break}}$, which is the inter-particle distance at which AGES cross-links break. We investigate the effect of changing these parameters on the mechanical properties of collagen fibrils with different AGES densities (see Fig. 7.2). Our results show that various aspects of the macroscopic fibril behavior change significantly with changing stiffness and bond breaking length of the cross-links. Generally, the stress-strain curves show a two-phase behavior as observed in previous works [97]: at low strains $\varepsilon < \varepsilon_0 \approx 0.15$, where $\varepsilon_0$ defines the limit of the linear elastic regime, the fibril presents a linear deformation behavior. This initial linear behavior is independent of the cross-link parameters and density, i.e. the fibril stiffness is the same for all cases. Beyond the limit of $\varepsilon_0$, the fibril shows different mechanical behavior depending on stiffness $k_1$ and bond breaking length of the cross-links. At low $N_{\text{AGE}}$ (0-1 AGES/TC) and with low $k_1 < 24$ and $r_{\text{break}} < 30$ (see Fig. 7.2a, b, c, d, e, f, g, h, k, j), we observe a softening mechanism in the fibrils, where the stiffness reduces continuously until it does not have any remaining load bearing capacity, and hence failed. With larger $k_1$ and $r_{\text{break}}$ (e.g., Fig. 7.2i, l) at the same $N_{\text{AGE}}$ (i.e. 0-1 AGES/TC) the behavior is slightly different, where the stiffness only reduces temporarily, but then increases further to finally reach the maximum stress before failing somewhat more abruptly. This suggests that this type of AGES may activate a different deformation mechanism of the fibril. Finally, fibrils with high AGES densities, i.e. $N_{\text{AGE}} = 2 - 40$ AGES/TC, show a very different post-$\varepsilon_0$ behavior that is strongly dependent on stiffness $k_1$, breaking distance $r_{\text{break}}$ and cross-link density $N_{\text{AGE}}$. At low $N_{\text{AGE}}$ (0-1 AGES/TC) and with low $k_1 < 24$ and $r_{\text{break}} < 30$, the fibril shows a failure behavior with a smooth softening mechanisms, whereas stiff and strong AGES may lead to fibrillar stiffening at $\varepsilon > \varepsilon_0$ depending on $N_{\text{AGE}}$.

These results show clearly that the AGES properties, specifically their stiffness and critical failure length, have direct effects on the mechanical properties of the collagen fibril, and the extent of these effects depends on $N_{\text{AGE}}$. For example, the peak stress $\sigma_{\text{peak}}$ increases with increasing $k_1$, $r_{\text{break}}$ and $N_{\text{AGE}}$, see Fig. 7.2 and Fig. 7.3. Furthermore, $\sigma_{\text{peak}}$ presents saturation with $k_1$ and $r_{\text{break}}$, where further increases in $k_1$ and $r_{\text{break}}$ do not lead to higher $\sigma_{\text{peak}}$, i.e. $\sigma_{\text{peak}} \leq \sigma_{\text{sat}}$. This saturation limit depends on the combination of $k_1$ and $r_{\text{break}}$. For example at $N_{\text{AGE}} = 2$AGES/TC (see Fig. 7.3a), the saturation is reached at $r_{\text{break}} \approx 45$ at $k_1 \approx 5.0$, but with increasing $k_1 \approx 16$, $\sigma_{\text{sat}}$ is already reached at $r_{\text{break}} \approx 35$. Similar trends can be observed for other AGES densities (see Fig. 7.3). However, we note that first, $\sigma_{\text{sat}}$ depends strongly on $N_{\text{AGE}}$ and increases for higher $N_{\text{AGE}}$ (see Fig. 7.3). Second, the saturation limit moves to lower $k_1$ and $r_{\text{break}}$ values.
Figure 7.3: Peak stress $\sigma_{\text{peak}}$ of collagen fibril for varying tensile stiffness $k_1$ and bond breaking distance $r_{\text{break}}$ of AGEs cross-links at (a-d) AGEs densities of 2, 5, 10, 40 AGEs/TC, respectively.
Figure 7.4: Elastic to peak stress change $\Delta \sigma$ of collagen fibril for varying tensile stiffness $k_1$ and bond breaking distance $r_{\text{break}}$ of AGEs cross-links at (a-d) AGEs densities of 2, 5, 10, 40 AGEs/TC, respectively. Dash-dotted line indicates theoretical limit for the existence of the stiffening regime, following Eq. 7.6.
The appearance of the stiffening regime with changing parameters is important, since this demonstrates a change in mechanical behavior of the collagen fibril. To characterize the onset of the stiffening regime, to evaluate its effect on $\sigma_{\text{peak}}$, and to analyze the AGEs properties causing it, we computed the elastic-to-peak stress difference $\Delta\sigma = \sigma_{\text{peak}} - \sigma_0$, with $\sigma_0 = \sigma(\varepsilon_0)$. Accordingly, $\Delta\sigma \geq 0$ indicates stiffening of the fibril, and $\Delta\sigma \approx 0$ corresponds to a fibril that does not stiffen before failure. Furthermore, a larger $\Delta\sigma$ indicates more pronounced stiffening, and since the linear regime is independent of the AGEs density, this also means that such a fibril presents a larger $\sigma_{\text{peak}}$ (compare Fig. 7.3 with Fig. 7.4). In general, we only observe significant stiffening for $r_{\text{break}} > 25\text{Å}$ and for fibrils with $N_{\text{AGE}} \geq 2\text{AGEs}/\text{TC}$ (see Fig. 7.4).

In our previous work [97], we showed that the stiffening of collagen fibrils with high AGEs density occurs because a high number of AGEs leads to small average forces within the AGEs and hence small AGEs deformation, which results in large deformations of the TC molecules. In other words, high AGEs density causes force transmission to be predominantly through the TC molecules (see Appendix A.4), and hence fibrillar stiffness approaches the stiffness of the TC molecules. Whether this transition to TC-deformation-governed state occurs is the result of the relative stiffness between the AGEs and the TC molecules, which explains the above-discussed effect of $k_1$ on the stiffening limit (see Fig. 7.4). However, relative-stiffness solely is not sufficient to explain the link between the AGEs properties and their effect on the fibril behavior. If the AGEs are weak, fibrillar stiffening cannot occur because the AGEs break before they can activate the TC-deformation-governed state. Hence, a criterion combining both AGEs properties, $k_1$ and $r_{\text{break}}$, is required to describe the stiffening limit of collagen fibrils with different types of AGEs. Here, we suggest that this criterion is expressed in terms of energy capacity, which is defined as

$$W(k_0, k_1, r_0, r_1, r_{\text{break}}) = k_0/2 (r_1 - r_0)^2 + k_0 (r_1 - r_0) (r_{\text{break}} - r_1) + k_1/2 (r_{\text{break}} - r_1)^2,$$  \hspace{1cm} (7.5)

as also shown in Fig. 7.1d, and which is used to compute the loading energy capacity $W_{\text{TC}}$ and $W_{\text{AGE}}$ of the TC molecules and AGEs cross-links, respectively.

We argue that the balance between the energy capacity of a TC molecule and its attached cross-links, as expressed by

$$W_{\text{TC}} = N_{\text{AGE}} \cdot W_{\text{AGE}}$$  \hspace{1cm} (7.6)

provides a reasonable criterion to describe the occurrence of collagen fibrillar stiffening (see the grey line in Fig. 7.4a-d). We observe that this balance is consistent with the onset of the stiffening regime. Specifically, for $W_{\text{TC}} < N_{\text{AGE}} \cdot W_{\text{AGE}}$ the
TC-deformation-governed regime is activated. This observation suggests that the macroscopically observed changes are a direct result of modifications to the deformation mechanism at the nanoscale as caused by the presence of AGEs cross-links, which is best described in terms of relative loading energy capacity. It can be expected that such changes also effect energy absorption in the collagen fibril and, hence, their failure mechanism.

### 7.3.2 Failure mechanisms, energy dissipation and toughness

In Sec. 7.3.1, we showed that macroscopic changes in the fibrillar stiffness and strength are the result of different deformation mechanisms at the inter fibrillar level. Similar reasoning should also apply to the failure mechanism of the collagen fibril. It has been suggested that the deformation and failure mechanism is directly affected by various types of energy absorption in the collagen fibril, which are more or less dominant depending on the AGEs density [3, 5, 52, 70, 176, 216]. The two most important mechanisms for failure are stretching of the TC molecules, and energy dissipation through sliding between TC molecules.

We compute the various contributions to the fibrillar energy absorption to determine their overall implication in the failure process of the collagen fibril. The energy being absorbed in stretching of the TC molecules at a given global strain level \( \varepsilon \) is given by

\[
E_{\text{stretch}}(\varepsilon) = \int_{\Delta r_{TC}(\varepsilon)}^{\Delta r_{TC}(\varepsilon^*)} F_{\text{stretch}} d\Delta r_{TC} ,
\]

where \( F_{\text{stretch}} \) is the average force within TC bonds, directly computed in the simulations, and \( \Delta r_{TC} \) is the average change in length per collagen bond with respect to the equilibrium bond length \( (\Delta r_{TC} = r_{TC} - r_0) \) at a specific global strain level \( \varepsilon \). We compute energy absorption by TC stretching starting from \( \varepsilon^* \), where fibrillar strain is equal to the average strain in the collagen fibril \( (\varepsilon = \varepsilon_{TC}) \), which is approximately the transition point between TC unfolding and TC stretching-governed deformation mechanism.

Further, we estimate the cumulative energy dissipated through sliding using

\[
E_{\text{slide}}(\varepsilon) = \int_{\Delta g(\varepsilon^*)}^{\Delta g(\varepsilon)} F_{\text{slide}} d\Delta g ,
\]

where \( \Delta g \) is the change in gap size (see Fig. 7.1e-f) and \( F_{\text{slide}} \) is the force due to friction of TC molecules. We note that this is a heuristic measure for sliding dissipation, which we use for simplicity. Specifically, the force \( F_{\text{slide}} \) is the average force per TC molecule within the fibril that is not arising from bond forces of collagen bonds or cross-links and, hence, it is an estimate calculated from the globally applied tensile force \( F \) on every TC molecules reduced by the forces due to
stretching of the molecule. The change in gap size $\Delta g$ was obtained from the global collagen fibril strain $\varepsilon$ by approximating it with the average change in bond and gap length as

$$
\varepsilon = \frac{\Delta g + n_{TC} \cdot \langle (r_{TC} - r_0) \rangle}{\langle g \rangle + n_{TC} \cdot r_0},
$$

(7.9)

where $n_{TC}$ is the number of bonds per TC molecule, $\langle g \rangle$ the average initial gap length, and $\langle (r_{TC} - r_0) \rangle$ the average change in length of the collagen bonds (see Fig. 7.1e-g).

