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The influence of mechanical manipulation on the mechanics, charge distribution and morphology of collagen fibrils on the nanoscale

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KING'S COLLEGE LONDON

DEPARTMENT OF PHYSICS

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

**The influence of mechanical
manipulation on the mechanics, charge
distribution and morphology of collagen
fibrils on the nanoscale**

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October 12, 2021



A ma famille

Abstract

The physical properties of collagen, and in particular individual collagen fibrils have been extensively studied in the past. These physical properties include the mechanical behaviour, and to a lesser extent the electrostatic behaviour of single fibrils. The influence of different loads, strains or the influence of the chemical environment on the stiffness or surface potential of collagen fibrils is experiencing an increase in interest in the community due to the recent advancements in the field of tissue engineering. Collagen, due to its inherent bio-compatibility is deemed as a good scaffold material for tissue engineering. Since these scaffolds are then transferred into the human body it is important to understand how the scaffolds, and hence single collagen fibrils, behave when subjected to different stress. First, I developed a novel technique to stretch and compress individual collagen fibrils which consists in depositing fibrils on a highly stretchable polymer-based substrate. By stretching the substrate, or depositing the fibrils on a pre-strained substrate and releasing the substrate from strain, the fibrils are either strained along with the polymer or compressed. The stretching technique presented in this thesis significantly expands the strain range of tensile tests of individual collagen fibrils. With this technique many fibrils are deposited on the substrate which means that a large number of fibrils can be studied in parallel. Since the fibrils are lying flat on the substrate, the fibrils are still accessible to AFM imaging and probing, and the nanometric resolution is preserved during AFM imaging. This technique also does not require any specialised hardware and can be implemented by most labs at a low cost. By compressing individual collagen fibrils, I was able to show that simply imaging the resulting buckling patterns with an Atomic Force Microscope (AFM) is sufficient to calculate the Young's modulus of the individual fibrils.

I then showed that axial loading of individual fibrils, with an AFM tip on a soft substrate does not only result in indentation of the fibril but also in bending of the fibril inside the polymer-based substrate, which can make the determination of the Young's modulus more accurate as bending is a more defined mechanical test compared to indenting. Contrary to AFM nanoindentation, models for the bending of collagen fibrils do not require the user to determine the exact contact area between the tip and the sample. In this thesis, I studied the stiffness of individual collagen fibrils under strain. To do this, single fibrils were strained up to roughly 25% and I used an AFM tip to apply an axial load to single fibrils. I found that through a possible remodelling of the inter-molecular crosslink network, fibrils undergo strain-stiffening followed by strain-softening. Finally, I studied the change of the surface potential of single fibrils as a function of strain and found that fibrils first become more positively charged when strained up to roughly 12% strains and then become less positively charged with greater strains. I also discovered that when fibrils are released from strain their surface potential does not return to the native state surface potential which is indicative, once again, of a remodelling of the inter-molecular crosslink network.

Declaration

All the work in this thesis is my own. If information was taken or derived from other sources it is mentioned directly in the thesis.

Publications

Research articles:

- **Gachon E**, Mesquida P. *Mechanical strain alters the surface charge of collagen fibrils* / Submitted to ACS Nano (2021)
- **Gachon E**, Mesquida P. *Buckling of biological nano fibers* / Submitted to Nano Letters (2020)
- **Gachon E**, Mesquida P. *Stretching Single Collagen Fibrils Reveals Nonlinear Mechanical Behavior*. Biophys J. 2020; 118(6):1401–8.
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Strain-induced stiffening and charging of collagen fibrils in various forms, presented at:

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- Microscience Microscopy Congress, Manchester UK, July 2019 (poster)
- International Scanning Probe Microscopy Conference, Louvain-la-Neuve, Belgium, May 2019 (poster - Nanoscale Prize Winner)

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Chapter 1

Introduction

1.1 Objectives

Collagen is a protein that forms structures which constitute the basic building blocks of the extracellular matrix (ECM). These structures provide the human body its shape and its strength but at the microscopic scale, collagen forms fibrillary structures which act as a scaffold for the cells inside the ECM. These structures are often subjected to different mechanical stresses and strains which will be discussed in the following section. The aim of this thesis is to gain a better understanding of how the physical properties of single collagen fibrils (surface topography, stiffness and surface charge) change in response to structural changes imposed by external physical and chemical stimuli. Cells are sensitive to the physical properties of their environment and therefore, the results presented in this thesis are important in understanding, predicting and fine-tuning cellular behaviour. The formation of collagen fibrils, their role in the ECM and the stresses they are subjected to will be discussed in the following sections.

1.2 Collagen

Collagen is the most abundant protein in animals and makes up roughly 30% of the total protein mass in humans [1][2]. It is a class of proteins that constitutes a major building block of connective tissues in vertebrates. So far, 29 different types of collagens have

been discovered, each of them having a different polypeptide sequence [3]. Different collagens can also have different functions such as fibril-forming collagens or network-forming collagens [3].

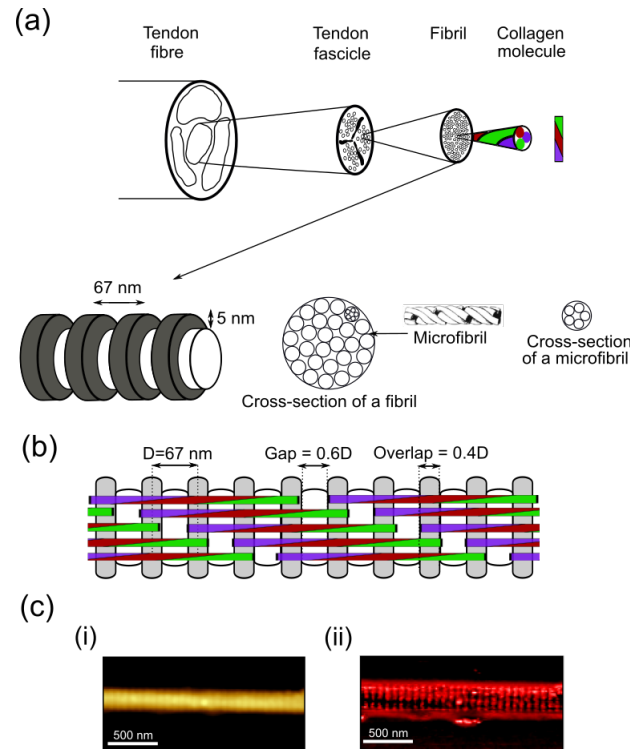


Figure 1.1: (a) Hierarchical structure of a tendon (diagram adapted from [4]). Fibrils have a characteristic surface pattern known as D-banding that has a 67 nm periodicity. Atomic Force Microscopy imaging showed that the banding pattern is made up of peaks that are roughly 5 nm in height in air. According to the Smith model, fibrils are made up of microfibrils. (diagram of a microfibril is from [4]). Five collagen molecules are arranged concentrically to make up a microfibril. Microfibrils are then arranged to form a collagen fibril. The cross-section of a collagen fibril shows molecules arranged into a hexagonal or pseudo-hexagonal lattice [5]. (b) Molecular organisation inside collagen fibrils as described by the two-dimensional Hodge and Petruska model [6]. Collagen molecules are roughly 300 nm long and form layers inside the collagen fibril. Each layer has a 234 residue offset with the overlying and underlying layer [7], which recreates the 67 nm D-banding pattern with overlap regions that are roughly 27 nm and 40 nm long gap regions. (c) (i) Atomic Force Microscopy (AFM) height image of a collagen fibril and the corresponding phase image (ii).

Collagen-based connective tissues have a very hierarchical structure. These structures range from the nanometer scale to the macroscopic scale. The mechanical behavior of such tissues therefore depends on, and is the result of the interactions between all levels of the hierarchy. For instance, tendons at the smallest scale are made of tropocollagen molecules, which assemble into microfibrils, which assemble into collagen fibrils, and finally into the collagen fibres (Figure 1.1 a).

Different types of collagens are present in different tissues and have different supra-molecular structures. Most of the collagen found in animals is in a fibrillar form and is synthesized by specialized cells. The predominant type of collagen in vertebrates is the type I fibril-forming collagen. Type I collagen fibrils have been extensively studied and

found in a wide range of tissues such as skin, bone, teeth, ligament and tendons [8][9][10]. Type I collagen fibrils are therefore able to create different ultra-structures of different dimensions, mechanical properties and ultimately different functions. These fibrils are well-known for providing the strength and maintaining the structural integrity of tissues [11]. They also interact and act as a functional scaffold for the cells inside the ECM [1]. Collagen is therefore involved in many cellular processes such as cell adhesion and cell motility [12][13][14]. Cells interact with the underlying substrate upon which they are deposited thanks to cell binding proteins which bind trans-membrane receptors of the cells to the ECM. The dynamic interaction between the ECM and the cells is involved in tissue remodeling during growth and wound healing [15][16][17].

One characteristic that sets type I collagen fibrils apart from other macromolecules or other fibrillar structures is their easily identifiable axial periodicity, known as *D-banding*. The D-banding pattern occurs every 67 nm and is made up of peaks, known as *overlap regions*, that are roughly 5 nm high in air and *gap regions*. Collagen type II and III also have a 67 nm banding pattern. This D-banding pattern has clearly been observed in Scanning Electron Microscopy (SEM), Atomic Force Microscopy and X-ray diffraction data [18][19][20].

Type I collagen fibrils provide structure to the ECM of many tissues in the human body, which is why they are the type and structure of collagen that were studied in this thesis. Furthermore, the integrity of the fibrils and their strain can be easily assessed thanks to the D-banding pattern at the surface of the fibrils. In fact, D-banding can be used as a marker for strain: if a D-banding length significantly greater than 67 nm is found at the surface of a fibril, the fibril has been strained. In the following section, I will detail how these fibrils are formed in vivo.

1.2.1 Type I collagen fibril formation

Collagen molecules are composed of three polypeptide chains, known as α -chains. These chains are identified by the following classification : $\alpha_n(N)$, where N refers to the type of collagen and n refers to the number of the α -chain [11]. Fibrillar collagen molecules can

be *homotrimeric*, consisting of three identical α -chains or *heterotypic*, consisting of up to three different α -chains. Type I collagen molecules are heterotypic with two identical $\alpha_1(I)$ -chains and a different third $\alpha_2(I)$ -chain. Each α -chain in type I collagen molecules contains a central region with a repeating sequence of roughly 300 Gly-X-Y amino acid triplets, where X and Y can be any amino acid but are most often Proline and Hydroxyproline respectively [7]. In fact, in human fibrillar collagen, Proline and Hydroxyproline make up roughly 20% of the total amino acid content. The central regions of the α -chains then fold into a tight left-handed helix due to steric repulsion between the Proline and Hydroxyproline residues in the repeating Gly-X-Y segments [21]. This process is highly dependant on post-translational alterations of the amino-acid sequence, like hydroxylation of the Proline amino acids for instance. In these helical regions, Glycine residues are present every three residues and form a row at the surface of the helix. *Procollagens* are the result of the trimerization and formation of a right-handed triple helix by three α -chains. This process begins with chain recognition via the Glycine row on each of the α -chains. Procollagens have all the Glycine residues packed inside the coil and the other residues at the surface of the structure [21]. Glycine is therefore essential to the stabilisation of the triple helical structure. Gene substitution assays have shown that a substitution of the X and Y amino acids do not prevent the formation of a helical quaternary structure whereas the substitution of a Glycine residue impairs such folding and can lead to various congenital diseases which will briefly be addressed below[22].

In this form, procollagens are secreted from the fibroblasts (Figure 1.2). Enzymatic removal of the N- and C-non helical ends of the procollagens, known as propeptides, are performed by BMP1/Tolloid-like and ADAMTS metalloproteinases and by furin-like protein convertases [11]. With the removal of propeptides, procollagen molecules become *tropocollagen* molecules. The tropocollagen molecule still has some remaining non-helical structures at the N- and C- terminal ends, known as *telopeptides*. Tropocollagen molecules are very reactive and self-assemble into collagen fibrils in an entropy-driven process (Figure 1.2).

The most well accepted model to explain tropocollagen molecule packing inside the

collagen fibril is the 1964 *Hodge and Petruska model* (Figure 1.1) [6]. In this model, tropocollagen molecules are aligned and form layers. These layers are staggered in the axial direction and each layer has a 234 residue offset with its overlying and underlying layer. This model has been shown to accurately reproduce the D-banding pattern in two dimensions with overlap and gap regions that are roughly 27 nm and 40 nm in length respectively (Figure 1.1 a) [6]. Fibrils are three dimensional structures and the Hodge and Petruska model cannot be extended into three dimensions. Many attempts at extending the Hodge and Petruska model into three dimensions were made over the years. One of the most important contributions corresponds to the *Smith model* and the *compressed fibril model* (Figure 1.1) [23][24]. The Smith model, is of particular interest because it was the first to describe a potential three dimensional packing of collagen molecules into a subfibrillar structure known as the *microfibril*. According to the Smith model, microfibrils are hollow tubes made up of five concentrically arranged collagen molecules [23]. In the longitudinal direction, the collagen molecules inside the microfibrils form a left-handed helix (Figure 1.1 a). Despite being the first model to expand the Hodge and Petruska model into three dimensions, the Smith model had limitations of its own. Namely, the Smith model could only account for fibrils as big as 3.5 nm in diameter, which does not fit the complete range of size for collagen fibrils. The Smith model was then combined with the compressed fibril model which was mainly based on X-ray diffraction data [24]. This model suggests that when looking at the cross-section of a collagen fibril, the microfibrils are compressed so that the collagen molecules are arranged on a pseudo-hexagonal lattice (Figure 1.1 a). Since 1968, the presence of microfibril-like structures packed inside collagen fibrils has been shown experimentally [25][26].

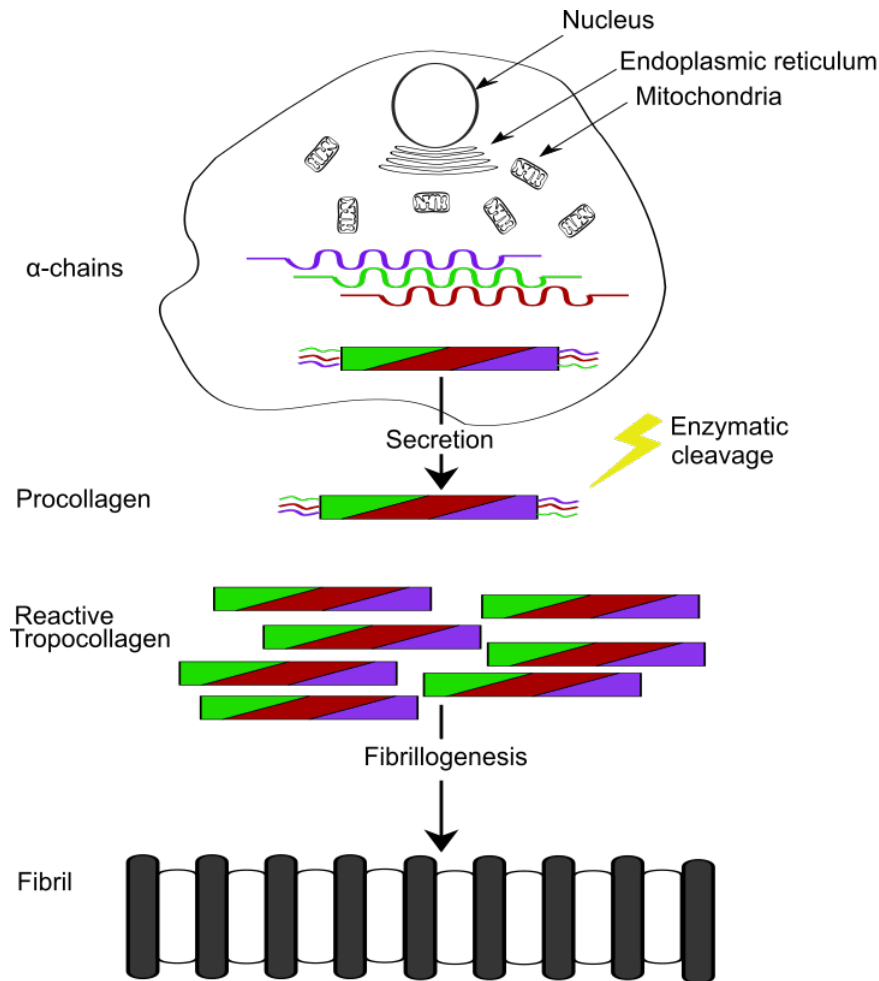


Figure 1.2: Formation of collagen fibrils. α -chains are synthesized in the cells by the endoplasmic reticulum. Three identical or different α -chains intertwine to form a left-handed helix procollagen molecule. Procollagen is then secreted from the cell, and through some post-translational modifications, collagen fibrils self-assemble through an entropy-driven process.

Collagen fibrils are not solely made up of collagen molecules. Type I collagen fibrils are stabilised by various types of inter and intra-molecular crosslinks and in vivo, fibrils are hydrated. The presence of water inside is not inconsequential to the fibrils' structure, surface topography and mechanical properties. Finally, collagen fibrils make up the organic matrix of structures such as bone, cartilage, and teeth. To provide the stiffness of these structures, the collagen fibrils in question are mineralised. All three of these aspects will be discussed in the following sections.

1.2.2 Crosslinks

Covalent crosslinking is essential to stabilising the supra-molecular structure of collagen fibrils. Two different types of crosslinks exist inside collagen fibrils : enzymatic and

non-enzymatic crosslinks.

Enzymatic crosslinks for the most part, are created by enzymes from the lysyl-oxidase family. These enzymes, act on either Lysine or Hydroxylysine residues in the telopeptide regions of reactive tropocollagen (on the N- and C- terminal ends) to convert them into the corresponding peptidyl aldehyde: lysine aldehyde or hydroxylysine aldehyde [11][27]. Once formed, the very reactive aldehydes react with other surrounding aldehydes or un-reacted Lysine and Hydroxylysine groups to form immature intra- and inter-molecular crosslinks.

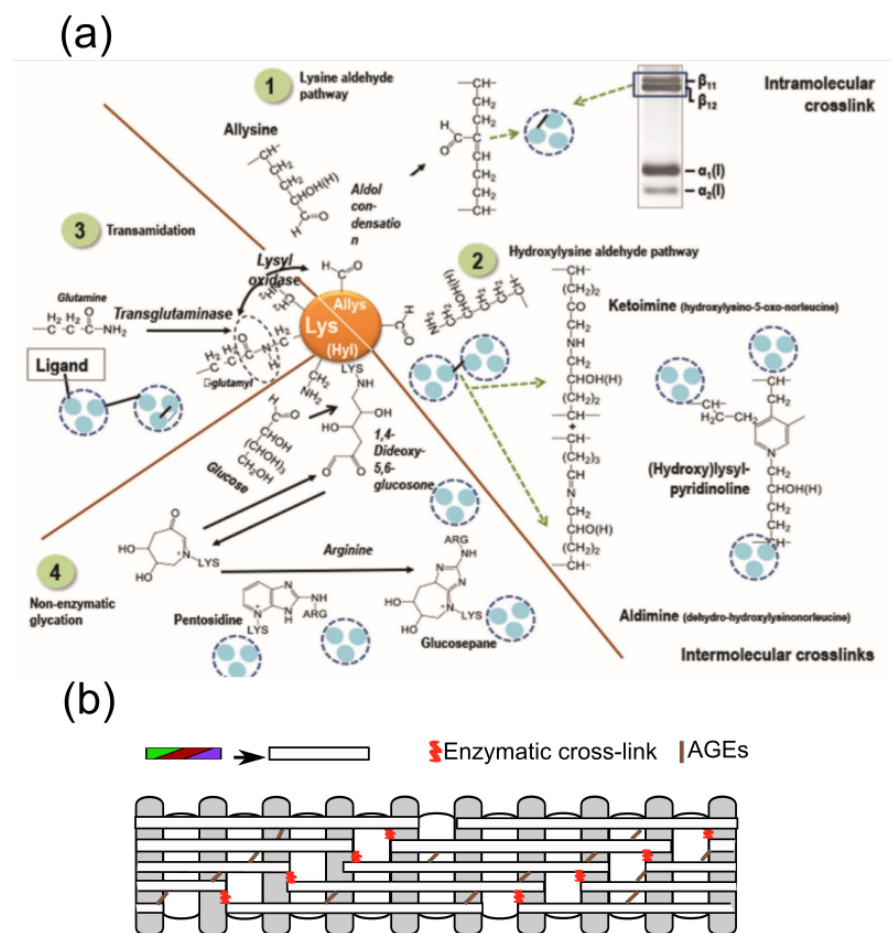


Figure 1.3: (a) Chemistry of the different crosslinking pathways. 1) Lysine oxidase pathway which leads to the formation of Allsines. 2) The Hydroxylysine pathway creates aldimine and ketoimine crosslinks. 3) The transglutaminase pathway which only affects collagen types II, V, VII, and VIII and which mainly links covalently non-collagen ligands to collagen. 4) The non-enzymatic pathway which involves the glycation of collagen by a reducing sugar leading to the formation of Schiff bases with Lysine and then a ketoimine product. These then react with other amino acids and go on to form crosslink adducts known as AGEs such as glucosepane or pentosidine. Image obtained from [27]. b) Schematic representation of different types of crosslinks inside the collagen fibril. Intermolecular enzymatic crosslinks are formed between two telopeptide regions of two adjacent collagen molecules. Intramolecular enzymatic crosslinks form α -chain dimers by covalently binding the telopeptide regions of individual α -chains. Non-enzymatic crosslinks, such as AGEs can be formed between two different collagen molecules.

Immature crosslinks, are important components of collagen fibrils as they provide the fibrils' tensile strength. Two immature crosslinks can also react and form a covalent bond

known as *mature crosslink*. Mature crosslinks, are stabilising agents inside the fibril and are non-reducible.

The lysyl-oxidase crosslinking pathway can generate both intra-molecular and inter-molecular crosslinks. Intra-molecular crosslinks create α -chain dimers by creating covalent bonds between the telopeptide regions of two α -chains from the same collagen trimer (Figure 1.3 (a) 1) [27]. Inter-molecular crosslinks occur from the reaction between the telopeptide region of a collagen molecule and one of the potential binding sites on the helical region of a surrounding collagen molecule. One potential site is an Aldehyde which leads to the creation of an aldimine group (Figure 1.3 (a) 2)). Another binding site is either a Lysine or Hydroxylysine group which creates reactive bivalent crosslinks. Another type of enzymatic crosslinking process is the transglutaminase mediated crosslink formation which has been shown for collagen type II, V, VII, and type VIII (Figure 1.3 (a) 3) [28][29][27].

Covalent crosslinks can also be formed through non-enzymatic processes. Crosslinking of collagen through prolonged exposure to ribose and glucose produces Advanced Glycation End-products (AGEs) (Figure 1.3 (a) 4) [30]. These crosslinks play an important role in the aging process and even occur in diseases like diabetes due to the abnormally high rate of glycosylation of long-lived proteins such as collagen. Glycosylation is the first step of the Maillard reaction which is the chemical reaction which occurs between amino acids and reducing sugars in the presence of heat which results in the browning of food [27]. The glycosylation adducts form AGEs and inter-molecular crosslinks which alter the mechanical properties of collagen making it less flexible and less soluble.

1.2.3 Role of water

In physiological conditions, collagen fibrils are very hydrated. Water has a profound effect on collagen fibrils both structurally and mechanically [31]. Structurally, fibrils swell radially and their volumes increase by five-folds when hydrated [32]. The volume taken up by water molecules in hydrated collagen fibrils is greater than the volume taken up by actual collagen molecules. Therefore, when looking at the structure and mechanics of collagen fibrils, water cannot be ignored.

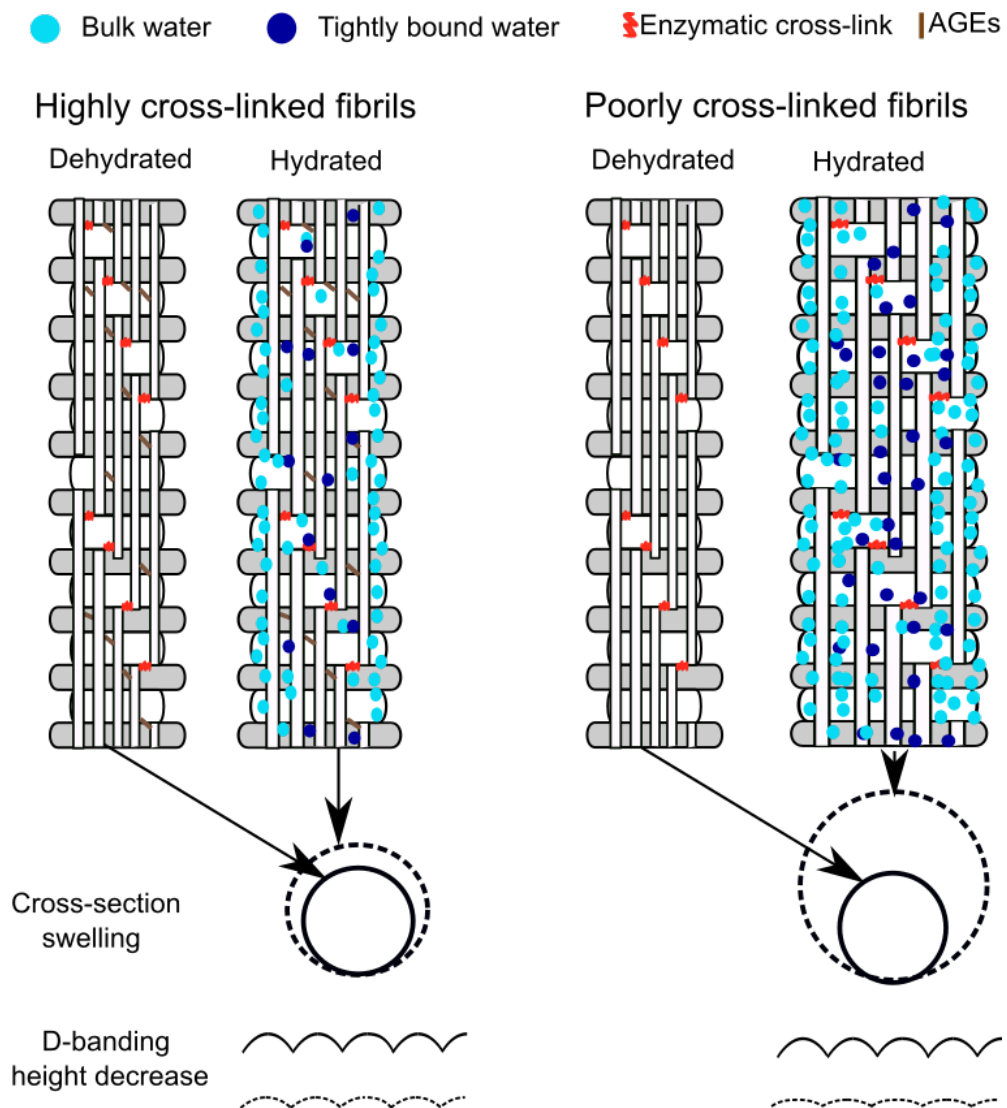


Figure 1.4: Effect of hydration on fibril morphology based on degree of cross-linking density. Highly cross-linked fibrils contain more AGEs than poorly cross-linked fibrils i.e the layers of collagen molecules in highly cross-linked fibrils are more tightly bound to each other than poorly cross-linked fibrils. In the case of non-hydrophilic cross-links, hydration leads to greater radial swelling in poorly cross-linked fibrils compared to highly cross-linked fibrils. The D-banding height in air for highly and poorly cross-linked fibrils is of roughly 5 nm. The D-banding height of hydrated fibrils decreases compared to the D-banding height of dehydrated fibrils. This decrease is more pronounced in poorly cross-linked fibrils compared to highly cross-linked fibrils.

Studies have found that water has a profound effect on the inter-molecular structure of

collagen fibrils and have identified different types of water inside the fibrils. At roughly 25% or less relative humidity, water binds to specific sites along the collagen molecule [33]. This *tightly bound water* has a different melting and freezing temperature than bulk water and has been shown to actively stabilise the triple helical structure of collagen molecules by participating in the H-bond backbone of these molecules [34]. At levels of relative humidity greater than 25%, additional water molecules constitute what is known as *bulk water*. Bulk water does not act merely as a lubricant inside collagen fibrils but encourages long-range interactions and can act as a glue in between hydrophilic surfaces separated by up to 4 nm [35].

The presence of water inside collagen fibrils has to be taken into account when studying the mechanics of collagen fibrils. Crosslinking is known to increase the amount of covalent bonds inside structures and to render them less susceptible to deformation. However, a study showed that the water uptake in the presence of hydrophilic crosslinks is so great that it actually increases the distance between adjacent molecules rendering the entire structure softer [32].

1.2.4 Fibril mineralisation

Collagenous structures such as bone, cartilage or teeth are made up of an organic matrix made of collagen, and an inorganic matrix made of apatite crystals and water associated with collagen [36]. In cartilage, mineralisation can be initiated by cells or matrix vesicles by the formation of large spherical crystal aggregates. However, in bone and teeth, fibril mineralisation starts at the surface of the the gap regions of the fibrils or inside the fibril itself [37]. These crystals are calcium phosphate based and can also contain small amounts of other cations. The general formula for these calcium based crystals is $\text{Ca}_5(\text{PO}_4)_3(\text{OH}, \text{F}, \text{Cl})$ [38]. If the hydroxyl group, OH^- is inserted in the crystal lattice this will create hydroxyapatite. If the fluorine, F^- , or chlorine, Cl^- ions are substituted in the lattice this will lead to fluorapatite or chlorapatite respectively. In the first instances of mineralisation these crystals follow the same 67 nm periodicity as the D-banding pattern by growing in the gap regions without changing the diameter of the fibrils [39][40][41]. These crystals

then form plates that are roughly 4 nm thick and that extend into adjacent overlap regions [41]. Mineralisation can also extend into the inter-fibrillar space and grow from one fibril to the other.

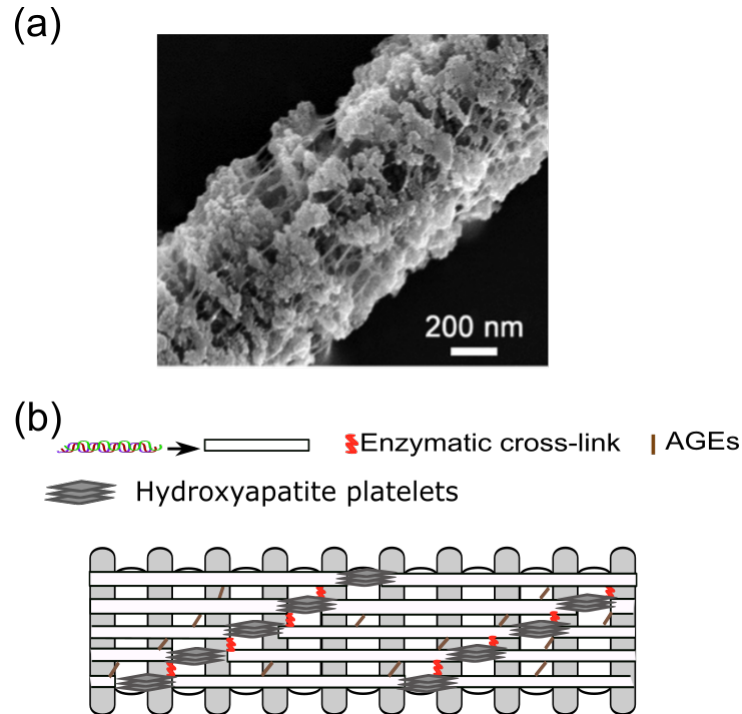


Figure 1.5: (a) SEM image of a mineralised collagen fibril [39]. (b) Schematic of the beginning of the mineralisation process in collagen fibrils with hydroxyapatite platelets forming initially in the gap regions.

Mineralised or un-mineralised collagen fibrils *in vivo* are part of a complex and intricate network of fibrous proteins. In the following section, I will focus on un-mineralised collagen fibrils and describe how collagen networks in the ECM are formed.

1.2.5 Collagen and the ECM

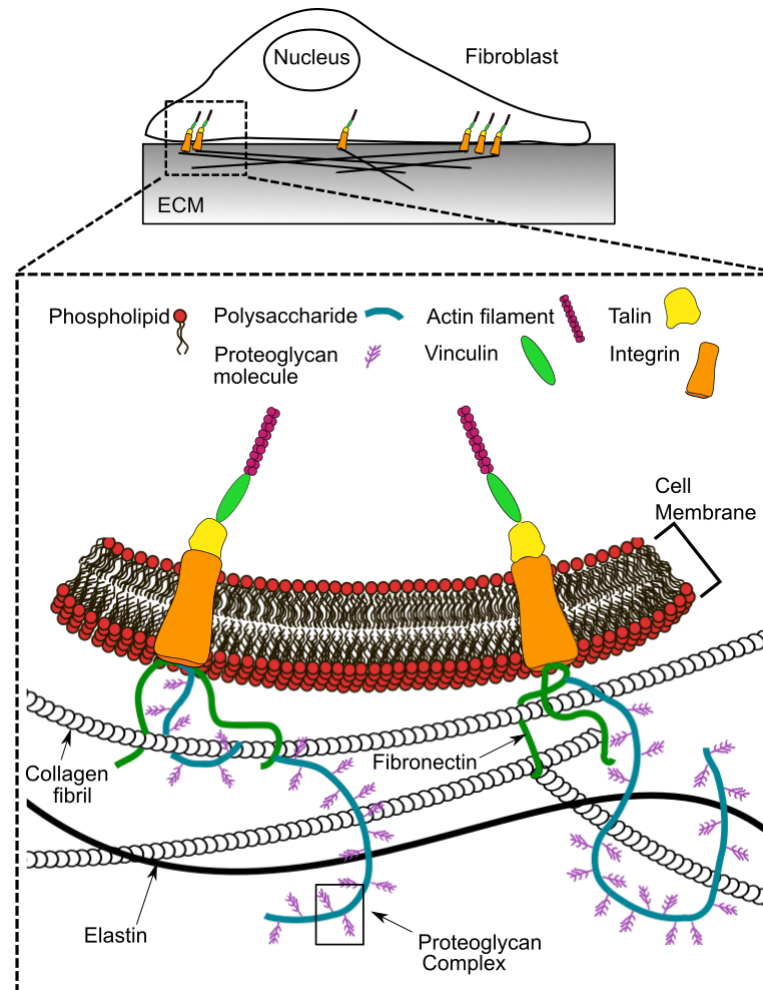


Figure 1.6: Schematic of a cell in the ECM. The zoomed in section represents the interface between a cell membrane and some intra-cellular and extra-cellular proteins.

The assembly of a collagen fibril network *in vivo* is dependent on the mediation from the surrounding ECM and adjacent cells. The ECM is mainly made up of two different types of macromolecules: fibril-forming proteins (collagen, elastins, fibronectins and laminins) and proteoglycans. Fibronectin is of particular interest when studying collagen fibril networks because it has an Arg-Gly-Asp peptide sequence that is both a collagen-binding site but also an integrin-binding site [17]. Integrins are receptors that are found at the cellular surface. Integrins and fibronectins are known as *fibril organizers* and play an active role in the remodelling of the collagen fibril network [42]. In fact, fibronectin fibrillogenesis by surrounding cells is necessary for collagen fibril matrix assembly. Once fibronectin fibrils are created, they are able to interact with integrins which engage with newly assembled

collagen fibrils. The newly assembled fibronectin network can then act as a template for the assembly and growth of a collagen network. Cells via their integrin receptors generate tensile and compressive strains on the fibres inside the ECM and are able to align, re-order and remodel the collagen fibril network [43].

Cellular adhesion to the ECM is essential to the functioning of healthy tissues. The ECM is the host of many adhesion sites for the cells' receptors and also provides mechanical cues to the cells inside tissues which can have an impact on their behaviour [43]. These mechanical cues depend in part on the fibrous protein in question but also on the mechanical state in which they are in, which can in turn be influenced by the cells themselves.

Cells in the human body are embedded inside the complex fibrous network of the ECM. In this thesis it was hypothesised that collagen fibrils inside ECM do not only act as a passive scaffold or substrate for the cells that inhabit it. In fact, many studies have shown that the stiffness and morphology of the fibres inside the ECM can be sensed by the cells and dictate the cells' behaviour. The impact of surface topographies of different patterns and sizes, ranging from the nanometer-scale to the micrometer-scale, on cell attachment, morphology and alignment has been the subject of extensive investigations in the past [12][44][45][46]. Evidence of this impact will now be discussed.

Good cell attachment is crucial for cell growth and proliferation, whereas poor cell attachment can lead to cell quiescence and even apoptosis in some instances. Cell attachment has been studied on substrates with different roughness, stiffness, and patterns with different lateral spacings or of different heights.

Cells are sensitive to the nanoscale roughness and topography of their substrate [12][47]. The effect of substrate roughness in the range of 10-250 nm on neuron cell attachment was studied and showed that optimal attachment occurred in the roughness range of 20-100 nm. Nanodots and microcraters were synthesized to study the effect of lateral spacing on cell attachment [48]. These studies showed that smaller microcraters resulted in worse cell attachment, whereas for nanodots with lateral spacings of 28 nm, 58 nm, 73 nm or 85 nm, cells showed reduced cell attachment at the critical spacing of 73 nm.

Interestingly, this spacing is only 6 nm greater than the average native D-banding length of collagen fibrils, suggesting that collagen fibril topography, and more importantly that the specific D-banding length of the fibrils, could also have an impact on cell attachment. Finally, nanoislands of heights ranging from 11-350 nm showed the best cell attachment for a height of 11 nm[49].

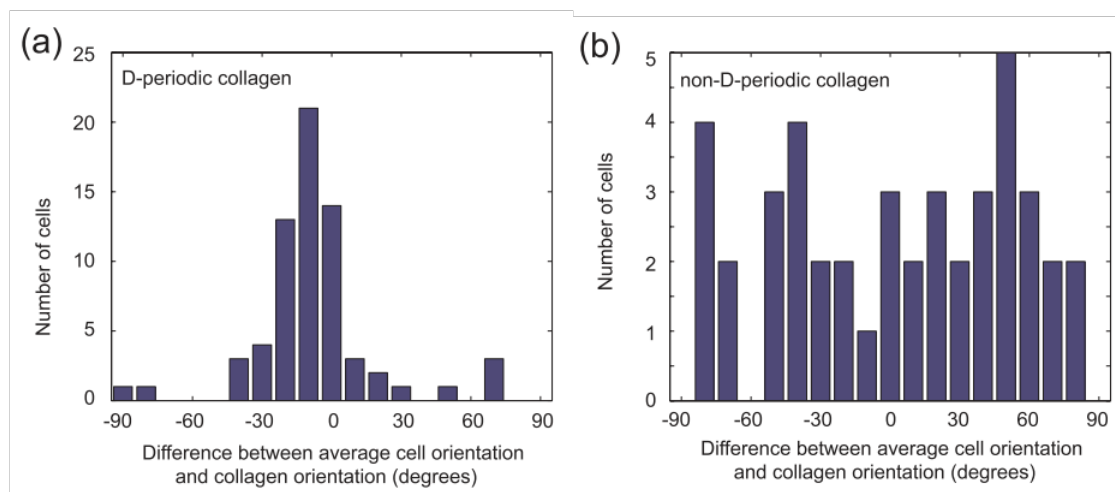


Figure 1.7: Cell orientation is dependant on the presence of D-banding, a 67 nm topography at the surface of the collagen fibrils. Graphs from [12].

Cells were also seeded on gratings and on electrospun fibres to study the effect of the substrates' topography on cell spreading, morphology and alignment. Cell conformation has been shown to not only be sensitive to the geometry of the topography but also, to the dimensions of these topographies [50][51]. A study showed that human mesenchymal stem cells can sense the difference between micro-scale and nano-scale features [52]. The cells' nuclei and cytoskeleton were more aligned on nanoscale gratings compared to micro-scale gratings [52]. Cells seeded on electrospun fibres covered with fibronectin had a greater cell surface area and aspect ratio when the fibres had a diameter greater than $1\mu\text{m}$ compared to those that had a diameter smaller than $1\mu\text{m}$ [50]. Finally, in a study focusing on type I collagen fibrils, primary neonatal mouse dermal fibroblasts were seeded on surfaces coated with D-banded collagen fibrils and fibrils with no D-banding [12]. This study showed that cell elongation, correlated with the presence of the 67 nm D-banding pattern [12]. In that same study, it was also shown that the direction of cellular migration is also influenced by the D-banding pattern [12]. Cells seeded on D-banded collagen fibrils,

on average, were aligned with the collagen fibrils' orientation whereas cells seeded on the non-D-periodic collagen fibrils showed no particular direction in alignment (Figure 1.7) [12].

Cell spread, motility, alignment and differentiation can also be predicted by the mechanics of the ECM. Despite important cell to cell variability, it is now generally accepted that cells on stiffer substrates have stiffer and more organised cytoskeletons with more stable focal adhesion points compared to softer substrates. Mechanical cues have also been shown to guide early stage embryonic development and stem cell fate [53]. Studies where cells were seeded on polyacrylamide substrates of different rigidity showed that cells had lower levels of spreading, increased rates of motility and lamellipodia activity on the more flexible substrates [54]. The cells also had irregular shapes and very dynamic focal adhesion points compared to cells on stiffer substrates. Hydration of collagen fibrils as described earlier makes them on average 1000 times softer than dehydrated fibrils [55][56]. Smooth muscle cells seeded on dehydrated fibrils failed to manipulate the collagen-fibril film. As a result, the cells were more proliferative and had a greater surface area compared to when they were seeded on hydrated fibrils. It was hypothesized that this resulted from the fact that softer substrates made up of hydrated collagen fibrils are much easier for cells to manipulate [56].

The studies discussed above make it obvious that there is a clear and direct link between stem cell differentiation, cellular attachment and behaviour and the stiffness, nanotopography and chemistry of the environment in which the cells are placed. In natural environments, collagen fibrils experience stresses such as longitudinal strain or compression [43]. At the macroscopic level for instance, tendons can be strained and cartilage in the knee is compressed. At a microscopic level cells can physically rearrange collagen fibrils by "tugging" on them [57]. It is clear that in order to understand, and more importantly predict cellular behaviour, the effect of strain and compression on the nanotopography, stiffness and surface potential of single fibrils need to be investigated and understood. In this thesis, each of these aspects have been considered at the single fibril level. When possible, the study of these properties for the gap and overlap regions

were investigated independently as we are now aware that cells can sense different structures that are smaller than 30 nm in length and in height. As described earlier, there is a stark difference between the mechanical properties of hydrated and dehydrated fibrils. The question of hydration and the impact that it has on the physical properties of single collagen fibrils as a function of strain will also be addressed in this thesis. The investigations carried out in this thesis provide a building block in understanding how the cellular environment can impact the cells' behaviour. In the following sections I will address how collagen is involved in diseases and how the findings exposed in this thesis could provide insight for the development of novel therapeutics to treat these diseases. Recently, artificial scaffolds made up of natural, or synthetic fibres, or a combination of both, have been developed in an effort to restore, maintain and improve damaged or lost tissues without having to use tissue grafts. I will therefore explain why collagen based scaffolds are experiencing a growing popularity and why studying the physical properties of such scaffolds could help predict the success of tissue restoration.

1.2.6 Disease

Collagen is a key component of the ECM and provides strength and shape to most of the connective tissues inside the human body. Collagen also plays an important role in a wide range of diseases, like diabetes [30][58], cancer [59][60], osteogenesis imperfecta [22] and even plays a role in the aging of tissues such as skin [30][58][61]. Collagen's role in these diseases can be direct and usually stems from either a mutation in the collagen-coding genes [22], or indirect where either collagen is manipulated by cells and facilitates the progression of diseases such as in cancer [59], or where the collagen's structure is altered by external factors such as UV exposure or extensive crosslinking [30][62].

Mutations in the collagen-coding genes result in a wide range of life-debilitating diseases such as, osteogenesis imperfecta (also known as *brittle bone syndrome*), some forms of chondroplasias, Alport syndrome, arterial aneurysms and Bethlem myopathy to name a few [63]. Osteogenesis imperfecta for instance, is a dominant autosomal disorder which is the result of a mutation in either the COL1A1 or COL1A2 genes. These mutations

usually result in the mutation of a Glycine residue for another larger amino acid [22]. As mentioned earlier, α -chain recognition and formation of the collagen molecule's triple helix is initiated by the recognition of the row of Glycine residues on each of the α -chains. A mutation of a Glycine residue prevents the correct folding of the molecule into a triple helix which leads to this life-altering bone disease.

Cancer cells manipulate and can alter collagen formation inside the surrounding ECM. In vivo, cancerous cells have been shown to outgrow their tumor by depositing matrix, re-aligning the matrix fibres, and secreting growth factors to cause mechanical stress and enhance cellular proliferation [64][65]. By manipulating the surrounding collagen fibrils, cells change both the topography and the mechanics of their surroundings. As described earlier, cells can sense their environment and receive external signals which trigger a cascade of intra-cellular signals. These external signals, then become internal signals which can have an impact on protein production and other cell functions. These cell-collagen loops gradually foster cancer progression [59]. For instance, the p53 pathway regulates the formation of tumor-associated aligned collagen fibril bundles that form 90° at the edge of the cancer cell front, which supports cancer cell progression [59]. Human breast cancer progression generates an increase in collagen fibril deposition resulting in a thickening of the interstitial collagen. These newly deposited fibrils show an increased alignment at the aggressive front [60]. The alignment of these collagen fibrils by one cell facilitates the progression and migration of further cells. Cancer cells have also been shown to create single cell invasion tunnels by secreting matrix metallo-proteinases (MMPs) that degrade the surrounding collagen to facilitate the migration of subsequent cells through the ECM [64].

Collagen can also be altered by external factors like exposure to UV or high levels of reducing sugars such as glucose. The skin's exposure to UV generates highly reactive free radicals which can damage the skin cells and the surrounding ECM leading to premature skin aging or even skin cancer. Studies have shown that exposing collagen fibrils to UV radiation, leads to a decrease in the D-banding height [66]. Exposure of collagen fibrils to UV has also been shown to lead to increased levels of crosslinking in

collagen fibrils [67]. Diseases such as diabetes also affect collagen fibrils. Connective tissue stiffness increases with age and diabetes [58][68][69]. This tissue stiffening has been associated with non-enzymatic, oxidative reactions between glucose and collagen which lead to the formation of previously described AGE crosslinks [27][30]. So far however, there is no direct experimental evidence that shows that increases in AGE content results in an overall stiffening of collagen fibrils. In fact, AGEs seem to affect the mechanics of collagen fibrils by preventing molecular sliding between layers of collagen molecules inside of the fibrils but not to alter the overall stiffness of collagen fibrils [70]. A study even showed that fibrils extracted from diabetic mice tissue were softer than healthy fibrils. The collagen fibrils extracted from these diabetic tissues also had altered structures with reduced cross-sectional areas [71]. Collagen is also very much involved in the aging process, which affects all systems in the human body. In skin for instance, aging is characterised by wrinkling and sagging. As tissues age, enzymatic crosslinking plateaus and AGE crosslink-formation in collagen fibrils increases. Cells on substrates made of aligned aged collagen fibrils were shown to have decreased contractibility [72].

Collagen also plays a role in a wide range of disorders and auto-immune diseases. Scleroderma for instance, is an auto-immune disease which results in thickening and hardening of the skin and formation of fibrous tissue inside organs such as the heart, lungs, kidneys or the gastrointestinal tract [73]. These symptoms are due to an increased production and accumulation of collagen. Other studies investigating the mechanical properties of rheumatoid arthritis tissue showed that the Young's modulus of collagen fibrils in the articular surface and the cartilage-bone interface were of 3.11 ± 0.91 GPa and 5.64 ± 1.10 GPa respectively compared to 2.65 ± 0.31 GPa and 3.70 ± 0.44 GPa in healthy collagen fibrils from articular surfaces and cartilage-bone interfaces [74].

It is clear that alterations in the mechanical properties of collagen fibrils either play a role or are the result of diseases. Studying collagen fibril mechanics and crosslinking could help us gain a better understanding of these diseases and potentially be an interesting avenue for therapeutic intervention. Another avenue where collagen fibrils could play a role is tissue engineering. In medical instances where a damaged organ needs to

be restored or replaced, the current technique that has been widely used is tissue grafting. Nowadays, tissue engineers are developing fibrous scaffolds to promote cell, and ultimately, tissue growth in vitro or in vivo. In the following section, I will discuss this technique further and explain why collagen fibrils are deemed a good scaffold material by the tissue-engineering community.

1.2.7 Role of collagen in tissue engineering

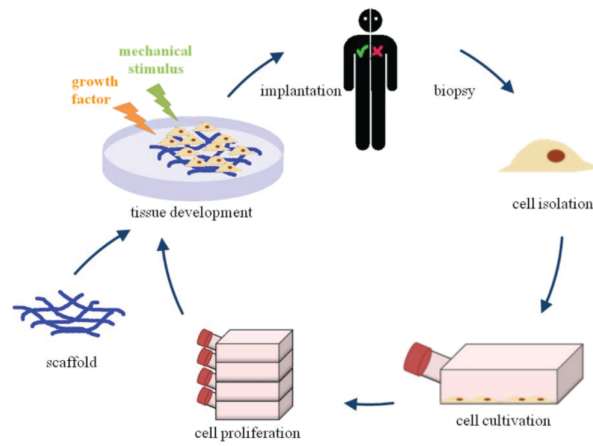


Figure 1.8: Tissue engineering process. Cells used to regenerate tissues are extracted from a patient through a biopsy. The cells are then cultivated and proliferated before being introduced into a polymeric scaffold. Through mechanical stimuli and growth factors the tissue begins to regenerate and is then reintroduced inside the human body. Imaged obtained from [75].

The term tissue engineering was introduced in 1988 at UCLA by Professor Robert Nerem and was defined as the development of bio-substitutes to restore, maintain and improve damaged or lost tissues [76]. In the past, tissue grafts were used for tissue restoration or substitution. Tissue grafting is a delicate technique which presents some limitations: it is dependant on the availability of donor samples, the procedure is very costly for both the donor and the recipient, and both present high risks of infections and tissue morbidity [76]. Tissue engineering on the other hand, is a unique and promising technique: with the aid of a bio-engineered scaffold and chemical fine-tuning, specific cell-lines, can be grown in vitro or in vivo if the scaffolds are implemented directly in the body. The bio-engineered scaffolds are made of natural or synthetic polymers, or a blend of both, and are tailor-made to mimic the mechanical, topographical, and chemical properties of the native ECM of specific tissues for complete regeneration of the tissue [77]. To assure the survival

of the tissue, the scaffolds have a controlled porosity that allows vascularisation and cell growth [78]. Another crucial aspect to consider is the immunogenicity of the polymers used to engineer the scaffolds. If the polymers induce a too big immune response from the recipient, the body could reject the scaffold. In order to decrease the amount of surgeries or interventions a patient would have to go through, scaffolds should also be able to biodegrade in a controlled amount of time [76].

Two different types of polymers are currently used for scaffold designs: natural and synthetic polymers [79]. Synthetic polymers include polycaprolactone (PLC) [80], polyanhydride [81], polypropylene fumarate (PPF) [82] where as natural polymers include fibrin [83], hyaluronic acid [84], chitosan [85], fibroin silk [86], and collagen [87][88] to name a few. Scaffolds can be engineered as pure natural polymer scaffolds, natural/natural polymer scaffolds, natural/synthetic polymer scaffolds, or pure synthetic polymer scaffolds [76]. Figure 1.9 details the different methods that have been developed to fabricate these scaffolds.

Fabrication technique	Advantages	Disadvantages	
Conventional fabrication techniques	1. Freeze-drying	1. Use in a variety of purposes 2. Capability of obviating high temperatures 3. The pore size is manageable to be controlled by changing the freezing method [20]	1. High energy consumption 2. Long-term timescale 3. The use of cytotoxic solvents 4. The generation of small 5. Irregular size pores (usually in the range of 15 to 35 μm)
	2. Solvent casting and practical leaching	1. Fits thin membranes of thin wall three-dimensional specimens 2. High porosity (50–90%) 3. Lost cost technique	1. Time consuming since thin membranes are only used 2. The widespread use of very toxic solvents
	3. Gas foaming	1. Porosity up to 85%	1. If the fabrication process did not change, the product obtained might have a closed pore structure or a solid polymeric skin
	4. Electrospinning	1. Essential technique for developing nanofibrous scaffolds for the TE 2. Homogeneous mixture made of fibers with high tensile strength	1. Used solvents can be toxic 2. Problematic to obtain 3D structures as well as sufficient size of pores needed for biomedical applications 3. Process depends on many variables
	5. Thermal-induced phase separation	1. Construction of the thermoplastic crystalline polymer scaffold 2. Low temperature can be utilized for the integration of bioactive molecules 3. The porosity of fibers is more than 98% a higher surface-to-volume ratio than those constructed	Only used for thermoplastic
Rapid prototyping (RP)	1. Stereolithography (SLA)	1. Enables to overcome the challenges related to wastage in subtractive fabrication methods 2. High resolution 3. Uniformity in pores interconnectivity	1. Has limitations in the process of photopolymerization 2. Requiring massive amounts of monomers and postpolymerization treatment to improve monomer conversion
	2. Selective laser sintering (SLS)	1. Using ultrahigh-molecular-weight polyethylene 2. Provides user excellent control over the microstructures of the produced scaffold by an adapting SLS process parameters 3. Utilized to obtain the preferred properties of the created scaffold	1. Steps after processing the spin of the phase are required to remove injected powder 2. High operating temperature
	3. Solvent-based extrusion freeforming (SEF)	1. It can be utilized to make ceramic, metal, and metal/ceramic composite part 2. It can directly or indirectly function in printing the actual part or a mould 3. It is a new fabrication method for tissue engineering that can be utilized for precise control of scaffold structure at the micron level 4. Success involves the ability to strictly follow the structure of the natural tissue and the mechanical characteristics of the scaffold	1. Temperature extrusion. Their design includes a change to the factors affecting extrusion pressure, including nozzle length-to-diameter ratio, a paste formulation, and the extrusion velocity
	4. Bioprinting	1. Low costs 2. Higher accuracy and greater shape complexity 3. The high speed of printing with the capability of supporting parallel high cell viability (80/90%)	Depends on existence of cells
	5. Fused deposition modeling (FDM)	1. Useful in the scaffold design under the different aspect of scaffold fabrication. Low-temperature deposition	Has limitations in its application to biodegradable polymers [13]

Figure 1.9: Table detailing the different methods for scaffold production. The table was produced by and obtained from [76].

One of the advantages of using natural polymer-based scaffolds is that they can be cell-independent since they are direct elements of the ECM which decreases their preparation times. These types of scaffolds can be created in a matter of minutes or hours compared to weeks for synthetic based scaffolds that have very long cell-seeding stages [89]. Fibril forming collagen such as collagen type I, II and III are of particular interest for collagen based scaffolds because they already contribute to the scaffolding and architecture of the ECM in natural environments. Collagen is inherently bio-compatible, it is also porous, biodegradable and compared to other natural polymers, it exhibits less immunogenicity. One of the challenges associated with collagen-based scaffolds is that

collagen fibrils absorb a significant amount of water altering their mechanical properties. These fibrils lack the mechanical stability that is required of scaffolds for tissue engineering. To overcome this, collagen blends with other natural and synthetic polymers have been created [90] and physical [91][92] or artificial chemical crosslinking of collagen has been used [93].

As mentioned earlier, three different types of collagen-based scaffolds can be created: pure collagen scaffolds [94][95] ; collagen/natural polymer scaffolds [96][97]; collagen/synthetic polymer scaffolds [98][99][100].

Collagen-based scaffolds show great bio-compatibility and have sufficient mechanical properties to produce scaffolds that promote cell growth during tissue engineering. However, more work needs to be done to optimise the scaffolds' structural and mechanical properties to fine-tune cellular behaviour. Just as the ECM's mechanical properties and topography influences cellular behaviour and differentiation, the scaffolds' stiffness, pore sizes and topography also has an impact on cellular behaviour and stem cell fate. Stiffer collagen/hydroxyapatite blend scaffolds promote the proliferation of rat mesenchymal stem cells compared to softer scaffolds of the same 3D structure [101]. Moreover, pore sizes and pore surface areas influence cellular attachment, with bigger pores resulting in an increase in cell attachment [102]. It is therefore important to study and understand how the properties of single fibrils are influenced by different stresses and environmental conditions and how this in turn would affect cellular behaviour. Tissue-engineered scaffolds for tendon repair for instance, will most likely be subjected to tensile stresses and strains. Understanding how these strains can impact the local stiffness of the fibrils in these scaffolds could have an impact on cellular attachment, migration and stem cell differentiation.

In this thesis, mechanical testing of the fibrils was conducted : stresses and strains were applied to single collagen fibrils in order to establish their impact on the stiffness and surface charge of the fibrils. Key concepts of continuum elastic theory such as stress, strain, elastic constants, Poisson's ratio, and finally, elasticity and stiffness which are needed to understand the mechanical probing underwent by the fibrils will be defined in

the following sections.

1.3 Continuum elastic theory

1.3.1 Stress, Strain

The goal of mechanical testing is to determine how a solid behaves when it is subjected to different loads. For a specific body, *stress*, σ is a measure of the forces applied, $\vec{F}$, to imaginary surfaces inside or on the surface of the body, $\vec{A}$. The amount of deformation per unit length caused by a stress is known as *strain* and is denoted as ϵ .

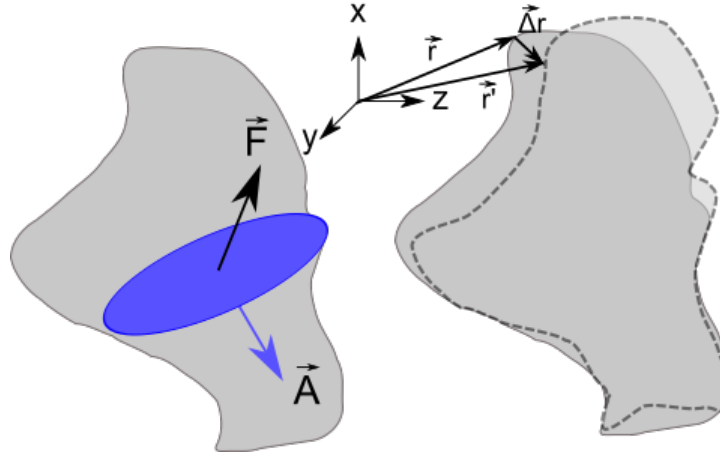


Figure 1.10: Deformation of a solid due to an applied force $\vec{F}$ on an area $\vec{A}$ inside the solid. $\vec{r}$ and $\vec{r}'$ are position vectors and $\vec{\Delta r}$ is the deformation vector.

Stress, σ , can be defined in several ways. *Normal stress* occurs when a force is applied along a single axis perpendicular to the cross-sectional area where the stress is applied : uni-axial tensile or uni-axial compressive forces. *Shear stress* stems from the force vector component that is tangential to the cross-sectional area. *Plane stress* is a two-dimensional state of stress in which all of the stress is applied in a single plane. Normal stress, shear stress and plane stress can be defined as the ratio of a force, F , acting on a surface, A :

$$\sigma = \frac{F}{A} \quad (1.1)$$

Strains, ϵ , quantify the deformation of a body by stress. As illustrated by figure 1.10, the

position vector $\vec{r}$ becomes $\vec{r}'$ due to the deformation of the body. $\vec{r}'$ is proportional to ϵ (Equation 1.2) and the difference between $\vec{r}$ and $\vec{r}'$ corresponds to the deformation vector, $\vec{\Delta r}$. However, just like stress, strains can be described by different definitions. *Uni-axial strain*, ϵ_l , is the longitudinal deformation of a material relative to its initial length when the body is subjected to a normal stress. Shear stress, however, does not change the length of a material but changes its shape. *Shear strain*, ϵ_s , is defined as the change in the angle between two material lines that were initially perpendicular prior to being subjected to shear stress.

$$\vec{r}' = \epsilon \vec{r} \quad (1.2)$$

Stresses are not applied, and materials do not deform, only uni-axially. Therefore both stress and strain need to be described as tensors: σ_{ij} and ϵ_{ij} . These tensors completely define the state of a stress of any point in the loaded material. In these tensors, i corresponds to the direction that is normal to the plane of the cross-sectional area onto which the stress is applied and j , is the direction of the force. The stress tensor relates a unit length direction vector, $\vec{e}_n$ to a force vector acting on a surface [103].

$$\sigma = \begin{pmatrix} \sigma_{xx} & \sigma_{xy} & \sigma_{xz} \\ \sigma_{yx} & \sigma_{yy} & \sigma_{yz} \\ \sigma_{zx} & \sigma_{zy} & \sigma_{zz} \end{pmatrix} \epsilon = \begin{pmatrix} \epsilon_{xx} & \epsilon_{xy} & \epsilon_{xz} \\ \epsilon_{yx} & \epsilon_{yy} & \epsilon_{yz} \\ \epsilon_{zx} & \epsilon_{zy} & \epsilon_{zz} \end{pmatrix} \quad (1.3)$$

When i and j are equal, the tensors describe a normal stress or a uni-axial strain. If i and j are not equal, the tensors correspond to a shear stress or a shear strain. When one of the principal stresses (or eigenvalues) of the stress tensor is null, the material is said to be under plane stress.

Perhaps the simplest mechanical test is the tensile test (Figure 1.11). Collagen fibrils can be characterised as cylinders and when such a body is subjected to a uni-axial tensile force the body will expand along the direction of the applied force and contract perpendicularly to the direction of that force (Figure 1.11). On the other hand, if a body is subjected to a uni-axial compressive force, the body will contract along the direction of

the applied force and expand perpendicularly to the direction of the force. The uni-axial strain experienced by the material, ϵ_l , can be calculated longitudinally or radially with equation 1.4.

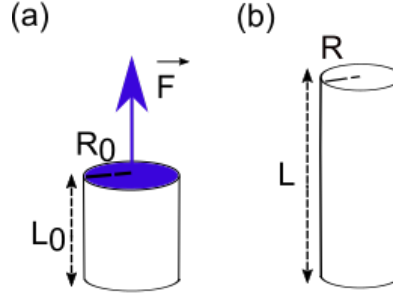


Figure 1.11: Deformation of a cylinder when subjected to a uni-axial tensile force. (a) $\vec{F}$ acts on the blue cross-sectional area of the cylinder, and applies a stress, σ , to the surface: $\sigma = \frac{F}{\pi R_0^2}$ which deforms the cylinder (b). L_0 : initial length, L : Length under strain, R_0 : initial radius, R : radius after deformation, F : tensile force.

$$\epsilon_l = \frac{R - R_0}{R} = \frac{L - L_0}{L} \quad (1.4)$$

In this thesis, individual collagen fibrils will be subjected to strains. These strains will be quantified from the longitudinal expansion of each fibril using equation 1.4. This process is explained in great detail in chapter 4. Fibril strains will then be calculated and used in chapters 4, 6 and 7.

1.3.2 Elastic constants

The relationship between stress and strain for a linear, elastic material is characterised by Hooke's law where E , is the Young's modulus and describes a material's ability to withstand linear stress and deformation [103].

$$\sigma_{ij} = E\epsilon_{ij} \quad (1.5)$$

In crowded physiological systems, materials are not subjected to only uni-axial stresses but can also experience uniform pressure and shear stresses. The *shear modulus*, G , is the material's resistance to shear strains. Similarly to the Young's modulus, the ratio between shear stress, σ_s and shear strain, ϵ_s , corresponds to the shear modulus:

$$G = \frac{\sigma_s}{\epsilon_s} \quad (1.6)$$

The *bulk modulus*, K , quantifies the material's resistance to volumetric deformations and is therefore defined as the pressure, p that is needed to produce a change in volume, V :

$$K = -V \frac{\partial p}{\partial V} \quad (1.7)$$

Materials that are linearly elastic and have a definite, constant composition (homogeneous) and the same mechanical properties in all directions, have elastic, shear and bulk moduli (E , G , and K) that can be combined as [103]:

$$E = \frac{9 \cdot K \cdot G}{3K + G} \quad (1.8)$$

The Young's modulus of individual collagen fibrils will be calculated in chapters 3 and 5 using buckling and bending models respectively. The theoretical framework of these models will be presented in the introductions of these chapters.

1.3.3 Poisson's ratio

As described earlier, when a uni-axial tensile force is applied, most materials will expand in the direction of the force and contract in the direction perpendicular to the force. The Poisson's ratio, ν , is a measure of this effect and corresponds to the negative of the ratio of the axial strain of a material to the lateral strain when a material is subjected to a uni-axial stress (Equation 1.9). Tensile load, illustrated in Figure 1.11, is considered positive whereas compressive load is considered negative.

$$\nu = -\frac{(R - R_0)L_0}{R_0(L - L_0)} \quad (1.9)$$

ν is related to all three moduli through the following equation [104]:

$$\nu = \frac{3K - 2G}{6K + 2G} = \frac{E - 2G}{2G} \quad (1.10)$$

Most materials have a Poisson's ratio between 0 and 0.5 and thus become narrower when uni-axially stretched (Figure 1.11) and expand when compressed. The two extremes, $\nu=0$ and $\nu=0.5$, correspond to materials that do not expand when subjected to a compressive strain and perfectly incompressible isotropic materials, respectively. However, isotropic linear elasticity theory predicts the Poisson's ratios of any object free of constraints, to be in the range of -1 to 0.5 [104]. Materials with a negative Poisson's ratio are called *auxetic* materials. When these materials are subjected to a positive strain, they do not contract axially but rather, expand. Materials with a Poisson's ratio greater than 0.5 are anisotropic.

Knowing the exact Poisson's ratio of biological tissues, such as collagen for instance, is necessary to accurately calculate the mechanical properties of said tissues. The Poisson's ratio of the gap and overlap regions are known to be different, but the exact values of these ratios are unknown [105]. Since most materials have a Poisson's ratio in the 0-0.5 range, in this thesis, collagen fibrils are assumed to be perfectly incompressible and a Poisson's ratio of 0.5 is used in the simulations in chapter 5 as the average Poisson's ratio of the fibril. The limitations associated with this are that if we simulate the straining or deformation of the fibrils, a Poisson's ratio of 0.5 indicates that the D-banding height will remain constant with increasing strain which has been shown to be false [106]. Hence, to limit the influence of the topography on the mechanical measurements obtained from the simulations conducted in chapter 5, fibrils were simulated as smooth cylinders (no D-banding at the surface).

1.3.4 Elasticity and stiffness

Studying the deformation of materials subjected to various stresses enables the determination of the materials' mechanical properties. When a material deforms under stress but returns to its initial state, it is said to be *elastic*. When the material is not able to return to its initial dimensions, the material is said to be *plastic*. Elasticity is an intensive material

property: a property that does not depend on the shape and size of the material. Linear elastic materials return to their initial shape and dimension after being loaded without dissipation of energy. The behaviour of elastic materials is described by Hooke's law where stress increases linearly with strain (Equation 1.5). This linearity is an approximation as most materials exhibit some kind of non-linear behaviour.

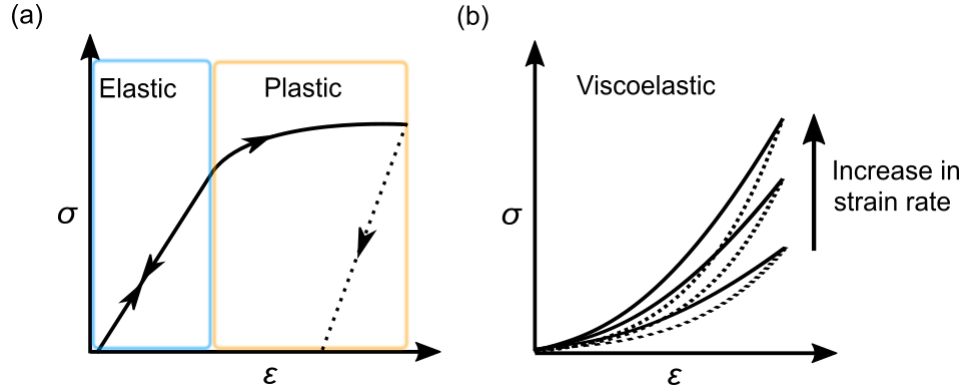


Figure 1.12: (a) Stress-strain curves for elastic and plastic materials. The full line corresponds to the loading of a material and the dashed line corresponds to the unloading. Elastic materials, when unloaded, retrieve their original dimensions. Linear elastic materials conserve their energy during loading and unloading, which is why the unloading curve is not visible as it is the same as the loading curve. The hysteresis that can be seen in nonlinear pathways corresponds to the amount of energy that is lost. The slope of the stress-strain curve for elastic materials corresponds to the Young's modulus, E . Plastic materials when unloaded relax back to a strain greater than their native strain. (b) Viscoelastic materials have a time dependent response to stimuli and the stress-strain curves vary depending on the frequency of deformation.

Stiffness, k , is an extensive material property and describes an elastic material's resistance to deflection or deformation. In the case of the extension of the cylinder in figure 1.11, the axial stiffness can be described as [103] :

$$k = \frac{\pi R^2}{L} E \quad (1.11)$$

Unlike elastic materials certain materials return to their initial dimensions with a delayed response to external stimuli. The time dependence of the reversal of deformation is characterised as *viscoelastic behavior*. Many polymers and biological tissues, such as collagen, are viscoelastic at room temperature and in physiological conditions. In this scenario, stress depends on the strain, ϵ , on the rate of strain, $\frac{d\epsilon}{dt}$, and on the viscosity of the system η [41]. The time dependence of the mechanical response of viscoelastic materials substantiates through two different processes: *creep* and *relaxation*. Under a maintained constant load, creep occurs when a material progressively deforms. Stress relaxation corresponds to a gradual decrease in stress under a constant deformation. Col-

lagenous tissues are known to exhibit viscoelastic behaviour [107][108]. Such tissues being very hierarchical in nature, it can be expected to observe viscosity between different levels of the hierarchy. However, viscoelasticity has also been observed in individual sub-structures of collagenous tissues, such as collagen fibrils [109].

In this work, the assumption is made that the collagen fibrils probed are elastic. In fact, fibrils were axially loaded with an AFM tip with loading rates in the range of 1 Hz - 5 Hz. Previous studies have shown that the mechanical and physical properties of collagen fibrils such as the Young's modulus and the indentation depth do not show any dependency on the strain rate used for strain rates in the range of 1 Hz - 10 Hz [34]. Hence, in this thesis, when collagen fibrils are axially loaded with an AFM tip, the deformation of the fibrils is deemed to be elastic.

Chapter 2

Instrumentation used and underlying principles

The experimental work in this thesis is based on *Atomic Force Microscopy* (AFM). The physical properties investigated are the topography, stiffness and surface potential of collagen fibrils, all of which were studied by either AFM imaging and mechanical loading with an AFM tip or Kelvin-probe Force Microscopy (KFM). This chapter is dedicated to these techniques and their underlying principles.

Atomic Force Microscopy is a technique that is part of the Scanning Probe Microscopy (SPM) family. The first SPM was the Scanning Tunneling Microscope (STM), and was developed by Bining and Rohrer in 1983 [110]. In 1986, Bining and Rohrer obtained the Nobel Prize for their invention and developed the first Atomic Force Microscope (AFM) [111]. SPMs produce images by scanning the surface of a sample with a sharp probe generally referred to as *tip*.

2.1 AFM components

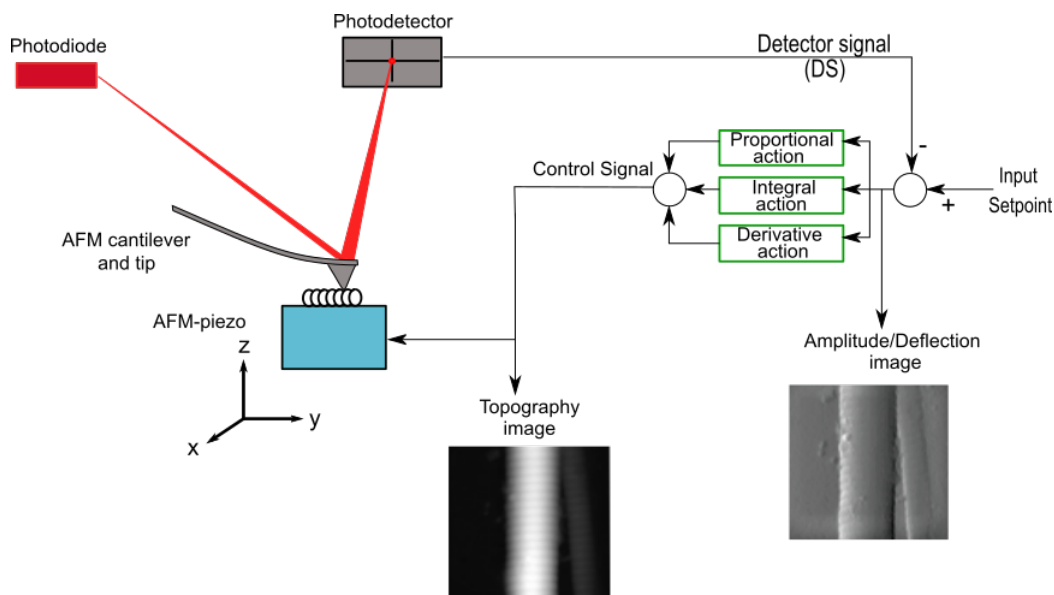


Figure 2.1: General schematic of an AFM. A tip scans the surface of a collagen fibril line by line, controlled by the AFM-piezoelectric transducers. A deflected photodiode beam is reflected off of a cantilever and onto a photodetector. The position of the beam on the photodetector is fed back to the controller and the position of the tip is then adjusted to either maintain a constant distance between the sample surface and the AFM tip, or a constant deflection of the cantilever. These adjustments made by the feedback produces the topography images. The difference between the user-chosen setpoint and the signal detected on the photodetector is the error signal. The error signal is used to produce amplitude error images in intermittent contact mode which will be discussed below.

2.1.1 AFM cantilevers

AFM probes are made up of a micrometer-sized cantilever upon which is attached a smaller tip. The resolution of AFM imaging is dependent on the knowledge of the geometry of the tip used during imaging. The stiffness of the cantilever used is also an important parameter to take into account when using the AFM to probe the mechanical properties of the underlying sample. The shape and size of cantilevers vary. They are either V-shaped or rectangular. Generally, cantilevers are hundreds of micrometers long, roughly $10\text{ }\mu\text{m}$ - $60\text{ }\mu\text{m}$ in width and $0.5\text{ }\mu\text{m}$ - $0.8\text{ }\mu\text{m}$ in thickness. The stiffness of the cantilevers can also vary and typically are in the range of 0.01 N/m to roughly 100 N/m . In practice, softer cantilevers are used to image soft samples while stiffer cantilevers on the other hand are used to image stiff samples.