First, we consider the effect of AGEs cross-link properties on the fibrillar energy absorption mechanisms. Specifically, we compute the energy absorption that occurs until failure initiates using Eq. 7.7 and 7.8 with $\varepsilon = \varepsilon_{peak}$, where $\sigma_{peak} = \sigma(\varepsilon_{peak})$. We normalize the respective energies with $E_{slide}^0$ and $E_{stretch}^0$, the energies of a collagen fibril with an AGEs density of $N_{AGE} = 0$, as it provides an AGEs-parameter-free reference value, and is expected to be the most extreme sliding case. We observe that $E_{slide}$ and $E_{stretch}$ are strongly dependent on both $r_{break}$ and $k_1$ (see Fig. 7.6a-d and 7.5a-d). Following the arguments presented in Sec 7.3.1, we suggest that the most important mechanical property of the AGEs cross-link is the loading energy capacity $W_{AGE}$, which combines $r_{break}$ and $k_1$. If $E_{slide}$ and $E_{stretch}$ are reported with respect to $W_{AGE}$, the energy absorption is well described and can be directly linked to the AGEs property and the AGEs density (see Fig. 7.6e-h and 7.5e-h). Nevertheless, we note some limited scattering of the data, in particular in the sliding energy at low $N_{AGE}$ (e.g., Fig. 7.5e), which is likely due to the used heuristic measure that estimates the forces on the TC molecule due to sliding via force balance. Hence, tetrahedral forces, for instance, cannot be excluded from this estimate, and contribute indirectly to the sliding energy. These effects are stronger for cases with lower $N_{AGE}$ because overall forces in the fibril are lower. Despite this imprecision, the overall trend and importance of the AGEs energy capacity $W_{AGE}$ for the fibrillar behavior is evident, and allows us to describe and compare the effect of the stretching or sliding mechanisms on the collagen failure properties.
Figure 7.5: Sliding energy in collagen fibrils for different types of AGEs. (a-h) Sliding energy $E_{\text{slide}}$ computed up to global strain at peak stress $\varepsilon_{\text{peak}}$ following Eq. 7.8 normalized by the sliding energy $E_{\text{slide}}^0$ of a collagen fibril with 0 AGEs per TC molecule. (a-d) The normalized sliding energy is shown as function of AGEs tensile stiffness $k_1$ at varying $r_{\text{break}}$ for different AGEs densities, respectively. (e-h) The normalized sliding energy is shown as function of the AGEs loading energy capacity $W_{\text{AGE}}$ for different AGEs densities, respectively.
Figure 7.6: Stretching energy in collagen fibrils for different types of AGES. (a-h) Stretching energy $E_{\text{stretch}}$ computed up to global strain at peak stress $\varepsilon_{\text{peak}}$ following Eq. 7.7 normalized by the stretching energy $E_{\text{stretch}}^0$ of a collagen fibril with 0 AGES per TC molecule. (a-d) The normalized stretching energy is shown as function of AGES tensile stiffness $k_1$ at varying $f_{\text{break}}$ for different AGES densities, respectively. (e-h) The normalized stretching energy is shown as function of the AGES loading energy capacity $W_{\text{AGE}}$ for different AGES densities, respectively.
For further evaluation of the influence of different $N_{AGE}$ and $AGEs$ cross-link properties, we report the summarized and normalized data for stretching and sliding energy in Fig. 7.7. We see that sliding and stretching at lower levels of $W_{AGE}$ are dependent on both parameters (indicated by the dotted lines) up to a certain saturated value that is only dependent on $N_{AGE}$ (indicated by the dashed lines). The failure mechanism is either dominated by sliding or stretching and the relation between the two (compare Fig. 7.7a&b): We observe that with decreasing normalized sliding energy $E_{slide}$, normalized stretching energy $E_{stretch}$ increases, representing the relation of sliding and stretching as failure mechanism. At lower levels of $W_{AGE}$ and $N_{AGE}$ sliding is dominant whereas stretching is very limited. With both parameters increasing, sliding becomes less pronounced and stretching of the TC molecules is the more dominant mechanism. Both mechanisms then reach a saturation level dependent on the $N_{AGE}$ of the fibril. This shows that higher levels of $N_{AGE}$ and $W_{AGE}$ cause stiffening within the collagen through reduction of sliding between the TC molecules and by increasing their stretching. We note that since the sliding energy is expected to decrease with higher $N_{AGE}$, the reported $E_{slide}/E_{slide}^0$ should theoretically satisfy $<1$. While most of our reported $E_{slide}$ are consistent with this limit, some results for cases with $N_{AGE} = 2$ exceed it (see Fig. 7.7a). The likely cause is our heuristic approach to calculate $E_{slide}$, as discussed above, this may lead to relatively significant errors in low force situations such as fibrils with $N_{AGE} = 2$.

Since the induced stretching might eventually lead to fracture of the collagen bonds within the TC molecules, we evaluated the quantities of broken $AGEs$ cross-links and broken collagen molecules in a collagen fibril at failure. Higher numbers of broken TC molecules indicate more abrupt failure of the collagen fibril. We observe a pronounced decreasing trend of broken $AGEs$ with increasing energy capacity of $AGEs$ (see Fig. 7.8a). While the numbers of broken $AGEs$ is dependent on $N_{AGE}$ for low energy levels, this trend vanishes for increasing energy levels. The decrease in broken $AGEs$ cross-links with increasing load capacity is correlating with an increase in the number of broken TC bonds (see Fig. 7.8b). In fibrils, where their stretching has been fully activated due to the force transmission to the TC molecules, the saturated level of TC bonds to break is reached once their energy capacity is depleted. This results in fracture of the TC molecules, not the $AGEs$ cross-links. At high energy capacities, the percentage of broken bonds in the TC molecule is highly dependent on $N_{AGE}$, due to the load transfer from $AGEs$ to TC molecules (see Appendix A.4). In summary, our results show that higher levels of $N_{AGE}$ and $W_{AGE}$ cause stiffening within the collagen fibril through reduction of sliding and enhancing of stretching of the TC molecules’ backbone. This change in deformation mechanism is eventually causing fracture of the collagen bonds in the TC molecules by force transmission from $AGEs$ cross-links to the TC molecules (see Appendix A.4).
On the macroscopic fibril level, we observe that fibrils that reach the stiffening regime, and hence present a high peak stress, also appear to fail in a significantly more brittle manner, as also noted in our previous study \[97\]. This behavior manifests itself as steep post-peak slopes in the stress-strain curves of the fibril (e.g., see red and black curves in Fig. 7.2c,f,i,l). To estimate the fracture toughness of collagen fibrils, we consider the energy dissipated through sliding instead of the commonly applied measure of work to failure, which is not an appropriate measure for brittleness. Accordingly, our results show that the sliding energy, and hence collagen fibril toughness, decreases with \(W_{AGE}\) and \(N_{AGE}\), with the latter being the determining factor for the extent of this reduction. Since, such a deformation mechanism leads to more stretching of the TC molecules, their absorbed energy causes higher energy release at fibril rupture, resulting in a brittle failure of the entire collagen fibril. The \(W_{AGE}\) accounts for different types of AGES and we see, that especially at low levels of \(N_{AGE}\), the type of AGE has a significant influence on the collagen deformation and failure mechanism by changing intrafibrilar deformation mechanisms.

Figure 7.7: Energy absorption in collagen fibril. (a) Dissipated energy through sliding \(E_{slide}\) normalized by \(E_{0}^{slide}\), where \(E_{0}^{slide}\) is the reference energy dissipated by sliding of fibrils with 0 AGES. Dotted lines fitted to data up to minimum value of \(E_{slide}/E_{0}^{slide}\). Dashed lines show the saturated value, fitted to data from minimum value of \(E_{slide}/E_{0}^{slide}\). (b) Energy \(E_{stretch}\) absorbed by stretching of the bonds within the TC molecules normalized by \(E_{0}^{stretch}\), where \(E_{0}^{stretch}\) is the reference energy absorbed via stretching of fibrils with 0 AGES. Dotted lines linearly fitted to data where \(E_{slide}/E_{0}^{slide} < 0.75 \max(E_{slide}/E_{0}^{slide})\). Dashed lines linearly fitted to data where \(E_{slide}/E_{0}^{slide} > 0.75 \max(E_{slide}/E_{0}^{slide})\).
7.4 Discussion

7.4.1 General limitations

With our model of a simplified collagen fibril, we can directly observe the effects of critical parameters of AGEs cross-links on the mechanical response of the fibril during tensile testing. Nevertheless, in physiological conditions numerous other factors contribute to its varying mechanical behavior. For example, tissue hydration regulated via osmotic pressure is possibly a crucial factor of collagen mechanics in the body [8], which we did not account for. Another factor influencing the mechanical properties of the collagen fibril in addition to AGEs are enzymatic cross-links, which form via an enzymatic reaction mediated by lysil oxidase between the telopeptide ends of the TC molecules and the helical domain of adjacent molecules [158]. Since AGEs are prone to occur in mature tissue, enzymatic cross-links are naturally present. In the absence of AGEs cross-links, the quantity of enzymatic cross-links controls strength and stiffness of the collagen fibril [28, 42, 176]. However, in the presence of AGEs cross-links, as previously studied [97], it was observed that enzymatic cross-links have a minor quantitative influence on the onset of the stiffening regime caused by AGEs cross-links. Consequently, enzymatic cross-links have not been included in this study.

Figure 7.8: Broken bonds after failure of collagen fibril with AGEs cross-links. (a) Percentage of broken AGEs cross-links and (b) ratio of broken collagen bonds with respect to number of TC molecules, shown as function of the loading energy capacity $W_{AGE}$ of AGEs. Broken bonds are counted after failure of the collagen fibril. The ratio may exceed 100% because a given TC molecule can break more than once.
Further, we note that our model considers AGES distribution as random, since, with the exception of one specific AGE (glucosepane) [64], the exact location of AGES along the TC molecule remains still unknown. In general, the lack of information about different types and, more importantly, quantities of AGES cross-links present in specific tissues is a major problem. Currently, it is known that there are more than a dozen AGES cross-link types that might occur in different combinations and quantities depending on the tissue type, but their mechanical properties are mostly unknown. Given the limited available information, we used glucosepane ($k_1 = 8.00; t_{break} = 31.72$, see Tab. 7.1), one of the most abundant cross-links, as a reference case and varied the mechanical properties of the cross-links with respect to it. The underlying idea is that the difference between various types of AGES cross-links manifests itself as changes in their mechanical properties such as stiffness, equilibrium length, critical hyperelastic length, and bond breaking length.

As we have shown here, the critical AGES cross-link property for the fibril behavior is the loading energy capacity $W_{AGE}$ of the AGES cross-links, which combines all of these factors. Furthermore, the AGES densities applied in our study exceeds the measured AGES quantities for a single type of AGES [166]. However, the observed mechanical behavior in our numerical model is still likely to occur, since 1) there are more than one type of AGES simultaneously present within the collagen fibril, and their effects add up, and 2) other types of AGES with higher loading capacity $W_{AGE}$ cause similar effects to the fibril at lower AGES densities, as we have shown here.

Our results show that, apart from cross-link density $N_{AGE}$, the loading energy capacity $W_{AGE}$ of the AGES cross-links, a nanoscale property, is the second contributing factor to changes in fibril deformation behavior. We found that the AGES cross-link properties affects the level of AGES density at which the collagen fibril experiences stiffening and how abrupt it fails, which depends on the energy absorption mechanism activated by the AGES cross-links. Although our approach to estimate energy absorption via sliding is only heuristic, given the available information, it still allows us to qualitatively characterize the deformation and energy absorption mechanism, and it enabled us to demonstrate a causal link between an absence of frictional dissipation and fibril brittleness. Nevertheless, we note that an experimental validation of our results is needed to link our qualitative findings to quantitative observations in physiological tissue. Finally, we should also note that collagen tissue is not only built from collagen I, but also other types of collagen. This might additionally have influence on the material behavior at larger length scales and, hence, should be considered when applying our results to larger models.

In biological conditions, AGES density and type, and hence their properties, are also dependent on their host tissue. Specifically, the number of present AGES is dependent on the half life or turnover of the tissue, since AGES form and accumulate
during aging when tissue is exposed to increased glycation levels. This increase of \( \text{AGEs} \) on the nano-scale is likely to influence tissue mechanical properties on the macro-scale. The variability of collageneous tissues is wide and the unknown quantity of \( \text{AGEs} \) (cross-links) and types, apart from other contributing factors, prohibit currently any quantitative statements about collagen fibril mechanics. Nevertheless, our model reveals the general trends and the mechanisms caused by increased \( \text{AGEs} \) content and varying \( \text{AGEs} \) mechanical properties, and allows for interpretation of their influence on the macro-scale.

In diabetic tissue, other factors might also contribute to changes in mechanical properties. For instance, various non-collageneous proteins, such as osteocalcin and osteopontin, play an important role in bone homeostasis and regulate the control of biomineralization. In diabetic tissue, it has been observed that these proteins are out of balance, and may cause severe changes in tissue composition, \textit{e.g.}, altering bone matrix formation, which affects its mechanical properties \([90, 102, 109, 183]\). In addition, it has been hypothesised that \( \text{AGEs} \) decrease osteoblastic cell differentiation and function causing diabetic osteopenia \([99]\). However, none of these mechanisms is included in our model.

Another important factor in coarse-grained molecular dynamics is the choice of the applied force field. Our model of the particle interactions was obtained from literature \([42]\), where they investigated the influence of enzymatic cross-links on the tensile behavior of the collagen fibril. They observed similar mechanisms before failure, \textit{e.g.}, when applying a higher cross-link strength, they also observed a stiffened regime. However, We note that the applied boundary conditions differ significantly between our approaches. Here, we use \textit{steered} molecular dynamics instead of periodic boundary conditions to allow for in-depth failure mechanism investigations. Nevertheless, the quantitatively measured peak stress and associated strain are in the same range. Furthermore, the used force-field accounts for an implicit solvent, but hydration levels of tissue may also affect the mechanical response of the collagen fibril and its building components. While simplifications associated to the force field definition are likely leading to quantitative differences compared to in-vitro, the qualitative mechanical deformation and fracture mechanics of the collagen fibril in in-silico simulations are expected to be consistent. Therefore, the obtained results can provide fundamental insight into the link between different \( \text{AGEs} \) cross-link properties and collagen mechanics, which is currently not possible in laboratory experiments due to size and resolution limitations.

### 7.4.2 Impact of \( \text{AGEs} \) on biomechanical behavior of collagen fibrils

Our model of the collagen fibril aims at the nanoscale of collageneous tissue, where we perform tensile testing on the main building constituent, the collagen fibril. We account for the different types of \( \text{AGEs} \) cross-links by varying their mechanical
7.4 Discussion

Properties (stiffness and bond breaking length) and reveal how these properties influence the deformation and failure behavior of the collagen fibril on the next larger scale. The original deformation and failure mechanism of collagen is sliding of the tropocollagen molecules within the fibril. Our results showed that the loading energy capacity $W_{\text{AGE}}$ of the AGEs cross-link combining both factors, stiffness $k_1$ and the breaking length $r_{\text{break}}$, is the second critical factor influencing the mechanical response of the collagen fibril apart from AGEs density, which has already been shown before [97]. When $W_{\text{AGE}}$ increases, the governing deformation and failure mechanism changes from sliding to stretching of the TC molecules, where forces are transmitted from one TC molecule to another via the AGEs cross-links. Since friction is limited within the collagen fibril during this deformation mechanism due to the reduced sliding, this leads to decreased energy dissipation, and the energy is absorbed by the TC molecules via stretching, which consequently causes the fibril to be more brittle at fracture.

From these observations, we conclude that it is not only AGEs density but also the mechanical properties of AGEs – specifically the loading energy capacity – that are responsible for changes in deformation and fracture behavior. This suggests that it is crucial 1) to determine the mechanical properties, such as $W_{\text{AGE}}$, of various types of AGEs, and 2) to focus on identifying the AGEs densities in priority for AGEs with high $W_{\text{AGE}}$ in a given tissue, as they are expected to have a stronger effect on the mechanical behavior of the collagen fibril.

7.4.3 Implications of results on tissue level

AGEs are a major concern when it comes to impaired properties of collageneous tissue, especially in patients with diabetes. The mechanisms leading to inferior mechanical performance in their presence remain poorly understood. In particular, knowledge about the types of AGEs that are present in various types of tissue, and in which quantities, is very limited. Consequently, it is also unknown how the properties of the AGEs affect collagen behavior and, for instance, which types of AGEs are the most critical.

The mechanical properties of collagenous tissues are highly dependent on the behavior of the collagen fibril, which is their main building constituent. Cross-link density and type are believed to be factors influencing collagen fibrils behavior and therefore, at the larger scale also the mechanical properties of tissue. For instance, brittleness in bone is most likely not only related to cross-link density but also cross-link type. Still, neither of them has been quantified in a laboratory study so far. It appears that the cross-link density is the governing factor determining the final strength of the fibril and the extent to which sliding between the TC molecules is reduced or stretching induced. Our results suggest that AGEs cross-links with increased $W_{\text{AGE}}$ have stronger influence on the reduction of sliding and toughness.
Therefore, it would be important to quantify and evaluate the types of AGEs present in tissues, such as bone, to determine their effects on the impaired properties at the tissue level. The effect of different AGEs is important to understand the failure mechanism of collagen fibrils, and hence its consequences on the behavior of collagenous tissues, e.g., on the toughness of the bone. Our results support the perspective of [216] and [3, 5], that AGEs cause bone brittleness by reducing sliding and thereby the natural energy dissipation mechanisms in bone. Additionally, the collagen fibril might not be the origin of fracture in bone with high AGEs content, since its stiffness and strength are increased. Instead, fracture might be initiated due to load transfer to the mineral component surrounding the fibrils [170]. The mineral in bone is known to be brittle and these mechanisms might contribute to the changes in fracture behavior. Further models including mineral should target these changes in bone collagen fibril mechanics.

Other tissues also show changes in mechanical behavior that correlate with the accumulation of AGEs. In tendons, for instance, an increase in failure stress (and strain) was observed. Specifically, on the fascicle and fibril level, the peak modulus was increased, as was also shown in our previous study [97]. Interestingly, [70, 107, 177] suggest that AGEs reduce tissue elasticity in tendons by limiting fiber-fiber and fibril-fibril sliding, which is consistent with our findings, whereas [213] did not observe any statistically significant relationship between AGEs content and mechanical parameters, and claim that mechanical properties of tendons rather change due to collagen disorganisation than AGEs content. These observations need further investigations, since as stated, AGEs quantity and properties are highly dependent on the tissue. Another example are intervertebral discs, in which increased AGEs levels were shown to correlate with functional changes such as increased stiffness, increased torque range and increased torque failure [103, 201]. Furthermore, in cartilage, tensile properties are changed and AGEs cause increased stiffness and strength, effects that we also observed, and decrease the failure length leading to brittleness [35, 193], but it appears that cartilage network embrittlement at the nanoscale is responsible for increasing stiffness [122], which still needs further investigation, since also other influences might contribute to changes in tissue mechanics.

Natural collagenous tissues are complex materials influenced by many factors, where AGEs are only one of them. Still, we can provide an insight into collagen fibril mechanics on the nano-scale and contribute to a fundamental understanding. Nevertheless, we lack consistent data of AGEs function, quantity and type (mechanical properties) in order to get further insight into collagen fibril mechanics.
We performed a parameter study on an in-silico destructive tensile test of collagen fibrils at the nano-scale to analyze the effect of mechanical properties of AGEs cross-links, as they vary from AGEs type to type, on the collagen fibril behavior. We specifically focused on the deformation and fracture behavior of the collagen fibril and the associated energy absorption mechanisms. We found that apart from AGEs density, the other crucial factor for the stiffening of the collagen fibril is the loading energy capacity of the AGEs cross-links. Further, we showed that AGEs with higher loading energy capacity have a stronger impact on the fibrillar mechanics, leading to stiffening of the fibril and more brittle failure. We demonstrated that this effect is due to a change in the energy absorption mechanisms at smaller scales, where the presence of AGEs reduces inter-molecular sliding, which leads to less energy dissipation, and increases stretching of the TC molecules. Consequently, the fibril fails by fracture of the TC molecules, which is a low-toughness failure mechanism compared to failure by sliding within the fibril. These effects are generally more prominent in collagen fibrils with higher AGEs content, but the critical amount of AGEs cross-links decreases for AGEs types with higher loading energy capacity. Therefore, our results show that apart from the AGEs density, knowledge about the type of AGEs with their specific mechanical properties, is crucial for a better understanding of how the presence of AGEs impairs the behavior of collagenous tissue.

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MINERAL AND CROSS-LINKING IN COLLAGEN FIBRILS: THE MECHANICAL BEHAVIOR OF BONE TISSUE AT THE NANO-SCALE

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KEY FINDINGS:

• Mineral position and morphology influence the mechanics of the collagen fibril.
• At low mineral contents, AGEs cross-links are governing the mechanical response.
• At high mineral contents, the mechanical response is dominated by the mineral.
• Minerals change the fracture mechanics: a high number of collagen molecules ruptures.
• Collagen fibril mechanics are possibly highly adjusted via mineral content.

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* This is a pre-print, differing from the submitted paper only in terms of layout and formatting.
The mineralized collagen fibril is the main building block of hard tissues and it directly affects the macroscopic mechanics of biological tissues such as bone. The mechanical behavior of the fibril itself is determined by its structure: the content of collagen molecules, minerals, and cross-links, and the mechanical interactions and properties of these components. Advanced Glycation End-products (AGEs) cross-linking between tropocollagen molecules within the collagen fibril is one important factor that is believed to have a major influence on the tissue. For instance, it has been shown that brittleness in bone correlates with increased AGEs densities. However, the underlying nano-scale mechanisms within the mineralized collagen fibril remain unknown. Here, we study the effect of mineral and AGEs cross-linking on fibril deformation and fracture behavior by performing destructive tensile tests using coarse-grained molecular dynamics simulations. Our results demonstrate that after exceeding a critical content of mineral, it induces stiffening of the collagen fibril at high strain levels. We show that mineral morphology and location affect collagen fibril mechanics: The mineral content at which this stiffening occurs depends on the mineral’s location and morphology. Further, both, increasing AGEs density and mineral content lead to stiffening and increased peak stresses. At low mineral contents, the mechanical response of the fibril is dominated by the AGEs, while at high mineral contents, the mineral itself determines fibril mechanics.

8.1 introduction

Type 2 Diabetes Mellitus (T2DM) is widely recognized as a significant contributor to serious health complications in the human body. In addition to adverse effects on the cardiovascular system and other organs such as kidneys and eyes, researchers have found that individuals with T2DM have an increased risk of bone fractures. [115, 131, 142, 143, 174, 182]. The standard clinical metric for assessing fracture risk is bone mineral density (BMD). Typically, decreased bone mineral density (BMD) correlates with conditions like osteoporosis or osteopenia, indicating a higher risk of fractures. The fracture risk in T2DM patients also remains elevated despite exhibiting a normal to slightly elevated BMD [2, 120, 194, 209]. The potential explanation for this paradox could lie in the high content of Advanced Glycation End-products (AGEs) in T2DM individuals. AGEs are built via glycosylation within the collagen fibrils of tissue in the presence of sugars, a process also known as the Maillard reaction [14]. Due to increased glucose levels in the system, the prevalence of these molecules is increased in diabetic patients [197–199]. This increased content of AGEs has been shown to correlate with an increased fracture risk and brittleness in bone at the macro-scale and with deterioration of the collagen fibril’s ability to deform at the nano-scale [5, 208]. However, the precise mechanisms through which
AGEs influence bone mechanics have not been fully elucidated, including the extent to which AGEs contribute to the impairment of material behavior.