2.1.2 AFM tips

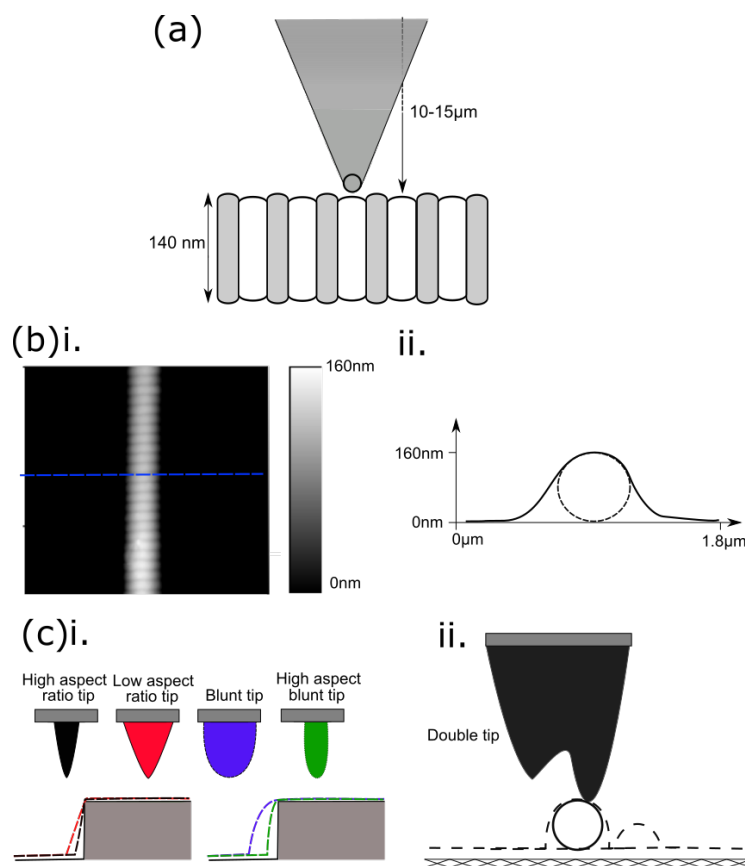


Figure 2.2: (a) To scale representation of a tip imaging a collagen fibril. (b)i. AFM topography image of a collagen fibril. The blue dotted line corresponds to the line profile shown in ii. The dotted circle corresponds to the true outline of the collagen fibril and hence lateral artefacts can be seen in this profile. The diagrams in (c) show that the shape of the tip has an impact on the topography image obtained with an AFM. (c)i. Schematic representation of the line profiles of a step acquired with a high aspect ratio, low aspect ratio, blunt and double tip. (c) ii. To scale representation of a double tip imaging a cross-section of a collagen fibril of 140 nm. The dotted lines represent the line profile produced by the tip.

AFM tips are sharp probes that also vary in size and geometry. Most common AFM tips are pyramidal or tetrahedral and range in length from about 3 μm to 20 μm [112]. The geometry, aspect ratio, and radius of the tip can have a dramatic impact on the quality of the image produced during AFM imaging. In fact, the topography image produced by the microscope is a convolution of the geometry of the AFM tip and of the geometry of the underlying sample's surface topography (Figure 2.2 c). This convolution results in a decrease of the lateral resolution of the image of the topography imaged by the probe (Figure 2.2 (b) ii and (c)i). Consequently, the measurement of the width of sharp edges or curved features are not reliable as they will be greatly exaggerated by this artefact. Artefacts in the height recordings are not as common but can occur when studying soft, compressible samples or due to the adhesion between the sample and the tip [112].

2.1.3 Piezoelectric transducers

AFMs use piezoelectric transducers to move the sample in the sub-nanometric range relative to the position of tip. Piezoelectricity is defined as the ability of certain materials to generate a potential difference across opposite faces of a material when that material is subjected to a mechanical stress and deformation. The inverse piezoelectric effect is used during AFM imaging: a voltage is applied to a lead zirconate titanate (PZT) compound which deforms in a certain direction to either move the sample or the AFM probe in a certain direction with nanometer resolution. The ideal relationship between the strain experienced by a piezoelectric transducer and the applied voltage is linear; in practice, however the behaviour of the transducers diverge from linearity. Some of these non-linearities will be discussed below.

Piezoelectric transducers are subject to artefacts which can impact the topography images produced by the AFM but also limit imaging speeds [113]. The main artefacts found with piezoelectric scanners are *creep* and *hysteresis* which can lead to positioning errors. *Hysteresis* corresponds to a non-linear relationship between the user-defined scan size and the piezoelectric transducer's expansion. Piezoelectric sensitivity, corresponds to the ratio of the expansion or contraction of the piezoelectric scanner to the applied voltage [114]. In the case of atomic force microscopy imaging, hysteretic behaviour means that the piezoelectric transducer's expansion does not only depend on the applied voltage but also on the history of the internal state of the system [113]. When a voltage is increased from an initial voltage of 0 V to a finite value and then decreased again to 0 V, and the piezoelectric deformation is plotted as a function of the applied voltage, the ascending curve will not match the backwards path [114]. The hysteresis of a piezoelectric scanner is quantified by the ratio of the divergence between these two curves to the maximum deformation that the applied voltage can create. In practice, hysteresis occurs when the AFM tip is scanning over the same scan line i.e. over the same feature, but in different directions. If not corrected, hysteresis can cause feature distortions in AFM images [113]. *Creep* on the other hand, is characterised by the material's delayed return to its initial dimensions despite being disconnected from the voltage source. This non-linearity is par-

ticularly visible when using an AFM to image a step with sharp edges [113]. When the tip approaches the edge of the step a sudden change in voltage is applied and the piezoelectric transducer deforms in two consecutive steps. The first step takes place in less than a millisecond [114] and the material deforms under an applied voltage fit to accommodate the full height of the step. The second step is much longer and is responsible for the creep because the piezoelectric transducer continues to deform. To counteract this creep and keep the tip in contact with the sample surface an opposite voltage has to be applied. These artefacts are compensated by strain gauges which correct for displacement errors.

2.2 AFM imaging modes

AFM imaging relies on a few very simple principles. A sharp tip is *raster-scanned* over the sample surface and is maintained by the piezoelectric scanners at either a constant distance from the sample surface, in order to obtain force information, or the cantilever deflection is maintained constant to obtain height information. AFMs contain an optical detection system and the AFM tip which is attached to a reflective cantilever. A photodiode beam is focused on the cantilever and is reflected onto a four-quadrant *photodetector* (Figure 2.1). As the tip is moved across the sample surface in the $x - y$ plane, the cantilever deflects and the tip moves in the z direction following the surface topography. This deflection and change in position results in a movement of the reflected photodiode beam onto the photodetector which is recorded. This activates a feedback mechanism which re-adjusts the position of the tip to either maintain it at a constant distance from the sample surface or maintain the cantilever deflection constant [113].

Since 1986, different modes have been created to maximise the AFM's imaging resolution in certain environments and for different samples. In this thesis the three most common and widely used AFM imaging modes will be discussed : *contact mode*, *non-contact mode*, and *intermittent-contact mode*. However, it should be borne in mind that other imaging modes exist such as small amplitude intermittent- contact mode [115].

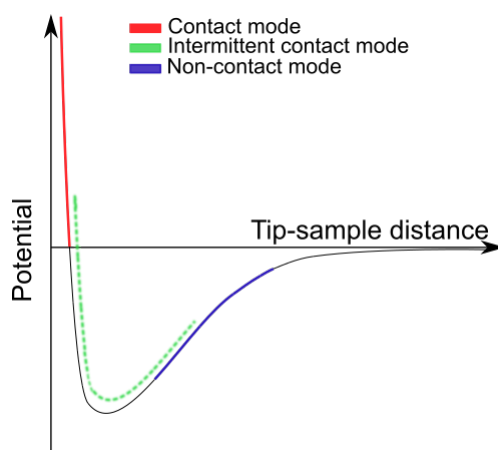


Figure 2.3: The Lennard-Jones potential provides a qualitative representation of the tip-sample interactions as a function of the tip-sample distance. The three main imaging modes which are presented in this thesis are represented based on the tip-sample surface distance. However, other imaging modes exist such as the small amplitude intermittent-contact mode [115].

2.2.1 Contact mode

In contact mode, the AFM probes typically used have a low spring constant of around 0.01 N/m-5 N/m. The lateral resolution of this mode is of roughly 1 nm and the height resolution is of about 0.1 nm. As suggested by the name of this imaging mode, the AFM probe is maintained in constant contact with the sample surface (Figure 2.4). As the tip is scanned across the sample, strong repulsive forces act between the tip and the sample and the feedback loop and piezoelectric transducers maintain a constant deflection of the cantilever i.e. a constant force between the sample and the tip. By being in direct contact with the sample, working in this mode has the potential of disrupting and damaging the surface of very soft samples [113].

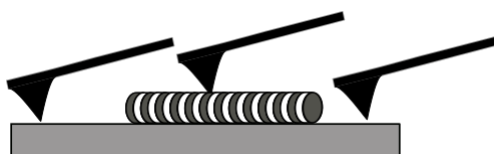


Figure 2.4: Contact mode imaging. The tip is always in contact with the sample surface and images are formed by maintaining a constant deflection of the cantilever.

2.2.2 Non-contact mode

As mentioned earlier, contact mode is not best suited for working with softer samples such as organic samples or polymers. In non-contact mode, the tip is oscillated above the sample surface (Figure 2.5). When the tip oscillates above the sample, the tip-sample

interactions change the resonant frequency of the tip to an effective resonant frequency and also alters the amplitude of the oscillations. The feedback loop maintains a constant distance between the sample and the tip, by either adjusting the resonant frequency of the oscillations and maintaining them at a constant value (*frequency modulation mode*) or by maintaining the amplitudes of the oscillations constant (*amplitude modulation mode*) [113].

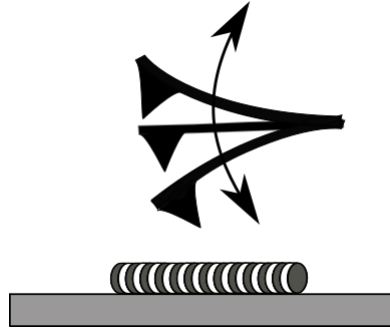


Figure 2.5: Non-contact mode imaging. The tip is oscillated at a distance above the sample, never coming into contact with it.

2.2.3 Intermittent-contact mode

Intermittent contact mode is also known as *tapping mode*. This mode is most well-suited for imaging samples in ambient conditions [113]. The cantilever is oscillated at a distance above the sample surface and intermittently comes into contact with the sample surface (Figure 2.6). This mode requires the use of cantilevers with a spring constant greater than 5 N/m so as to prevent the tip from sticking to the sample surface when they come into contact. Greater spring constants also allow greater scanning speeds and produce less noise in the data. In this mode it is the amplitude of the oscillations that are determined and tracked. When scanning a surface, the amplitude of the oscillations change in accordance with the topographical features of the sample. The feedback system works to adjust and maintain the amplitude of the oscillations constant. This mode is ideal for softer fragile samples and assures a 5 nm lateral resolution and a 0.1 nm height resolution [113]. This is the imaging mode that was used in the works presented in this thesis.

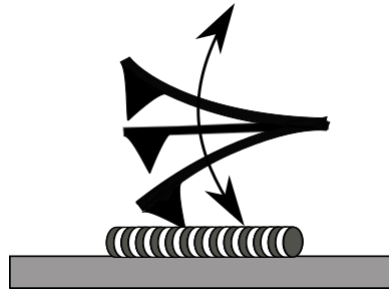


Figure 2.6: Intermittent contact or tapping mode imaging. The tip oscillates and periodically comes into contact with the surface of the sample. Images are generated in this mode by maintaining the amplitude of the oscillations constant.

2.3 Force measurements

2.3.1 Nanoindentation

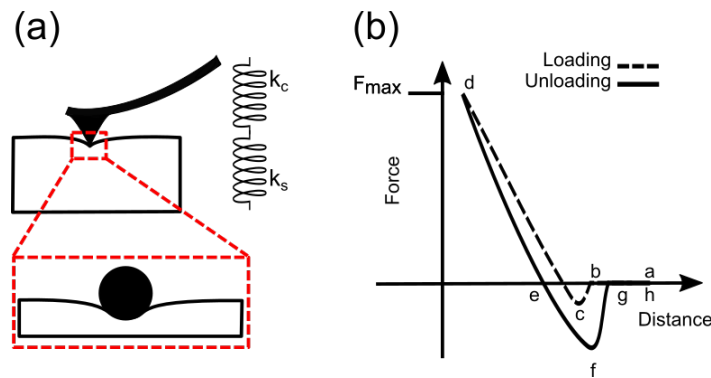


Figure 2.7: (a) Schematic of an AFM tip indenting a substrate. When the tip penetrates the surface of the sample, the AFM tip and the sample act as two springs in series. The stiffness of the cantilever and of the sample are, k_c and k_s respectively. (b) Typical force curve obtained during a nanoindentation experiment on an elastic-plastic sample. a-b: The distance between the sample and the tip decreases. b-c: Attractive forces between the sample and the tip force the cantilever to snap towards the sample surface. c-d: The tip makes contact with the surface and indents it. As the tip penetrates the surface of the sample, the cantilever deflects up to a maximum user-defined deflection. d-e: The maximum load applied to the sample, F_{max} , is reached and the tip retracts from the surface. The tip-sample distance increases until an equilibrium is reached between the attractive and repulsive forces. e-f: The tip-sample distance increases further and attractive forces force the cantilever to deflect down towards the sample. f-g: The distance between the sample and the tip increases further and the tip breaks free from the attractive forces and snaps upwards away from the sample. g-h: The cantilever is no longer deflected and the tip-sample distance keeps increasing.

During conventional nanoindentation, the cantilever is lowered by the z-piezoelectric transducers close to the sample surface. The AFM tip comes into contact with the sample and then indents it until a maximum deflection (i.e maximum load) is reached, this process is called *loading* of the sample. The tip then retracts (*unloading* of the sample) and the deflection of the cantilever returns to its initial state. These experiments lead to force vs. z-piezo displacement curves. By recording the z-piezoelectric extension and the deflection of the cantilever as a function of time, force vs. z-piezo displacement curves (also known as *force curves*) are created. These curves reflect the interactions between the tip and the

sample. The maximum cantilever deflection is user-defined and the corresponding force applied to the sample is calculated by using Hooke's law.

The total force felt by the tip is composed of various long-range and short-range forces. The most important of these contributions when imaging in air will be described here. *Capillary forces* arise from the presence of a liquid vapor in the environment surrounding the tip and the sample which creates a water layer on the surface of the sample and of the tip due to the condensation of water molecules. As the tip-sample distance decreases, the water layers form a water meniscus between the tip and the sample. The meniscus condensates around the tip creating an attractive force on the tip. For very small tip-sample distances, when the tip is either on the cusp of coming into contact with the sample or pulling away from the sample, these forces can be the main contribution to the overall force felt by the tip. Furthermore, capillary forces are proportional to the radius of the tip and can hence be even stronger than van der Waals interactions for large tips and small tip-sample distances [116]. *van der Waals interactions*, and more particularly London dispersion forces are attractive forces that are felt by the tip for distances up to 10 nm away from the sample surface. These interactions arise from the spontaneous and temporary formation of electric dipoles between neutral atoms or molecules that do not have a permanent dipole moment [116]. van der Waals interactions are dependent on the distance between the sample and tip, h , on the radius of tip ($F \simeq r^{-7}$), and sample shape, but also on the thickness of the adsorbed liquid layer at the surface of the sample. In fact, it was determined that for h greater than the liquid layer thickness, van der Waals interactions are dominated by the properties of the sample whereas for h smaller than the liquid layer thickness, the interactions depend largely on the properties of said liquid layer. Tip shape has also been shown to impact the measured attractive force on the tip. In fact, van der Waals interactions are additive and hence the atoms of the tip apex are not the only ones to contribute to the van der Waals interactions. Ideally, tips are sufficiently sharp such that they can be approximated as spheres coming into contact with a flat surface. In practice, the effect of the side walls of the tip on the attractive forces felt by the tip cannot be neglected. In fact, the wider the tip, the greater the measured force will be. For

tip-sample distances greater than 1 nm, van der Waals interactions are dominant. For tip-sample distances smaller than those in a chemical bond, the electron shells of the atoms from the tip and the surface start to repel each other. Finally, *Electrostatic forces* also known as Coulomb forces, are very strong and long range interactions that act between two charged particles [116]. An attractive force occurs when two oppositely charged particles are close to one another, and a repulsive force, also known as Pauli repulsion, occurs between two particles of the same charge sign. The attractive electrostatic force between the two ions of opposite charges becomes repulsive when the distance between the two decreases and their electron shells start to overlap and interpenetrate. In practice, when dealing with uncharged surfaces, if the tip and sample have been exposed to and are used in air, no net charge is expected to persist on the tip or sample and hence, the resulting electrostatic force is zero.

In liquid other forces related to solvation, hydrophobicity and electrical double-layer forces come into play. Hydrophobic tips for instance have been manufactured to decrease the tip-sample interactions and adhesion during the measurement of the mechanical properties of the sample [117]. The electrical double-layer forces are attractive or repulsive forces that are felt for distances of up to 10 nm. They are generated when two charged surfaces are brought closer together when immersed in an electrolyte solution. Most surfaces become charged when immersed in such a solution which creates a concentration gradient in the medium surrounding the surface called the electrical layer. When the two surfaces are then brought closer, the respective electrical layers overlap leading to either attractive or repulsive forces on the tip [116]. This is of particular interest when studying biological matter, such as collagen for instance, in its physiological medium.

2.3.1.1 Determination of the stiffness of a sample

During the acquisition of a force curve, the cantilever and the sample are like two springs (with their respective spring constants) in series (Figure 2.7 a). Once a force curve is acquired it is possible to extract from it certain mechanical properties of the sample that is studied. In the repulsive regime (the top quarter of the unloading curve), the slope of the

unloading curve is proportional to the combined stiffness, k_{tot} , of the cantilever, k_c , and of the sample, k_s :

$$\frac{1}{k_{tot}} = \frac{1}{k_c} + \frac{1}{k_s} \quad (2.1)$$

During the loading of a sample, both elastic and plastic deformation occurs. During the unloading process only the elastic contribution of the deformation reverts to its original state. The stiffness is therefore only determined from the slope of the top quarter of the force vs. displacement unloading curves.

In order to extract the stiffness of samples, k_s , by nanoindentation the exact stiffness of the cantilever used, k_c needs to be determined. To do so, three different types of methods can be used: *Static methods*, *dynamic methods* and *dimensional methods*.

Static methods rely on applying a constant static force on a cantilever and measuring the cantilever's resulting displacement. To do this, reference masses are added to the cantilever [118]. The uncertainty linked with this method depends on the knowledge of the properties of the reference mass and its accurate placement on the cantilever. These methods have uncertainties of roughly 10% [119]. *Dimensional methods* rely on semi-empirical formulas to calculate the cantilever's spring constant which depend on the cantilever's exact dimensions, material properties, and resonant frequency. The main drawback and source of uncertainty with these methods comes from having to measure the specific dimensions of the cantilever. Since not all cantilevers from the same production batch are exactly the same, a time-consuming, precise measurement of the cantilever's dimensions needs to take place. *Dynamic methods* use theoretical models to link dynamic cantilever properties to its stiffness. One of these methods is the Sader method [120]. In this method, the hydrodynamics of the medium surrounding the cantilever (air or liquid) are modelled and related to the cantilever's spring constant. For a rectangular cantilever for instance, its resonance in vacuum is compared to its resonance in fluid. Then, using the hydrodynamic function, which depends on the fluid's Reynolds number, and on the cantilever's length and width, the cantilever's spring constant can be calculated. One of the advantages of this method is that the length and width of the cantilevers do not vary

much from tip to tip in the same production batch compared to the thickness which can vary based on the wafer used to create the cantilevers. However, this method assumes that the cantilever's resonance quality factor is greater than 1. This is true for fluids such as air but not liquids such as water. This method can achieve uncertainties of 15% to 20% [121].

Another dynamic method known as the thermal noise method, which is the method used in this thesis, uses energetic considerations. In this method, cantilever deflections are recorded. Hence, in order to obtain cantilever deflection values in units of length, the output voltage from the photodetector is translated into a value quantifying the deflection of the cantilever. To do this, the *deflection sensitivity* of the photodetector needs to be measured before thermal tuning of the cantilever. As described earlier, AFM imaging and probing depends on the deflection of the cantilever as the tip scans the surface of a sample or indents a sample. A photodiode beam is focused on a reflective cantilever and is reflected onto a photodetector. The photodetector is actually a semiconductor which converts light into an electrical signal. To calibrate the output photodetector signal so that it can be translated into cantilever deflection data, the deflection sensitivity of the cantilever needs to be determined. Deflection sensitivity is a conversion factor between the photodiode signal and the cantilever bending data. The deflection sensitivity of the cantilever can be measured by performing a force curve on a very stiff substrate, such as glass (Figure 2.8). The unloading curve is linear (Figure 2.8) and the inverse of the slope of the unloading curve in the repulsive domain corresponds to the deflection sensitivity of the cantilever.

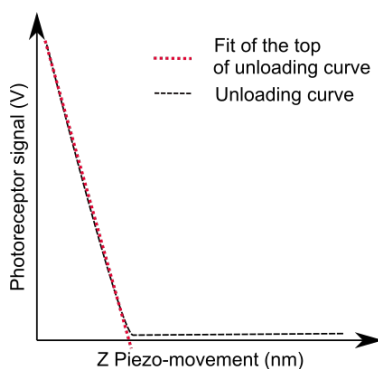


Figure 2.8: Calibration of the deflection sensitivity of a cantilever. The cantilever performs a force curve on an unindentable substrate. The inverse of the slope (red dashed line) of the linear fit of the unloading curve corresponds to the deflection sensitivity of the cantilever.

Once the deflection sensitivity of the cantilever is obtained, the cantilever is maintained at a specific distance above the sample. Brownian motion of the air particles in the environment around the cantilever results in random impacts with the cantilever. These collisions lead to the deflection of the cantilever which is recorded over a specific time interval. This function of cantilever deflection vs.time is then Fourier transformed to obtain the Power Spectral Density (PSD) in the frequency domain. The area under the resonant peak in the spectrum is then integrated using a theoretical model of power spectral densities which allows us to obtain the power associated with the cantilever's resonance, P . In this thesis, the Lorentz power spectral density model was used. This step is automated in most AFMs and known as "thermal tuning".

$$k_c = \frac{k_B T}{\langle x^2 \rangle} = \frac{k_B T}{P} \quad (2.2)$$

Where, k_B is Boltzmann's constant, T is temperature, x is the displacement of the free-end of the cantilever's thermal motion and P the power associated with the cantilever's resonance. x is obtained by converting the photodiode voltages into cantilever deflection distances by measuring the cantilever's deflection sensitivity as described earlier.

The accuracy of the thermal noise method depends on multiple factors. For instance, non-thermal sources of noise might contribute to oscillations of the cantilever, such as electrical noise for instance. However the biggest source of uncertainty is the accuracy with which the power spectral density can be obtained. This depends in part on the resolution of the photoreceptor but also on the sampling rate at which the deflection of the cantilever is recorded during thermal tuning. The former mainly prohibits the use of very stiff cantilevers which produce smaller deflection signals. The Nyquist Sampling Theorem states that the accuracy of a Fourier Transform is maintained for frequencies up to half of the sampling rate. The sampling rate of the AFM controller used in this thesis (a NanoScope V Controller) is 64 kHz. Hence cantilevers which resonate at frequencies greater than 32 kHz can lead to inaccurate power spectral densities. With this method, which is the one used in this thesis, only the fundamental peak of the power spectrum is

considered. When ignoring the higher modes, uncertainties have been estimated to be of up to 30% [119][122]. Such high uncertainties must be considered when measuring the mechanical properties of samples, such as the stiffness or Young's modulus using nanoindentation since the measurement of the same sample with two different cantilevers of the same spring constant could lead to very different values. The effect of the use of different AFM probes to measure the stiffness of a single sample was considered in this thesis and is presented in chapter 6.

2.3.1.2 Determination of the Young's modulus

Stiffness describes a samples' ability to deform under load while still returning to its initial shape. The stiffness of a sample depends on the sample's modulus of elasticity, or Young's modulus which, as described earlier, is the ratio of stress to strain for small strains. Stiffness is proportional to the elastic modulus.

Experiments that involve the indentation of the sample surface and the resulting force curves contain important nano-mechanical information about the sample's material properties. In 1992, the Oliver and Pharr model [123] was developed and has since been widely used to determine the elastic modulus for a wide range of biological samples probed by nanoindentation. As mentioned earlier, during an indentation experiment the tip and sample behave as two springs in series. The behaviour of two solid bodies brought into contact and subject to elastic deformation can be expressed by the following expression [124].

$$\frac{1}{E_r} = \frac{1 - \nu_c^2}{E_c} + \frac{1 - \nu_s^2}{E_s} \quad (2.3)$$

where, ν_c and ν_s are the Poisson's ratios of the tip and the sample respectively. E_r , E_c , and E_s are the reduced elastic modulus, elastic modulus of the tip and of the sample respectively. However, in our experiments, the indenter is a silicon AFM tip which is much stiffer than any of our biological samples [125]. Hence, $E_c \gg E_s$ and equation 2.3 can be rewritten as :

$$\frac{1}{E_r} = \frac{1 - \nu_s^2}{E_s} \quad (2.4)$$

In this thesis, only the reduced modulus will be deduced from the nanoindentation data because the exact Poisson's ratio of collagen fibrils is not known [105]. The reduced Young's modulus of the sample can be expressed mathematically as [123].

$$E_r = \frac{k_s}{2 \cdot \beta} \cdot \sqrt{\frac{\pi}{A_c}} \quad (2.5)$$

Where k_s is the stiffness of the sample determined from the slope of the top quarter of a retract curve, and A_c is the contact area between the sample and the tip. β is a constant close to 1, which takes into account non-axisymmetry of the tip and potential large loads and strains applied to the sample. β being close to 1 and having a small influence on the overall value of E_r , it can be neglected.

For this model to be valid, certain assumptions have to be made. The first is that the sample is an infinite half space. This assumption can be made here because tips used in this thesis are at least ten times smaller than the fibril diameter. Another assumption is that the samples are homogeneous, isotropic and linearly elastic and that no creep or viscoelasticity occurs during the nanoindentation experiments. To calculate the Young's modulus of a sample using equation 2.5, the contact area between the sample and the tip needs to be estimated and hence, the precise radius of the AFM tip needs to be measured. Commercial AFM tips show a high variability in both geometry and size and AFM tips being nanometer-sized objects it is very difficult to visualise and measure their radii. Two indirect approaches have been developed to determine the contact area between the tip and the sample surface. The first approach is the *inverted imaging method*. This method consists in imaging a sample with a well-defined size and geometry. For instance, tip shape calibration samples consisting in arrays of sharp tips of known geometries, or Berkovitch tips can be used [126]. By analysing and comparing the convoluted-topography image obtained with the tip that will be used for nanoindentation to the known shape and topography of the sample, the radius of the AFM tip can be approximated. Another approach consists in imaging the imprint left behind by the tip onto the sample during a nanoindentation experiment. This second approach is less desirable because it involves a plastic deformation of the sample. This technique could also lead to contamination of the tip.

Once the radius of the tip is measured, and assuming that the tip of the indenter has a spherical shape, the contact area A_c is expressed as a polynomial function:

$$A_c = \pi(2Rh_c - h_c^2) \quad (2.6)$$

where A_c is the contact area, and h_c is the contact depth.

The contact depth is determined by [123]:

$$h_c = h_{max} - \epsilon \frac{F_{max}}{k_s} \quad (2.7)$$

Where k_s is the stiffness obtained from the top quarter of the slope of the unloading curve in the repulsive range and ϵ is a constant dependant on tip geometry. ϵ is equal to 0.73 for coned-shaped tips, 0.75 for Berkovitch tips and 1 for a flat punch. F_{max} is the user-defined maximum load applied to the sample and h_{max} is the corresponding maximum penetration depth of the tip into the sample. In practice, h_{max} should not exceed 10% of the total height of the probed sample so as not to sense the underlying hard substrate during loading.

2.3.1.3 Force volume maps

In this thesis, force volume maps were generated to correlate the mechanical properties of different samples to their topographical features. During force volume mapping, a scan size is chosen. Over the scan size, $n \times m$ force curves are performed. From these force curves, height data can be extracted by recording the absolute z-position of the point of contact between the sample and the tip. Mechanical data, such as stiffness, can also be mapped by measuring the slope of the unloading part of the force curves.

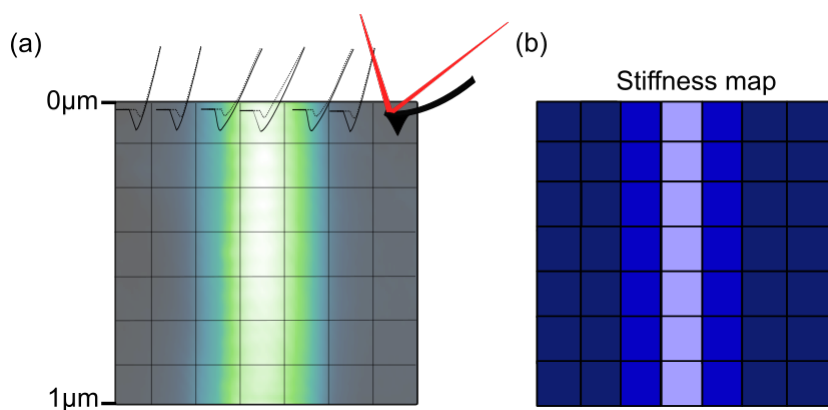


Figure 2.9: (a) Schematic of how a force volume map is obtained. A collagen fibril is first identified (represented by the AFM height image in (a) and in this case, a $1\text{ }\mu\text{m} \times 1\text{ }\mu\text{m}$ scan size is defined. 7×7 force curves are performed over the entire scan size, resulting in a total of 49 curves. Each unloading curve of the 49 force curves will be analysed to extract the stiffness. Each stiffness value will then be used to create a stiffness map as shown in (b). A color table for the stiffness map is chosen and the color of each pixel of the stiffness map corresponds to a specific stiffness value i.e. is an index in the color table.

In practice, force volume maps are not always obtained in an effort to save time and instead, single force curves are performed in a region selected by the user. This can be a problematic technique because due to creep, the crest of the collagen fibril might not always be selected. Hence, the contact area, A_c needed to calculate the elastic modulus of the sample cannot be accurately determined, leading to either an over-estimation or under-estimation of the Young's modulus of the sample.

2.3.1.4 The importance of matching the cantilever stiffness to the sample's stiffness

To optimize sensitivity and maximize the signal to noise ratio, tips with cantilever spring constants which match the stiffness of the studied sample are chosen to indent the sample. This requires that the user knows the stiffness of the sample prior to nanoindenting it or at least, has a general idea of the range of stiffness that the chosen cantilever can measure. In practice this is rarely the case and a series of time-consuming trial-and-error runs with cantilevers of different spring constants are performed prior to doing the actual experiment. In general, stiffer cantilevers provide a higher signal-to-noise ratio however nanoindentation depths are limited to small indentation depths which results in small cantilever deflections and hence, low accuracy of the measured stiffness of the sample. If a tip attached to a cantilever is selected to probe a much softer sample, the tip will indent the sample without any significant deflection of the cantilever. This would lead to a flat force-displacement curve which would give a false result that could not be

exploited : the underlying sample has a stiffness value of 0 N/m. On the other hand, a cantilever that is much softer than the probed sample will deflect without significantly indenting the sample, which would give the false impression that the sample is infinitely stiff. To get accurate stiffness and Young's moduli measurements, it is therefore crucial to select the appropriate cantilever. To facilitate this selection, the range of stiffnesses that can be measured with a specific cantilever can be calculated.

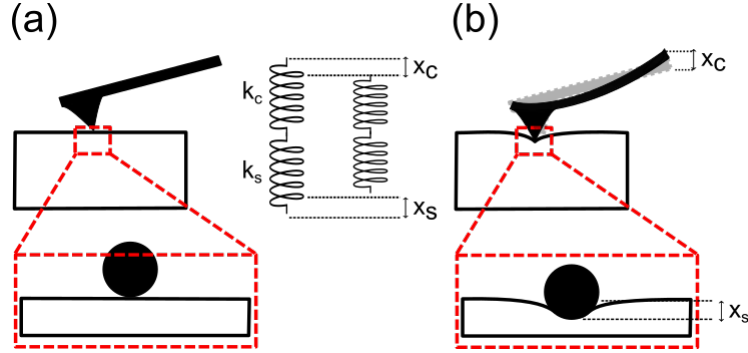


Figure 2.10: (a) The substrate and AFM tip can be approximated as two springs in series with spring constants of k_s and k_c respectively. The AFM tip is like a sphere indenting the surface of a substrate by a few nanometers (b). When the AFM tip indents the substrate by a distance x_s (b), the cantilever deflects in units of length by x_c . The gray AFM tip and cantilever correspond to the AFM tip and cantilever prior to indentation.

The cantilever and sample can be approximated as two springs in series (Figure 2.10).

This system can be described by Hooke's law:

$$F = x_{tot} \times k_{tot} \quad (2.8)$$

where x_{tot} is the total deformation of the system and is equal to $x_{tot} = x_c + x_s$ where, x_c and x_s correspond to the deflection of the cantilever and the deformation of the sample respectively. Equation 2.8 in combination with equation 2.1 can be re-written as:

$$x_{tot} = x_c + x_s = F \times k_{tot} = \frac{k_c \times x_c}{k_{tot}} = \frac{x_c \times (k_c + k_s)}{k_s} \quad (2.9)$$

As described earlier, in the case of indenting a collagen fibril, the sample should not be indented by more than 10% of the fibril's height, hence:

$$1 \text{ nm} < x_s < 10 \text{ nm} \quad (2.10)$$

$$x_c \times k_c > k_s > 0.1 \times x_c \times k_c \quad (2.11)$$

A typical deflection sensitivity is of 40 nm/V. If the smallest detectable voltage change by the photo detector is of roughly 0.1 V, the minimum detectable deflection of the cantilever is of around 4 nm. Hence if we set $x_c = 4$ nm we obtain:

$$4k_c > k_s > 0.4k_c \quad (2.12)$$

For a standard tip with a spring constant of 40 nN/nm, the stiffness of fibrils that can be measured is in the range of 16 nN/nm and 160 nN/nm. Using the Oliver and Pharr model described earlier this range translates into a measurable Young's moduli range of 34.7 GPa $> E_s >$ 1.17 GPa. The elastic moduli measured with an AFM cantilever with a spring constant of 40 nN/nm of a sample whose true elastic modulus is beyond that range would give a skewed result. Hence, it is important during nanoindentation to perform a series of trial-and-error tests to find the most suitable AFM cantilever to probe the sample of choice.

2.4 Kelvin-probe Force Microscopy

In 1991, Nonnenmacher developed a technique that is used to measure the local potential difference between a conductive AFM tip and a sample surface, coined *Kelvin-probe Force Microscopy* (KFM) [127]. At first, KFM was used to measure the surface potential of metals and semiconductors, but more recently, the technique has been used to study organic polymers [128].

2.4.0.1 Principles of KFM

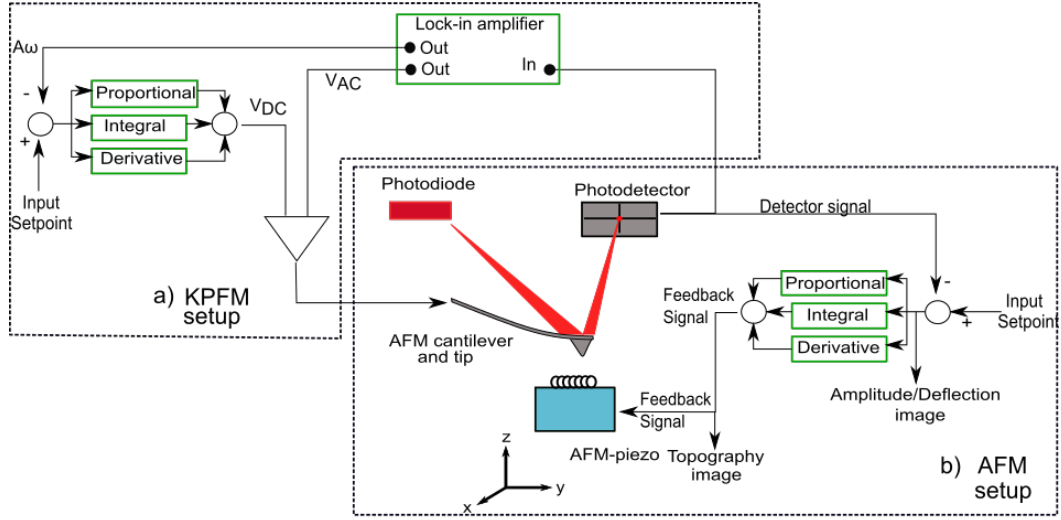


Figure 2.11: Diagram of a KFM setup. In the dotted box “b) AFM setup”, the setup described corresponds to the one showed in figure 2.1 for AFM topography imaging. The dotted box “a) KFM setup” corresponds to the setup used for surface potential imaging. $A\omega$ corresponds to the amplitude of the oscillations at frequency, ω . The input set-point in the KFM part of the diagram is the value used to nullify the amplitude of oscillations. V_{AC} is applied to the cantilever and generates oscillating electrical forces between the sample and the tip. V_{DC} is used to compensate and to nullify the oscillating electrical forces.

KFM is part of the SPM family of microscopy techniques and uses an AFM to measure a contact potential difference between a conductive AFM tip and a sample surface, V_{PD} (Equation 2.13). In this thesis, amplitude modulation-KFM (AM-KFM) was used.

$$V_{PD} = \frac{\phi_{tip} - \phi_{sample}}{-e} \quad (2.13)$$

ϕ_{tip} , and ϕ_{sample} are the work functions of the tip and sample respectively and e , is the electric charge. The tip is electrically excited at its resonance frequency, ω by an AC voltage and a DC offset (Equation 2.14). When scanning the sample surface, the tip is held at a small distance of a few nanometers, h , above the sample surface, known as *lift height*. At this distance, attractive electrical forces between the sample and the tip arise, $F_{ES}(h)$ (Equation 2.15) [129].

$$V(t) = V_{DC} + V_{AC}\cos(\omega t) \quad (2.14)$$

$$F_{ES}(h) = -\frac{1}{2}\Delta V^2 \frac{dC(h)}{dh} \quad (2.15)$$

$$\Delta V = V(t) - V_{PD} \quad (2.16)$$

ΔV is the potential difference between the contact potential difference, V_{PD} , and the voltage applied to the AFM tip, $V(t)$. $\frac{dC(h)}{dh}$ is the capacitance gradient between the tip and the surface of the sample.

By combining equation 2.14 - 2.16 , we obtain:

$$F_{ES}(h, \omega, t) = -\frac{1}{2}(V_{DC} + V_{AC}\cos(\omega t) - V_{PD})^2 \frac{dC(h)}{dh} \quad (2.17)$$

This equation can be split into three equations :

$$F_{DC} = -\frac{C(z)}{dz}(0.5(V_{DC} - V_{PD})^2) \quad (2.18)$$

$$F_{\omega} = -\frac{C(z)}{dz}(V_{DC} - V_{PD})V_{AC}\sin(\omega t) \quad (2.19)$$

$$F_{2\omega} = -\frac{C(z)}{dz}(0.75V_{AC}^2(\cos(2\omega t) - 1)) \quad (2.20)$$

and equation 2.17 can be re-written as :

$$F_{ES} = F_{DC} + F_{\omega} + F_{2\omega} \quad (2.21)$$

Where F_{DC} results in static deflection of the tip, F_{ω} is an oscillating force at the frequency ω used to measure the contact potential difference V_{PD} , and $F_{2\omega}$ is used for capacitance microscopy [129]. It can be seen from equation 2.19 that when $F_{\omega} = 0$, $V_{DC} = V_{PD}$. Hence, F_{ω} is recorded and tracked and the feedback electronics adjust the DC offset, V_{DC} , to nullify the amplitude of F_{ω} . The DC offset is therefore equal to the contact potential difference between the AFM tip and the sample's surface. V_{DC} is therefore recorded and used to create surface potential maps.

In practice, during an experiment each scan line is scanned twice. During the first

scan, the topography of the sample is acquired in tapping mode and the tip is mechanically oscillated at its resonance frequency, ω . During the second scan, also known as *interleave scan*, the tip is raised to the fixed height h above the sample surface, the mechanical drive signal used in tapping mode imaging is turned off and a voltage, $V(t)$, oscillating at the tip's mechanical resonance frequency ω is applied directly to the AFM tip. During this scan, the tip deflects only according to the electrical forces it senses.

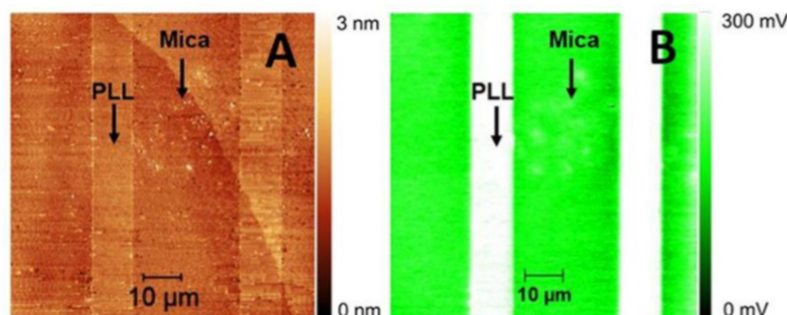


Figure 2.12: The topography image of Poly-lysine stripes on Mica is labelled “A”. The potential image of the same Poly-lysine stripes obtained in AM-KFM imaging mode is labelled “B”. Images were obtained from [130].

In this thesis, the mechanical and electrostatic properties of individual collagen fibrils were probed at different strains using AFM and KFM respectively. Imaging and axial loading of collagen fibrils with an AFM tip was performed in air under ambient conditions (apart from one experiment described in section 6.2.2.2) to test the mechanical properties of individual collagen fibrils. As described in chapter 1, water molecules soften the collagen fibrils considerably and cannot be merely described as a lubricant for the molecules inside the fibrils. Hence, the results presented in chapters 6 only give a qualitative idea of what the deformation behaviour or mechanical properties of collagen fibrils in water might be. Thus, the results presented here provide an initial building block in our understanding of the behavior of single collagen fibrils under strain. However, future work would have to focus on the effect of strain on these mechanical properties when fibrils are immersed in buffers mimicking the physiological environment and of different pHs. Furthermore, KFM experiments were conducted on individual collagen fibrils, to measure their surface charge as a function of strain. As of today, KFM experiments can only be performed in air but studies have shown that the surface potential values of collagen fibrils obtained in air are indicative of the surface potential of collagen fibrils in water

[131]. Hence, only the trends or changes of surface potential as a function of strain for individual fibrils measured in air and presented in chapter 7 reflect what is expected to occur in water and not the actual measured values themselves.

Chapter 3

Buckling of collagen fibrils

3.1 Introduction

The objective of the work in this chapter is to determine the average elastic modulus of single collagen fibrils by simple compression and AFM imaging without the need to do actual mechanical testing on the nanoscale, which would require the use of a force sensor.

In chapter 1, the reasons why the physical properties of individual collagen fibrils need to be determined were explained. In the past, the elastic, bending, and shear moduli of single collagen fibrils have been determined using a variety of techniques such as AFM nanoindentation or AFM-based force spectroscopy [132][133][134]. Nanoindentation is particularly relevant in the study of collagen fibrils as it is the only technique that has the spatial resolution to distinguish between the gap and overlap regions mechanically, but as described in chapter 2 it suffers from a number of practical and fundamental issues due to the small size of the fibrils and the testing setup. Other techniques involved laying single fibrils across a micro-channel and using the AFM probe to bend the freely suspended portion of the fibril to measure its bending modulus. Microelectromechanical systems were also used to strain single collagen fibrils and study their stiffness [135][136][137]. These techniques allowed users to test the mechanical properties of single collagen fibrils but had a low throughput as these fibrils were usually tested one at a time. Furthermore, many of these techniques consist of force measurements which rely on the complex analysis of the force curves obtained to determine the desired mechanical properties.

In this chapter, an entirely different approach was used which allowed me to study multiple fibrils at the same time all the while circumventing the issues posed by AFM nanoindentation and complex force curve fittings. I used buckling, which makes use of the Euler-buckling theory. In most cases, buckling occurs when a high aspect ratio column is subjected to a high compressive stress on one of its ends. Above a certain stress level, the column deflects perpendicularly to the direction of the applied force. Buckling is well-known in structural engineering, and was used to calculate the elastic modulus of individual collagen fibrils. The advantage of using the buckling technique presented in this chapter is that the elastic moduli are determined solely from AFM height images. To induce fibril buckling, many fibrils were deposited on a pre-strained stretchable elastomeric substrate. By releasing the substrate from strain, the fibrils were compressed along with it, thereby adopting easily recognisable buckling patterns.

Buckling pattern analysis of stiff beams with radii in the nanometer range has been performed by Khang et al. and Xiao et al. by depositing them on a pre-strained polydimethylsiloxane (PDMS) substrate and releasing the substrate from its initial strain to compress the beams [138][139]. The compression resulted in the non-linear axial (perpendicular to the PDMS plane) or lateral (parallel to the PDMS plane) displacement of the beams that can best be described by a sinusoidal waveform, $v = v_{max} \cos(kx)$, where k is the wave-vector, $k = \frac{2\pi}{\lambda}$, λ is the wavelength of the sinusoidal waveform and x is the direction along the beam. Continuum mechanics and elastic theory were used in these studies to describe the buckling behaviour as a function of the buckling wavelength, λ , and the radius, R , of the buckled structures. The total energy of the system is the sum of the bending energy of the beam, U_b , the membrane strain energy in the beam, U_m , and the strain energy in the PDMS, U_s , all of which can be described as a function of v_{max} , the amplitude of the waveform and k , minus the work across the beam/elastomeric substrate interface. By minimising the total energy with respect to v_{max} and k the following equation is obtained :

$$\left(\frac{E_{buckling} I}{\bar{E}_s} \right)^{\frac{1}{4}} \cdot k = \left(\frac{2\pi \left(\frac{1}{2-\nu_s} - \gamma - \ln kR \right)}{\left(\frac{3-\nu_s}{1-\nu_s} - 2\gamma - 2 \ln kR \right)^2} \right)^{\frac{1}{4}} \quad (3.1)$$

where $E_{buckling}$ is the Young's modulus of the beam, $\bar{E}_s$ is the reduced Young's modulus of the elastomeric substrate, I is the second moment of area of the beam, and ν_s is the Poisson's ratio of the elastomeric substrate. For a nearly incompressible substrate, such as PDMS, $\nu_s \approx 0.5$. These studies have shown that the buckling wavelength of the nano-structures increases linearly with their radii and that these two variables alone can be used to calculate the Young's modulus of the beams [138][139].

Such a sinusoidal buckling of stiff, long and slender bodies attached to a softer substrate upon release from strain described by equation 3.1 is related to Euler buckling where columns buckle after being subjected to a compressive force on both ends [140]. In fact, the buckling behavior of slender rods, such as single or multi-walled carbon nanotubes, silicon nanowires, or carbon nanotube bundles, studied by Khang et al. and Xiao et al. and the buckling behavior of collagen fibrils that is studied in this thesis correspond to a special case of Euler buckling [138][139]. In this case, the rods are supported along their entire length by a soft underlying foundation and the compressive force is applied along the entire length of the rod that is in contact with the foundation. The buckling model described by equation 3.1 assumes that the columns have an aspect ratio in the 10 - 100 range and that the Young's modulus of the fibrils is much greater than that of the underlying softer foundation and that there is a strong adhesion between the column and the underlying foundation. Type I collagen fibrils in air are widely accepted to have a Young's modulus in the GPa range in air, whereas the PDMS substrate used in this study to deposit the fibrils on has a Young's modulus of 1.32 ± 0.07 MPa [141]. The collagen fibrils used in this work have a diameter in the nm range and are at least $20 \mu\text{m}$ long. Collagen fibrils on PDMS can therefore be characterized as long stiff beams on a softer underlying foundation. Furthermore, Euler buckling assumes that the studied columns are structurally and mechanically isotropic. As described in chapter 1, collagen fibrils are anisotropic, meaning that the longitudinal Young's modulus of a fibril is different than the radial Young's modulus. With regards to buckling however, the radial Young's modulus is not considered. In fact, bending of the fibril corresponds to applying a localised strain and tests the longitudinal properties of the fibrils. Moreover, the presence of D-banding

reveals a longitudinal structural and mechanical anisotropy (see chapter 6). The length scales of the buckling pattern are however an order of magnitude greater than collagen fibrils' D-banding lengths and hence, equation 3.1 will only be able to provide an average longitudinal Young's modulus of the fibril. Finally, equation 3.1 and the Euler buckling model assumes that the deformation of the columns is in the linear, elastic deformation regime of the column. That is certainly the case here because the deformation of the column itself upon buckling is very small. In fact, any material that is not auxetic or that does not have an unusual molecular structure is considered to have an elastic regime for small strains. Collagen fibrils are a natural material and empirical evidence has shown that for small strains the deformation of collagen fibrils is elastic. The break of the linear regime of the stress-strain curves observed in chapter 6 is the result of mechanical probing of the fibrils at high strains.

In this work, I imaged individual collagen fibrils deposited on a pre-strained film of PDMS and once the PDMS film was released from its pre-strain. Fibrils naturally adhere very strongly to the PDMS film and hence, releasing the PDMS film from strain lead to a compression of the fibrils. The buckling wavelength and the radius of individual collagen fibrils were obtained from the AFM height images and used as variables in equation 3.1 to calculate the average Young's modulus, $E_{buckling}$ of multiple fibrils.

3.2 Methods

3.2.1 PDMS film preparation

PDMS substrates were prepared by mixing Sylgard 184™ polymer with the Sylgard 184™ crosslinking agent (Dow Corning Co., Midland, MI, USA) to a 10:1 mass ratio [141]. The mixture was poured into a glass petri dish to obtain foils of 1-2 mm thickness. The mixture was then cured at room temperature for at least 48 h. Curing under such conditions allows the PDMS to achieve approximately 150% longitudinal strains without tearing (Figure 3.2) [141].

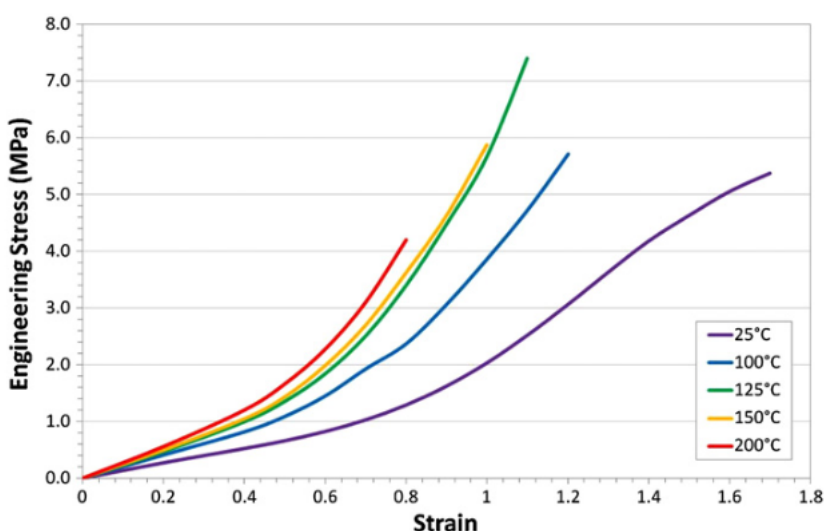


Figure 3.1: Average stress strain curves for PDMS foils cured at different temperatures. Graph obtained from [141]. A strain of 1 corresponds to a strain of 100%. Thin blocks of PDMS cured at a room temperature of 25°C were strained to more than 160%.

3.2.2 Collagen fibrils

Type I collagen fibrils were obtained from a Wistar wild-type, adult rat tail tendon, which was a by-product of teaching-lab courses. All local laws and regulations pertinent to research with vertebrate tissue were observed. Approval was not required for this study. First, the skin was removed from the tail to expose the underlying tendons. Pieces of tendon were extracted using a scalpel and were kept in a Phosphate Buffered Saline solution (PBS, Sigma-Aldrich Company Ltd., Dorset, United Kingdom) for a maximum of four months at 4°C.

Fibrils with different levels of crosslinking were prepared by exposing small pieces of rat tail tendon to glutaraldehyde (GA, Sigma-Aldrich), a strong and well-known protein crosslinking agent [142]. Pieces of tendon extracted from a dissected rat tail tendon were pulled apart and loosened with tweezers before being put in either a 1% or 2% GA solution at 37°C for 40 h. The pieces of crosslinked fibrils were then rinsed with deionised (DI) water in order to remove the excess GA and kept in a PBS solution for a maximum of four months at 4°C.

3.2.3 Sample preparation

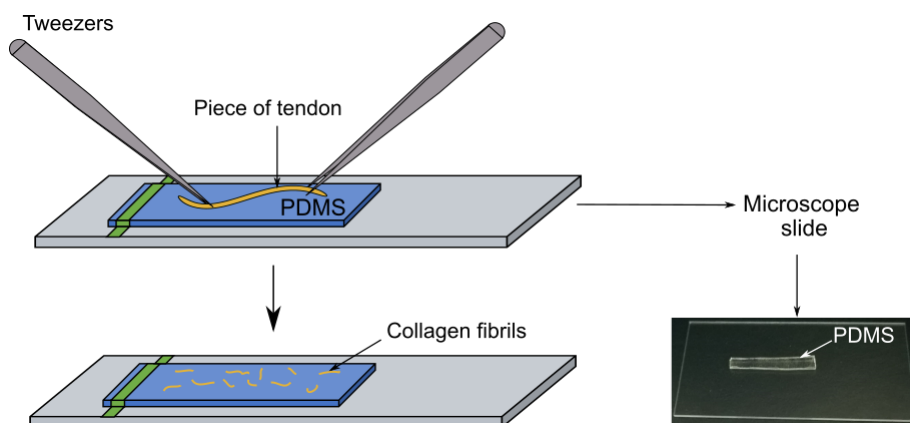


Figure 3.2: Sample preparation process. A small piece of tendon extracted from a rat tail tendon is smeared across a small piece of PDMS, leaving behind a trail of individual collagen fibrils and fibril bundles.

Samples were prepared by first rinsing a piece of native tendon, or tendon exposed to GA, with DI water. The washed piece of tendon was then drawn with tweezers over a rectangular and 1 mm-2 mm thick piece of stretchable PDMS (see section 3.2.1) which was taped strained to 50% to a glass microscope slide. Fibrils being naturally very sticky, drawing a piece of tendon over PDMS leaves a trail of fibril bundles and individual fibrils (Figure 3.2). These fibrils can be identified through AFM topography imaging [105]. The sample was subsequently dried using a stream of nitrogen.

3.2.4 Compressing collagen fibrils

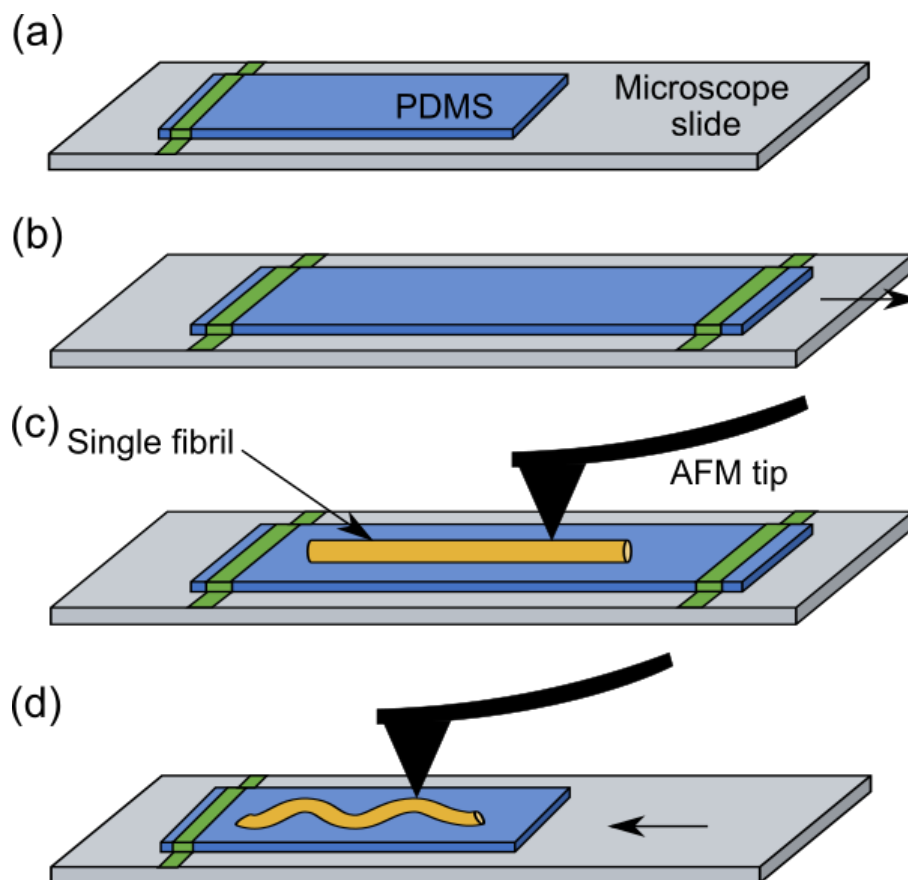


Figure 3.3: Sample preparation and compression of collagen fibrils. (a) A piece of PDMS film is taped at one end to a microscope slide. (b) The free end of the PDMS substrate is pulled with a pair of tweezers and until the PDMS film reaches a strain of 50%. The loose end of the PDMS film is then also taped down to the microscope slide, maintaining the PDMS film in its strained state. (c) A piece of tendon is drawn over the PDMS film leaving behind a trail of un-strained collagen fibrils. AFM height images of the fibrils are obtained. (d) The PDMS substrate is released from strain, and fibrils are compressed, by detaching one end of the film from the microscope slide and returning the film to its original length. AFM height images of the buckled fibrils are obtained.

After being probed in the un-strained state, fibrils were compressed by un-taping one end of the piece of PDMS from the microscope slide and returning the PDMS film back to its original length. Fibrils that had adhered to the strained PDMS in their un-strained states were therefore compressed when the PDMS was released from strain (Figure 3.3 d). After compression, the sample was left overnight before being imaged.

3.2.5 Imaging of the fibrils

All measurements were performed in air and under ambient conditions (relative humidity $\approx 30\%$, temperature $\approx 23^\circ\text{C}$). Topography images of both native fibrils and fibrils artificially crosslinked in either a 1% or 2% GA solution were taken on a Dimension Icon

AFM (Bruker Corporation, Billerica MA, USA). Topography imaging was performed in Tapping-Mode using Budget Sensors Tap300-G tips ($f=50$ kHz; $k=40$ N/m). AFM height and phase images of individual native fibrils were obtained prior to compression. These images were analysed in the Gwyddion Image Processing Software (available free under <http://gwyddion.net/>). To remove any imaging artifacts in the raw AFM images such as line skips for instance, the raw AFM height images were first plane-leveled. The plane is computed by the software from all the pixels in the image and is then subtracted from the data.

3.2.6 Evaluating the wavelength and radius of buckled fibrils

Three samples were created and imaged (see section 3.2.4): one with 1% GA crosslinked fibrils, one with 2% GA crosslinked fibrils and a third with native fibrils. On each compressed sample, individual buckled fibrils were identified and imaged by AFM. Native fibrils buckle perpendicular to the PDMS film plane. The wavelength of native buckled fibrils is obtained by using the "profiles along arbitrary lines" function in Gwyddion. The line is traced along the length of the fibril. From the line profile, peaks and troughs are visible and the distance along at least 5 wavelengths i.e. the distance from one trough or peak to the fifth trough or peak, is obtained. The average wavelength is then obtained by dividing the length value by the number of wavelengths (Figure 3.4 a). Crosslinked collagen fibrils buckle parallel to the PDMS plane. The average wavelength is obtained by tracing an arbitrary line from one maxima of the wave formed by the fibril and over at least five maxima of the fibril (corresponding to five wavelengths). The average wavelength is then obtained by dividing the length measured over multiple wavelengths by the number of wavelengths (Figure 3.4 b). For both native and crosslinked data-sets, the error associated with each average wavelength value was obtained by measuring the length of at least five consecutive wavelengths in a single image and corresponds to the standard deviation of these single wavelength values.

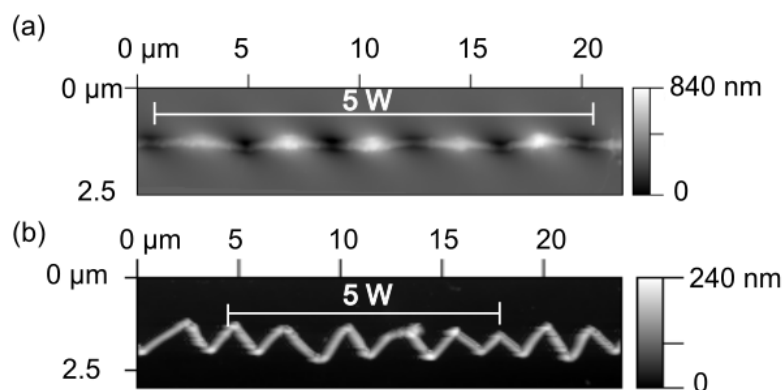


Figure 3.4: AFM height images of (a) native collagen fibrils and (b) 2% artificially crosslinked fibrils. By tracing an arbitrary line along the fibril, the length of five wavelengths can be measured. By dividing that length by the number of wavelengths, the average wavelength of the buckled structure is obtained.

The average diameter of the fibril is obtained by using the "row/column statistical" function. With this function, the line profiles of a user-defined number of rows are averaged. In these experiments at least eight rows of pixels across the fibril are averaged (Figure 3.5). The radius of native fibrils after compression could not be measured due to out-of-plane buckling (Figure 3.5 a) and hence, individual native fibrils were identified on the PDMS film prior to compression and imaged (Figure 3.3 c). This allowed me to measure the radius of these fibrils in their non-buckled states. The PDMS foil was then released from strain and the exact same fibrils that had been imaged prior to compression were found and imaged in the buckled state on the compressed PDMS film (Figure 3.3 d). The radius of in-plane buckled, crosslinked fibrils, however, could be measured by obtaining the average line profile of the fibril in between two peaks. The radii of ten crosslinked fibrils after compression were compared to the radii of these same fibrils prior to compression and were not found to be significantly different. Assuming a circular cross-section, the radius of individual fibrils can be determined accurately by measuring the peak height from the cross-sectional line profiles (Figure 3.5 b).

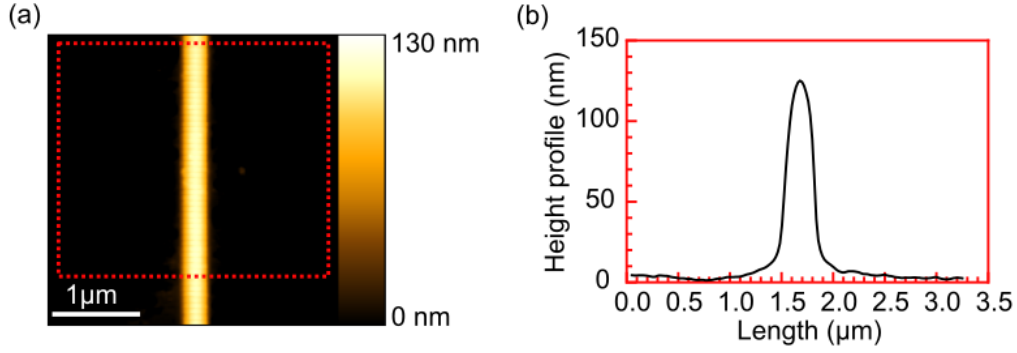


Figure 3.5: (a) AFM height images of a native fibril prior to compression. The red rectangle corresponds to the user-defined area over which the line profiles of each row of pixels are averaged. (b) Corresponding average line profile.

3.2.7 Calculating the Young's modulus of buckled fibrils

The radii and buckling wavelengths of individual collagen fibrils were used as variables in equation 3.1 to calculate the Young's modulus of these fibrils, $E_{buckling}$. It is assumed that the PDMS substrate is a semi-infinite solid because its thickness is of 1 mm-2 mm whereas the diameter of collagen fibrils are on the nanometer scale. The reduced modulus of the PDMS film, $\bar{E}_s$, is calculated by using the following equation:

$$\bar{E}_s = \frac{E}{1 - \nu_s} \quad (3.2)$$

where E is equal to 1.32 ± 0.07 MPa [141] and $\nu_s \approx 0.5$.

3.2.8 Statistical analysis

In this work, the Young's modulus of twelve native fibrils, eight crosslinked fibrils in a 1% GA solution and seven crosslinked fibrils in a 2% GA solution were calculated using the measured λ and R values from their buckled patterns. The statistical comparison between the calculated $E_{buckling}$ values for all three data-sets was done using an ordinary one-way analysis of variance (ANOVA) test. The null hypothesis for this test is that there is no statistical difference between the mean of all three data-sets. During this test, the F ratio and p values are calculated. The F ratio is expected to be close to 1 if the null hypothesis is true. Large F ratios indicate that the differences between the means of each data-set is greater than what we would expect to obtain by chance.

$$F = \frac{\frac{\sum n_j \cdot (\bar{X}_j - \bar{X})^2}{k-1}}{\frac{\sum \sum (\bar{X} - \bar{X}_j)^2}{N-k}} \quad (3.3)$$

n_j is the size and, $\bar{X}_j$ the mean of the j^{th} data-set. $\bar{X}$ is the overall mean, k is the total number of data-sets and N the sum of the sizes of all of the data-sets.

3.3 Results and Discussion

Native fibrils and fibrils artificially crosslinked in either a 1% GA or 2% GA solution were compressed to study their different buckling patterns. AFM height images of compressed native collagen fibrils and artificially crosslinked collagen fibrils showed distinct out-of-plane(perpendicular to the PDMS film plane) and in-plane (parallel to the PDMS film plane) buckling patterns, respectively (Figure 3.6).

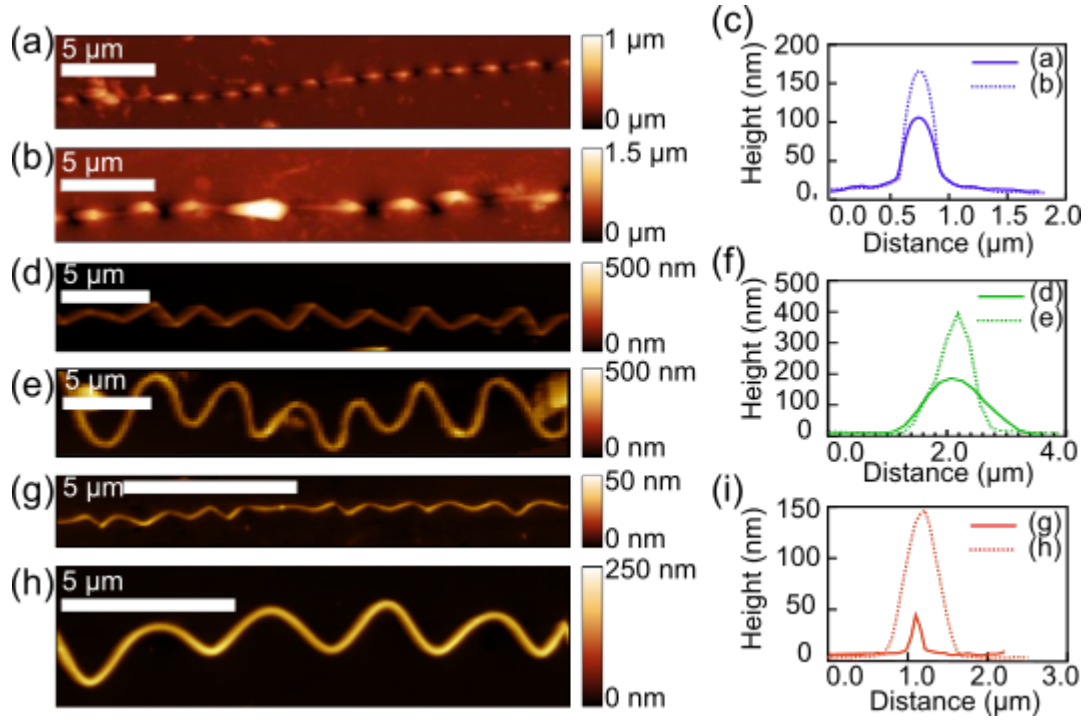


Figure 3.6: (a) and (b) AFM height images of two different native fibrils buckling out of the PDMS plane and their corresponding height profiles obtained prior to compression (c). (d) and (e) AFM height images of 1% GA crosslinked collagen fibrils and their corresponding height profiles (f). (g) and (h) AFM height images of 2% crosslinked collagen fibrils and their corresponding height profiles (i). All artificially crosslinked fibrils buckle parallel to the PDMS plane. For both native and artificially crosslinked fibrils, the fibrils with smaller radii (fibril shown in a, d and g) have a smaller buckling period than the fibrils with larger radii (fibril shown in b, e and h).

The buckling patterns were observed over long sections of individual fibrils, sometimes over 100 μm. Sections greater than 60 μm could not be mapped in one image because of the limited, horizontal range of the AFM scanner. Figure 3.6 shows that both the shape (Figure 3.6 g, h) and amplitude (Figure 3.6 a, b) of the patterns vary from one fibril to another. More rounded patterns were observed (Figure 3.6 e, h) as well as fibrils exhibiting saw-tooth-like patterns (Figure 3.6 d). Despite these variations from one fibril to another, the wavelength along individual fibrils remains relatively constant and can be determined from the AFM height images along with its appropriate uncertainty. The ra-

dus, R , of individual fibrils can be determined accurately as described in section 3.2.6. Obtaining height images, and extracting the λ and R values of many fibrils on the same sample results in a relation between λ and R . Qualitatively, it can also be observed in figure 3.6 that the wavelength of the buckling-pattern is greater for fibrils with a greater radius as expected from the theory (Equation 3.1).

To investigate this behavior more quantitatively, I performed the experiment with many fibrils deposited on the same PDMS film. Figure 3.7 shows the experimentally determined buckling-wavelengths, λ , versus the radius, R , of all the fibrils investigated. In order to assess whether the theory characterised by equation 3.1, describes the observed buckling-behavior, I simplified equation 3.1. For the range of λ and R obtained experimentally, the right-hand side, RH, of equation 3.1 is approximately constant to within an error of 2%. This is a smaller, relative error than the one due the natural variation of λ and R between fibrils. Hence, we can estimate the average to be $RH = 0.804$ and simplify and rearrange equation 3.1 to :

$$\lambda = 0.205Pa^{1/4}E_{Buckling}^{1/4}R \quad (3.4)$$

Equation 3.4 describes a simple, linear relation between λ and R from which the average Young's modulus of a data-set can easily be extracted. Equation 3.4 was used to fit the native, 1 % GA-crosslinked, and 2% GA-crosslinked fibrils data-sets (Figure 3.7 a-c). The high R^2 values show that equation 3.4 describe the relation between λ and R reasonably well indicating that the buckling-theory described above is applicable.

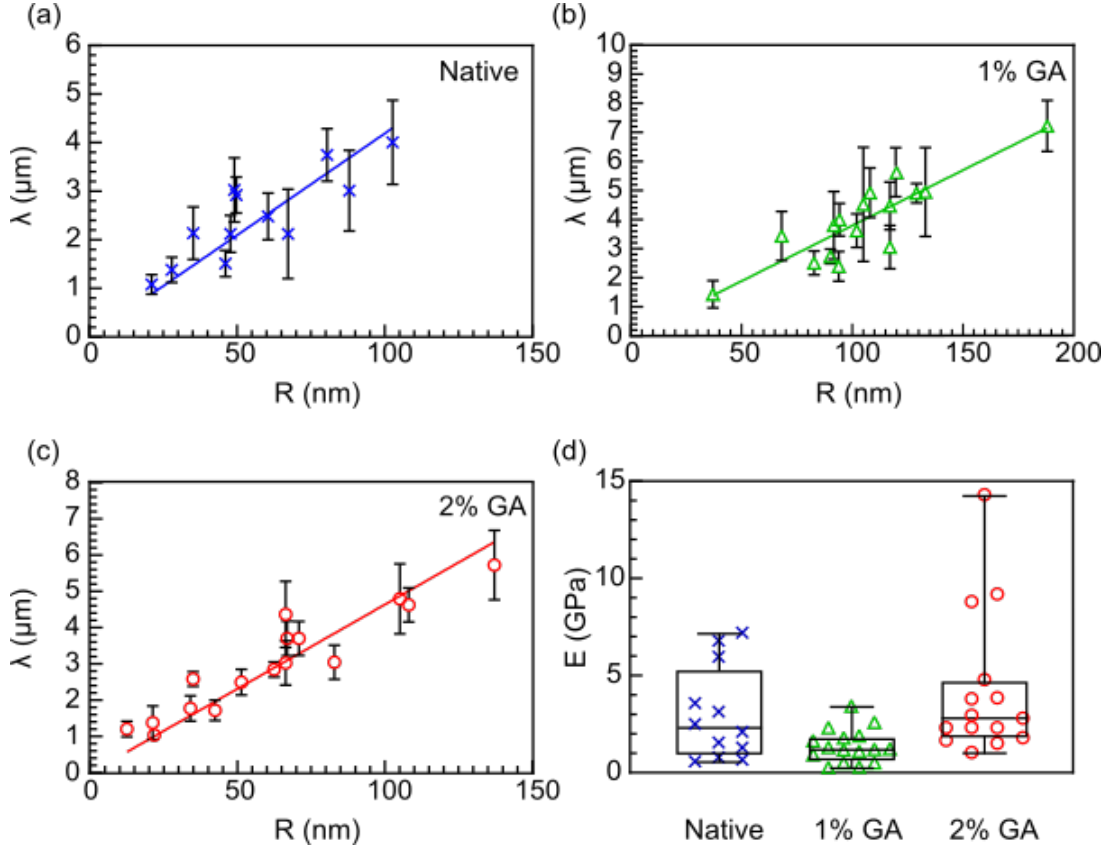


Figure 3.7: Graphs of the wavelength dependence on the radius of (a) native fibrils, (b) 1% GA-treated and (c) 2% GA-treated fibrils. (a) Native fibril linear fit slope = 39.08 ± 3.227 , $R^2 = 0.64$; (b) 1% GA linear fit slope = 38.91 ± 6.511 , $R^2 = 0.74$; (c) 2% GA linear fit slope = 47.91 ± 5.222 , $R^2 = 0.75$. (d) Young's modulus, $E_{buckling}$, of fibrils obtained from equation 3.1. The middle, horizontal bar represents the median value, the top and bottom of the boxes show the 75th and 25th percentile, respectively, and the upper and lower whiskers show the 95th and 5th percentile, respectively.

From the slopes of the linear regression fits in figure 3.7 (a)-(c), it cannot be concluded that the average tensile modulus of the native fibrils was significantly different from any of the GA-treated fibril data-sets. However, the increase of the slope values from the 1% GA-crosslinked fibrils data-set to the 2% GA-crosslinked fibrils data-set (Figure 3.7 b, c) seems to be significant taking into account the estimated uncertainties. We can, thus, conclude that the GA treatment used in this study has a weak yet significant effect on the buckling-behavior of collagen fibrils and, hence, on their tensile modulus. This is in agreement with the action of GA, which is a well-known crosslinking agent for proteins [142]. As GA reacts with collagen, crosslinks are formed between collagen molecules within the fibril and increase the Young's modulus of the fibril. This leads to the observed change in the λ -vs- R relation. GA-crosslinking is believed to prevent intermolecular sliding between different layers of collagen molecules within a collagen fibril. This effect on the tensile modulus of collagen fibril bundles has been studied in the past and shown

to be weak [143].