The hierarchical composite structure confers bone with unique material properties that are highly adapted to its function, such as providing support for movement and protection of vital organs [151, 187, 202]. Apart from the organic constituents, with the collagen fibril as the basic unit, bone also comprises a mineral phase and water. Collagen type I is the most abundant protein in the extracellular matrix of bone, comprising about 95% of the collagen content, which makes it the most important structural unit [129, 195]. Its basic building blocks are tropocollagen (TC) molecules bundled up to form the collagen fibrils, with a diameter between 20 to 500 nm and a length of about 100 μm [84, 86, 87]. TC molecules consist of three coiled peptide helices with non-helical telopeptide areas at each end of the molecule [86]. In the longitudinal direction, their structural built-up displays the collagen-specific staggered pattern with five gap and overlap zones per TC length [205]. AGEs are usually formed at helical regions of the TC molecules, but very little is known about their exact location, which also depends on the AGE type. We distinguish between non-cross-linking AGEs attached to the TC molecules and cross-linking AGEs linking two neighboring TC molecules within the collagen fibril structure. The cross-linking AGEs are suspected of preventing the deformation behavior of the collagen fibril at the nano-scale level of tissue, but the exact mechanisms remain unknown [5, 52, 77, 107, 153, 154, 191, 216]. Since the collagen fibril is the main building component of bone, alterations in its deformation behavior resulting from elevated AGE contents are expected to influence the behavior of bone on the tissue level. However, the precise mechanisms remain unclear.

In previous studies, it has been shown that AGE cross-linking significantly changes the deformation and fracture behavior of the non-mineralized collagen fibril [96, 97]. In particular, it was observed that the strength and stiffness of the fibril increases with increasing AGE densities and AGEs’ loading energy capacity. The deformation behavior of the fibril presents a stiffening at high strain levels when the cumulative loading energy capacity of all AGEs exceeds the loading energy capacity of collagen bonds within the collagen molecules. Further, it was demonstrated that changes in the failure mechanism causing this stiffening eventually resulted in a more brittle failure, i.e., energy is rather absorbed via stretching than dissipated via inter-molecular sliding of the collagen molecules in the presence of high AGE contents. This leads to a more sudden energy release when the stretched bonds break. While these results have revealed the origin of brittle failure in non-mineralized collagen fibrils in the presence of AGEs, they do not provide direct and clear evidence for impaired bone tissue behavior since the collagen fibrils in bone are mineralized.

Mineralization of collagen fibrils occurs in a process in which mineral crystals are deposited onto the bone matrix, facilitating the development and strengthening of
the bone [44]. The addition of mineral content confers bone with exceptional elastic properties, mostly stiffness, leading to higher strength [215]. It has been shown that an increasing amount of mineral correlates with increasing elastic modulus and yield stress [200]. The mineral crystals are apatite, similar to hydroxyapatite (HAP) but have a less perfect structure. Still, it is generally referred to as HAP. Different theories exist regarding the exact location and distribution of minerals within bone tissue. It is generally believed that the nucleation of minerals starts in the gap zones of the collagen fibril [11, 184, 203]. Nevertheless, the precise arrangement remains a subject of ongoing debate given that the mineral represents about 65% of hydrated bone weight. However, the gap zones of the collagen fibril do not provide sufficient space to accommodate this large amount of material. It is extremely challenging to extract mineralized collagen fibrils from bone for mechanical tensile testing and the question of how minerals and their distribution and structure influence fibril mechanics on the small scale and tissue on the larger scale has not been answered yet. Some techniques using small-angle X-ray scattering (SAXS) during in situ tensile tests of bone samples have been applied to reveal the loss of collagen deformation capacity in the presence of high AGEs contents [5, 77]. In addition to experiments, in-silico testing provides a valuable tool for investigating the mechanical behavior of collagen fibrils: Depalle et al. [42, 43] and Tavakol and Vaughan [181] have used coarse-grained molecular dynamics to investigate the influence of different parameters such as enzymatic cross-linking and mineralization on the collagen fibril deformation behavior, but the effect of AGEs in the mineralized fibril has not been investigated so far.

Here, we aim to provide numerical evidence for the influence of both, AGEs cross-linking and minerals, on the mechanics of collagen fibrils. We perform in-silico destructive tensile tests on mineralized collagen fibril models with different AGEs densities and mineral contents using coarse-grained steered molecular dynamics simulations. We investigate the influence of mineral content and morphology and AGEs cross-linking on collagen fibril deformation and fracture behavior, revealing the effect of these parameters on the nano-scale mechanics of bone.

### 8.2 Material and Methods

We use a 3D coarse-grained steered molecular dynamics model of a representative fibril during destructive tensile testing. The AGEs are inserted randomly between neighboring TC molecules in their helical regions. The model is based on our previous studies [96, 97], where force-field parameters are obtained from [42, 43]. The specificity of the collagen fibrils considered here is their mineral content, for which the modeling approach is described in detail below.
Figure 8.1: Schematic overview of model implementation and evaluation of deformation mechanisms. (a) Hodge-Petruska Model for collagen fibril: Displaying characteristic banding pattern with gap and overlap zones of TC molecules. (b) Coarse grained molecular dynamics model: The mechanical behavior of the TC molecules is represented by a string of particles mimicking the mechanical response that has been extracted from full scale simulations [42]. Mineral particles are shown in red, and collagen in grey. AGEs cross-links orange. (c) Schematic representation of insertion of mineral particles: mineral content $c_{\text{mineral}}$ is defined as $c_{\text{mineral}} = c_{\text{min}} \cdot 100\%$. Red arrows show the direction of mineralization. (d) Changes in nucleation of minerals: Mineralization starts from the center of the gap region. (e) Changes in the mineral pattern. Decreasing and increasing mineral particle density. (f) Schematic representation of our implementation of a representative collagen fibril geometry: 5 gap and overlap zones; AGEs cross-links were randomly inserted between TC molecules (red) with different densities; fibril is strengthened at the ends (blue area) to guarantee smooth force transmission. (g) Definition of bond interactions (collagen bonds in TC molecules and AGEs cross-links): trilinear bond behavior, where the force depends on the distance $r$ between two particles. The varied parameters in our simulations are $k_1$ and $r_{\text{break}}$, the loading energy capacity of a single bond, e.g., $W_{\text{AGE}}$ indicated by orange area. (h) Definition of non-bonded particle interactions due to van-der Waals forces: Soft-core Lennard-Jones Potential.
8.2.1 Geometry implementation of the collagen fibril

We build the geometry of the mineralized collagen fibril to represent the biological configuration of collagen type I including mineral in the fibrillar structure. First, we create the collagen fibril geometry without mineralization, where we use the same approach as described in [96, 97]. The TC molecules are arranged in the collagen-specific 5-staggering pattern with gap and overlap zones with a periodicity of $D = 67\,\text{nm}$, with a gap size of $0.6 \cdot D$ and an overlap of $0.4 \cdot D$ (see Fig. 8.1a). The TC molecules are represented by a string of particles, where the bonded interactions between these particles represent the behavior of the TC molecules following a coarse-grained molecular dynamics approach [28, 29, 42, 43]. The geometry of these TC molecules is obtained from Protein Data Bank entry 3HR2, the atomistic structure of TC extracted by X-ray crystallography [134], and 218 particles are placed equidistantly along its longitudinal backbone. The fibril consists of 155 TC molecules per cross-section, resulting in a diameter of 200 nm. For smooth force transmission during tensile testing, the ends of the fibril are extended with 40 particles per TC molecule and the bonds between these particles are strengthened. After creating the geometry of this collagen type I fibril, the mineralization is performed.

8.2.2 Mineralization of the collagen fibril

We insert mineral particles into the model to account for the mineralization of collagen fibrils in hard tissues like bone. The question of where mineral i.e. HAP crystal, is exactly located in bone has not been answered to date and different opinions and theories exist at this point (see Discussion 8.4.1). Some claim that it is intrafibrillarly located between the TC molecules and in the gap zones [11, 130, 172], while other experimental studies have shown that the mineral is also located extrafibrillar between the collagen fibrils [105, 112, 175, 184].

In our approach, the mineralization of the fibrils starts from the ends of the TC molecules at the sides of the gap zones and does not extend into the overlap region. This is similar to the approach of Nair et al. [124], who performed full-atomic simulations, with the mineral nearly exclusively located in the gap zones. This is in agreement with their experimental studies showing that the mineral phase is nucleated in the first section of the gap region after the transition from overlap to gap zones. We mineralize the gap zones by equidistantly placing HAP particles starting from each overlap/gap transition point towards the inside of the gap (see Fig. 8.1c) at the equilibrium distance $r_0^{\text{HAP}}$ ($r_0^{\text{HAP}} = 2^{1/6} \cdot \sigma_{\text{HAP}}$). The distance at the collagen/mineral transition (between the last collagen and the first mineral particle) is the equilibrium distance $r_0^{\text{col-HAP}}$ ($r_0^{\text{col-HAP}} = 2^{1/6} \cdot \sigma_{\text{col-HAP}}$) where forces are 0, following the Lennard-Jones-Potential between collagen and HAP (see Fig. 8.1c, h
and Tab. 8.1). This mineralization pattern is considered as the reference state of our mineralized collagen fibril. In addition, we created other models where mineral insertion was started from the center of the gap zones (see Fig. 8.1d) to evaluate the influence of the mineral nucleation position. Even more, for investigating how the morphology of minerals influences fibril mechanics, models with less mineral density were implemented. In these cases, either only every second particle position with respect to the reference state was occupied or two lines of mineral particles in an equidistant scaffold were added (see Fig. 8.1e). The mineral content is defined via the percentage of gap length that is occupied by mineral particles $c_{\text{mineral}} = m/l \cdot 100\%$ (see Fig. 8.1c-e).

8.2.3 Insertion of AGEs cross-links

The insertion process of AGEs cross-links is consistent with the one used in Kamml, Acevedo, and Kammer [96] and Kamml et al. [97]. AGEs are inserted into the mineralized collagen fibril after the first equilibration of 20 ns. Aside from computational studies concentrating on individual AGEs [37, 64], their exact location and where they act as cross-linking or non-cross-linking AGEs is unknown. Hence, we insert them randomly between the central 95% helical regions of the collagen fibril. The AGEs content $N_{\text{AGE}}$ is measured per TC molecules, where AGEs are inserted per TC molecules to avoid accumulation effects. Further, since AGEs types and contents have not been quantified in bone, the content we apply in our models cannot be verified with experimental data, and, hence, we vary the content to investigate its effect.

8.2.4 Definition of the particle interactions

We use coarse-grained Molecular Dynamics for simulating the destructive tensile tests on the collagen fibrils. The total energy of the system is defined as the sum of all force field terms. For our mineralized collagen fibril, this translates to

$$ E_{\text{total}} = E_{\text{bond}} + E_{\text{angle}} + E_{\text{non–bonded}} $$

$$ = \sum_{\text{bond}} \Phi_{\text{bond}}(r) + \sum_{\text{angle}} \Phi_{\text{angle}}(\phi) + \sum_{\text{non–bonded}} \Phi_{\text{non–bonded}}(r), \quad (8.1) $$

where $E_{\text{bond}}$ is the bond energy due to stretching, $E_{\text{angle}}$ the dihedral bond interactions energy due to bending, and $E_{\text{non–bonded}}$ the pairwise interaction energy due to molecular interactions such as Van-der-Waals forces.
The interactions between particles are expressed through forces, and these forces between particles are calculated via particle distance

\[ F = -\frac{\partial \Phi(r)}{\partial r} \]  

or angle

\[ F = -\frac{\partial \Phi(\phi)}{\partial \phi} \]

as the negative derivative of the potential energy.