The measured λ and R values were used as variables in equation 3.1 to calculate the Young's modulus of each individual fibril presented in figure 3.7 (a)-(c), without resorting to the simplification of equation 3.4. The median value of the Young's modulus for native fibrils is approximately 2 GPa, which is consistent with values reported in the literature for dehydrated collagen fibrils obtained by nanoindentation and other mechanical testing methods. One can see that the natural variation of Young's moduli between individual fibrils is very large which has also been reported in the literature [61][144]. As with the slope values, the only significant increase of average modulus that could be observed occurred between the 1% and 2% GA-treated fibril data-sets (Figure 3.7 b, c).

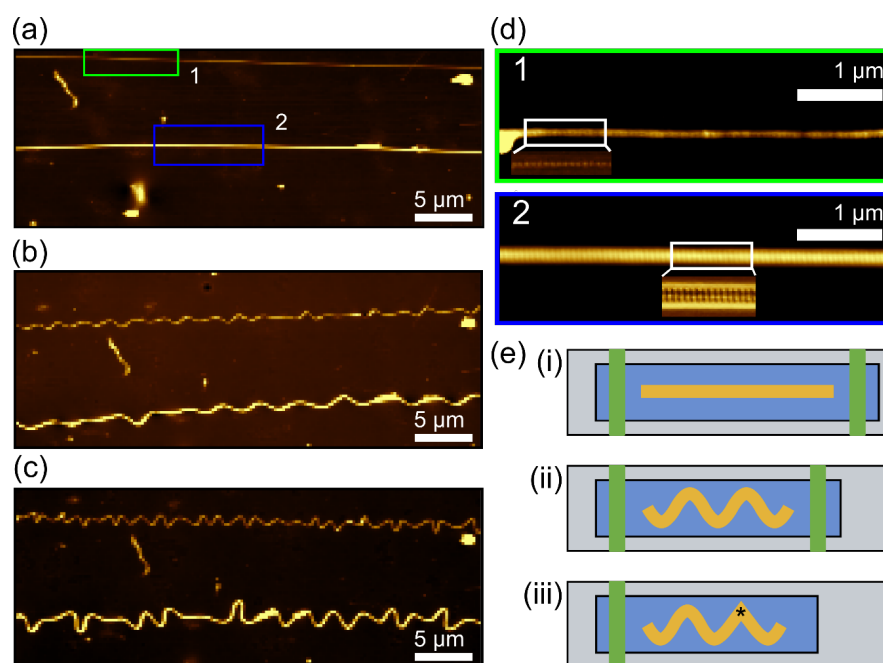


Figure 3.8: (a) AFM height images of two collagen fibrils (fibrils 1 and 2) deposited on a PDMS film pre-strained to 25% prior to compression (color scale = 100 nm). The D-banding patterns are visible in the height images in (d) but AFM phase images of selected regions are added in to make the D-banding pattern more obvious. (b) AFM height images of fibrils 1 and 2 after having been compressed (color scale = 100 nm). The PDMS film was released from strain by roughly 12%. (c) AFM height images of fibrils 1 and 2 after having compressed the PDMS film by 25 % (color scale = 100 nm). The PDMS film was fully released from strain and returned to its original, native length. (e) Putative sequence of stages: (i) collagen fibrils are deposited on a strained piece of PDMS. (ii) 12% compression of the PDMS film leads to a wave-like buckling pattern of the PDMS whereas further compression leads to local breaking along the collagen fibril resulting in a visible kink marked, * in (iii).

As mentioned earlier, some artificially crosslinked fibrils (Figure 3.6 d,e), exhibit a more saw-tooth-like buckling pattern than other fibrils. This seems to indicate that fibrils on the same PDMS film exhibit different degrees of compression. To compress collagen fibrils, fibrils are deposited on a strained piece of PDMS. Compression cannot be quantified at the single fibril level but instead is a measure of how much the PDMS film is

released from strain. Some fibrils on the PDMS film are compressed and exhibit either a sinusoidal buckling pattern or a more triangular buckling pattern. Other fibrils on the other hand, most likely detached from the underlying PDMS film and were not compressed. Hence, these fibrils did not exhibit any buckling behaviour. To investigate more closely how the different wave patterns arise, I tracked individual fibrils at different stages of compression (Figure 3.8). The topography was imaged before compression, after small compression (roughly 12% compression), and after large compression (once the PDMS film is returned to its original length which corresponds to a 25% strain). As it is not easy to track specific fibrils in between these different steps because the foil has to be manipulated manually several times, only a small number of fibrils could be investigated in this way. Figure 3.8 illustrates this by showing AFM height images of two fibrils tracked in the three different stages of compression. The sections of fibrils which were tracked could be identified to within a few hundred nanometer at each stage due to the presence of debris and occasional holes in the foil. To facilitate geometrical analysis, we used fibrils undergoing in-plane deformation, that is, fibrils crosslinked in a 2% GA solution. Figure 3.8 (a) shows two fibrils before compression. They are straight over a length of more than 30 μm . Their radius is 21.1 nm (Figure 3.8 d, 1) and 42.3 nm (Figure 3.8 d, 2). After releasing the PDMS film such that it is 12% shorter macroscopically, the same two fibrils were imaged again (Figure 3.8 b). The fibrils showed a more rounded buckling pattern, with varying amplitude but consistent wavelength ($\lambda_1 = 1.89 \pm 0.183 \mu\text{m}$, $\lambda_2 = 3.07 \pm 0.316 \mu\text{m}$). The film was then returned to its original length which corresponded to a macroscopic shortening of 25%. The fibrils showed a more pronounced wave pattern with greater amplitude and a consistent wavelength ($\lambda_1 = 1.97 \pm 0.261 \mu\text{m}$, $\lambda_2 = 3.10 \pm 0.271 \mu\text{m}$). At this stage of compression, variation of the shape of the wave along each fibril was observed. There was no evidence of detachment from the surface, fraying or any damage to the morphology of the fibrils.

A possible explanation for this process is described in figure 3.8 (e). Fibrils are initially deposited on a pre-strained PDMS film and can be considered as straight rods (i). After partial foil release, the critical buckling-load is exceeded but the material yield

strength is not yet exceeded (ii). This results in column buckling which creates a rounded wave pattern with a defined wavelength, λ . This wavelength only depends on the tensile modulus of the fibril and its radius, R . After complete foil release (iii), the material yield strength is exceeded in certain locations along the fibrils (represented by a sharp kink marked (*) in figure 3.8 e iii). Unlike free-stranding columns, in these locations the fibril breaks but cannot collapse because it is stuck to the PDMS film along its entire length. It should be noted that, at all stages, the microscopic foil strain immediately around the fibril is likely to be much less than the macroscopic foil length reduction. This is because the fibril is much stiffer than the film even in its buckled state and probably prevents it from fully relaxing locally [145].

The interpretation of the results as a form of column-buckling requires a critical analysis, especially regarding the particular experimental conditions and to what extent the assumptions of buckling theory are fulfilled. First, regular wave patterns are observed (Figure 3.6) when the fibrils are compressed. Imaging these patterns and retrieving each buckled fibrils' λ and R values led me to observe a linear λ -to- R relationship (Figure 3.7). Similar wave-like patterns have been observed with silicon nanowires, single-walled carbon nanotubes, multi-walled carbon nanotubes, and carbon nanotube bundles attached on a soft PDMS substrate [138][139]. These nanowires and nanotubes were at least 25000 times stiffer than the underlying PDMS [139]. By releasing the PDMS from strain, a longitudinal load was applied to the nanowires and which either buckled perpendicularly to the PDMS plane (process coined out-of-plane buckling) or parallel to the PDMS plane (process coined in-plane buckling). I observed the same phenomenon here with collagen fibrils, which are long rods and which are much stiffer than the underlying PDMS and hence, a similar interpretation was made.

Such a buckling of stiff, long and slender bodies attached on a less stiff substrate upon release from strain can be described as a kind of column-buckling related to the well-known Euler-type column-buckling in civil engineering [140]. Euler-type column-buckling theory relies on the assumption that the force is applied between two ends of the column and is only applied on one side. In my experiments however, fibrils are stuck

along their entire length to the PDMS film and hence the force is applied along the entire length of the fibril. Despite this discrepancy, the physical process remains unchanged: a longitudinal force is applied to a long, slender body, resulting in beam-bending when a critical load is reached. Using a Newtonian analytical mechanics model and/or energy variation method [138][139], the elastic modulus of each body can be calculated (Figure 3.7).

As shown in figure 3.8 b and c some fibrils exhibit non-uniform wave patterns. It is possible that fibrils could partly detach or slide when the PDMS film is being manipulated. Detachment from the PDMS film however, would be noticeable from AFM height images. In fact, even if sections of fibrils slid horizontally on the surface, they would still be subjected to a longitudinal load by other, more tightly attached sections of the fibril, and buckling-theory would still be applicable. It is also possible that the load applied by the released PDMS is not uniform and that some areas of the PDMS exert a higher load than other areas. Buckling-theory is still applicable however, because λ is independent of the actual, external force that is applied on the individual beams.

Just like Euler-type buckling, increasing the applied load leads to an increase in the “amplitude” of the wave pattern but not to a significant change in wavelength (Figure 3.8). Increasing the load bends the fibrils even more, until their yield strength is reached, at which point, material failure starts to occur. This can be seen, for instance, in Figure 3.8 d, where the fibril shows a more pronounced saw-tooth pattern. In these experiments, AFM height images show a snapshot of a particular buckling state. In fact, even if the material yield strength is exceeded, fibrils cannot collapse completely as would a free column because they are held back by the PDMS. In these experiments, the PDMS freezes the fibrils in a specific compression state. This means that a meaningful wavelength value can still be retrieved from the AFM height images and be assigned to fibrils whose yield strength is exceeded (Figure 3.8 c), provided the compression is not excessive. This mechanism is illustrated in Figure 3.8 e.

As with all other mechanical measurements on collagen fibrils (e.g., nanoindentation, tensile testing), an important factor that needs to be taken into account is that fibrils are

not isotropic and homogenous. As described in chapter 1, collagen fibrils consist of a hierarchy of substructures such as microfibrils [146] and have sometimes been described as wound-up ropes [147]. This means quantities such as the elastic modulus in the strict sense corresponds to the precise quantification of an inherent, intensive, physical property of a material, and must be used with caution. What I have determined in the present work is the tensile- or bending-modulus, $E_{buckling}$, of collagen fibrils as a structural element, not necessarily as a material per se. This method shares this limitation with any other nanomechanical measurement technique. In practice, this is of little relevance because the structure of collagen or similar biological nanofibers is given, and investigators are often more interested in the tensile properties of their specific material and how it changes when undergoing chemical treatment or physical manipulation. If major structural changes occurred (e.g. separation, unravelling of the fibril or fraying of sub-fibrils) these would need to be investigated with other methods (e.g. microscopy). This method, however, has the great advantage to be much more accessible to non-specialized labs and achieves much greater throughput since many fibrils can be compressed at once.

3.4 Conclusion

In summary, buckling under longitudinal load was used to determine the Young's modulus, $E_{Buckling}$ of nanometer scale collagen fibrils from simple AFM topography images. This was achieved without the need of knowing the actual load applied and without performing an actual, mechanical test such as in nanoindentation of the fibrils. This feature offers us a great advantage for systematically investigating biological nanofibers in terms of, both, fundamental and practical aspects. The method is easy to apply, it does not require specialist expertise beyond basic AFM imaging, and it does not need any special hardware other than a basic, commercially available AFM. The method, therefore, has the potential to be adopted much more widely by non-specialized labs, especially in the field of bionanomaterials. However, it should be born in mind that this technique does not allow us to differentiate the mechanical properties of the gap and overlap regions. Such resolution can only be achieved by AFM nanoindentation. This technique is less precise

than nanoindentation but provides an accurate estimate of the fibrils' average Young's modulus and could be used, for instance, to check if a sample has undergone crosslinking. It will be used again in chapter 5 as an easy way of obtaining the average Young's modulus of many fibrils.

Chapter 4

Stretching of collagen fibrils

4.1 Introduction

The aim of the work in this chapter is to stretch multiple fibrils to different strains in a single experiment, and to quantify the strain at the single fibril level.

As described in chapter 1, collagen fibrils are able to provide mechanical cues that influence cellular behaviour and even stem cell differentiation [44]. Collagen fibrils in the ECM or in scaffolds used in tissue engineering are subjected to strains and stresses from movement, but also from the cells that can physically tug and align the fibrils. If collagen fibrils are able to fine-tune cellular behaviour, it is important to understand how their structure and physical properties are impacted by strain. To study the physical properties of individual fibrils in the strained state, such as stiffness of surface charge, fibrils need to be strained to different strains all the while being available to AFM probes for imaging and axial loading or KFM probing.

In chapter 3, it was shown that the PDMS film technique could be used to compress many fibrils at once and that the resulting buckling patterns could be used to calculate the elastic moduli of these fibrils. In other works, stretchable films were used to stretch and not compress single collagen fibrils. These techniques relied on the use of a motorised stage [134] or a home-built devices [105] and had the great advantage of straining single fibrils to different strains in a single experiment all the while maintaining them accessible to AFM probes for imaging, and axial loading. This allowed them to image and probe the

mechanical properties of single collagen fibrils at different strains and along an expanded strain range.

In this chapter, the same type of PDMS film that was used in chapter 3 was used to stretch multiple fibrils at once. Fibrils were first deposited on an un-strained PDMS film. Fibrils being naturally very sticky, they adhere strongly to the PDMS film and were thereby stretched when the PDMS film was manually strained macroscopically to 50%. The PDMS film was then taped stretched to a microscope slide which rendered the stretched fibrils accessible to AFM imaging. To verify whether the fibrils had successfully been strained, the fibrils were imaged by AFM and their D-banding lengths were measured. The technique presented here allowed me to strain fibrils on a single PDMS film to many different strains along an expanded strain range. In fact, some fibrils were stretched up to 26% strain. In this chapter, individual fibril's average D-banding length was used a marker of the local strain experienced by individual collagen fibrils.

4.2 Methods

4.2.1 Sample preparation

Type I collagen fibrils were obtained from the same wild-type, adult rat tail tendon (RTT) mentioned earlier in section 3.2.2. Pieces of tendon were extracted using a scalpel and were kept in a PBS solution (Sigma-Aldrich Company Ltd., Dorset, United Kingdom) for a maximum of four months at 4°C. Unstretched samples were prepared as described in section 3.2.3.

4.2.2 AFM imaging

Tapping-mode imaging was performed using a Dimension Icon AFM (Bruker Corporation, Billerica MA, USA). Three different AFM tips were used to image the fibrils at different strains (Table 4.1). Each tip had a nominal apex radius of less than 10 nm. All of the AFM images were obtained in air and under ambient conditions (humidity $\approx 30\%$, temperature $\approx 23^\circ\text{C}$), on fibrils of at least 100 nm in diameter.

Tip	Tip type	f (kHz)	k (N/m)
A	BudgetSensors All-in-One	350	40
B	Bruker RFESP-75	75	3
C	Budget Sensors Tap300-G	350	40

Table 4.1: Tips used for imaging collagen fibrils in air on a PDMS substrate. The supplier for these tips was Windsor Scientific Ltd., Slough, UK.

4.2.3 Stretching of collagen fibrils

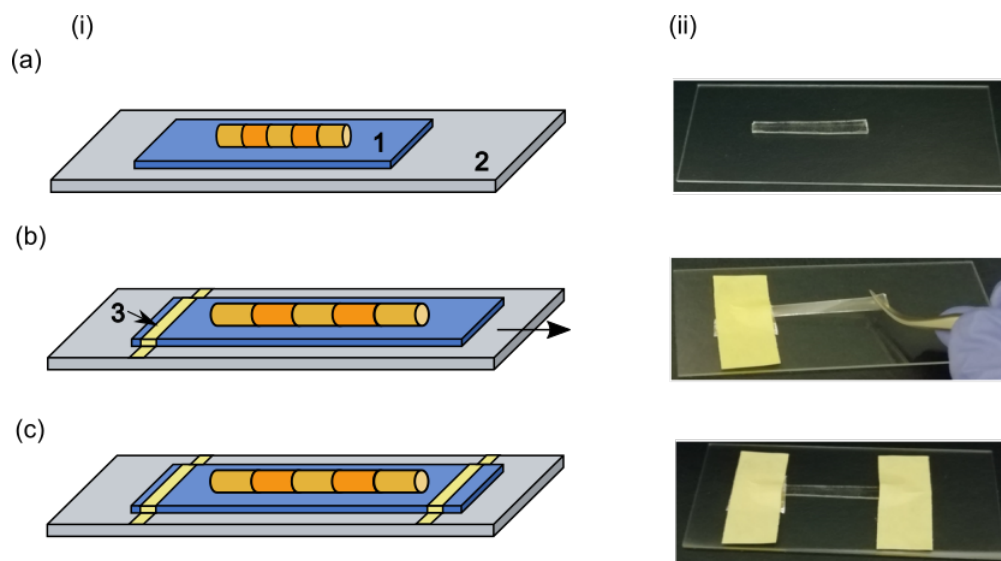


Figure 4.1: Stretching process of the collagen fibrils. (i) Diagrams of the stretching process and the corresponding pictures (ii). (a) Collagen fibrils are deposited on a PDMS film. (b) One end of the PDMS is taped down to the microscope slide and the other end is manually stretched by being pulled with a pair of tweezers to a macroscopic strain of 50%. Fibrils adhere to the PDMS and are stretched along with it. (c) The loose end of the PDMS foil is taped to the microscope slide in the stretched state maintaining the fibrils strained. 1) PDMS film 2) Glass microscope slide 3) Tape.

Once fibrils were deposited on a piece of PDMS, one end of the PDMS film is taped down to a microscope slide. The other end is pulled and stretched manually to a strain of 50% (Figure 4.1 b). By stretching the PDMS, the fibrils stretch along with it. The loose end is then also taped down to the microscope slide and the sample is therefore, maintained stretched (Figure 4.1 c). The sample is then left overnight before AFM imaging to avoid any creep.

4.2.4 Data analysis

The Gwyddion Image Processing Software was used for image analysis (available free under <http://gwyddion.net/>). To remove any imaging artifacts in the raw AFM images resulting from a sample tilt or line skips for instance, raw AFM height images were first plane leveled. The line profile of the fibril is obtained by averaging a few rows of pixels at the center of the fibril, all the while avoiding the edges of the fibril (Figure 4.2). The average line profile, provides us with the D-banding pattern of the fibril. From this profile, both the strain applied to the fibril and the D-banding height were measured and calculated (Figure 4.2).

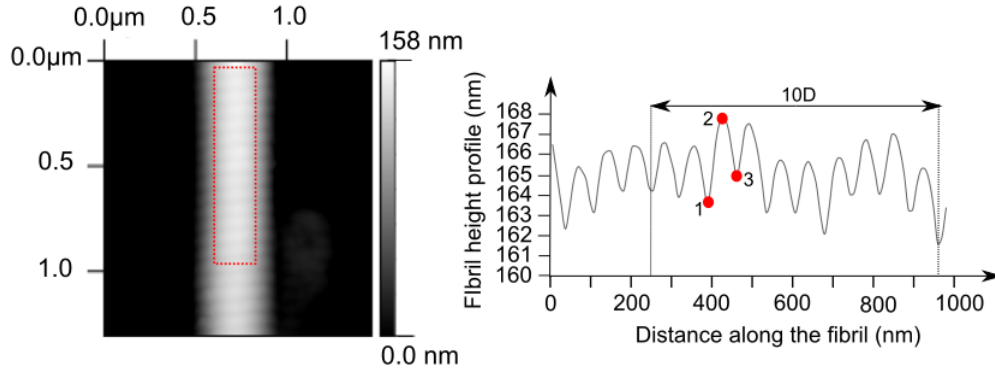


Figure 4.2: AFM height image of a collagen fibril. The red rectangle corresponds to the rows used to create the average line profile to the right of the image. From the line profile, the length of 10 D-bands is measured. The average D-banding length is obtained by dividing the length of ten D-bands by 10. The red dots labelled 1-3 correspond to the height of gap and overlap regions used to calculate the D-banding height using equation 4.2.

The D-banding period, D , for a given fibril was determined by dividing a specific distance by the number of peaks along that distance. To minimise the error associated with the measured D-banding length, the minimum number of peaks used was ten. The size of the images obtained were of $1.5\mu\text{m} \times 1.5\mu\text{m}$ and hence even when stretched, the collagen fibrils exhibit at least ten D-bands in the images. The fibril strain is then given by:

$$\text{Strain} = \left(\frac{D - D_0}{D_0} \right) \times 100 \quad (4.1)$$

Where D_0 is equal to 67 nm and corresponds to the initial average D-banding length of unstretched collagen fibrils.

D-banding height, h , was calculated for individual overlap regions by averaging the height difference between a gap region to the left of an overlap region with the height difference between that same overlap region and the gap region to its right (Figure 4.2):

$$h = \frac{H_{1/2} + H_{1/3}}{2} \quad (4.2)$$

$H_{1/2}$ and $H_{1/3}$ correspond to the banding height to the left and right of an overlap region. This process was repeated for at least ten overlap regions. The ten banding heights were then averaged to give the average D-banding height of the fibril.

4.3 Results and Discussion

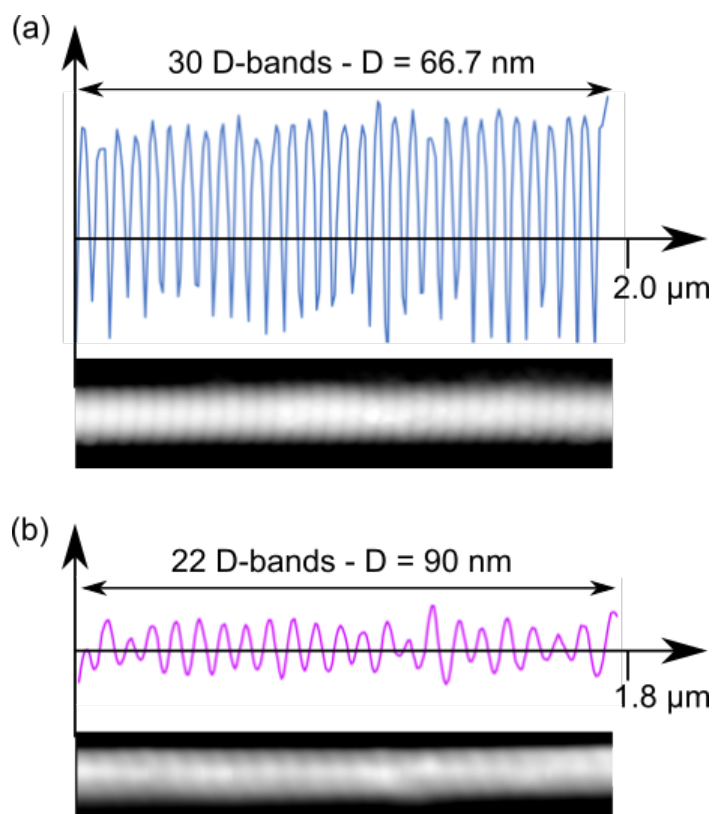


Figure 4.3: (a) D-banding height profile of an unstrained collagen fibril on PDMS. (b) D-banding height profile of a collagen fibril stretched to roughly 22% on PDMS.

As can be seen from Figure 4.3, fibrils deposited on an unstrained PDMS film are stretched along with it when it is macroscopically strained to 50%. The stretching of individual collagen fibrils is quantified by the increase in the effective D-banding length of individual collagen fibrils determined from AFM height images (Figure 4.3). The biggest local strain prescribed to our fibrils was of roughly 25% (Figure 4.4). Strain is measured on a fibril-per-fibril basis by directly measuring the D-banding length of individual fibrils. The strain applied to the piece of PDMS is of 50%, however none of the fibrils reached D-banding lengths of 100 nm or greater. Fibrils are stiffer than the underlying PDMS. During stretching of the PDMS the fibrils most likely locally hold the PDMS back and prevent it from stretching or they might stretch along with the PDMS film and then detach, hence never stretching to a final strain of 50%. It is also worth noting that different fibrils were stretched to different strains, thereby covering in a single stretching experiment, the entire strain range from 0% to 26%.

Fibrils lay flat on the PDMS, and are attached to it by the molecules that are at the bottom surface of the fibrils. This experiment is therefore different than AFM-based force spectroscopy experiments where fibrils are stretched by being pulled on one end by an AFM tip, for instance. In this test, the strain is prescribed more or less uni-axially by pulling on one end of the piece of PDMS and the strain is prescribed to the fibrils by applying a strain along the entire length of the molecules in contact with the PDMS. It is assumed here, that the strain applied by the PDMS to the bottom layer of collagen molecules propagates throughout the entire fibril and translates into an equally strained layer of collagen molecules at the top of the fibril, leading to a specific D-banding length. It is believed that the strain applied to the bottom layer of collagen molecules propagates through the entire volume of the fibril through the crosslinking network. If the strain is not propagated equally from the bottom layer of collagen molecules to the top, a resulting gradient of strain, or unequal distribution of strain would appear. In the case of a large gradient, the internal structure that results in observable D-banding would be in disarray and the D-banding pattern would be disrupted. However, for a sufficiently small gradient, it would be possible for the fibrils to retain a D-banding structure. Finally, if the molecules in contact with the PDMS were strained more than the molecules in the overlaying layers, with the top layer being the least strained, this would by definition correspond to a bending of the fibrils and curling of the fibrils would be expected to be visible. In these experiments, the fibrils are laying flat against the PDMS and the D-banding pattern is visible in AFM topography images (Figure 4.3). It is therefore believed that during our experiments, molecules inside individual collagen fibrils are strained equally.

As observed in previous studies, the D-banding height decreases with increasing strain (Figure 4.4) [105]. The decrease of D-banding height with increasing strain is quantified by the slope of the linear fit which is of -8.64 ± 1.12 nm. Taking into consideration the spread of the data, due to the natural variability between individual fibrils, the slope value is similar to previously reported values [105]. These results also show that the strain prescribed to the PDMS film is not that of the individual collagen fibrils present on the surface of the film. The measurement of each fibril's D-banding length quantifies by how

much each individual fibril has been strained and were thereby used as a marker for strain in future experiments presented in this thesis.

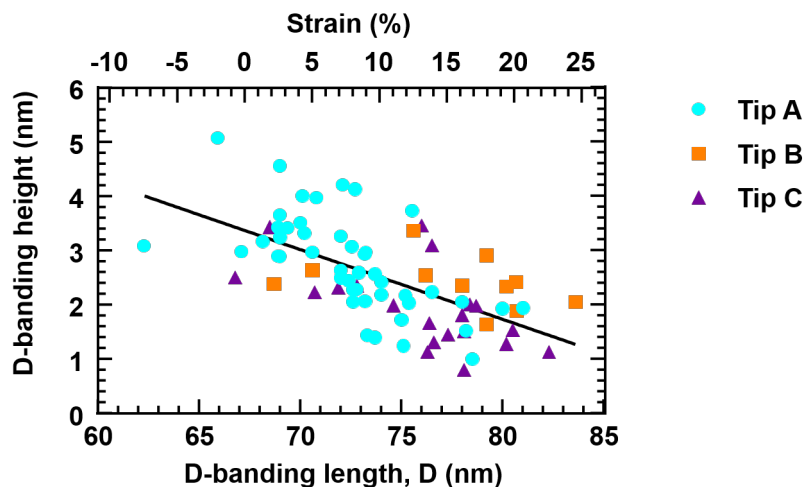


Figure 4.4: D-banding height as a function of strain. Each data point corresponds to an individual collagen fibril. Three different tips were used to obtain the AFM height images from which the D-banding lengths and heights were determined. The type of tip used did not seem to impact the overall trend observed. A linear regression was obtained and the slope was calculated according to the D-banding height and strain values. Linear fit: slope = -8.64 ± 1.2 nm, R^2 -value = 0.39.

The local strains applied to the fibrils in this experiments exceed the strains that collagen fibrils are subjected to under physiological conditions. In fact, when physiological strains were applied to collagenous tissues during small-angle X-ray scattering (SAXS) experiments, collagen fibrils were shown to only experience small strains. In fact, at tissue failure, D-banding length analysis revealed that individual fibrils within the tissue had only been strained to a maximum of 5% strain [70]. The work presented here showcases a technique that allows the fibrils to be strained to much greater lengths. These strains are most likely never achieved under physiological conditions, however since collagen is increasingly being considered and used as a material for tissue engineering purposes or wearable electronics, it is important to characterize collagen fibrils as an engineering material and therefore probe their topography, mechanics and electrostatic properties along an extended strain range [148][90].

4.4 Conclusions

In this work, I was able to show that the same type of PDMS film previously used to compress fibrils can be used to apply up to 26% strains to individual fibrils. As opposed

to tensile tests, this technique strains the fibrils by stretching the entire length of the fibril that is in contact with the PDMS film. Individual collagen molecules inside collagen fibrils are assumed to be strained equally since the characteristic D-banding pattern was visible for all fibrils in the studied strain range of 0-26% and no curling of fibrils were observed. As previously reported, the D-banding height decreases with increasing strain and is projected to become null for strains of about 40% [105] whereas the D-banding length increases with increasing strain. D-banding length was therefore systematically used as a marker for fibril strain in all future experiments described in this thesis. As opposed to other works [134][105], no specialised hardware is required to strain fibrils with this technique, making it easily implementable by most labs. This approach maintains the accessibility of fibrils to AFM axial loading allowing users to maintain nanometric resolution. In the next chapters, this technique was used in combination with axial loading and KFM probing to probe the both the mechanical and electrostatic properties of single collagen fibrils along an expanded strain range.

Chapter 5

Bending of collagen fibrils

5.1 Introduction

As described in chapter 1, collagen fibril's D-banding is of particular interest because its length is in the range of topographies that can be sensed by cells. Its presence or not on the surface of collagen fibrils impacts cellular behaviour [12] and the gap and overlap regions are known to have different mechanical properties [105][149]. Hence it is important to be able to measure the elastic modulus of these regions independently. The only technique that allows such spatial resolution is nanoindentation with an AFM probe. Nanoindentation is a convolution of radial compression and longitudinal extension. The local strain applied by the AFM tip during nanoindentation is small and hence, it is of particular interest to be able to expand the strain range. In chapter 4, fibrils deposited on a PDMS film that was subsequently stretched reached strains up to 26%. This technique proved to be particularly useful to mechanically manipulate many fibrils, and cover an expanded strain range in a single experiment. Combining this stretching technique, with nanoindentation would be ideal to probe the stiffness of single collagen fibrils along an expanded strain range. However, axially loading fibrils on a soft PDMS film does not fulfill one of the assumptions upon which nanoindentation is based, i.e. that the probed structures are deposited on a stiff substrate. This leads to one question: are collagen fibrils deposited on PDMS indented or do the fibrils bend into the softer substrate when they are axially loaded with an AFM tip (Figure 5.1)?

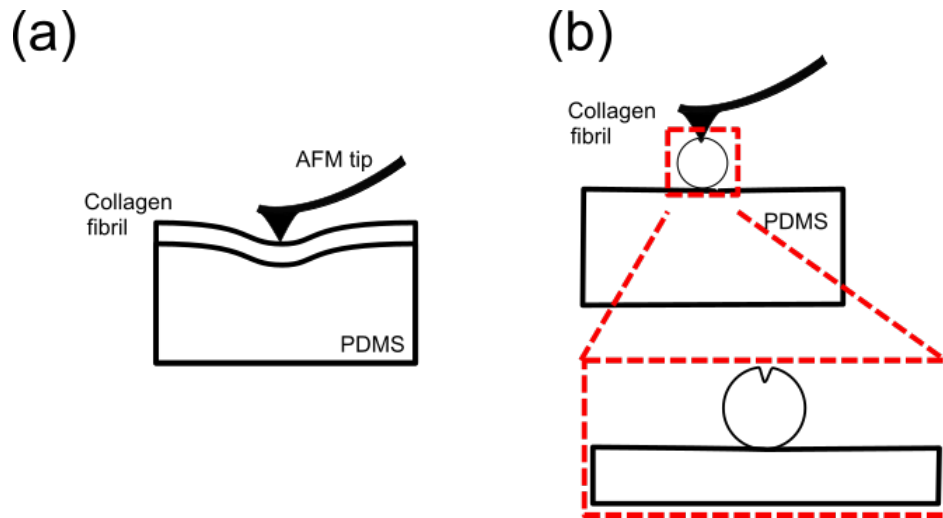


Figure 5.1: Schematics showing possible deformation behaviours for a collagen fibril on a PDMS film : The fibril can either (a) bend into the PDMS substrate or be indented (b) when subjected by a radial force by an AFM tip or a combination of both.

This question cannot be answered easily due to the inherent limitations posed by the size of the fibrils. The deformation of the fibrils cannot be directly observed with an optical microscope. Imaging of the fibrils post loading shows that the fibrils are not deformed plastically i.e. no scarring is clearly visible on the fibrils' surface. This means that AFM imaging of the fibrils post-loading cannot give the user any information on the effect of loading on the deformation of the fibrils. In an effort to understand the deformation of collagen fibrils on PDMS, two different approaches were used. First, the shape of the force curves resulting from the axial loading of fibrils deposited on glass and on PDMS were compared. This comparison allowed me to investigate whether the deformation behaviour of the fibrils on these two substrates differed, however it could not rule in favour of a specific type of deformation. Hence, finite element simulations of fibrils deposited on glass and on PDMS were conducted to recreate the experiments. These simulations produced visual representations of the deformation of the fibrils subjected to a force and created force vs. displacement curves which were compared to the ones obtained experimentally. Hence, the type of deformation that collagen fibrils on PDMS undergo when axially loaded was determined. Finally, the Young's moduli of many collagen fibrils were calculated using a bending-based model (the Winkler foundation model) and were compared to the values obtained using the buckling model described in chapter 3. I will now explain the theoretical framework of the Winkler foundation model.

5.2 The Winkler foundation model

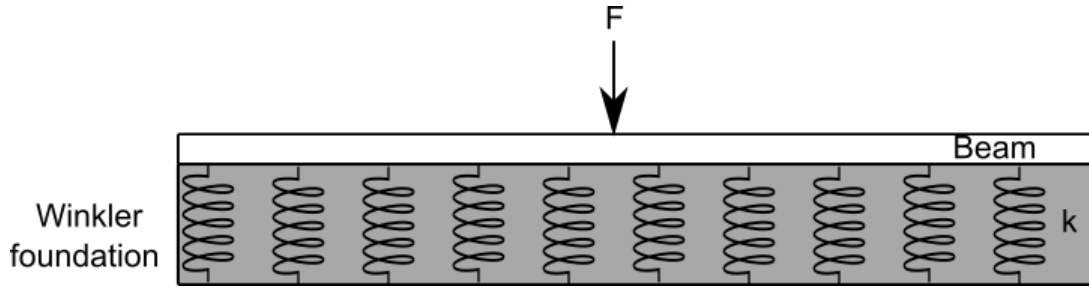


Figure 5.2: Schematic of a beam on a Winkler foundation. The Winkler foundation is made of uniformly spaced springs and has a spring constant of k . The beam is rested along its entire length along the fibril and is subjected to a load F .

The behaviour of macroscopic stiff beams on a softer compliant foundation has been described in engineering by the Winkler foundation model. Winkler foundations can be characterised as a bed of uniformly-spaced springs which are independent and do not interact with each other (Figure 5.2). The deformation of a Winkler foundation when loaded is proportional to the force applied to the foundation (Equation 5.1) [150]. The deformation created by a load on a Winkler foundation only develops directly under the point of contact (Equation 5.1).

$$h(z) = \frac{F(z)}{k} \quad (5.1)$$

h is the bending depth of the foundation in the direction of the applied load (in this case, the z -direction), F , and k is the stiffness of the foundation.

When an infinitely long stiff beam on a Winkler foundation is axially loaded with a sharp probe, the beam bends into the foundation. By knowing the force that is applied to the beam, F , and by measuring the maximum deflection or maximum bending depth of the beam inside the foundation, h_{ind} , the Young's modulus of the beam, $E_{Winkler}$, can be calculated [141]:

$$E_{Winkler} = \frac{F_{max}^4}{2^{44} \bar{E}_s^3 I h_{ind}} \quad (5.2)$$

Where $\bar{E}_s$ is the reduced Young's modulus of the foundation, I is the second moment of area of the beam.

Collagen fibrils have a Young's modulus in the GPa range whereas, PDMS has a Young's modulus in the MPa range [141]. The collagen fibrils studied are at least 60 μm long and are mechanically loaded with an AFM probe that has a nominal tip radius of 10 nm. In these experiments, collagen fibrils can therefore be considered as stiff beams on a soft foundation mechanically probed by a concentrated load. Hence, the Young's moduli of the fibrils were obtained using equation 5.2.

5.3 Methods

5.3.1 Axial loading of collagen fibrils

As described in section 3.2.2, native fibrils were extracted from an adult Wistar rat tail tendon and some of the extracted fibrils were crosslinked in a 2% GA solution. Two separate samples were prepared according to section 3.2.3: one with native fibrils and a second with 2% GA-crosslinked fibrils. Tapping-mode imaging of individual collagen fibrils and axial loading were performed on both samples with a Dimension Icon AFM (Bruker Corporation, Billerica MA, USA). First, a height image of a fibril was obtained. Then, three force curves were performed along the length of the fibril using the “Point and Shoot” mode of the Nanoscope 8.10 AFM operating software. The “Point and Shoot” mode allows the user to choose the locations where to mechanically and axially load a sample in a previously mapped height image. Mechanical loading was performed with a Budget Sensor’s Tap 300Al-G tip on three randomly chosen overlap regions in the height image with loads ranging from 50 nN to 4 μ N . For all of the experiments presented in this chapter, a loading rate of 1 Hz was chosen. The depth of the indentation marks left behind by the indenter on the fibril surface were studied with the “Extract profile along arbitrary lines” function in Gwyddion.