The bond energy \( E_{\text{bond}} \) includes collagen bonds between the particles of the TC molecules and AGEs cross-links. The bonds are modeled as trilinear springs with a regularization after bond breakage

\[
F_{\text{bond}}(r) = \begin{cases} 
-k_0(r - r_0) & \text{if } r < r_1 \\
-k_1(r - r_0) & \text{if } r_1 \leq r < r_{\text{break}} \\
z \cdot k_1(r - r_0) & \text{if } r_{\text{break}} \leq r < r_{\text{break}} + a \\
0 & \text{if } r \geq r_{\text{break}} + a
\end{cases}
\]  

where \( F_{\text{bond}} \) is the force acting between two particles that are connected with a bond, \( k_0 \) and \( k_1 \) are the respective spring constants of the bond deformation, \( r_0 \) is the equilibrium distance between the two bond particles and \( a \) is defined as \( a = z \cdot (r_{\text{break}} - r_1) \), with \( z \) as the regularization factor to avoid any discontinuities and provide computational stability (see Fig. 8.1d).

We account for angle bending between a set of three particles with forces defined as

\[
F_{\text{angle}}(\phi) = -k_B(\phi - \phi_i) \cdot \phi,
\]

where \( \phi_i \) are the varying equilibrium angles obtained from the initial TC molecule geometry, and \( k_B \) is the bending stiffness of the molecule [29, 32, 42].

Van-der-Waals forces \( E_{\text{inter}} \) define the potential between the different kinds of non-bonded particles: The collagen-to-collagen (col) interaction, the mineral-mineral (HAP) interaction and the collagen-mineral interaction (col – HAP) are modeled via a Lennard-Jones-Potential with a soft core following

\[
F_{\text{non-bond}}(r) = \begin{cases} 
F_{\text{LJ}}(r) & \text{if } r \geq \lambda \sigma_{\text{LJ}} \\
F_{\text{LJ}}(\lambda \sigma_{\text{LJ}}) & \text{if } r < \lambda \sigma_{\text{LJ}}
\end{cases}
\]  

where

\[
F_{\text{LJ}}(r) = \frac{1}{r} \left[ 48 \epsilon_{\text{LJ}} \left( \frac{\sigma_{\text{LJ}}}{r} \right)^{12} - 24 \epsilon_{\text{LJ}} \left( \frac{\sigma_{\text{LJ}}}{r} \right)^{6} \right].
\]
with $\epsilon_{LJ}$ as the well depth between two particles, $\sigma_{LJ}$ is the distance at which the intermolecular potential between the two particles is zero and $\lambda$ is the parameter to adjust the critical force associated to the soft core (see Fig. 8.1e).

The parameters applied in our simulations for calculating particle interaction are displayed in Tab. 8.1. For cross-link modeling, we use the mechanical properties of glucosepane, extracted from full-atomistic simulations with a reactive force field, since glucosepane is the most abundant cross-link in tissue and has been shown to influence mechanics of non-mineralized collagen fibrils [96]. Additionally, to investigate the influence of AGEs cross-link mechanics, we increased the stiffness by a factor of 2.

8.2.5 Simulations

We perform destructive tensile tests on the models of the mineralized collagen fibrils in LAMMPS [141]. In the first step, different contents of mineral are inserted into the gaps of the collagen fibrils to account for mineralization, followed by an equilibration for 20 ns in an NPT ensemble (300 K, 0 Pa) simulating an infinitely long fibril with periodic boundary conditions. After the insertion of cross-links, the tensile tests are performed, using steered molecular dynamics at a constant velocity of 0.0001 Å/fs ($=10$ m/s) in an NVT ensemble at a temperature of 300 K. The time step is $\Delta t = 10$ fs in equilibration and tensile test simulation. The ends are moved apart, and the required force is measured to calculate the engineering stress within the collagen fibril.

8.3 RESULTS

8.3.1 Effect of mineral on collagen fibril deformation behavior and strength

With our first set of simulations, we investigate the effect of changing mineral content in the gap zone, expressed in gap-filling percentages (see Fig. 8.1c – reference state). We observe that the mechanical behavior of the collagen fibril changes drastically in various aspects with increasing mineral content (see Fig. 8.2a). All fibrils show the same elastic behavior for strains up to $\epsilon \approx 0.1$ independent of their mineral content. Fibrils with low contents, i.e. $c_{\text{mineral}} \leq 10\%$, present a softening behavior after reaching the peak stress $\sigma_{\text{peak}}$, eventually leading to zero strength. This behavior results from sliding between TC molecules, which is manifested in the observation that the collagen bonds within the TC molecules do not break (see Fig. 8.2b). The fibril with mineral content $c_{\text{mineral}} = 15\%$ presents a different behavior: after reaching the limit of linear deformation at $\epsilon_0 \approx 0.15$ the modulus decreases. After reaching peaks stress $\sigma_{\text{peak}}$, it softens, and the behavior is characterized by a unique phenomenon within mineralized collagen fibrils – the so-called
Figure 8.2: Mechanics of mineralized collagen fibril with varying contents of mineral. (a) Stress-strain curves of tensile tests until rupture of a representative collagen fibril with different mineral contents $c_{\text{mineral}}$ in [%]. (b) Number of broken bonds in TC molecules at fibril strain. (c) Average gap length at fibril strain (the average gap length is the average distance between the particles at the end of the TC molecules at both sides of the gap).
“sawtooth” effect [43]: The stress-strain curve is not smooth, but presents multiple relatively large stress-drops. These drops coincide with a slightly undulated behavior (see Fig. 8.2c) of the average gap length $l_{\text{gap}}$, as defined in Fig. 8.1f, which is the length of the gap zone between the particles at the ends of two TC molecules. At the same time, the number of broken collagen bonds remains relatively constant (see Fig. 8.2b), which suggests that this sawtooth behavior is the result of (dynamic) sliding events between the TC molecules.

Fibrils with mineral contents of $20\% \leq c_{\text{mineral}} \leq 40\%$ show a slight decrease in modulus after reaching the limit of the elastic behavior at $\varepsilon_0$, followed by stiffening with increasing stress, starting at $\varepsilon \approx 0.3$. These fibrils finally reach their peak stress at $\sigma_{\text{peak}} \approx 2.5 \text{ MPa}$. Fibrils with $c_{\text{mineral}} \leq 30\%$ still show a sawtooth behavior, while at higher $c_{\text{mineral}}$, the stress drops from $\sigma_{\text{peak}}$ directly to 0, which is a sign of brittleness. We also observe that higher contents of minerals lead to more broken bonds in the TC molecules (see Fig. 8.2b), where high drops in stress generally appear when a larger amount of bonds breaks. The bond breaking within the TC molecules indicates that the mineral blocks the sliding between the TC molecules. In general, mineral makes the fibril stronger i.e. increases the peak stress $\sigma_{\text{peak}}$ up to a mineral content of $c_{\text{mineral}} \approx 40\%$ when $\sigma_{\text{peak}}$ reaches its saturation level. Mineral contents of $c_{\text{mineral}} \leq 40\%$ do not lead to a further increase in $\sigma_{\text{peak}}$. We also note that the strain at peak stress $\varepsilon_{\text{peak}}$ increases up to $c_{\text{mineral}} \approx 30\%$ and then does not show any further significant changes. All of these results show that changes of $c_{\text{mineral}}$ have a stronger influence at lower mineral contents ($c_{\text{mineral}} \leq 30\%$) than at higher content, where the behavior does not change significantly when the mineral contents change.

In the following, the fibril with $c_{\text{mineral}} = 0\%$ that neither contains mineral nor cross-links is considered as the state of pure collagen molecules sliding. When observing the changes in the average length of the gap zone $l_{\text{gap}}$, this state of pure sliding shows a constant change of $l_{\text{gap}}$. The slope of the curves is generally decreasing with increased $c_{\text{mineral}}$, indicating that the TC molecules within the fibrils are stretched. When $c_{\text{mineral}} \geq 20\%$, the failure of the fibril and the larger amounts of broken collagen bonds in TC molecules coincide with the onset of a sudden change in slope at $\varepsilon_{\text{peak}} \approx 0.3$. The higher $c_{\text{mineral}}$, the less pronounced the changed modulus. This is an indicator that at a mineral content of $c_{\text{mineral}} = 40\%$, the collagen molecules are barely sliding, but the fibril breaks via a condensed break of collagen molecules. This is another indicator for increased brittleness of the collagen fibril with increasing mineral content.

### 8.3.2 Effect of mineral nucleation position

We now investigate the effect of the position of the nucleation of the mineral. In the reference state presented in Sec. 8.3.1, nucleation of minerals starts at the respective
Figure 8.3: Effect of different mineral nucleation points on the mechanical response of the collagen fibril. Mineralization starting from the edges of the gap and growing towards the center are depicted with dashed lines, and correspond to the reference case shown in Fig. 8.2. Mineralization starting from the gap center and growing outwards are depicted with solid lines. (a) Stress-strain response of the collagen fibrils with different nucleation procedures. Mineral contents in [%]. (b) Comparison of peak stress $\sigma_{\text{peak}}$ in collagen fibrils with different mineral nucleation starting points. (c) Comparison of post-failure work $W_{\text{PF}}$ in collagen fibrils with different mineral nucleation starting points.

ends of the gap zones. We compare this reference fibril to a fibril where the mineral nucleation starts in the center of the gap (see Fig. 8.1c). We observe that nucleation in the center intensifies the effect of the mineral on the collagen fibril mechanics. Specifically, the fibril demonstrates changes in mechanical behavior already at lower contents of the mineral when the mineral is nucleated from the center of the gap (see Fig. 8.3). With a mineral density of $c_{\text{mineral}} = 15\%$, the fibril with mineral extending from the center of the gap exhibits a behavior similar to the fibril with $c_{\text{mineral}} = 20\%$ in the reference fibril. The peak stress $\sigma_{\text{peak}}$ reaches its saturation level already at a mineral content of $c_{\text{mineral}} \approx 20\%$ (see Fig. 8.3a&b). Further, we consider the post-failure work $W_{\text{PF}}$, which is defined as

$$W_{\text{PF}} = \int_{\varepsilon_{\text{peak}}}^{\varepsilon_{\text{PF}}} \sigma(\varepsilon) \, d\varepsilon$$

(8.8)

with $\varepsilon_{\text{peak}}$ corresponding to the strain at peak stress $\sigma_{\text{peak}}$ and $\varepsilon_{\text{PF}}$ being the strain when $\sigma \approx 0$ post fibril failure. We observe that $W_{\text{PF}}$ is generally smaller at the same mineral contents (see Fig. 8.3c). At the same $c_{\text{mineral}}$, the mineral nucleated from the center reaches higher stresses faster, but also loses the sawtooth behavior, leading to a more abrupt failure. With increasing mineral content, this difference is getting smaller, since the fibril has an increased $\varepsilon_{\text{peak}}$, but also more abrupt rupture, causing a decrease in $W_{\text{PF}}$. Since the nucleation position has mostly quantitative but no qualitative effects on the fibril behavior, we can infer that the mineral content is
more important than its position but our results do not provide direct evidence on the location of mineral in the collagen fibril.