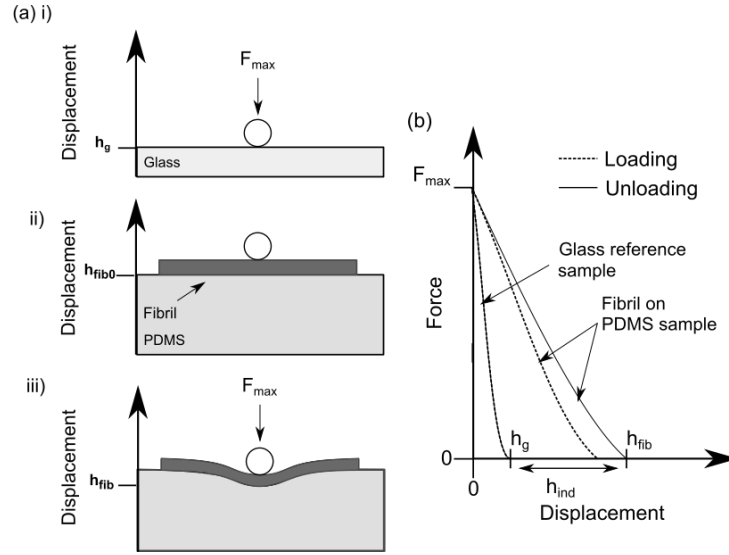


Figure 5.3: (a) Schematic representing i) an indenter applying a force $F_{\max}$ to a glass substrate. ii) Schematic representing a collagen fibril on PDMS being axially loaded by an indenter with a force $F_{\max}$ and iii) subsequently bending into the underlying PDMS foundation. (b) The contact point between the indenter and the glass, h_g is the intersection between the unloading curve of the force curve with the displacement axis for a null force. The maximum displacement of the fibril inside the PDMS is h_{fib} . The difference in contact point positions between the unloading curves on a glass substrate and on a fibril on a PDMS film corresponds to the maximum bending depth.

The Young's modulus was calculated using equation 5.2 where F is the maximum load applied to the fibril and is equal to 50 nN. $\bar{E}_s$ is the reduced Young's modulus of the PDMS film and is equal to 2.65 ± 0.04 MPa [141], I is the second moment of inertia of a collagen fibril in the radial direction, $I = \frac{\pi R^4}{4}$, and h_{ind} is the maximum displacement of the fibril upon loading. The maximum displacement of the fibril inside the PDMS foundation is defined as the difference in the contact point positions between loading a glass reference sample, h_g and loading the fibril on the PDMS film substrate, h_{fib} (Figure 5.3 b). Three force curves were performed per fibril, thus yielding three Young's moduli that were then averaged to obtain the average Young's modulus of an individual fibril.

5.3.2 Finite element simulations

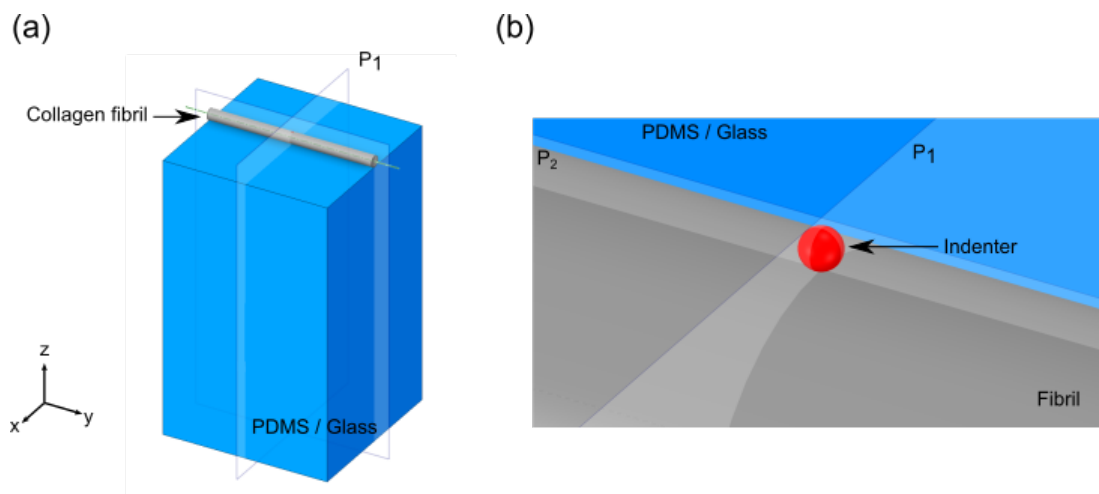


Figure 5.4: 3D representation of the geometry of the finite element simulations. (a) A gray cylinder which represents a collagen fibril is positioned on either a glass or PDMS substrate. P_1 and P_2 correspond to the symmetry planes of the system. (b) Zoom on the fibril where the two planes intersect. A red spherical indenter is clearly visible at the surface of the fibril.

Finite element simulations of the axial loading of collagen fibrils on either a PDMS or glass substrate were conducted in an effort to reproduce the experiments performed. The simulations allow the user to zoom in on the structure and visualise the simulated deformation of the structure: whether the fibrils on either substrate is being indented or pushed into the underlying substrate. The general purpose, free student 2021 R1 version, finite element analysis software, ANSYS®, was used to study the deformation of simulated fibrils on glass and on PDMS when axially loaded with different forces. Glass and PDMS substrates were designed as $5\text{ }\mu\text{m} \times 3\text{ }\mu\text{m} \times 3\text{ }\mu\text{m}$ rectangular blocks. The collagen fibrils were designed as $3\text{ }\mu\text{m}$ long cylinders. Some simulations replicated the loading of specific fibrils that had been axially loaded with an AFM probe experimentally. In those instances, the diameter of the cylinder matched the diameter of the collagen fibril in question that was obtained from the AFM height image (see section 3.2.7) and will be specified in the text. When simulations did not recreate the axial loading of any specific fibrils that had been axially loaded experimentally, the diameter of the cylinder in the simulations was set to 200 nm . The indenter was designed as a sphere with a 20 nm diameter. The geometry of our system is symmetrical along the planes P_1 and P_2 (Figure 5.4). The system was therefore sliced along those planes. Only using a quarter of the system reduced the total amount of nodes in the mesh of the system, which allowed me to create a denser mesh in

regions of high deformation, and reduced the computation time.

The system was made up of three different bodies: a substrate (made from either glass or PDMS), a collagen fibril, and a spherical indenter. Each body was designed as a solid element which were only permitted to deform along the direction of the applied load. Element material properties follow an elastic law and behave isotropically. The mechanical properties used in these simulations for all three bodies are detailed in the table below (Table 5.1). Once again, when recreating the axial loading of specific collagen fibrils, the Young's modulus of those fibrils were obtained experimentally using the previously described buckling model and those values were used in the simulations and will be specified in the text. The natural fibril to fibril variability leads to a broad range of measured Young's modulus values. Static nanoindentation revealed a Young's modulus range of 5-11 GPa [105] and tensile tests an average value of 5 ± 2 GPa for type I collagen fibrils [151]. In our buckling tests presented in chapter 3 an average value of 2.47 ± 1.67 GPa was obtained. Hence, when simulations did not recreate the axial loading of any specific fibrils a Young's modulus of 5 GPa was used.

	Glass slide	PDMS	fibril	indenter
Young's modulus	140 GPa	1.32 MPa	5 GPa	179 GPa
Poisson's ratio	0.2	0.5	0.5	0.265

Table 5.1: Mechanical properties of the different bodies used in the simulations.

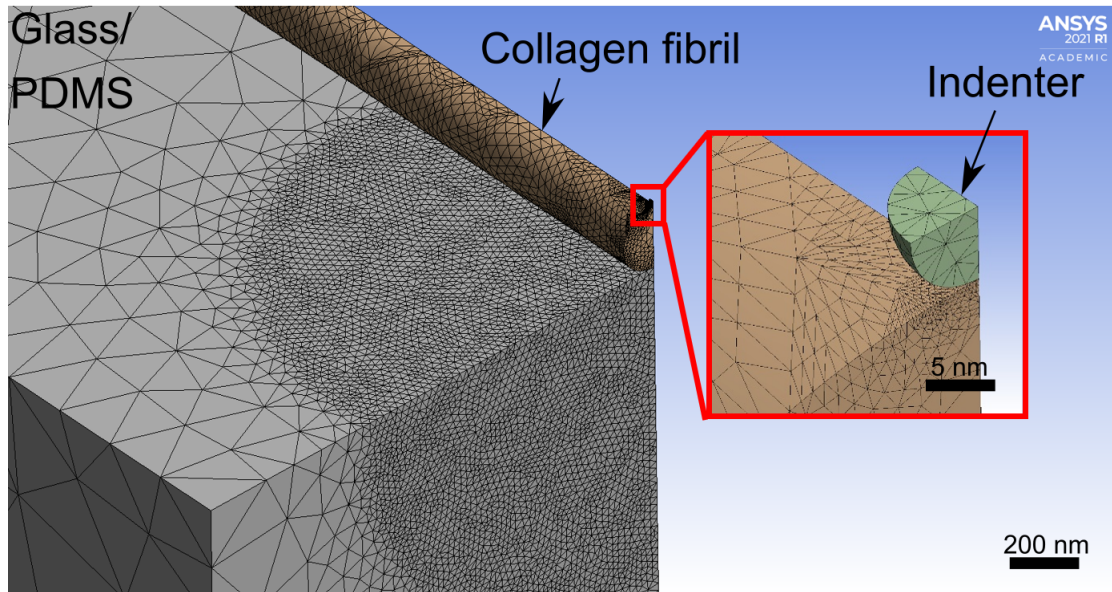


Figure 5.5: Meshing of all three elements in the simulation. The meshing consists of tetrahedrons with a greater density in the regions of high deformation.

The meshing of all three elements is made of tetrahedrons. Two contact regions are present and are handled using surface to surface contact elements: one between the fibril and the glass or PDMS substrate, and one between the spherical indenter and the fibril. Denser meshing in the contact region between the indenter and the fibril was created and the element size was set to 2 nm. When simulating fibrils on PDMS, denser meshing in the contact region between the fibril and the PDMS substrate directly under the indenter was generated using vertex sizing. The sphere of influence of the vertex sizing had a radius of 1 μm and the element size in this region was set to 20 nm.

5.3.3 Statistical analysis

In this work, the Young's modulus of eight native, and five 2% GA-crosslinked collagen fibrils were calculated using equation 5.2 from the Winkler foundation model prior to compression. These fibrils had been deposited on two separate PDMS films pre-strained to 50%. After axial loading, the PDMS film was released from its pre-strain. The exact same fibrils that had previously been axially loaded were imaged once compressed and their Young's moduli were calculated once again using the radii and buckling wavelengths determined from the height images (see chapter 3). It should be noted that the Young's moduli obtained from the study of the buckling behavior of collagen fibrils presented

in this chapter were obtained from AFM height images of buckled fibrils that were not used in chapter 3 and were obtained during a separate experimental session than the data obtained and presented in chapter 3. The statistical comparison between the calculated $E_{buckling}$ and $E_{Winkler}$ values within each data-set was done using a paired Student's t-test where both the t-value, t_{paired} and p-value, p , were calculated.

$$t_{paired} = \frac{\bar{d}}{\sqrt{\frac{s^2}{N}}} \quad (5.3)$$

$\bar{d}$ is the difference between the means of two data-sets, s^2 is the sample variance, and N is the sample size. The statistical comparison of $E_{buckling}$ and $E_{Winkler}$ between the native and crosslinked data-sets was done using an unpaired Student's t-test where both the t-value, $t_{unpaired}$ and p-value, p , were calculated;

$$t_{unpaired} = \frac{m_1 - m_2}{\sqrt{\frac{S^2}{n_1} + \frac{S^2}{n_2}}} \quad (5.4)$$

where :

$$S = \frac{\sum(x - m_1)^2 + \sum(x - m_2)^2}{n_1 + n_2 - 2} \quad (5.5)$$

m_1 and m_2 are the means and n_1 and n_2 are the sizes of the two different data-sets being compared.

5.4 Results and Discussion

5.4.1 Experimental loading of fibrils

Native collagen fibrils on glass and on PDMS were loaded with forces in the range of 500 nN to 4 μ N. As mentioned in chapter 2, nanoindentation is limited to forces that will result in indentation depths that are 10% or less than the diameter of the fibril so as to avoid plastic deformation and sensing of the underlying sample. The forces used in this experiment are much greater than the ones typically used to probe collagen fibrils in air and so plastic deformation was expected.

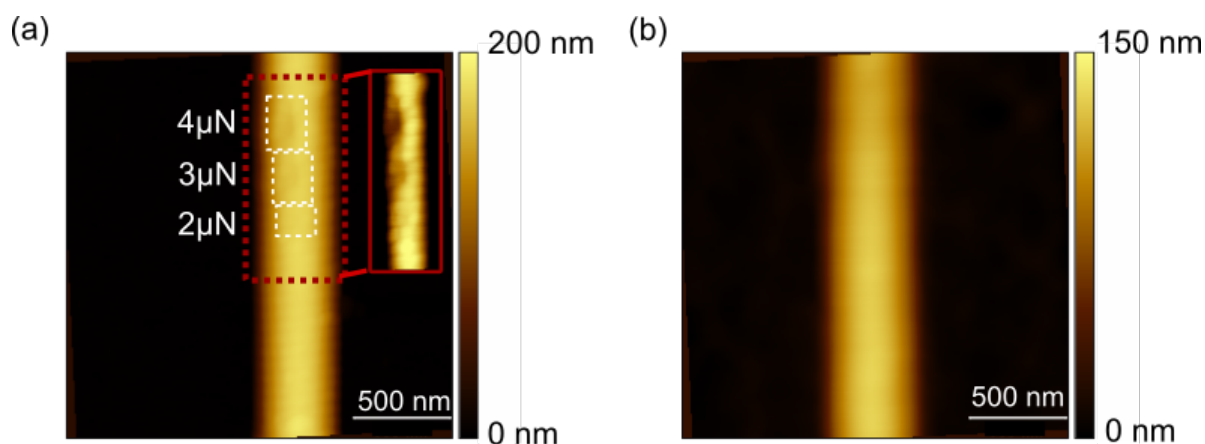


Figure 5.6: AFM height image of a collagen fibril on glass (a) or on PDMS (b) after having been loaded with forces ranging from 500 nN to 4 μ N. Visible scarring due to plastic deformation is present at the surface of the fibril on glass. The red inset in (a) is a zoom with a modified scale to clearly show the scarring of the fibril (color scale = 160 nm-190 nm).

AFM height images of collagen fibrils with similar diameters were obtained on either a glass (Figure 5.6 a) or PDMS film substrate (Figure 5.6 b). Qualitatively, it can be seen that visible scarring due to plastic deformation occurred to the fibril deposited on glass but did not to the fibril deposited on a soft PDMS film when probed with the same forces (Figure 5.6). AFM height images of both fibrils were obtained before and after axial loading. Line profiles along the length of both fibrils were extracted, from the AFM height images obtained before (Figure 5.7 a, b, green line) and after axial loading (Figure 5.7 a, b red line). When comparing the line profiles obtained from the height images of the fibril on glass, prior to and after axial loading, the scarring is clearly visible in the regions where the fibril was loaded with high forces (Figure 5.7 a). The depth of the scars increase with increasing load (Figure 5.7 a). The AFM height image of the

fibril deposited on PDMS showed no scarring on the surface of the fibril (Figure 5.6 b). However, by comparing the line profiles of the fibril before and after loading, a 7 nm deep scar is visible where the fibril was loaded with a 4 μN load (Figure 5.7 b). Figure 5.7 shows that when probed with equal loads, fibrils on stiff substrates, such as glass are subjected to greater plastic deformation resulting in greater indentation depths than fibrils on softer substrates such as PDMS.

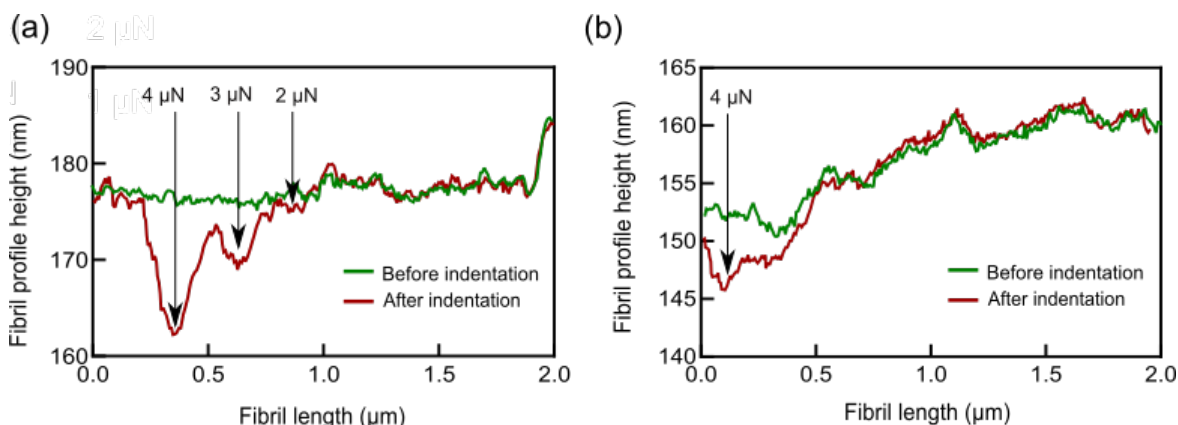


Figure 5.7: Comparison of the line profiles along the fibril on glass (a) and on PDMS (b) before (green line) and after (red line) being axially loaded with different forces.

Force vs. distance curves were obtained from the axial loading of the two fibrils shown in figure 5.6. The force curves from the fibril on glass (Figure 5.8 a, c) show large hystereses for loads in the range of 1 μN -4 μN (Figure 5.8 a), which attests to the plastic deformation of the sample. Furthermore, the bottom half of the retract curves for the fibril on glass are non linear. This is characteristic of nanoindentation. The force curves obtained from the fibril on PDMS show some non-linear behaviour for the 4 μN and 3 μN loads (Figure 5.8 b), however lighter loads result in more linear force curves suggesting that the fibrils are not being indented but rather, pushed into the underlying PDMS substrate (Figure 5.8 b, d).

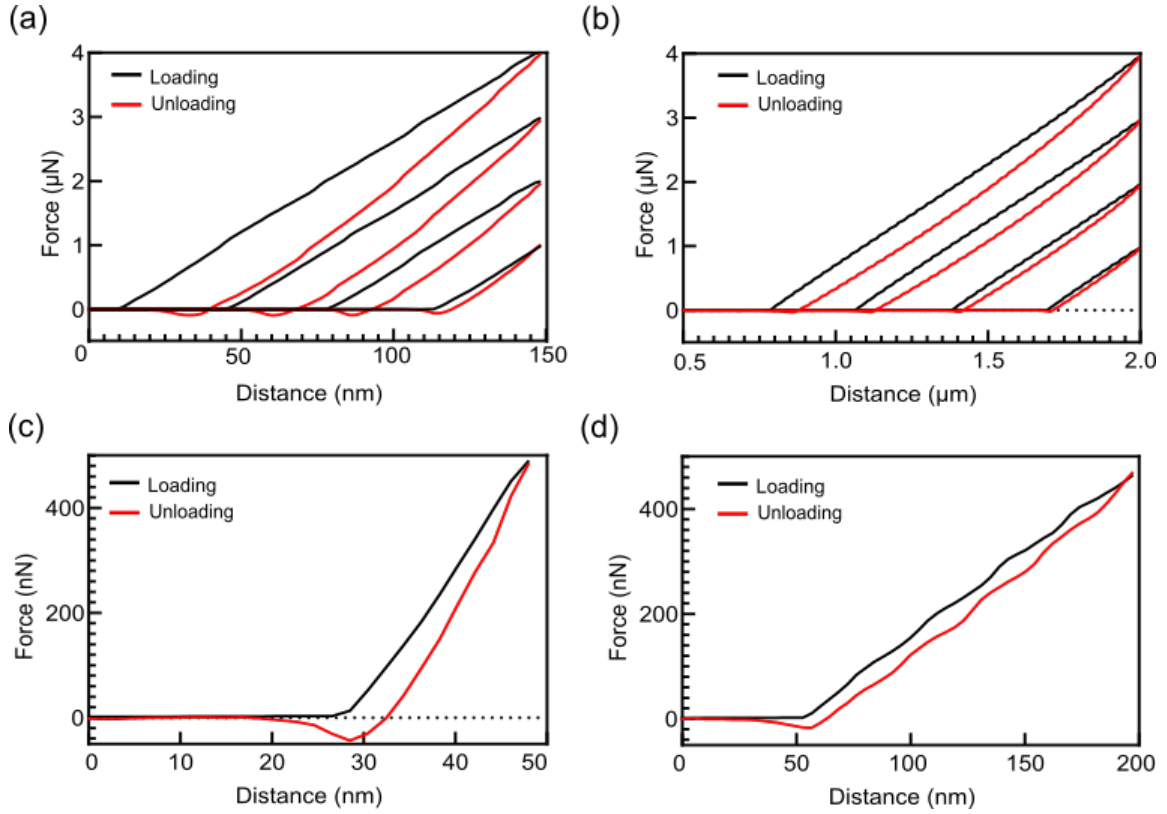


Figure 5.8: Force curves obtained from loading a fibril on glass a) and on PDMS b) with forces ranging from 1 μN to 4 μN . c) and d) are the force curves obtained from loading the same fibrils with a 500 nN load.

The indentation or bending depths, h_{ind} are measured from the force curves as described in section 5.3.1. Figure 5.9 shows that h_{ind} obtained from the force curves from the fibril on PDMS are all at least one order of magnitude greater than those obtained from the fibril on glass. For loads greater than 1 μN , h_{ind} exceeds the height of the fibril itself. During nanoindentation, such great indentation depths would lead to plastic deformation, and would undoubtedly lead to visible scarring of the fibril surface and tip contamination. This indicates that fibrils on PDMS are not being indented when a force curve is performed but that a different type of deformation is at play. A possible hypothesis is that the fibrils on PDMS first bend into the soft substrate when small forces are applied with an AFM tip and only start to be indented after having been significantly pushed into the soft substrate at very high loads.

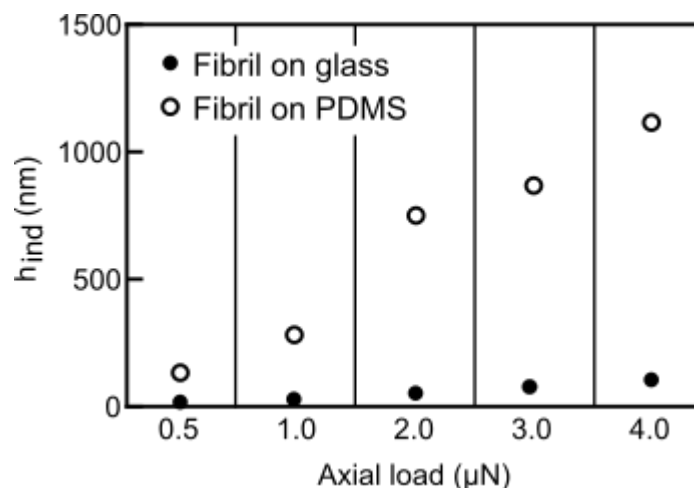


Figure 5.9: h_{ind} values obtained for a fibril on glass and on PDMS subjected to forces in the 500 nN to 4 μN range.

Fibrils deposited on a glass or on a PDMS substrate undergo different types of deformation when they are subjected to axial loads. This is visible in both the shape of the force curves and the resulting h_{ind} values. Smaller hystereses between the loading and unloading curves of fibrils axially loaded on PDMS indicate that these fibrils undergo less plastic deformation than the fibrils on a glass substrate probed with the same load. Despite being less plastically deformed, fibrils on PDMS have much greater h_{ind} values than fibrils on glass, sometimes exceeding the diameter of the fibril itself. This indicates that the distance that the tip is pushed into the sample when loading the fibril on PDMS greatly exceeds the distance that the tip is able to travel into a fibril on a glass substrate for the same applied load. This behaviour seems to indicate that when loading a fibril on PDMS, the fibril predominantly bends into the underlying, soft, substrate.

5.4.2 Simulations of axially loaded collagen fibrils

Force curves provide useful insight on the deformation behaviour of the fibril being probed but cannot definitively rule in favour of a specific type of deformation. In order to assess whether fibrils deposited on PDMS bend into the underlying substrate when subjected to an axial load, finite element simulations mimicking the experiments detailed above were conducted. Simulations have the great advantage of providing a visual result where the deformation of the simulated fibril can be directly observed.

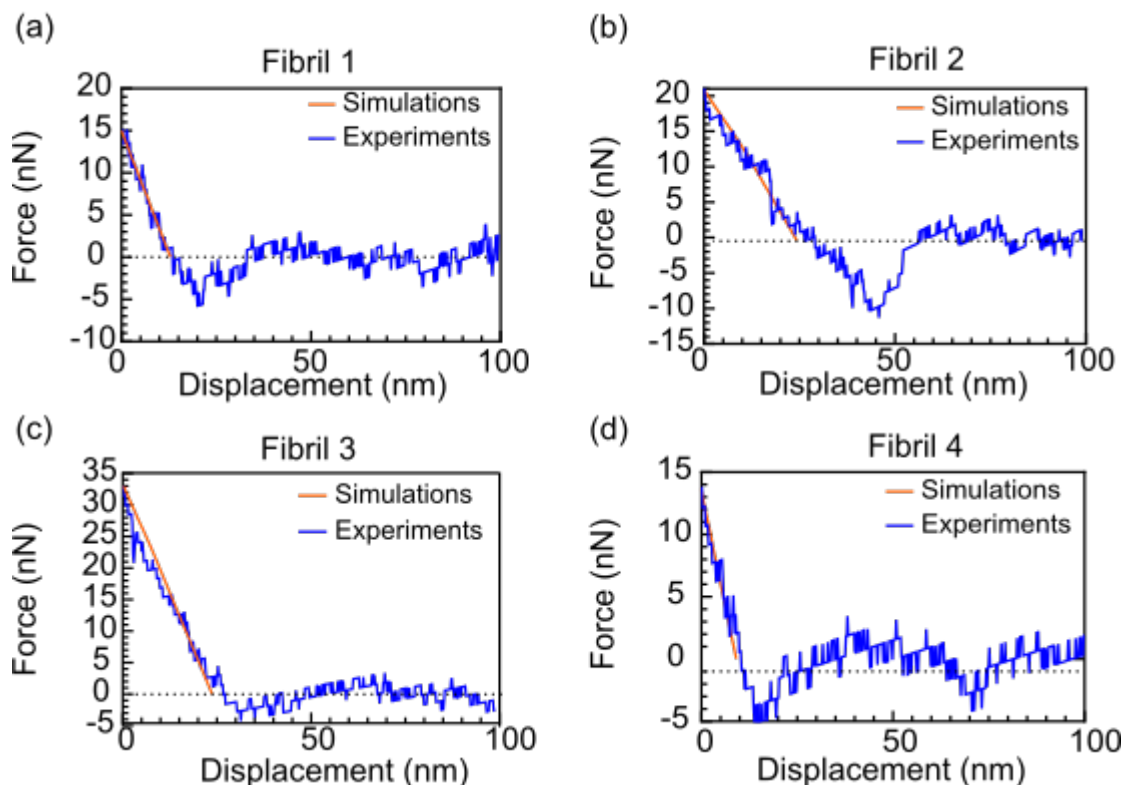


Figure 5.10: (a)-(d) Comparison of the experimental unloading curves obtained on four different fibrils (blue line) with the simulated unloading curve of these exact same fibrils loaded with the same load (orange line). To estimate if the simulated retract curves are a good linear regression of the experimental force curves, the R^2 value was calculated. (a) Collagen fibril properties: Young's modulus=1.06 GPa, radius=67.3 nm ; $R^2=0.96$; (b) Collagen fibril properties: Young's modulus=3.79 GPa, radius=32.1 nm; $R^2=0.89$; (c) Collagen fibril properties: Young's modulus=3.71 GPa, radius=80.5 nm; $R^2=0.74$; (d) Collagen fibril properties: Young's modulus=0.394 GPa, radius=93.6 nm; $R^2=0.85$

First, native fibrils were deposited on a PDMS film pre-strained to 25%. Then, four fibrils were found, imaged and axially loaded with an AFM probe with a specific force (Figure 5.10). Using the method described in section 3.2.6 the radius of each fibril was measured. Then, the PDMS was released from strain and the fibrils were compressed (see section 3.2.4), resulting in out-of-plane buckling of the four fibrils. From each buckling pattern, a buckling-wavelength was obtained and using equation 3.1, the average elastic modulus of each fibril was calculated. This allowed me to obtain two independent values of Young's modulus for a single fibril which are independent from one another. Each of these four fibrils were then simulated with the radii and elastic moduli values obtained experimentally. Experimentally and prior to compression, each fibril was loaded with a certain load and those loads were applied to the fibrils in the simulations. Hence, the resulting unloading curves from the simulated and experimentally obtained force vs. displacement curves could be directly compared to assess whether the simulations accurately

reproduced the experiments that had been conducted (Figure 5.10). As highlighted by the R^2 values in figure 5.10 the unloading curves of the simulations are good regressions of the experimental unloading curves, resulting in similar h_{ind} values. This ascertains that the simulations accurately reproduced the experimentally obtained data. This result is of particular interest because it shows that a simple continuum finite element model can be used to accurately describe the deformation of collagen fibrils when axially loaded with an AFM tip. In the simulations, the structural and mechanical longitudinal anisotropy of collagen fibrils is neglected. The results presented in figure 5.10 show that this anisotropy does not significantly impact the deformation of fibrils under a point load. The same experimental protocol where fibrils deposited on a pre-strained PDMS film are first identified and axially loaded prior to fibril compression and analysis of the resulting buckling behavior was repeated to obtain Young's moduli values obtained from both the Winkler foundation model and the buckling model (detailed in chapter 3). The resulting Young's moduli obtained from each model are independent of each other.

Further simulations were then conducted and the substrate upon which a fibril with a 200 nm diameter is deposited was alternatively set to be glass or PDMS. These simulations showed that collagen fibrils subjected to axial forces on glass, unsurprisingly, always resulted in nanoindentation of the surface of the fibril (Figure 5.13) whereas axially loading a fibril on a PDMS substrate predominantly results in bending of the fibril inside the PDMS substrate (Figure 5.12).

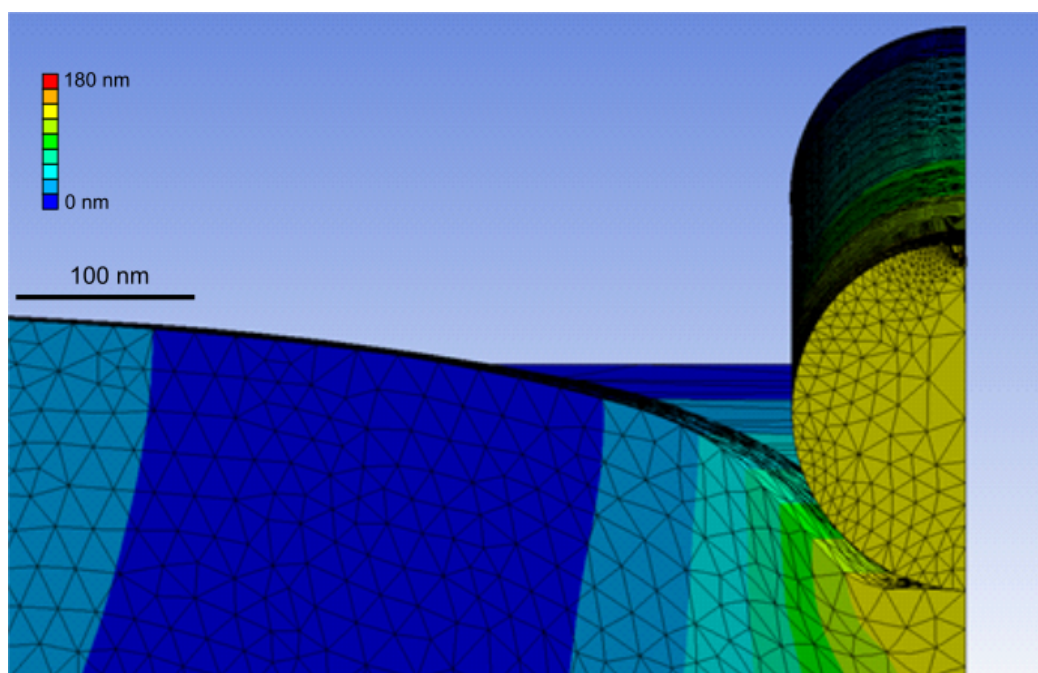


Figure 5.11: Bending of a collagen fibril with a diameter of 200 nm into the underlying PDMS substrate when subjected to a 1 μ N axial load.

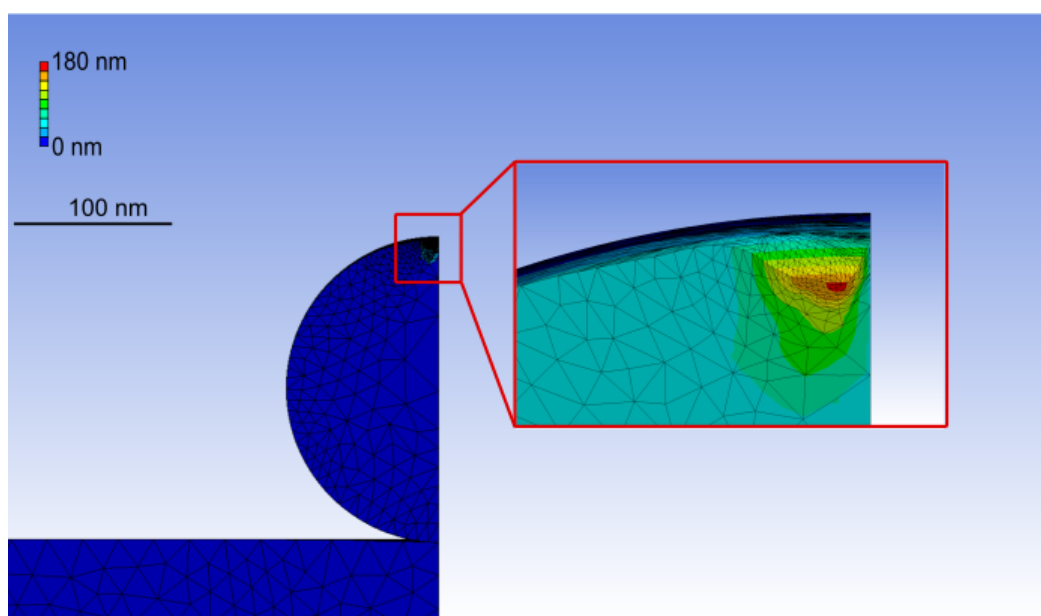


Figure 5.12: Indentation of a collagen fibril on a glass substrate with a diameter of 200 nm when subjected to a 1 μ N axial load.

Axial forces ranging from 50 nN to 4 μ N were applied to a simulated collagen fibril with a diameter of 200 nm on PDMS. Figure 5.11 shows that the predominant type of deformation for this fibril is bending and not indentation. As the applied load increased, the nanoindentation depth of the fibril increased as well and only starts to become significant for loads of 1 μ N and greater (Figure 5.13). Nanoindentation experiments are limited to

indentation depths that are 10% or less of the probed structure's diameter. In this experiment, nanoindentation is only achieved for loads of roughly 1000 nN which is much greater than any forces used in typical nanoindentation studies and in this thesis (Figure 5.13). The small indentation depths for forces smaller than 4 μN provides a clear reason why no visible scars were left at the surface of the experimentally probed fibril on PDMS shown in figure 5.6.

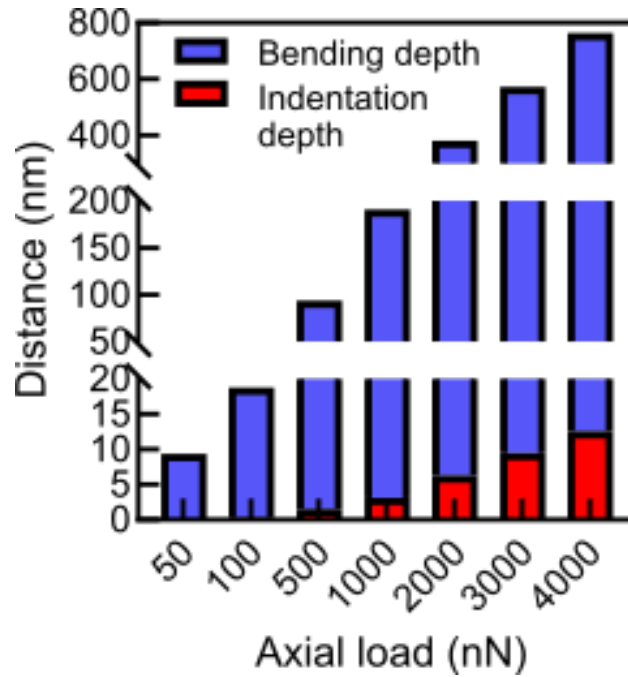


Figure 5.13: Comparison of bending and indentation depths for a fibril with a 200 nm diameter on PDMS. Axial loads ranging from 50 nN to 4000 nN were applied to the simulated fibril. The total height of each column corresponds to h_{ind} . This h_{ind} value is made up of bending and indentation which are represented as the blue and red components of each column respectively.

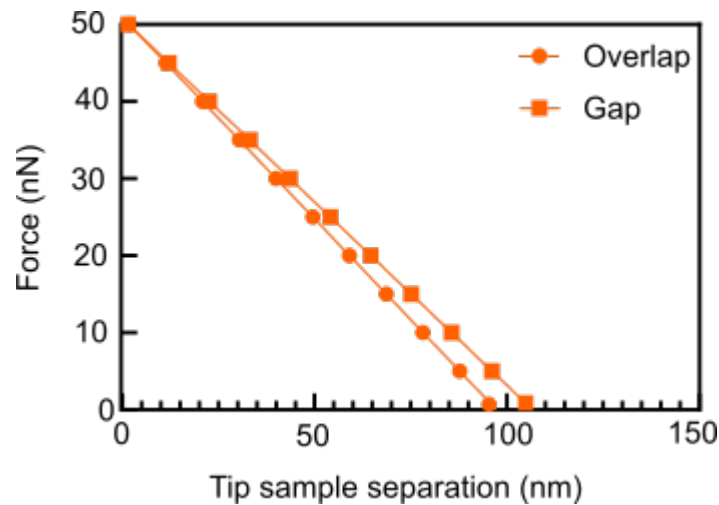


Figure 5.14: Simulation of a 50 nN load being applied to an overlap region of a fibril with a Young's modulus of 5 GPa and to a gap region of the same fibril with a Young's modulus of 4.25 GPa on a PDMS substrate.

The gap and overlap regions are mechanically distinct regions along the length of the collagen fibril. In order to assess whether the gap and overlap regions could be mechanically differentiated when loading a fibril on a PDMS substrate, a 50 nN load was applied to the same, previously described, simulated fibril on either a gap or overlap region. These regions had the same lengths as gap and overlap regions but the D-banding height was not present in our simulations. Hence, the influence of topography could be ruled out when analysing the force vs. displacement data obtained from the simulations. The gap regions are on average softer than the overlap regions by 15% [134]. The overlap regions were therefore simulated with a Young's modulus of 5 GPa and gap regions with a Young's modulus of 4.25 GPa. Despite the overlap and gap regions only being indented by 0.9 nm and 1.1 nm respectively, this is enough to translate into a noticeable difference between the slopes of the unloading curves of the gap and overlap regions (Figure 5.14). The slopes of these curves are directly related to the stiffness of the gap and overlap regions. Hence, the stiffness of both regions can still be differentiated when a collagen fibril is mechanically loaded with an AFM tip on a soft substrate such as PDMS.

5.4.3 Comparison of buckling values and loading values

For applied loads typically used during nanoindentation (50 nN-150 nN), collagen fibrils predominantly bend into the underlying substrate and hence, nanoindentation models cannot be used to calculate the Young's modulus of individual collagen fibrils. The Winkler foundation model is commonly used in engineering to characterise the deformation of a stiff beam resting along its entire length on a soft Winkler foundation when it is subjected to an axial force. This model was applied to the bending data of collagen fibrils on PDMS as a way to obtain the elastic modulus of individual fibrils.

Twelve native, and five 2% GA-crosslinked collagen fibrils were deposited on two separate pre-strained pieces of PDMS film (macroscopically strained to 25%) and then loaded with a maximum force of 50 nN with an AFM tip. By measuring the maximum vertical deformation value, h_{ind} , of the collagen fibrils into the PDMS from the force curves, equation 5.2 could be used to calculate the Young's modulus for each individ-

ual fibril. The pieces of PDMS were then released from strain, resulting in buckling of the fibrils. The fibrils that had been loaded prior to compression were tracked and their buckling patterns were studied as described in chapter 3, in order to calculate each fibril's elastic modulus and compare it to the one obtained with the Winkler foundation model.

The Young's moduli calculated for the native and crosslinked fibril data-sets with both techniques are coherent with other experimentally obtained Young's moduli values of dehydrated collagen fibrils [152][34][32]. Figure 5.15 (a) shows that the average Young's moduli of fibrils calculated from the buckling patterns, $E_{Buckling}$, or from the bending data, $E_{Winkler}$ are not significantly different for both data-sets (Table 5.2). Figure 5.15 (b) and (c) show the comparison of $E_{Buckling}$ and $E_{Winkler}$ for individual fibrils in each data-set. Statistical analysis comparing the elastic moduli obtained with the Winkler foundation model and the buckling model for each fibril in both the native and crosslinked fibril dataset showed that they were not significantly different (Table 5.3). The small sample sizes of both the native and crosslinked fibril data-sets combined with the large standard deviation in the data of both data-sets may have compromised the statistical power of the paired student t-test hindering its ability to detect a difference between the elastic moduli obtained from the buckling and the Winkler foundation model. However, similar trends for the Young's moduli values obtained with the buckling model and the Winkler foundation model for both native and crosslinked fibrils are clearly identifiable in figures 5.15 (b) and (c). Fibril 1 in figure 5.15 (b) and fibril 3 in figure 5.15 (c) seem to be outliers in both datasets. The Young's modulus values obtained from axial loading data for these fibrils are much greater than the other Young's modulus values obtained for the other fibrils in the datasets. This is most likely the result of using the "Point and Shoot" mode to apply an axial load to the fibrils. Due to drift, despite selecting a loading point on the crest of the fibril the actual loading might take place slightly to the side of the crest. Hence, the side walls of the tip could have made contact with the fibrils and the fibrils probably did not bend as much as they would have, had they been loaded on the crest of the fibril. This most likely lead to increased calculated Young's modulus values for these fibrils.

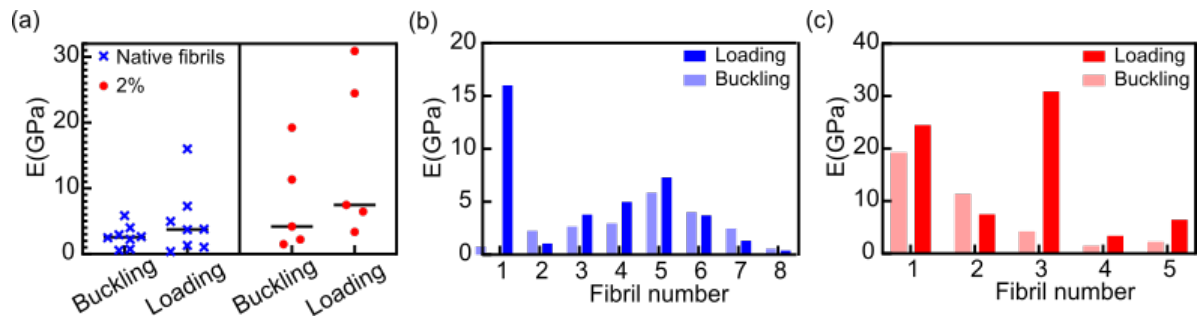


Figure 5.15: (a) Young's modulus of native and 2% GA-crosslinked fibrils calculated from buckling data or from loading data and the Winkler foundation model. (b) Direct comparison of E_{Buckling} and E_{Bending} for eight native collagen fibrils. (c) Direct comparison of E_{Buckling} and E_{Bending} for five 2% GA crosslinked collagen fibrils.

	A	B
Bending vs. Buckling	Native fibrils	2% GA fibrils
t-value	1.108	1.312
p-value	0.3046	0.2598
Null hypothesis	not rejected	not rejected

Table 5.2: Paired Student's t-tests. The null hypotheses are: calculating the Young's modulus from buckling data or with the Winkler foundation model does not produce different results for (A) native fibrils and (B) 2% GA-crosslinked fibrils.

	Native vs. 2% GA
t-value	2.361
p-value	0.0377
Null hypothesis	rejected

Table 5.3: Unpaired Student's t-tests. The null hypothesis is: artificially crosslinking fibrils does not result in an increase in the fibrils' Young's modulus.

The experimental and simulated data show that when collagen fibrils are deposited on a softer, compliant substrate such as PDMS and axially loaded with forces typically used in nanoindentation experiments [144] the fibrils predominantly bend inside the PDMS substrate. This effect is overwhelming compared to the indentation depths which is why the Winkler foundation model can be used to obtain a good estimation of the average Young's modulus of the bent fibrils. This technique is very easy to use and does not

require complex fittings of force curve and estimations of the tip-sample contact area as is required in nanoindentation models. However, unlike nanoindentation models, the Winkler foundation model only gives us the average Young's modulus value of a fibril and we lose the resolution needed to differentiate the gap and overlap regions mechanically. Furthermore, a critical analysis of the Winkler foundation model must be done. In fact, this model is based on the assumption that the PDMS substrate is a Winkler foundation and that the fibrils only bend into the underlying soft substrate. It was shown, however, that fibrils bend but are also indented, to a much smaller extent, when axially loaded on PDMS. The elastic moduli values obtained from this model are good estimations but must be used and analysed carefully.

5.5 Conclusion

In summary, the deformation of dehydrated collagen fibrils on both a stiff and a soft substrate under different loads were studied. This work showed that when fibrils on PDMS are probed with forces typically used during nanoindentation experiments, the fibrils are predominantly bent into the softer PDMS substrate and indented to a much lower extent (Figure 5.13). The bending of fibrils on PDMS has been shown to be consistent with the bending of stiff beams on a Winkler foundation. The Winkler foundation model used to study the bending of the fibrils yielded Young's moduli values that match those calculated from the buckling patterns of those same fibrils. It has hence been shown that loading a fibril on PDMS with low forces in the range of 50-150 nN (which are the range of forces used in this thesis) bends the fibril into the PDMS and from the load applied and resulting bending depth, the average Young's modulus of the fibril can be reasonably well calculated using the Winkler foundation model. To understand the bending of fibrils and measure the average Young's modulus of a fibril the structural complexity of the molecular arrangement inside the fibrils can be neglected and the fibrillar structure can be approximated to be an isotropic rod. As discussed in chapter 3, bending consists in probing the longitudinal properties of the fibrils and the Young's modulus corresponds to the bending modulus at 0% strain. However, with an increasing load the indentation depth in

the fibrils is more pronounced. As discussed in chapter 2, for small loads and small indentation depths the indentation is elastic whereas for greater loads and greater indentation depths, plastic deformation occurs and hence the approximation that the fibril is an elastic rod is no longer valid. However, it is of particular interest when working on collagen that the fibrils are being indented, even if by a negligible amount. In fact, the fibrils' gaps and overlaps have different Young's moduli and the mechanical distinction between the two during an AFM loading experiment can only be achieved if the two different regions are indented.

Chapter 6

Mechanics of collagen fibrils

6.1 Introduction

The aim of the work presented in this chapter is to determine the stiffness of single collagen fibrils, and of their gap and overlap regions, at different strains and along an expanded strain range.

As I showed in chapter 4, the PDMS film technique can be used to stretch multiple fibrils at once and to different strains all the while maintaining them accessible to AFM imaging and axial loading. Similarly, as presented in chapter 5 loading of fibrils on such soft films results in a combination of bending of the fibril into the underlying substrate and nanoindentation to a much lesser extent. However, it was shown that the mechanical properties of individual fibrils and of the gap and overlap regions independently, can still be calculated from the force displacement curves obtained. In this chapter, the stiffness of individual collagen fibrils was determined by combining axial loading with an AFM probe with the previously described pre-straining technique of individual collagen fibrils by stretching the PDMS film on which the fibrils are deposited. AFM axial loading is the only technique that offers the spatial resolution necessary to measure the mechanical properties of the gap and overlap regions independently [149] and hence, the stiffness of these regions too were measured along an expanded strain range by obtaining force volume maps. The great advantage of combining loading of the fibrils with stretching is that fibril stiffness can be determined at several pre-strains, thereby probing small segments of

the longitudinal stress-strain curve along an expanded range, while at the same time preserving the inherently high, spatial resolution of the AFM loading technique. Combining loading of fibrils with stretching consists in probing small segments of the longitudinal stress-strain curve along an expanded range which allowed me to assess whether collagen fibrils as a material behave linearly at high strains.

In this work, the stiffness of individual fibrils was determined but not the Young's modulus. As described in chapter 5, fibrils are both bent and indented when axially loaded on PDMS and hence, nanoindentation models cannot be used to extract the elastic modulus of the fibrils. In chapter 5, the Winkler foundation model, was used to determine the average elastic modulus of axially-loaded single collagen fibrils. However, this model relies on the assumptions that the PDMS is a perfect Winkler foundation, and that the fibrils only bend into the underlying foundation, which was determined not to be the case in chapter 5. The indentation of the fibrils, however small it might be, is the only way to distinguish between the gap and overlap regions mechanically. Hence, the Winkler foundation model was not applied in this work and only the stiffness of the fibrils and of the gap and overlap regions were determined. In fact, stiffness values correspond to the slopes of the force vs. displacement curves obtained from axial loading and can be determined without the use of any models. These findings could prove to be useful for the design of scaffolds in tissue engineering as changes in scaffold stiffness, induced by the surrounding cells or by external chemical or physical stimuli, could have an impact on cellular behaviour.

6.2 Methods

6.2.1 Collagen fibril sample preparation

Un-strained collagen fibril samples were prepared as described in sections 3.2.1 and 3.2.3. Two samples with crosslinked collagen fibrils were also prepared by exposing native collagen fibrils to either a 2% GA or a 4% GA solution as described in section 3.2.2. Native and crosslinked collagen fibrils were imaged in tapping mode using a Dimension Icon AFM (Bruker Corporation, Billerica MA, USA) and force curves on fibrils were performed in the un-strained state but also once strained. Straining of the collagen fibrils was performed as described in section 4.2.3.

6.2.2 AFM loading of the fibrils

6.2.2.1 In air

Tapping-mode imaging and loading of the fibrils was performed using a Dimension Icon AFM (Bruker Corporation, Billerica MA, USA). Loading measurements were performed in order to obtain the stiffness of collagen fibrils. First a $3\text{ }\mu\text{m} \times 3\text{ }\mu\text{m}$ height image of a fibril was obtained. Then, three force curves were performed along the fibril using the Point and Shoot mode of the Nanoscope 8.10 AFM operating software. Force-distance measurements were performed with a BudgetSensors All-in-One tip ($f = 350\text{ kHz}$; $k = 40\text{ N/m}$) on three randomly chosen overlap regions in the height image. A maximal force of 150 nN was applied to ensure that no plastic deformation of the fibrils would take place. For these experiments, a loading rate of 1 Hz was chosen. To differentiate the mechanical properties of the overlap regions from the gap regions, the tapping force-volume mode was used with a Bruker RFESP-75 tip ($f = 75\text{ kHz}$; $k = 3\text{ N/m}$). The force-volume mode provides force-distance measurements in a two-dimensional array along with topography information. First, a tapping-mode image was taken. Then, a map of 64×64 force curves were taken on a $1.15\text{ nm} \times 1.15\text{ nm}$ area, which means that at least four force curves were taken within any single D-banding period. The loading rate was 5 Hz . Different maximum forces (50 nN , 100 nN , 150 nN) were tested during nanoindentation on five

different fibrils (Figure 6.1) to assess whether or not the stiffness of the fibril, k_{fib} , is dependent on the trigger threshold used. It was found that k_{fib} did not depend on the maximal applied force in the range from 50 nN to 150 nN. That is, the unloading branch of the unloading curve was always linear, irrespective of the right-hand end point of the curve. A maximum force of 150 nN was used to probe fibrils in air.

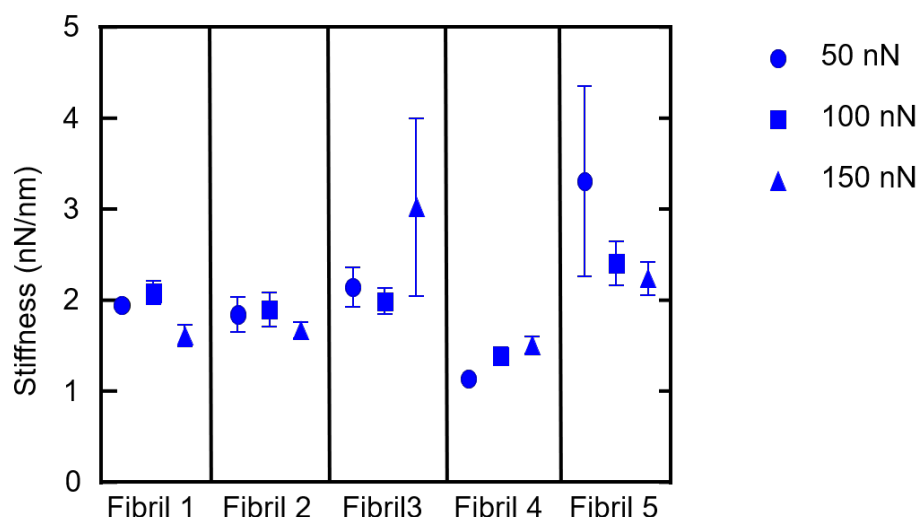


Figure 6.1: Stiffness of five collagen fibrils probed at three different loads: 50 nN, 100 nN and 150 nN. Each stiffness value corresponds to the average of three slope values from force curves done on individual fibrils.

6.2.2.2 In liquid

Tapping mode imaging and nanoindentation in liquid was performed using a MultiMode AFM (Bruker Corporation, Billerica MA, USA). A BudgetSensors All-in-One ($f = 350$ kHz; $k = 40$ N/m) tip was used to first image the collagen fibrils in air in order to measure the D-banding length of the strained fibrils prior to hydration according to section 4.2.4. Once the fibrils are imaged in air, the tip is replaced by a Bruker RFESP-75 tip ($f = 75$ kHz; $k = 3$ N/m). 60 μ L of PBS is placed on the sample to hydrate the fibrils. In order for the fibrils to get fully hydrated and for them to reach equilibrium, fibrils were not imaged or loaded for 5 minutes after hydration. Three force curves were then done along the hydrated fibril. A loading rate of 5 Hz and a maximum force of 5 nN was used to probe the fibrils.

6.2.3 Data analysis

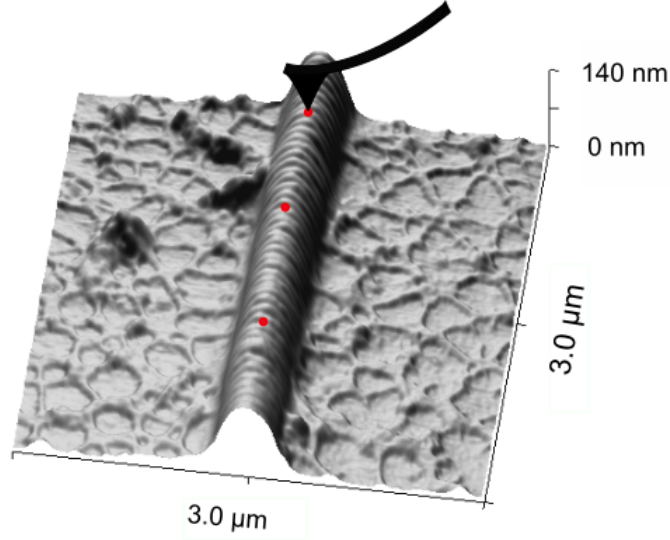


Figure 6.2: 3D AFM height image of a collagen fibril on PDMS. The red dots correspond to exemplary locations where the fibril is probed by nanoindentation.

Fibril stiffness was determined by performing three force curves on individual fibrils. A linear fit was done on the top quarter of the unloading curve of each deflection versus scanner z-position curves. The slopes are directly proportional to the stiffness of the fibril and the three values are then averaged. When the tip is in contact with the fibril, the cantilever/tip and the fibril behave as two springs in series. The cantilever spring constant, k_c , and the stiffness of the fibril itself, k_{fib} result in a total spring constant, k_{tot} . Using the series rule for ideal springs, k_{fib} can be calculated:

$$k_{fib} = \frac{k_c - k_{tot}}{k_c \times k_{tot}} \quad (6.1)$$

As described in section 2.3.1, the cantilever spring constant, k_c , was obtained by first performing a force curve on a glass microscope slide. Then a linear fit of the retract curve is done and the inverse of the slope is equal to the cantilever's deflection sensitivity in V/nm. Using the “thermal tune” function of the Nanoscope AFM software, the stiffness of the cantilever was obtained.

A MATLAB script (The MathWorks, Natick, MA) was used to reconstruct a stiffness map from a force-volume map by following the same process as described earlier[34].

Using Gwyddion, an average stiffness profile for each fibril was obtained by averaging multiple rows from the stiffness maps (similar to figure 4.2 in section 4.2.4 with height images). The peaks and troughs were identified, and their corresponding stiffnesses were averaged to obtain the average overlap and gap stiffness, $k_{overlap}$ and k_{gap} respectively, for each fibril.

6.3 Results and Discussion

6.3.1 Nonlinear mechanical behaviour of fibrils under strain

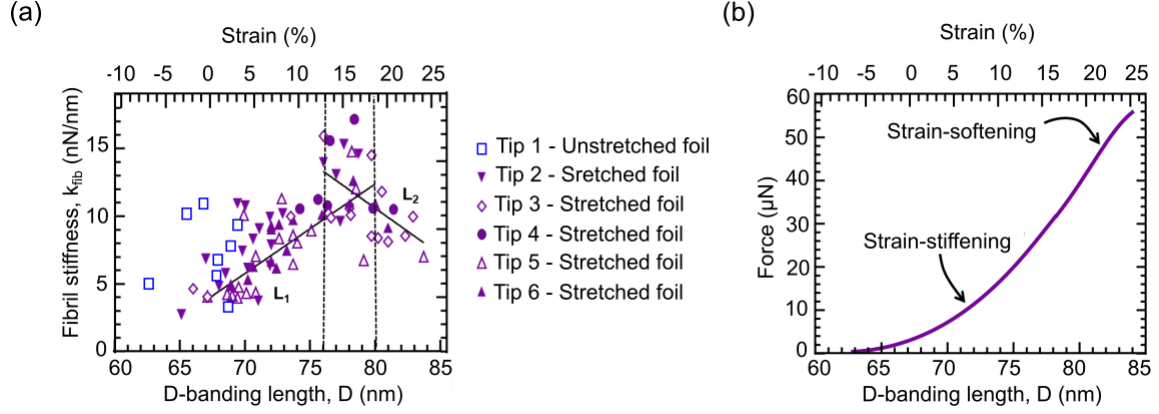


Figure 6.3: (a) Individual collagen fibril stiffness as a function of strain. Each data point corresponds to an individual collagen fibril. The linear fit L_1 of the data in the interval of $60 \text{ nm} < D < 80 \text{ nm}$ is as follows: slope = 0.51 nN/nm , $R^2 = 0.62$. The linear fit L_2 of the data in the interval of $76 \text{ nm} < D < 85 \text{ nm}$ is as follows: slope = -0.68 nN/nm , $R^2 = 0.26$. (b) By integrating the linear regressions in (a) a force versus strain curve that are typically obtained during tensile tests can be obtained.

Figure 6.3 (a) shows the stiffness, k_{fib} , of individual, native fibrils as a function of their individual D-banding length. No correlation was found between the diameter and stiffness of the fibrils. Although the data scatters considerably, reflecting the natural variation of k_{fib} of different fibrils, an overall trend can be observed: the stiffness, k_{fib} , increases from roughly 2 nN/nm to 14 nN/nm with strains up to 15% (*strain stiffening*), then decreases again to 8 nN/nm with strains up to 25% (*strain softening*). The overall trend is highlighted with the two trend lines, L_1 and L_2 . From the stiffness vs. strain data (Figure 6.3 a), a force versus-strain curve can be reconstructed (Figure 6.3 b) by the integration of the former with a force of zero (integration constant of zero) at the lowest strain. The result is similar to the curves often observed in polymeric and, especially, elastomeric materials. The exemplary AFM topography images taken at a high strain confirms that the overall integrity and structure of the fibrils are preserved. This is particularly visible due to the presence of the D-banding period on the surface of those fibrils at high strains. The fibrils were probed with a loading rate of 1 Hz. Collagen fibrils are viscoelastic [153], which means that the measured stiffness could be dependent on the loading rate. In our experiments, there was no significant difference between loading rates of 1 Hz and 0.5 Hz (Figure 6.4). However, a lower stiffness was measured when fibrils were indented at a

rate of 0.1 Hz. Such a tendency is common in the mechanical testing of elastomers and can readily be explained by the particular dynamics of slow, molecular rearrangements in viscoelastic materials [154]. Although interesting, the investigation of collagen fibril viscoelasticity at low loading rates, is outside the scope of this work, and the data in figure 6.4 suggests that loading rates of 1 Hz (for a given indentation depth) are on the “safe” side for determining elastic properties.

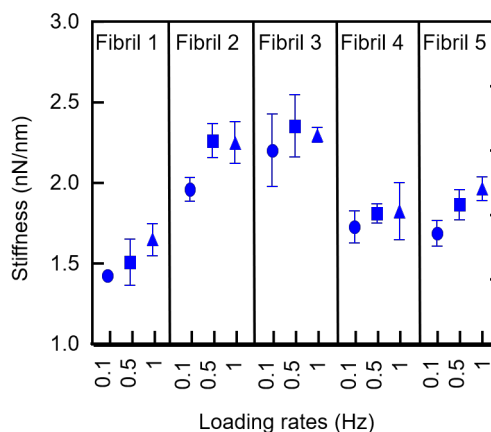


Figure 6.4: Stiffness of five collagen fibrils probed at different loading rates : 0.1 Hz, 0.5 Hz, 1 Hz. Each stiffness value corresponds to the average of three slope values from force curves done on individual fibrils.

Strain stiffening (Figure 6.3 a) could be explained by molecular straightening (Figure 6.5 a i-iii). There are domains within the fibrils where the molecules are more disordered and allow for straightening while still, overall, preserving the number and locations of intermolecular, native crosslinks. This is typical behavior for elastomers. As more and more of the disordered sections are straightened, the resistance of the fibril to an external tensile force increases. At some point, when most of the disordered sections are straightened, the stiffness reaches a maximum, and further strain starts to break crosslinks (Figure 6.5 iv). This then allows molecules to slide against each other, which results in a lower resistance to the external tensile force and leads to the strain softening part of the curve shown in figure 6.3 b.

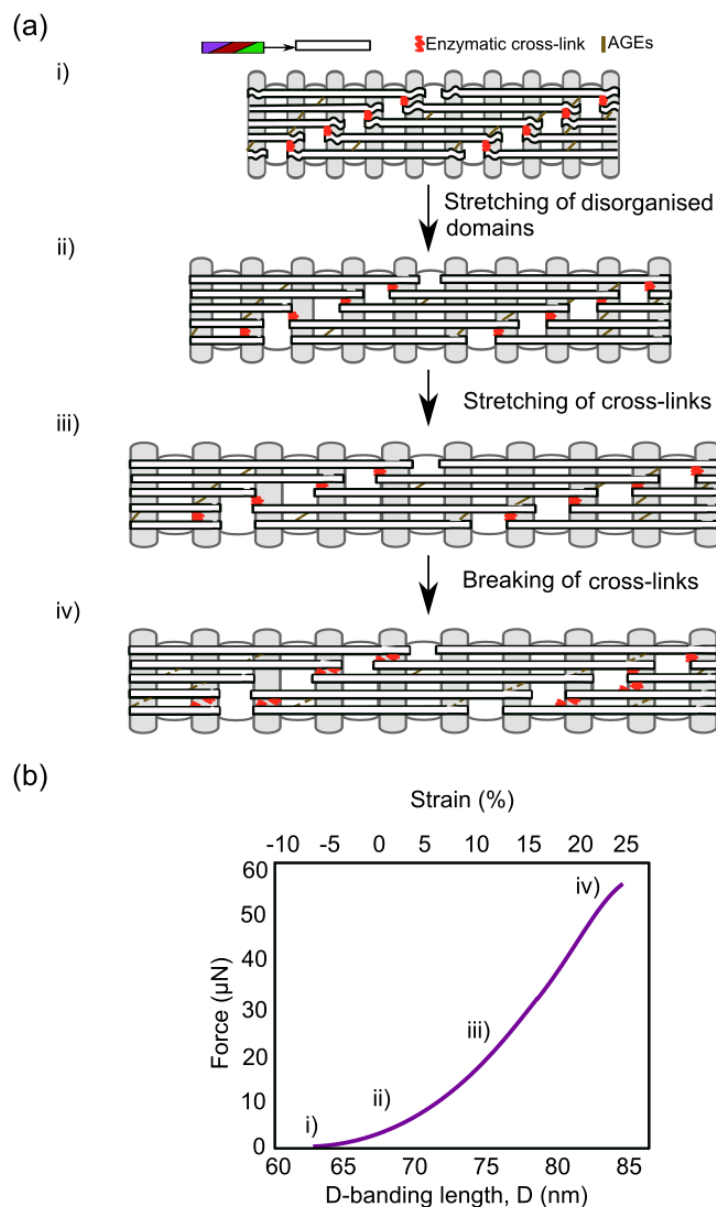


Figure 6.5: The impact of stretching of collagen fibrils on their internal molecular structure and integrity. i) Unstrained collagen fibril. ii) Fibrils start to get strained, which results in an unravelling and “stretching-out” of the disordered domains in the gap region. Further straining of collagen fibrils stretches the intermolecular crosslinks iii) and results in a stiffening of collagen fibrils. Straining of collagen fibrils past 15% strain results in probable breakage of intermolecular crosslinks leading to a softening of the fibril.

The experiments in figure 6.3 (a) were performed with different AFM tips of the same type and nominal geometry (same tip material, spring constant and tip shape). As describe in chapter 5, fibrils on PDMS subjected to loads are bent into the underlying PDMS and indented to a much lower extent. According to theoretical indentation models, the measured indentation stiffness scales with the square root of the projected tip-sample contact area [105], which means the stiffness is effectively dependent on tip shape and indentation depth. However, in figure 6.3 a), the data for k_{fib} does not show any obvious

correlation with different tips, and, if anything, the natural fibril-to-fibril variation of the data is greater than the variation between different tips. It can therefore be concluded that the error introduced by using different tips of the same type is not significant, and hence, measurement sets taken with different tips of the same type and geometry can be compared quantitatively.

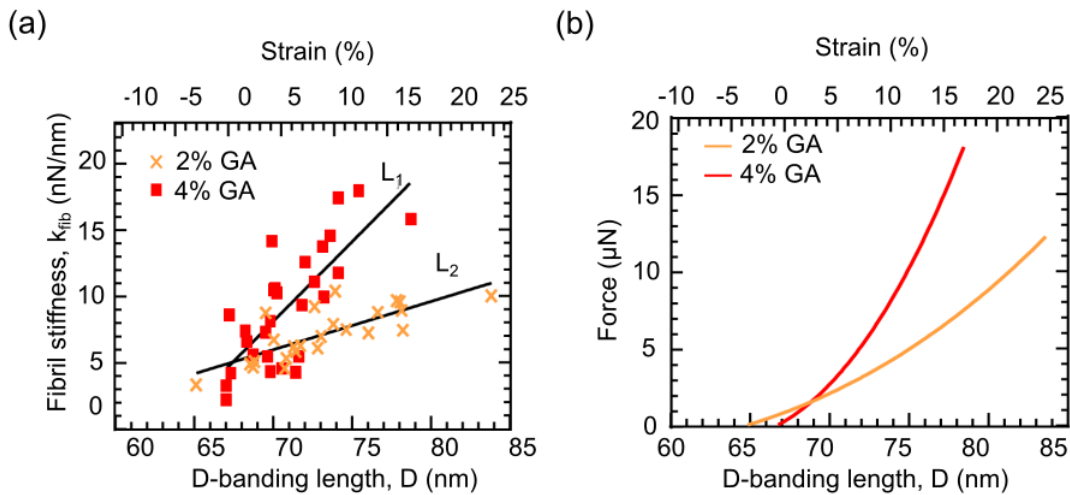


Figure 6.6: (a) Stiffness as a function of strain of fibrils crosslinked in either a 2% or 4% GA solution. Each data point corresponds to an individual collagen fibril. L_1 : slope = 0.2011 nN $R^2 = 0.58$; L_2 : slope = 0.06425 nN $R^2 = 0.59$. (b) Force versus strain curves derived from the stiffness versus strain curves in (a).

Figure 6.6 (a) shows the stiffness vs. strain data of fibrils artificially crosslinked in either a 2% or 4% GA solution. Just like native fibrils, an increase in stiffness was observed, but no strain-softening behavior could be detected. It was also observed that the increase in stiffness is higher for fibrils exposed to a more concentrated GA solution. The lack of strain-softening could be due to the reduced number of fibrils found and probed at D-banding lengths greater than 75 nm compared to native fibrils. GA is a strong protein crosslinking agent and hence, fibrils are harder to stretch to high strains when they are artificially crosslinked. It is also conceivable, and it has been shown in earlier experiments, that GA exposure increases the intrafibrillar crosslink density [142]. Overall, the observations indicate that, GA results in more crosslinks. In this case, the disordered sections of artificially crosslinked fibrils straighten upon strain just like native fibrils, but after most of these sections are straightened, the sheer number of crosslinks prevents strain-softening. Some crosslinks may well break, as in the case of native fibrils, but this is not sufficient to result in an overall strain-softening of the fibrils. These experimental results

agree with stress-strain simulations performed on collagen fibrils with different crosslinking densities [153]. In those simulations, fibrils with a low crosslink density strain stiffen up to a small yield strain and then strain soften [153]. However, highly crosslinked fibrils strain stiffen up to a greater yield strain and then exhibit a brittle-like fracture. To see the D-banding, the experiments had to be performed in air, that is, with (at least partially) dehydrated fibrils, which raises the question of how representative the results are for fibrils under physiological conditions. This will be discussed in section 6.3.2.

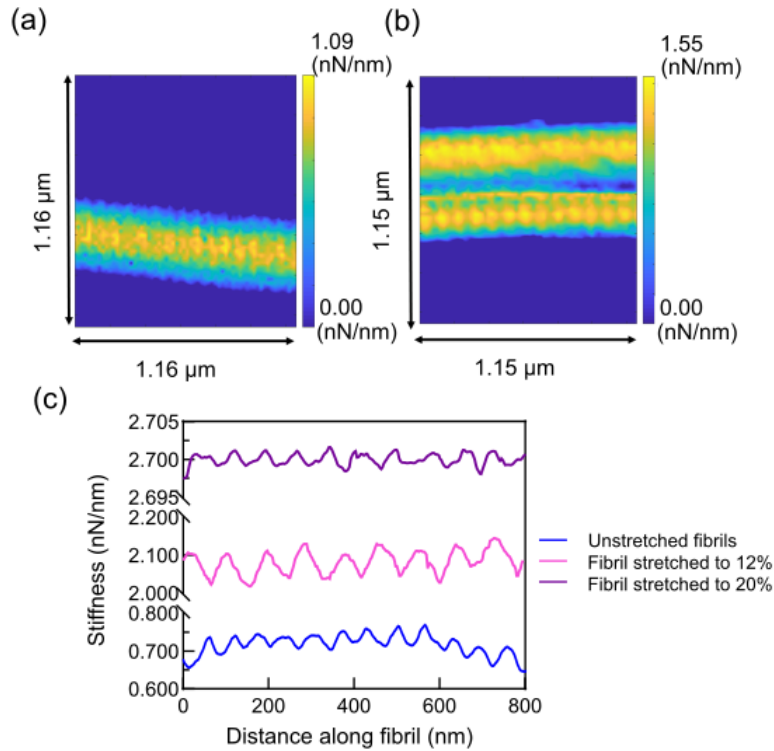


Figure 6.7: (a) Stiffness map reconstructed from force volume data of an unstrained fibril. (b) Stiffness map reconstructed from force volume data of two fibrils on a strained PDMS film. The banding pattern visible in the stiffness maps show that the bottom fibril is strained to a greater extent than the top fibril. (c) Stiffness profiles of three collagen fibrils: one unstrained, one strained to 12%, and one strained to 20%. The radial variation in stiffness matches the D-banding period observed in the height images and arises from the differences in the mechanical properties between the gap and the overlap regions.

As mentioned above, the great advantage of stretching the collagen fibrils by depositing them on a PDMS film is that the fibrils are accessible to AFM imaging and loading and that the spatial resolution of mechanical loading by AFM is preserved. Hence, tests that probe overlaps and gaps independently are possible. To this end, force-volume mapping was used, which is, essentially, a grid of many force-distance curves in a given area of the sample. Figure 6.7 (a) and (b) show two stiffness maps of unstrained and highly strained fibrils, respectively, in which the D-banding pattern is clearly visible.

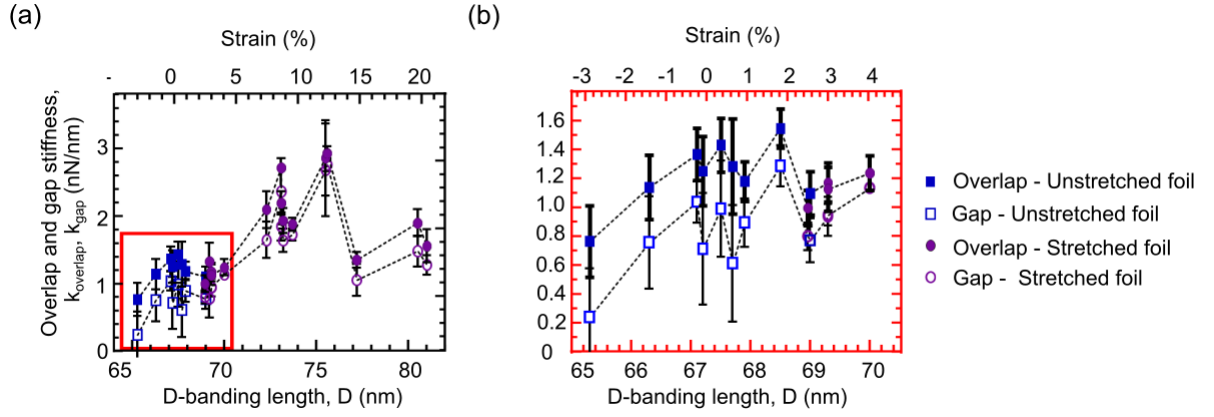


Figure 6.8: (a) Gap and overlap stiffness of individual collagen fibrils as a function of strain. (b) Zoom on the gap and overlap stiffness versus strain curve from (a) for strains up to 5% where the difference in stiffness between the gap and overlap regions can be more easily observed.