8.3.3 Effect of mineral pattern

To account for different forms and distributions of minerals, we additionally compare the reference fibrils’ mechanical behavior with fibrils with other mineralization patterns. First, we decrease the mineral particle density in the gap zone, i.e., only every second mineral particle position from the reference state is occupied (see Fig. 8.1e). In this configuration, the stress-strain curves show a decrease in the effect of minerals (see Fig. 8.4a). The stiffening is only initiated at higher mineral contents of about 40%. We observe that the peak stress saturation level is not reached at mineral contents of $c_{\text{mineral}} \leq 40\%$, but only at $c_{\text{mineral}} \geq 75\%$ (see Fig. 8.4a&b). Due to reaching the level of abrupt failure only at very high mineral contents, the $W_{PF}$ is constantly high and only reaches its minimum at $c_{\text{mineral}} = 100\%$ (see Fig. 8.4c). This shows that when the mineral is not perfectly organized and defects, such as inclusions (here represented as empty mineral slots), exist, it still contributes quite efficiently to the strength of the collagen fibril but does not affect the fibril brittleness to the same degree.

Second, we increase the mineral particle density by adding minerals in an equidistant scaffold in two rows (see Fig. 8.1e). We observe the opposite effect, where the fibrils stiffen already at a mineral content of $c_{\text{mineral}} \approx 15\%$ and reach their peak stress saturation already with $c_{\text{mineral}} \approx 30\%$. At mineral contents of $c_{\text{mineral}} \approx 50\%$, we see a drop in $\sigma_{\text{peak}}$, and at $c_{\text{mineral}} \approx 100\%$, the stress-strain behavior does not significantly differ from the reference fibril.

8.3.4 AGEs cross-linking in the mineralized collagen fibril

We investigate the influence of AGEs cross-linking on the collagen fibril mechanics at different mineral contents and varied not only AGEs density but additionally increased the stiffness of the AGEs by a factor of 2 to evaluate the influence of changing AGEs properties (see Fig. 8.6 and Tab. 8.1). Generally, when we observe AGEs and minerals separately, we observe that higher AGEs densities $N_{AGE}$ (without mineral) lead to higher peak stress $\sigma_{\text{peak}}$ and, depending on the AGEs properties, i.e., stiffness, leading to stiffening of the collagen fibril at the respective $N_{AGE}$ (at $k_1 = 8.0$ stiffening is initiated at $N_{AGE} = 40$, at $k_1 = 16.0$ at $N_{AGE} = 10$). The stiffening is initiated at $\varepsilon \approx 0.25$. At higher $N_{AGE}$ the failure of the fibril also is more abrupt, i.e. higher slope after reaching $\sigma_{\text{peak}}$. When observing the influence of $c_{\text{mineral}}$ (see Sec. 8.3.1), the mineral has similar effects with increasing peak stress $\sigma_{\text{peak}}$ and initiating a second stiffening. The $\sigma_{\text{peak}}$ reaches its saturation level at 2.5 GPa. The difference the stress-strain curve demonstrates is a reduction of the
Figure 8.4: The influence of mineral morphology on the mechanical response of the collagen fibril. Results from simulations with mineralization patterns with one continuous line of minerals are depicted with dashed lines and correspond to the reference case shown in Fig. 8.2. Simulation results with mineralization where every second mineral position is empty are depicted with solid lines. (a) Stress-strain response of the collagen fibrils with different mineral morphologies. Mineral contents in [%]. (b) Comparison of peak stress $\sigma_{\text{peak}}$ in collagen fibrils with different mineral morphologies. (c) Comparison of post-failure work $W_{PF}$ in collagen fibrils with different mineral morphology.

Figure 8.5: The influence of mineral morphology on the mechanical response of the collagen fibril: Mineralization pattern with one solid line of mineral (dashed lines) – reference case, see Fig 8.2 – and two solid lines of mineral (solid lines) (a) Stress-strain response of the collagen fibrils with different mineral morphologies. Mineral contents in [%]. (b) Comparison of peak stress $\sigma_{\text{peak}}$ in collagen fibrils with different mineral morphologies. (c) Comparison of post failure work $W_{PF}$ in collagen fibrils with different mineral morphology.
modulus after the linear regime. At this point, the subsequent stiffening is initiated but remains less pronounced for higher $c_{\text{mineral}}$. Further, the most striking difference is the sawtooth behavior: after reaching $\sigma_{\text{peak}}$ at a mineral content of $c_{\text{mineral}} \approx 15$. When $c_{\text{mineral}}$ is increased, the failure mechanism is more abrupt.

In all fibril configurations, the elastic modulus as observed from the initial fibril stiffness is independent of $c_{\text{mineral}}$ and $N_{\text{AGE}}$. Without minerals, AGEs cause the initiation of the second regime – the stiffening of the collagen fibril – which appears already at lower AGEs densities when the stiffness of the AGEs cross-links is increased (compare Fig. 8.6a&e).

When the mineral content $c_{\text{mineral}}$ of the fibril is at 15%, the peak stress $\sigma_{\text{peak}}$ increases further for all $N_{\text{AGE}}$, when the stiffness of AGEs $k_1 = 8.0$. In case of $k_1 = 16.0$, $\sigma_{\text{peak}}$ only increases up to a $N_{\text{AGE}} \leq 10 \text{AGEs/TC}$. At this higher level of stiffness, the fibril reaches the saturation level of strength at $\sigma_{\text{peak}} = 4.0 \text{ MPa}$. We observe that insertion of AGEs does not lead to the so-called sawtooth effect compared to insertion of mineral: while at lower mineral contents $c_{\text{mineral}} \leq 20\%$, we see also that $\sigma_{\text{peak}}$ increases with higher $c_{\text{mineral}}$, but the fibril shows this stick-slip behavior after having reached its peak stress. Insertion of AGEs at $N_{\text{AGE}} \geq 10 \text{ AGEs/TC}$ still leads to a more abrupt failure after reaching the same $\sigma_{\text{peak}}$. The higher the stiffness of the AGEs, the more pronounced the stiffening behavior of the fibril (compare Fig. 8.6b&f).

At a mineral content of $c_{\text{mineral}} \geq 30\%$, the influence of AGEs density on the mechanical behavior of the fibril is reduced compared to $c_{\text{mineral}} = 15\%$(see Fig. 8.6c, d, g, h). The fibril stiffens and fails abruptly, but the mineral at this stage is the dominating factor governing the deformation and failure behavior. Still, at higher $N_{\text{AGE}}$, the $\sigma_{\text{peak}}$ of the fibril is higher but reduces compared to the fibril at $c_{\text{mineral}} \leq 15$. When $c_{\text{mineral}} = 100\%$, we observe that AGEs do not affect the deformation behavior (see Fig. 8.6d&h).

Generally, we observe that AGE density influences collagen fibril mechanics at lower $c_{\text{mineral}}$, where the extent of manifestation is dependent on AGE stiffness, but when the mineral content increases to $c_{\text{mineral}} \geq 30$, the effect of AGEs reduces until no effect of AGEs can be observed (see Fig. 8.6d&h), independent of their stiffness.

When evaluating the breaking of bonds at different mineral contents $c_{\text{mineral}}$ and AGEs densities $N_{\text{AGE}}$, we observe that the higher the mineral content, the more mineral is dominating the mechanical response: While at low $c_{\text{mineral}}$, only at $N_{\text{AGE}} = 40 \text{ AGEs/TC}$ collagen bonds in the TC molecules break and also just very few, the more mineral that is inserted, the more bonds in the collagen molecules break (see Fig. 8.7a-d). At $c_{\text{mineral}} = 100$, around 500 collagen bonds are broken. With 155 TC molecules per cross-section of the fibril, this means that, on average, more than three fracture sites occur within every TC molecule. When we observe the breaking of AGEs cross-links, we see the opposite effect (see Fig. 8.7e-h): with increasing $c_{\text{mineral}}$, the number of AGEs cross-links breaking decreases massively,
from a maximum of 3800 cross-links in fibrils with $N_{AGE} = 40$ AGEs/TC at $c_{\text{mineral}} = 0\%$ to about 750 cross-links fractured at $c_{\text{mineral}} = 100\%$, which mean that at $c_{\text{mineral}} = 0\%$, more than 60% of the cross-links break, compared to only about 12% at $c_{\text{mineral}} = 100\%$. This indicates that at high mineral contents, the mineral in the gap zones acts like glue, attaching the molecules at the end of the TC molecules to the gap. This is in agreement with the measurements of the length of the gap zones in Fig. 8.8. We observe that at $c_{\text{mineral}} = 100\%$ the average gap length at a fibril strain of 0.5 is only one-third of the gap length at $c_{\text{mineral}} = 100\%$ (compare Fig. 8.8a&d). The average gap length constantly decreases with increasing $c_{\text{mineral}}$ (see Fig. 8.8a-d). Additionally, at increasing mineral, a sudden change of mineral length becomes more pronounced (see Fig. 8.8c) that finally disappears when the fibril reaches $c_{\text{mineral}} = 100\%$ where the average gap length is very low (see Fig. 8.8d).
Table 8.1: Parameters used in coarse-grained molecular dynamics mesoscale model of mineralized collagen fibrils [42, 43, 97].

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Figure 8.6: The influence of AGEs cross-link density and stiffness on the mechanical response of the mineralized collagen fibril. Stress-strain curves of mineralized collagen fibrils (and the non-mineralized fibril as reference (a&e)) at different mineral contents. (b&f) Mineral content of 15%. (c&g) Mineral content of 30%. (d&h) Mineral content of 100%.
Figure 8.7: Development of bond breaking of collagen bonds within TC molecules and AGEs at various fibril strain levels with different AGEs densities $N_{\text{AGE}}$ at different mineral contents of $c_{\text{mineral}} = 0\%$ (a&e), $c_{\text{mineral}} = 15\%$ (b&f), $c_{\text{mineral}} = 30\%$ (c&g), and $c_{\text{mineral}} = 100\%$ (d&h). The tensile stiffness of the cross-links is $k_1 = 8.0$.

Figure 8.8: Measured length of gap region during tensile testing at different mineral contents $c_{\text{mineral}}$ including different AGEs densities. The tensile stiffness of the cross-links is $k_1 = 8.0$. 
8.4 Discussion

8.4.1 Limitations in fibril geometry and mineralization procedure

Our models of mineralized collagen fibrils allow us to observe the changes in mechanics and deformation behavior of the fibril as a function of changing mineral content and form and varying AGEs cross-link density. These models provide a groundwork for assessing the influence of cross-linking and mineralization. However, various other parameters and environmental conditions might contribute to collagen fibril mechanical behavior, and some of these factors have not yet been quantified or determined. For example, the form and distribution of minerals within the gap zones of the collagen fibrils have not been determined precisely, and the mineralization process concerning mineral nucleation, location, and distribution within and between collagen fibrils and fibers is a question of ongoing debate: McNally et al. [112] used transmission electron microscopy to study the topological distribution and relation of collagen and minerals. They state that 70% of the mineral occurs extrafibrillar being placed between and oriented parallel to the collagen fibril’s longitudinal direction, in agreement with other studies [105, 175, 184]. The remaining 30% are located in the gap zones. This is contradictory to other theories, stating that the mineral is also placed intrafibrillar between the TC molecules, extending to the overlap regions of the fibril [11, 130, 172]. Since the quantities and morphology of minerals have not been determined precisely, we used several different densities and patterns of mineral distributions in the gap zones. Still, by taking several factors and distributions into account, the fibril models are comprehensive enough to provide a reference for estimation of the influence of AGEs cross-links and mineral density in collagen fibrils in the future when parameters have been quantified.

Hydration has been shown to be an important factor regulating collagen fibril mechanics [8–10, 53], which we did not account for by modeling collagen fibrils without the influence of water molecules. The mechanical strength of cross-links is likely to be sensible to pH [149], a fact that we do not account for in our simulations.

Although AGE cross-links occur in physical conditions only in the presence of enzymatic cross-links, we do not include them in the present study. We have shown in previous work that enzymatic cross-links do not significantly change the mechanical response of the collagen fibril [97]. Still, it has been found that the function of enzymatic cross-links is more complex, especially of trivalent cross-links: one of their bonds might act as a sacrificial bond to maintain mechanical stability via energy dissipation and relaxation afterward, and bond breaking might activate tailored repair mechanisms before macroscopic failure [149, 212]. Apart from enzymatic cross-links, we did not account for different AGE types except by varying AGE cross-link stiffness since they have not been quantified in bone so
far. Instead, we used the mechanical properties of glucosepane, the most abundant AGEs cross-link in tissue. Further, the distribution of AGEs was considered random since the exact locations of AGE cross-links are not known. When these locations are known, a future study should be performed on the influence of their locations.

In addition to these factors, AGEs might not only influence the mechanical behavior of the collagen fibril via cross-linking but also via changing other important physiological functions like the hydration mechanism [8] or via imposing tissue resorption activities [46, 101].

### 8.4.2 Implications of the results

Our results indicate that up to a certain content of mineral $c_{\text{mineral}}$, AGEs density $N_{\text{AGE}}$ influences the mechanical behavior ($c_{\text{mineral}} \leq 30\%$), but after the $c_{\text{mineral}}$ has exceeded this value, the mineral is dominating the deformation behavior. This leads to the conclusion that the deformation mechanism within the fibril changes: mineral-collagen interactions are strong enough to retain many particles at the gap/collagen transition (the last particles before the gap). This leads to a change in the failure mechanism, where a higher amount of collagen bonds breaks due to the retaining of the gap particles.

The increase of the peak stress $\sigma_{\text{peak}}$ up to a certain value and the fact that at higher stiffness $k_1$ at $N_{\text{AGE}} = 40$ it does not increase at $c_{\text{mineral}} = 15\%$ compared to $c_{\text{mineral}} = 0\%$ indicates that the transfer of forces to TC molecules via reduction of sliding reaches a saturation level where tropocollagen bonds break. The mineralized collagen fibril is probably a highly tuned biological system. Diabetes might not only influence collagen fibril mechanics via AGEs cross-linking but disturb the metabolic process of bone resorption [120]: Indeed, studies show that the mineral content of bone in T2DM is increased [79, 111, 132], raising the question of whether it is the increased AGEs cross-linking or the increased mineral content that is disturbing the tuned mechanical properties (or a combination of both). We observe that both increased AGE density and mineral content cause a more abrupt failure of the fibril. The stiffening of the fibrils is caused by changes in deformation mechanisms. The mineral content as well as AGEs block the sliding of the TC molecules. Finally, we note that further investigation on the presence of different AGEs types, i.e. AGEs with different mechanical properties, are needed for a comprehensive understanding of tissue mechanics at the nano-scale and for a more profound study on AGEs cross-linking in mineralized collagen fibrils.

### 8.5 conclusion

With our model of the mineralized collagen fibril, we investigated the influence of both, mineral content and AGEs cross-link density on the mechanics of the fibril.
We demonstrated that both of these components generally increase the strength of the fibril. Furthermore, increasing their quantity leads to the onset of another deformation regime characterized by stiffening of the fibril and resulting in a more abrupt failure. We showed that at higher mineral content, the mineral dominates the deformation and failure mechanism of the fibril. In contrast, at lower contents of minerals, AGEs dominate the mechanisms of deformation. These results indicate that distortions in either the deposition of mechanical components (e.g., increased or reduced mineral content, or increased AGEs cross-linking) or of their mechanical properties alter the mechanical behavior of tissue on a larger scale by changing the collagen fibril mechanics.

8.6 **Funding**

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Part III

CONCLUDING REMARKS
SUMMARY AND CONCLUSION

The negative influence of diabetes on tissue has been in focus of research for decades, and with growing prevalence of the disease it is of great interest to develop suitable measures for treatment. Diabetes is known for causing hyperglycation in the body, leading to the formation of Advanced Glycation End-products (AGEs) at the nano-scale level of tissue. The small-scale levels of tissue – their structure and mechanical behavior during deformation – are still a matter of ongoing research. Numerous studies tried to reveal the hierarchical build-up using various imaging techniques. Collagen type I, arranged in collagen fibrils, has been shown to be the main building component of many tissues, e.g. tendon and bone. AGEs cross-links, one group of AGEs, are suspected to accumulate within the collagen fibril between the TC molecules, changing its deformation behavior and thereby influencing the mechanics of tissue at the macro scale. At this specific scale level, commonly used imaging methods are limited due to their resolution, but computational modelling provides a tool to gain insight into deformation and failure mechanics and how these are affected by different parameters. Building upon existing knowledge from previous studies of Depalle et al. [42, 43] and Buehler [27], this computational study provides a deeper understanding of the influence of AGEs cross-linking on collagen fibril mechanics i.e. how different amounts of AGEs and AGEs mechanical properties change the deformation and failure behavior.

In-silico destructive tensile tests reveal that the strength of the collagen fibrils increases with higher AGEs content. Fibrils stiffen at higher strain levels when the number of AGEs cross-links between TC molecules exceeds a critical value. This stiffening, indicating a second regime in the deformation mechanism, appears due to a change in the deformation mechanisms: the force is transferred via cross-links to the TC molecules and energy is rather absorbed by stretching than dissipated by sliding between TC molecules. Analyzing changes in force transmission i.e. changes in the force distribution throughout the fibril, within the different components (average forces in TC molecules and cross-links), the models demonstrate that higher loads are mainly carried by the TC molecules. The stress carried by the fibril only reaches higher levels via stiffening, when the content of AGEs cross-links is high
enough to cause a load transfer from cross-links to TC molecules: When cross-link densities are increased, the average force in cross-links decreases and the averages forces in TC molecules increase. This explains the stiffening behavior: At lower cross-link densities, force transmission happens to some extent through cross-links, but mainly through TC molecule interaction e.g. friction. At higher AGEs densities, forces are transmitted mainly through AGEs cross-links, and the global deformation of the fibril is mainly due to TC molecule stretching. This force transmission causes a change in the fracture mechanism: While at lower AGEs contents, AGEs are breaking, and the fibril breaks due to TC molecule sliding, at higher AGEs contents, the bonds within the TC molecules fracture, leading to a more abrupt failure of the fibril.

Cross-linking exists in different variations: AGEs cross-links, also called non-enzymatic cross-links, are suspected to impair the material behavior of tissue, while ECLs are known for collagen fibril stabilization. ECLs are important to notice in the context of AGEs since they are always present in collagen fibrils with AGEs due to the physiological assembly mechanism. They are located at the respective ends of the TC molecules and appear in different configurations: divalent, linking two TC molecules, and trivalent, linking three TC molecules. However, how different cross-link types exactly influence the collagen fibril deformation and their relationship interfering with each other is not well understood. The fibril models including both cross-link types show that ECLs increase the peak stress, with more ECLs increasing the change in peak stress compared to fibrils with no ECLs, but the change in peak stress remains relatively constant and independent of ECL configuration. Important is the effect of ECLs on the actuation of the stiffening regime: ECLs alone cannot initiate the fibril stiffening, but if the content of AGEs is high enough, ECLs, in particular trivalent ECLs, trigger the initiation of the stiffening that would not occur in fibrils without them. Generally, ECLs are responsible for an extended stiffening regime, but the qualitative deformation mechanisms are not changing due to their presence.

In order to account for the influence of different AGEs types on the fibril mechanical behavior, collagen fibril models with different mechanical properties of AGEs were tested. The loading energy capacity of AGEs has been found to be the determining factor apart from AGEs density for changing the deformation mechanism with entering the stiffening regime. Our results show that the origin of the stiffening of the fibril is a deformation mechanism that favors energy absorption via stretching rather than inter-molecular sliding between TC molecules. The energy is absorbed in TC molecules instead of being dissipated through molecular sliding. This is shown by comparison of the energy lost by sliding and stretching with respect to the respective energies in the fibril where no cross-links are present in the fibril i.e. the pure sliding case. Depending on the combined loading energy capacity of all
AGEs present in the fibril, the deformation mechanism changes and the dominance of sliding is superseded by stretching. When energy is mostly absorbed by the TC molecules via stretching, the failure is more brittle. This leads to the conclusion that it is crucial to know AGEs properties and quantities for understanding the nano-scale origin of impaired tissue behavior. Overall, AGEs loading energy capacity and AGEs density are the key properties for determining whether the failure mechanism is dominated by sliding or stretching, indicating whether fracture is ductile or brittle.

Most tissues have a highly hierarchical structure. To this point, we examined the mechanical behavior of pure collagen fibrils, but in hard tissues like bone, the collagen fibrils are mineralized. Therefore, we performed tensile test on the mineralized collagen fibrils with different AGEs cross-link densities to investigate their influence on the deformation behavior. Focusing on mineralized collagen fibrils, we demonstrate that, after reaching a critical content, the mineral induces stiffening of the collagen fibril at high strains. This critical amount is dependent on the location and morphology of the mineral. Both, increasing mineral content and AGEs density increase the peak stress and cause stiffening, but the deformation mechanism is changing: At low mineral content, AGEs are dominating the deformation and fracture behavior of the collagen fibril, while at higher contents of mineral, the mechanical response is dominated by the mineral itself. This indicates that the mechanical behavior of tissue is altered by changes in the collagen fibril mechanics, which are likely to result from distortions in either deposition of mechanical components (e.g., increased or reduced mineral content, or increased AGEs cross-linking) or of their mechanical properties.

To conclude this study, we could demonstrate that the deformation behavior of collagen fibrils i.e. tissue at the nano-scale level, is dependent on energy dissipation, and whether failure is rather happening due to TC molecular sliding or stretching. Stretching is favoured when forces are transmitted to TC molecules and energy is absorbed by TC molecules. This leads to a rather brittle failure with high energy release, which is potentially responsible for changes in tissue behavior at the larger scale. A fundamental understanding of tissue structure and behavior is needed for targeting future challenges in terms of treatment for impaired tissue behavior, and computational studies can provide an insight at scales where common techniques do not have access. However, for future studies and for obtaining quantitative valuable results, quantification of the input parameters – AGEs density and mechanical properties – is needed.
When you are at the end of your rope,
tie a knot and hold on.
— Theodor Roosevelt

Even though the prevalence of diabetes is increasing and the accompanying negative effects within tissue are a well-known fact, the origins still remain quite unclear, especially in bone. AGEs are suspected to be responsible for these issues, but very little is known about their influence on mechanical behavior. Here, we revealed the origins of the changes in deformation and failure mechanisms at the collagen fibril level due to AGEs cross-linking, but there are still many questions to be answered. Future research should concentrate on various aspects related to the lack of information on tissue and provide a further understanding of tissue mechanics. Relevant aspects are:

**Bone tissue structural characterization** For a profound understanding of tissue behavior, an exact characterization of the structure of tissue on different scales is needed. Especially in bone, a description of the arrangement of different constituents, structural properties, and the composition of the different scales is still missing and opinions are contradictory. For example, in the case of the basic building component of bone – the mineralized collagen fibril – the location and distribution of minerals are important. Whether mineral phases are located intra- or extrafibrillar or both and in which forms they occur is unclear, but can, as we have shown, have a large influence on collagen mechanics. In computational modeling, this aspect is crucial for correct geometry reconstruction for providing a model consistent with physiological conditions to obtain representative material properties.

**AGES identification and quantification** Future experimental investigations should focus on the identification, quantification, and localization of different types of AGEs and on their appearance in different kinds of tissue. We suggest that AGEs should be characterized via the quantification of their loading energy capacity, as this study could show that this is a key property for determining their influence on collagen fibril mechanics. Of particular interest is also their function, whether they appear as cross-linking or non-cross-linking AGEs, and whether or how they are responsible for adverse processes and changes in tissue.
OTHER ASPECTS AND INFLUENCES OF DIABETES IN TISSUE  
There are numerous additional aspects in the context of diabetes and collagen mechanics that need further investigation. AGEs may influence tissue metabolism by binding to certain receptors important for maintaining the remodelling process or activating an inflammation cascade responsible for impaired tissue behavior [160]. Also changes in hydration of the collagen fibril due to increased AGEs content have been observed [8], which is likely to change collagen fibril mechanics, since hydration is known to have a strong effect on the mechanical behavior of collagen. The influence of these factors on the mechanical properties of tissue as well as the interplay with AGEs cross-linking and to which degree they are responsible for impaired tissue behavior has to be investigated. Knowing the main origin could help to develop targeted treatment.

FUTURE IN DIABETIC TISSUE MODELING  
Natural materials are often very complex, displaying hierarchical structures that are highly adjusted to their functions. Bone is a very good example of this adjustment. Researchers suggested that each scale is potentially designed to provide optimal material properties on the macro scale. But especially on the small scales that are difficult to access for imaging techniques and larger scales derive their properties from the components on the smaller scales. Therefore, a comprehensive bottom-up approach by modeling (bone) tissue from the sub-nano-scale to the full scale could provide valuable insights about mechanical behavior at each scale, the initiation of failure, and the influence on the larger scale. Upscaling our findings to the next scale in bone, the fiber and lamellae level, would be of great interest to obtain further insights.
A.1 DEFINITION OF THE BOND POTENTIALS

Figure A.1: Force-distance relation of different bond types

The force-distance parameters for collagen bonds in the TC molecules and the enzymatic cross-links are based on Depalle et al. [42]. The mechanical properties of AGEs cross-links were obtained from full-scale molecular dynamics simulations on the geometry of glucosepane using the ReaxxFF protein force-field following Crippa [39]. Figure A.1 shows the relation of the three different bond types used in our coarse-grained model, where enzymatic cross-links are either modeled as divalent or trivalent bonds. We used 18.72Å as a general equilibrium distance for inserting cross-links after equilibration, as the bead diameter is defined as 14.72Å and the maximum cross-link length is estimated to be 3.8Å [64]. Cross-links were inserted with a distance criterion in order to stay close to their equilibrium position. Parameters are displayed in Table 6.1.

A.2 MECHANICAL PROPERTIES OF COLLAGEN FIBRILS IN TENSILE TESTS

From Fig. A.2, we obtain the mechanical properties from different simulations of destructive tensile tests on collagen fibrils with different AGEs and ECLs densities.
Figure A.2: Mechanical properties of collagen fibrils with varying combinations of ECLs and AGEs densities.
A.3 General Force Field Modelling and Parameterization

For a comprehensive description of the modelling procedure and the force field definition, please refer to [97] and [42]. Here we give a short overview of the parameters used for implementation.

In molecular dynamics the total potential energy $E_{\text{total}}$ of a system is defined by the sum of all contributing energies due to bonded and non-bonded interactions

$$E_{\text{total}} = E_{\text{bond}} + E_{\text{angle}} + E_{\text{inter}} = \sum_{\text{bond}} \Phi_{\text{bond}}(r) + \sum_{\text{angle}} \Phi_{\text{angle}}(\phi) + \sum_{\text{inter}} \Phi_{\text{inter}}(r), \quad (A.1)$$

where $E_{\text{bond}}$ is the bond energy due to stretching, $E_{\text{angle}}$ the dihedral bond interactions energy due to bending and $E_{\text{inter}}$ the pairwise interaction energy due to molecular interactions such as Van-der-Waals forces. Forces define the interactions between the particles and are calculated as the negative derivative of the potential energy, depending on the particle distance

$$F = -\frac{\partial \Phi(r)}{\partial r} \quad (A.2)$$

or angle

$$F = -\frac{\partial \Phi(\phi)}{\partial \phi} \quad (A.3)$$

For the definition of the bond interactions and the equations used for force calculation due to bonds, please refer to Sec. 7.2.2. The dihedral bond interactions due to the tropocollagen molecule geometry not being purely straight are modelled as

$$F_{\text{angle}}(\phi) = -k_B(\phi - \phi_0) \cdot \phi \quad , \quad (A.4)$$

with $\phi$ as the angle between three particles causing forces when not in equilibrium position $\phi_0$ and $k_B$ accounting for the bending stiffness.

The non-bonded interactions are modelled via a Lennard-Jones potential with a soft-core as follows

$$F_{\text{non-bond}}(r) = \begin{cases} F_{\text{LJ}}(r) & \text{if } r \geq \lambda \sigma_{\text{LJ}} \\ F_{\text{LJ}}(\lambda \sigma_{\text{LJ}}) & \text{if } r < \lambda \sigma_{\text{LJ}} \end{cases} \quad (A.5)$$

where

$$F_{\text{LJ}}(r) = \frac{1}{r} \left[ 48 \epsilon_{\text{LJ}} \left( \frac{\sigma_{\text{LJ}}}{r} \right)^{12} - 24 \epsilon_{\text{LJ}} \left( \frac{\sigma_{\text{LJ}}}{r} \right)^{6} \right]. \quad (A.6)$$
Table A.1: Parameters used in coarse-grained molecular dynamics mesoscale model of collagen fibrils [42]

<table>
<thead>
<tr>
<th>Parameters used in coarse-grained molecular dynamics mesoscale model of collagen fibrils</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibrium particle distance ($r_0$, Å)</td>
<td>14.00</td>
</tr>
<tr>
<td>Critical hyperelastic distance ($r_1$, Å)</td>
<td>18.20</td>
</tr>
<tr>
<td>Bond breaking distance ($r_{\text{break}}$, Å)</td>
<td>21.00</td>
</tr>
<tr>
<td>Tensile stiffness parameter ($k_0$, kcal mol$^{-1}$ Å$^{-2}$)</td>
<td>17.13</td>
</tr>
<tr>
<td>Tensile stiffness parameter ($k_1$, kcal mol$^{-1}$ Å$^{-2}$)</td>
<td>97.66</td>
</tr>
<tr>
<td>Regularization factor ($z$, -)</td>
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</tr>
<tr>
<td>Equilibrium angle ($\phi_0$, degree)</td>
<td>170.0 to 180.0</td>
</tr>
<tr>
<td>Bending stiffness parameter ($k_B$, kcal mol$^{-1}$ rad$^{-2}$)</td>
<td>14.98</td>
</tr>
<tr>
<td>Dispersive parameter ($\epsilon_{\text{LJ}}$, kcal mol$^{-1}$)</td>
<td>6.87</td>
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<tr>
<td>Dispersive parameter ($\sigma_{\text{LJ}}$, Å)</td>
<td>14.72</td>
</tr>
<tr>
<td>Soft core parameter ($\lambda$, -)</td>
<td>0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Particles at ends of TC molecules</th>
<th>Same parameters as collagen molecules except:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bond breaking distance ($r_{\text{break}}$, Å)</td>
<td>70.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameters used for ECLs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibrium particle distance ($r_0$, Å)</td>
</tr>
<tr>
<td>Critical hyperelastic distance ($r_1$, Å)</td>
</tr>
<tr>
<td>Bond breaking distance ($r_{\text{break}}$, Å)</td>
</tr>
<tr>
<td>Tensile stiffness parameter ($k_0$, kcal mol$^{-1}$ Å$^{-2}$)</td>
</tr>
<tr>
<td>Tensile stiffness parameter ($k_1$, kcal mol$^{-1}$ Å$^{-2}$)</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Trivalent Cross-links</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass of each mesoscale particle, atomic mass units</td>
</tr>
</tbody>
</table>

The parameter $\epsilon_{\text{LJ}}$ is the well depth between two particles, $\sigma_{\text{LJ}}$ the distance at which the intermolecular potential between the two particles is zero and $\lambda$ the parameter to adjust the critical force associated to the soft core. We obtained the parameters of the interactions in collagen molecules and for the LJ potential for our coarse-grained model from full-scale simulations in literature and adjusted the approach by adding a soft-core to the LJ Potential [28, 42]. Further, for the definitions of parameters of AGEs cross-links, we performed full-scale steered molecular dynamics tensile tests on glucosepane as a reference cross-link with a reactive force field. For parameters applied in our models, see Tab. A.1.

A.4 ADDITIONAL RESULTS

In the following, we provide additional results to give a comprehensive overview of the evaluated data. During the stiffening of the collagen fibril with increased AGEs cross-link content, we observe a force transmission from AGEs cross-link to the backbone of the TC molecules: Average forces at $\sigma_{\text{peak}}$ in AGEs decrease with
increasing $N_{AGEs}$, increasing $k_1$ and $r_{break}$ (see Fig. A.3), while average forces in bonds within the TC molecule increase (see Fig. A.4. This is the force transmission, that leads to stiffening of the collagen fibril and changed energy absorption via molecular stretching rather than sliding.

Figure A.3: Average force $\left(\frac{\text{kcal}}{\text{mol} \ \text{Å}^{-1}}\right)$ in AGES cross-links at maximum stress $\sigma_{peak}$ at different AGES densities and changing $k_1$ and $r_{break}$

A.5 EFFECT OF FIBRIL GEOMETRY RANDOMNESS ON FIBRIL MECHANICS

To evaluate the effect of the randomly chosen fibril geometry, we run multiple simulations with the same parameters but different random geometric configurations. The results show that the variations due to the random choice on the mechanical
Figure A.4: Average force \( \left( \text{kal mol}^{-1} \text{Å}^{-1} \right) \) in bonds within the TC molecule chains at maximum stress \( \sigma_{\text{peak}} \) at different AGEs densities and changing \( k_1 \) and \( r_{\text{break}} \) behavior are small compared to the effects of different AGEs cross-link densities (see Fig. A.5).
Figure A.5: Stress-strain curves for tensile tests of collagen fibrils including different densities of glucosepane cross-links using 7 different fibril geometries (different arrangements of TC molecules and different randomness of cross-links) – the thicker line corresponds to the used fibril geometry in our study, the thinner lines are other cases.


[9] Orestis G. Andriotis, Wiparat Manuyakorn, Jurgita Zekonyte, Orestis L. Kat-


[38] Christian Couppé et al. “Human Achilles tendon glycation and function in diabetes.” In: *https://doi.org/10.1152/japplphysiol.00547.2015* 120.2 (Jan. 2016), pp. 130–137. DOI: 10.1152/JAPPLPHYSIOL.00547.2015.


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