Figure 6.7 (c) shows an example of longitudinal line profiles through the stiffness maps, showing an overall stiffness increase from 0 to 12% strain and then a decrease from 12 to 20% strain. Figure 6.8 (a) shows that overlap and gap stiffness - $k_{overlap}$ and k_{gap} , respectively - individually follow the same general trend (strain-stiffening followed by strain-softening). Figure 6.8 (b) shows a zoom into data points on the bottom left of figure 6.8 (a) for clarity. Figure 6.8 (b) shows that in the first instances of strain, no significant change in gap and overlap stiffness occurs. However, both overlap and gap strain-stiffen from 4% strain up to roughly 12% strain and then strain-soften. Furthermore, the overlap always appears stiffer than the gap region, which is in agreement with the literature [149].

Figure 6.9 (a) is a reconstruction of the force-versus-strain curves from figure 6.8 (a). It should be noted that because the reconstruction is performed by integration of the stiffness data with an arbitrary integration constant, the vertical position of the force-versus-strain curves has no significance. Figure 6.9 (b) shows the difference between the the overlap and the gap stiffnesses, Δ_{stiff} , determined from Figure 6.9 (a) for the strain stiffening region (strains $\leq 12\%$). Here, a linear regression and statistical analysis of lower (L_1) versus higher strains (L_2) reveals that, at a lower strain, Δ_{stiff} decreases with increasing strain, whereas at a higher strain, Δ_{stiff} remains essentially constant.

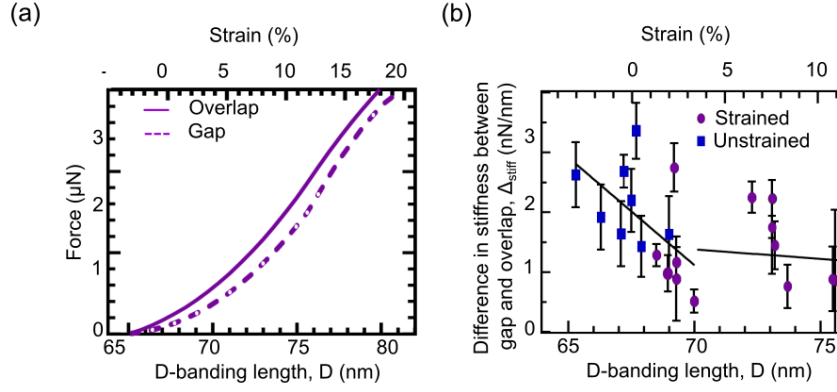


Figure 6.9: (a) Force vs. strain curve for gap and overlap regions respectively reconstructed from Figure 6.8 (a). (b) Difference in stiffness between overlap and gap regions as a function of strain up to roughly 11% strain. From 0-5% strain, the difference in stiffness between the two regions decreases significantly. The linear fit for data points in the interval $65 \text{ nm} < D < 70 \text{ nm}$ is as follows: slope = -0.07 nN/nm^2 , $R^2 = 0.33$. The linear fit for data in the interval $70 \text{ nm} < D < 76 \text{ nm}$ is as follows: slope = -0.006 nN/nm^2 , $R^2 = 0.82$.

This observation indicates that overlaps and gaps become mechanically and structurally more similar with increasing strain. The decrease in difference in stiffness between the gap and overlap regions occurs mainly the 0% to 4% strain range highlighted in figure 6.8 (b). This is particularly interesting because it corresponds to the strain range observed physiologically. The most plausible interpretation for our observations is that, in native, unstrained fibrils, the gap contains more disordered sections than the overlap (Figure 6.5 a i), making the gap softer. Upon straining, disordered sections in both overlaps and gaps straighten (Figure 6.5 a ii). However, because there are initially more disordered sections in the gap, their “concentration” decreases faster upon straining relative to the concentration in the overlap. This leads the gap to stiffen faster than the overlap and thus to the observed reduction of Δ_{stiff} in figure 6.9 from roughly 0% to 3% strain. In figure 6.9, from roughly 3% to 12% strain, the “oversupply” of disordered sections has almost vanished so that the overlap and gap appear to have similar stiffnesses within the accuracy of the measurement. Above a 12% strain, however, not enough data points are available to determine the dependence of Δ_{stiff} on strain. Studies using Small Angle X-Ray Scattering (SAXS) experiments showed that when physiological strains are applied to type I collagen fibrils (strains up to 4%) removal of crimp and unkinking of disordered regions within the fibrils occurs [41]. This is consistent with the findings presented here. However, results from SAXS experiments also showed that the application of such small strains to fibrils does not result in a visible shift in the average D-banding period. In this

thesis, the only quantifiable way to measure the strain applied to collagen fibrils is through the increase in the D-banding period. It is therefore possible that fibrils take up a certain amount of strain before a visible shift in the D-banding period occurs and that the strains reported here are underestimated. Furthermore, in this work, individual single fibrils were not tracked in the unstrained and strained states. Hence the true native D-banding length for each fibril is unknown and the strain applied to the fibrils is calculated using the average D-banding length obtained from 8 fibrils on an unstrained PDMS block. This can potentially lead to either an overestimation or underestimation of the strain that individual fibrils are subjected to. Hence in this work, rather than focusing on actual strain values, it is more interesting to note that in the first instances of strains, prior to the straining of the intermolecular crosslinking network, the concentration of disordered domains in the gap regions seems to decrease.

6.3.2 Hydrated fibrils

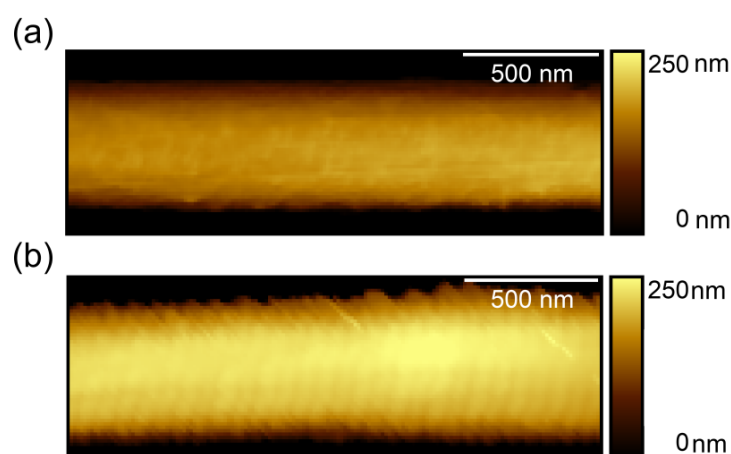


Figure 6.10: (a) Height image of a native fibril hydrated in PBS. No pattern at the surface of the fibril is visible. (b) Height image of a 2% GA-crosslinked hydrated collagen fibril. The D-banding pattern is visible at the surface of the fibril.

Hydrated fibrils were also imaged by AFM and mechanically loaded with an AFM tip. In liquid, the D-banding height of the fibrils is significantly smaller than the D-banding height of dehydrated fibrils. For some fibrils, the D-banding height of hydrated collagen fibrils is not visible at all [31]. The cross-sectional diameter of collagen fibrils immersed in PBS increases by 1.5 folds compared to their diameters in air [32]. As water molecules penetrate the fibril, the bulk water content increases and pushes the collagen molecules

apart. The water uptake inside the fibrils has been shown to be heterogeneous. The amounts of bound and free water inside the gap and overlap regions are different and the gap regions absorb more water than the overlap regions which results in a smaller or null D-banding height when fibrils are hydrated [31]. This uptake of water and consequently the swelling of the fibrils can be tuned through artificial crosslinking: increasing the content of hydrophilic crosslinks has been shown to result in a greater swelling of the fibrils compared to native fibrils [32].

In our experiments, native fibrils were shown to swell significantly in PBS and the D-banding pattern was no longer visible (Figure 6.10 a). Fibrils artificially cross-linked with GA also swelled when immersed but the D-banding pattern was still visible (Figure 6.10 b). GA is not a hydrophilic crosslink but creates covalent bonds inside collagen molecules and between adjacent collagen molecules. It is conceivable that increasing the GA crosslinking content limits the bulk water uptake inside collagen fibrils and the swelling inside the gap regions that decreases the D-banding height of collagen fibrils.

To test whether the mechanical behaviour of dehydrated collagen fibrils is representative of the mechanical behaviour of hydrated collagen fibrils, the stiffness of individual hydrated collagen fibrils was probed as a function of strain. Collagen fibrils were deposited on a pre-strained PDMS substrate. Since the D-banding pattern was not visible in the AFM height images, individual collagen fibrils were tracked before and after hydration. Before immersion in PBS, fibrils were identified and imaged in air. This allowed us to obtain visible D-bands in the height and phase images. The same fibrils were then identified once hydrated in PBS and probed by mechanical loading. It was assumed that when immersed, fibrils swell radially but do not expand longitudinally. Hence immersion was assumed to not strain fibrils longitudinally and the D-banding length obtained from the height and phase images in air was used as a marker for strain.

Hydrated fibrils were shown to be much softer than dehydrated fibrils (Figure 6.11). Hydrated fibrils, much like fibrils probed in air, strain-stiffened up to roughly 14% strain and then strain-softened for greater strains (Figure 6.11).

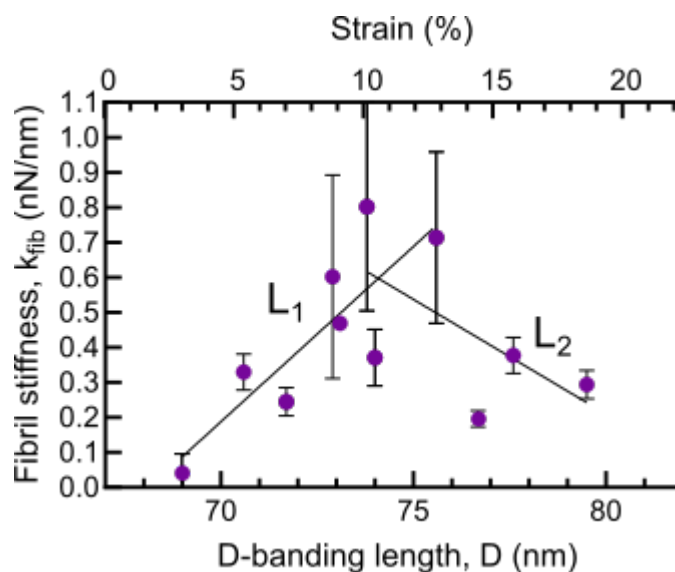


Figure 6.11: Stiffness of collagen fibrils hydrated in a PBS solution as a function of strain. L_1 : slope=0.100 nN $R^2 = 0.68$; L_2 : slope=-0.0654 nN $R^2 = 0.35$.

In terms of mechanical properties, the biggest difference is that hydrated fibrils are much softer than dehydrated fibrils which has been shown consistently in numerous studies, including nanoindentation as well as uniaxial, longitudinal testing [105][105][152][32], and is attributed to significant water uptake of hydrated fibrils. The simplistic picture is that water inside fibrils acts as a “lubricant” or “cushion” between the collagen molecules and facilitates molecular sliding, thereby leading to a lower stiffness (continuum model of water). However, and as described in chapter 1, it is also known that, on the few-nanometer scale, water molecules form structured networks, especially between hydrophilic molecules, and contribute in much more complex (and, to date, largely unknown) ways to protein interactions [35]. In this network model of water, it is conceivable that water molecules acts more like a “glue” between adjacent protein. The exact molecular mechanisms of collagen reorganization under load and the role of water in the fibrillar context are beyond the scope of this work. However both strain-stiffening and strain-softening have been observed in liquid suggesting that the results and interpretations in air are at least qualitatively representative of the mechanical response of individual regions of collagen fibrils under physiological conditions.

6.4 Conclusion

To summarize, a novel approach is presented here which significantly expands the strain range of tensile tests on single collagen fibrils while preserving the nanometer scale resolution. It can be implemented readily by most labs at low cost and effort. It was found that individual fibrils undergo strain-stiffening followed by strain-softening in both air and in PBS but that artificially crosslinked fibrils only strain-stiffened. Moreover, the slightly different stress-strain behavior of the gap compared with the overlap corroborates the hypothesis that the gap is molecularly more disordered than the overlap. A possible reason for this could be that the gap regions have evolved to take up strain so that fibrils are not structurally disrupted when a mechanical load is applied to them. An aspect that we did not investigate in this work is reversibility. Biological tissues such as tendons are often subjected to cyclic, mechanical loading. Such macroscopic loading-unloading cycles must translate somehow to the microscopic scale of collagen fibrils. The obvious question would be whether the nonlinear behaviors shown in figure 6.3 (a) and 6.11 is fully reversible, and, if not, how cyclic loading affects the stress-strain behavior.

Ultimately, the stress-strain behavior of collagen fibrils as integral constituents of the extracellular matrix is most significant in cell biology. As previously described in chapter 1, cells take mechanical cues from the extracellular matrix, which can direct their behavior and their differentiation routes [155]. Furthermore, in cancer progression, the structure and mechanical properties of the matrix have a crucial significance and are even thought to regulate metastasis [156]. This means that it is the extracellular matrix itself that could be a new and promising target for therapeutic intervention. These results could also have implications in the design of novel, collagen-based tissue scaffolds in tissue engineering. In principle, fibrillar collagen-based scaffolds should at least exhibit a similar strain-stiffening behavior as shown here with strains of up to 15%. Here, the stretching method presented could help in determining the right crosslinking level and possibly, because of its low cost and simple set-up, even be used in quality control.

Chapter 7

Strain-induced changes in surface charge of collagen fibrils

7.1 Introduction

Surface potential and charges play an important role in cellular processes. Hence, the electrostatics of biological tissues, their role in tissue regeneration and development has found an increasing interest in the scientific community. Studies looking into the cephalo-caudal electric potential of salamander embryos for instance, have shown that the potential changes depending on the stage of development [157]. Electric potentials can even be measured in the gelatinous membrane of eggs and be used as a predictor of the long axis of the future embryo [158]. Differences in the electric potential at the surface of normal and cancerous tissues in women with cervical cancer have also been observed and in general, tumors exhibit an overall more negative surface charge than healthy tissues [159]. Studies have shown that epithelial cells and fibroblasts' adhesion increases with increasing cell potential [160][161] in the range of $-187 \pm 7\text{mV}$ to $6.8 \pm 2.8\text{mV}$. This is believed to be the result of less electrostatic repulsion between a more positively charged surface and the negative cell membrane. Other studies have shown that cells spread more on more positively charged surfaces and are more mobile on negatively charged surfaces [162]. However, the cells' migration rates were faster on more positively charged surfaces, most

likely due to more electrostatic attraction between the surfaces and the cell membrane.

This highlights the importance of taking into account the surface charge or surface potential of the fibers making up the ECM when building scaffolds in tissue engineering. Their surface potentials are responsible for the integrity of the ECM and help regulate the cellular attachment and behaviour inside the ECM. For instance, laminin-dependant cells such as epithelial cells, would not adhere as well to a more negatively charged protein in the ECM, such as fibronectin.

As described earlier, collagen fibrils play a central role in regulating the architecture of the ECM through an interaction with integrins that possess both cell binding and collagen binding sites. Integrins bind to a specific GXOGER molecular sequence at the surface of the fibrils. The accessibility and exposure of these molecular sequences is essential to cell adhesion and for remodelling of the ECM. Glycation of collagen fibrils, which is a natural process that occurs during aging and can even be exaggerated in certain diseases like diabetes, has been shown to alter both the molecular packing inside collagen fibrils and the fibrils' surface charge [163]. The changes in surface charge are likely to alter the interactions between the collagen fibrils and the surrounding cells and proteins in the ECM.

Collagenous structures ranging from single collagen fibrils, to more complex structures such as whole tendons and even bone possess some form of electromechanical properties [13][164][165]. With the advent of probe-based methods, the effect has been studied down to the scale of individual collagen fibrils [165][166][167][168]. Originally, it was believed that the piezoelectricity of collagen fibrils could be at the origin of Wolff's law which is the empirical observation that bone is reinforced in locations where the mechanical load is the greatest [167][169]. Since then however, other mechanisms, in particular those that involve streaming potentials, were also suggested and a more complex interrelationship between mechanics, fluid dynamics, and surface charge was proposed [169].

Piezoelectricity is characterised as the electrical polarisation in certain materials due to the deformation of a material. The reverse is also true: the application of an electric

field onto these structures results in a physical deformation of the material. Collageneous structures' piezoelectricity is thought to be the result of the head to tail packing of the individual collagen molecules inside of the collagen fibrils. In fact, collagen molecules have positively and negatively charged N- and C- terminal ends respectively. Streaming potentials on the other hand, correspond to a convective flow of charged molecules due to the presence of pressure gradients. Structures such as bone have been shown to adapt to mechanical loads by creating strain-generated potentials which recruit osteoblasts and promote the production of collagen [170]. In dry bone, strain-generated surface potentials are the result of the piezoelectric nature of collagen whereas in hydrated bone, streaming potentials are the main contributors to the strain-generated potential differences [169].

The piezoelectric effect of collageneous structures has been extensively studied. Bending of a de-calcified bone matrix by insertion in a radial plastic tube resulted in polarisation with the strained surface of the matrix becoming more positively charged and the compressed surface more negatively charged [171]. Other studies tested both the piezoelectric effect and the converse piezoelectric effect of tendons by applying an AC current to a piece tendon or straining the piece of tendon, respectively [172]. These studies have allowed investigators to calculate the tissues' piezoelectric constants and showed that tendons charge under strain and conversely expand when an electric field is applied to it.

At the nanoscale, the converse piezoelectric effect was studied in fibrillar collagen from bone, teeth, and skin, using Piezoelectric Force Microscopy (PFM). It was found that predominantly, shear piezoelectricity occurs at the single fibril level and that the coefficients were up to an order of magnitude greater than those of macroscopic structures, which was attributed to the fact that not all fibrils in such macroscopic structures are oriented in the same way [172][173]. KFM studies of individual collagen fibrils have shown that in the native state, the overlap regions are more positively charged than the gap regions [128]. As mentioned earlier, collagen fibrils are considered a good scaffold material for tissue engineering. The fact that there is a link between mechanical strain and electrical surface charge is very important as fibrils are naturally subjected to continuously changing, mechanical loads, and cells "react" not only to the mechanical proper-

ties of their environment but also to the charge of the surfaces they attach to [160][161]. It is therefore important to study and understand how the surface potential of collagen can be altered through mechanical stresses. Studying charge alterations as a function of strain for single fibrils is a difficult task due to the size of the fibrils. Kelvin-Probe Force Microscopy has been applied to a wide range of biological materials to study DNA or lipid films for instance and map their surface charge distribution. It is therefore the ideal technique to study the surface potential of native and artificially crosslinked fibrils under strain.

Apart from piezoelectric measurements on single fibrils at the nanoscale, very little research so far has dealt with the electrostatic properties of collagen fibrils. In the present work, I investigated how strain alters the surface charge of single collagen fibrils. This can be interpreted as a direct piezoelectric effect but is not strictly identical to it, particularly as I focused on large strains greater than 10%. As such large strains are difficult to apply using force microscopy alone, I used the previously described method of depositing fibrils on a highly extensible PDMS film, which was then stretched, thereby straining fibrils along. Fibrils were strained to varying amounts and the D-banding lengths of each fibril measured from AFM height and phase images were used as a marker for the strain experienced by each individual fibril. This technique allowed me to cover a large range of strains in one single experiment. By performing KFM before and after straining I could determine how the electric surface potential of individual fibrils changes in response to mechanical strain. Most importantly, using this method, I was able to achieve strains on individual fibrils of up to 17% and, therefore, probe the electromechanical effects for a much greater strain range than previously reported. The method also allowed me to measure the surface potential after releasing fibrils from strain and, thereby, investigate whether the effects of strain of the electrostatic properties of the fibrils are reversible.

7.2 Methods

7.2.1 Sample preparation and stretching

Samples were prepared as described in section 3.2.1, except that 60 μL of silver paint was mixed in with the PDMS polymer and crosslinker before curing. This was done in an effort to render the thin PDMS film more conductive. Once cured, the PDMS substrate had visible flakes of silver paint embedded into it. The approximately 1.5 mm-thick block of PDMS was attached on a piece of highly doped, polished Si wafer (Figure 7.2). The conductive Si wafer was in electrical contact with the AFM sample support stage in all measurements, thereby ensuring that it was at ground potential of the KFM electronics at all times. Samples were then stretched as described in section 4.2.3. Both individual native and 2% GA-crosslinked collagen fibrils were imaged (see section 3.2.2) and their surface potentials were mapped prior to being strained (Figure 7.2 a), in the strained state (Figure 7.2 c) and once one end of the PDMS was un-taped from the Si substrate and the block returned to its native strain (Figure 7.2 e).

7.2.2 AFM and KFM imaging

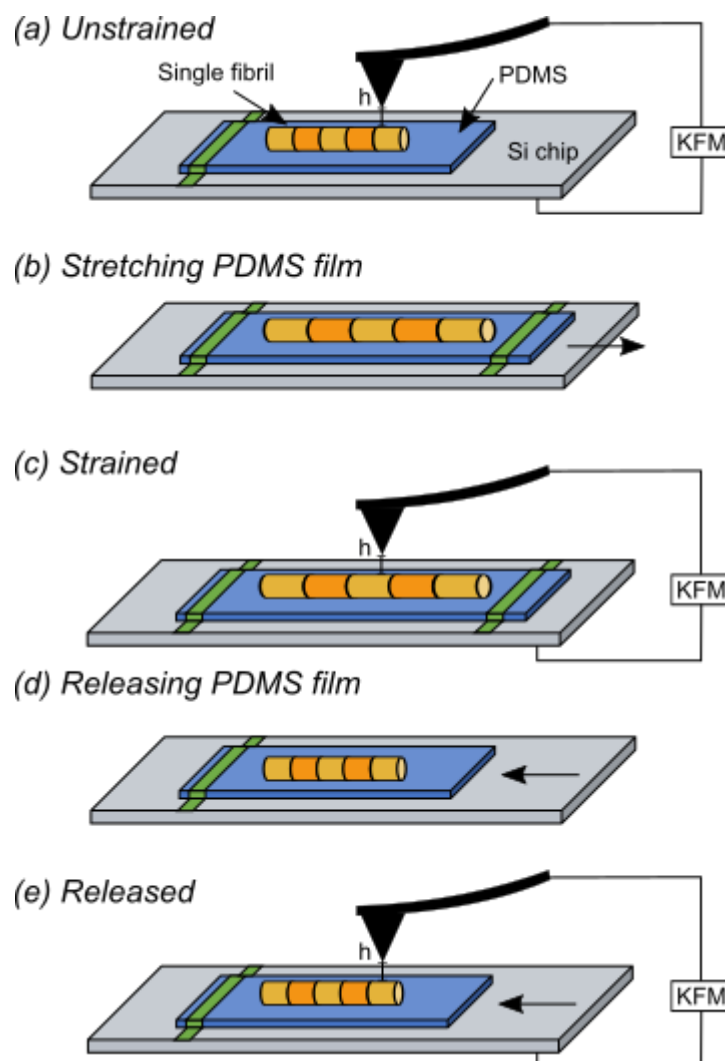


Figure 7.1: Stretching and measurement of the surface potential of individual collagen fibrils. (a) A piece of PDMS is taped at one end to a microscope slide. A piece of tendon is manually drawn over the block of PDMS leaving behind a trail of un-strained collagen fibrils. The two regions along the fibril represents the collagen fibril's D-banding. The surface potential of individual collagen fibrils is measured at a lift height, h , by KFM. (b) The free end of the PDMS block is pulled with a pair of tweezers and until the PDMS block reaches a strain of 50%. The loose end of the PDMS block is then also taped down to the microscope slide. The fibrils strongly adhere to the PDMS and stretch along with it. (c) KFM is performed on the stretched fibrils. (d) One end of the PDMS block is un-taped and the PDMS block is returned to its initial length. (e) Collagen fibrils released from strain are probed by KFM.

All of the measurements were done in ambient conditions (relative humidity $\approx 30\%$; temperature $\approx 23^\circ\text{C}$). The AFM used was a Dimension Icon AFM (Bruker Corporation, Billerica MA, USA). All AFM height and phase images were obtained in tapping-mode and Amplitude-Modulated KFM (AM-KFM) was performed at a lift height of 0 nm to increase the achievable, lateral resolution, and with a drive amplitude of 8000 mV. All of the topography, phase and surface potential images were obtained using BudgetSensors Tap300-G tips ($f = 350 \text{ kHz}$; $k = 40 \text{ N/m}$). Two different tips of the same type were used

to obtain the data presented in Figure 7.4 (a), one was used to obtain the data presented in Figure 7.4 (b) and another one was used to obtain the data in Figure 7.6. All of the images gathered were of $3\text{ }\mu\text{m} \times 3\text{ }\mu\text{m}$ and 512×512 lines.

During sample preparation, pieces of tendon needed to be hydrated with deionized water in order to be pulled apart with the tweezers and for individual fibrils to deposit on the PDMS film. Once the samples were prepared, they were dried in a stream of nitrogen and left overnight prior to imaging. This waiting time, and the largely constant ambient conditions in the lab (30% relative humidity, temperature = 23°C), ensured that drifting of the KFM signal, which can occur in the first few hours after removal of water [174], was negligible. No subsequent hydration of the fibrils was done prior to stretching of the PDMS film or releasing it from strain.

7.2.3 Data analysis

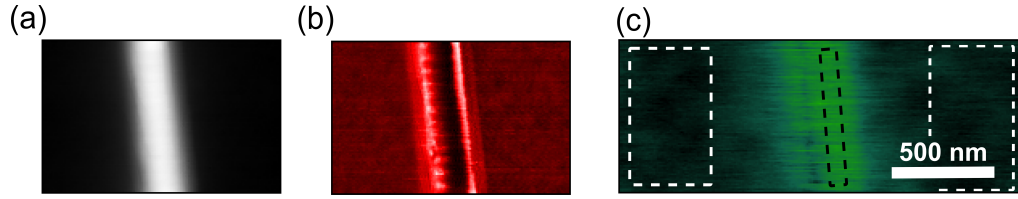


Figure 7.2: (a) Height image of a collagen fibril on an unstrained block of PDMS (color range = 150 nm). (b) Phase image of the same collagen fibril with the visible D-banding structure which was used as a marker for strain (color range = 30°). (c) Surface potential map of the same collagen fibril (color range = 100 mV). The black rectangle shows the area selected on the fibril during analysis with Gwyddion to obtain the average surface potential of the fibril. The edges of the fibril are omitted to minimize edge effects. The correct location of this area can be found from the corresponding AFM topography image. The white rectangles correspond to the regions selected to calculate the average surface potential of the PDMS. The relative fibril potential shown in (c) was calculated as the difference between the absolute fibril potential and absolute PDMS potential. Lighter pixels represent a more positive potential. The scale bar in (c) applies to all images.

All of the images were analysed using the free, data analysis software Gwyddion (gwyddion.net). Height images were analysed as described in section 4.2.4, and D-banding length was once again used as a marker for strain (Equation 7.1).

$$Strain = \frac{D_{S/RS} - D_0}{D_0} \times 100 \quad (7.1)$$

Where, D_0 is the D-banding length in the unstrained state, and $D_{S/RS}$ is the D-banding length in the strained or released state, respectively. The D-banding length of unstretched native or 2% GA-crosslinked collagen fibrils, D_0 , was calculated by taking the average D-banding length of eight native collagen fibrils ($D_0 = 67.1$ nm) and four crosslinked collagen fibrils ($D_0 = 67.1$ nm) lying on an unstretched block of PDMS. The calculated D_0 value is then used with the D-banding length of stretched fibrils, or fibrils released from strain in equation 7.1 to calculate the strain of individual fibrils. The error associated with the D-banding length measurement was calculated to be on average of 1.40 nm. To calculate this, the “read value under mouse cursor” function in Gwyddion was used to zoom in on a region of ten D-bands on an AFM height image. A pixel in the middle of the first overlap region was selected and the pixel in the middle of the 10th D-band was also selected. The uncertainty associated with the ability to select the middle of an overlap region was estimated to be of 1 pixel. The number of pixels in the images and the size of the images are known. Hence, 1 pixel can be converted into a length value. The uncertainty associated with the measurement of the length over ten D-bands, Δ_{10}

can therefore be obtained by combining the two length values associated with 1 pixel in quadrature. The uncertainty associated with the measurement of 1 D-band is hence $\frac{\Delta_{10}}{10}$. This value was calculated for a total of 6 individual fibrils and the average value obtained was of 1.40 nm.

Potential images were 1st-order line-levelled using the “align rows/median” function all the while excluding the fibril itself from the levelling by applying an exclusion mask function on the area occupied by the fibrils in the images. Individual fibrils’ surface potentials were extracted from the images by selecting a rectangle of roughly 8 pixels in width along the entire length of individual fibrils. The rectangle covers the middle of the fibrils as seen in figure 7.2 while avoiding the edges of the fibrils. The average potential was calculated using the statistical quantities function in Gwyddion. The surface potentials of the PDMS to the left and to the right of the fibrils were also calculated using the same process and then averaged to obtain the average surface potential of the PDMS. The average, absolute surface potential of the PDMS was then subtracted from the value of the absolute surface potential of the fibril to obtain the fibril potential relative to the one of PDMS. Care was taken to position the rectangle in the center of the fibril in order to avoid the edges of the fibril. At edges between two different materials, the KFM data signal is unreliable because of the limited resolution of KFM of approximately 20 nm – 30 nm. Also, the principle of constant lift height is not valid here because of the asymmetrical tip-sample geometry at the edge. This is similar to the edge effect in nanoindentation, where the assumption of a flat surface is not valid [55]. The rms-variation along the length of a fibril is taken as the error margin uncertainty of the relative surface potential of a fibril.

7.3 Results and Discussion

Figure 7.3 shows the effect of longitudinal strain on surface potential of a single fibril. Row (a) shows the unstrained fibril (corresponding to Figure 7.1 a), row (b) shows the strained fibril (corresponding to Figure 7.1 c), and row (c) shows the released fibril (corresponding to Figure 7.1 e). The images in column (i) show the height determined by tapping-mode AFM and the images in column (ii) show the corresponding tapping-mode phase images. Phase images are very sensitive to the overall morphology of fibrils and were therefore used as a control measurement to check for any damage to fibrils upon straining, and to measure the D-banding length of individual fibrils. In the present case, there is no indication of any such damage. Column (iii) shows the local surface potential mapped by KFM in air in each state. One can see that the surface potential of this individual fibril becomes more positive relative to the PDMS film upon stretching and then more negative again after releasing the film. In fact, the potential after release is even more negative than the potential before straining. This will be investigated more systematically below (Figure 7.6). The characteristic 67 nm-periodic D-banding pattern is visible in the phase images but is not resolved in the potential images. This is very likely due to the limited resolution of this setup. In fact, fibrils lie on a relatively thick (1 mm-2 mm), dielectric PDMS film. This is in contrast with other works, where fibrils were deposited directly on a conductive surface and showed the D-banding in the potential maps [163]. The PDMS film acts as an insulator and forms a large dielectric barrier between the fibril and the Si support chip. The Si chip acts as the counter electrode in the measurement. In terms of electrostatic field distribution between the tip and the counter electrode, this setup has the same effect as a large KFM lift height (tip-sample distance) which as described in chapter 2, decreases the achievable, lateral resolution. This is why the tip lift height was set to 0 nm in all of my measurements. Low resolution is also responsible for the large edge effects (spatial broadening of the potential) that can be observed in Figure 7.3 (iii).

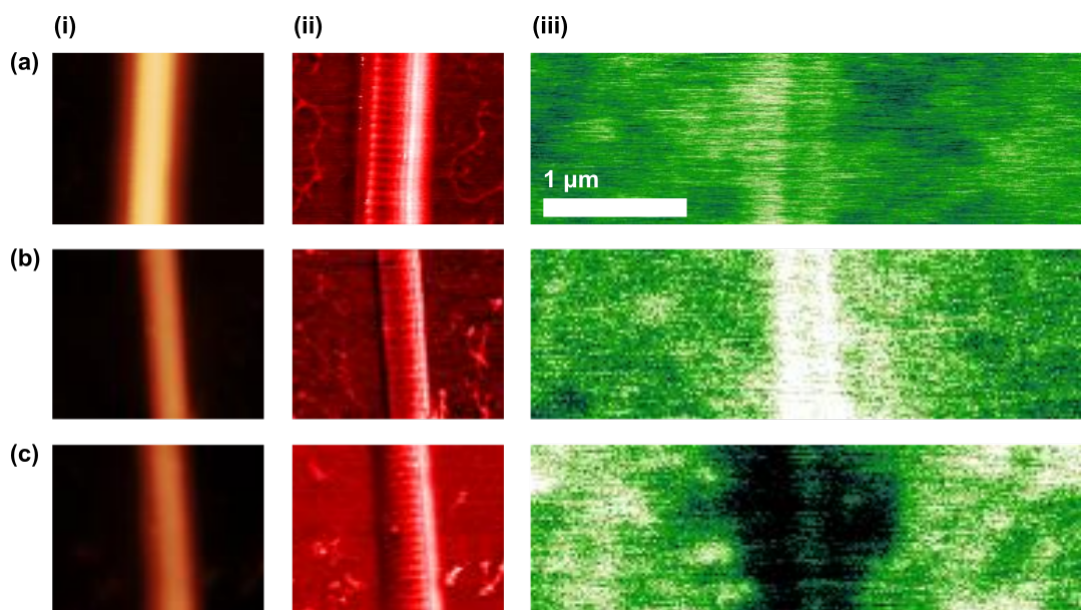


Figure 7.3: Stretching and releasing a native collagen fibril on a PDMS film. Rows: (a) = before film-stretching (un-strained), (b) = after film-stretching (strained), (c) = after film-releasing. Columns: (i) = tapping-mode height images (color range = 200 nm), (ii) tapping-mode phase images (color range = 20°), (iii) surface potential maps on same location as (i) and (ii) (color range = 100 mV). No buffer or deionized water was added to hydrate the fibrils prior to stretching the PDMS film or prior to releasing it from strain. The horizontal scale bar in (a, iii) applies to all images. The relative fibril potential shown in column (iii) was calculated as the difference between the absolute fibril potential and absolute PDMS potential. Lighter pixels represent a more positive potential.

It was described in chapter 6 that collagen fibrils undergo some structural changes when strained beyond approximately 15%, and that the behavior is different between native and GA-crosslinked fibrils [145]. I therefore used the same approach to determine how the surface potential changes with increasing strain. Fibrils were first deposited on a PDMS film and then stretched manually to a macroscopic strain of 50%. Measurement of individual fibrils' D-banding lengths showed that a range of individual fibril strains from 0% to approximately 15-20% was achieved [145]. The same type of AFM tip, i.e. tips with the same radius and of the same material, and the same imaging parameters (e.g. lift height) were used for all measurements to minimize artifacts and the uncertainty of the potential measurement.

Native fibrils and fibrils artificially crosslinked in a 2% GA solution were deposited on two separate PDMS films. Figure 7.4 shows the dependence of the relative surface potential of single fibrils with respect to PDMS film's surface potential on strain. Each data point represents a single fibril. The measurement was performed for native fibrils (Figure 7.4 a) and for GA-treated fibrils (Figure 7.4 b). In both cases, there is significant variation of the potential values, which reflects the variability of the overall physical

properties of individual fibrils. A similar variability was found for the stiffness of collagen fibrils in chapter 6 [145]. For native fibrils, the surface potential first increases to more positive values with strain of up to approximately 10%, but then decreases again with further strains of up to approximately 17%, which is the maximum strain that was found in this experiment. This electromechanical effect has never been reported in the past, and it is completely absent in GA-treated fibrils (Figure 7.4 b). A statistical analysis of the data-set for native fibrils (Figure 7.4 a) shows that the increase and subsequent decrease are significant (p-values of L1 and L2 < 0.0001). An analysis of the dataset for GA-treated fibrils (Figure 7.4 b) does not show a significant strain dependence (p-value > 0.05). In principle, the change of the relative surface potential of fibrils with respect to the surrounding PDMS (Figure 7.4 a) could be caused by a change of the absolute fibril potential, the absolute PDMS potential, or a combination of both. However, meaningful absolute potentials are difficult to determine by KFM, especially with dielectric samples. In fact, dielectrics do not have a defined potential with respect to a reference potential such as mass or earth potential in the KFM circuit. Figure 7.4 (c) and (d) show the absolute potentials of the fibrils (filled data symbols) and of the PDMS (open data symbols) that were used to calculate the data in Figure 7.4 (a) and (b), respectively. There is a large, random fluctuation of the absolute potential values over several hundred mV which seem to be partly correlated with the specific day that the measurements took place. Only by subtracting the absolute PDMS potential from the absolute fibril potential can the subtle change of the native fibrils' potential due to strain be detected (Figure 7.4 a). Also, no noticeable trend was visible in the GA-treated dataset (Figure 7.4 b). This absence makes it improbable that the result of Figure 7.4 (a) was caused by an alteration of the absolute PDMS potential when it was stretched. This would suggest that the PDMS potential far away from fibrils would behave differently depending on, or be affected by the type of fibrils that would be attached to it. It is also worth noting that the strain at which the maximum surface potential occurs in native fibrils seems to broadly correlate with the strain at which the maximum stiffness occurs (Figure 7.5) [145].

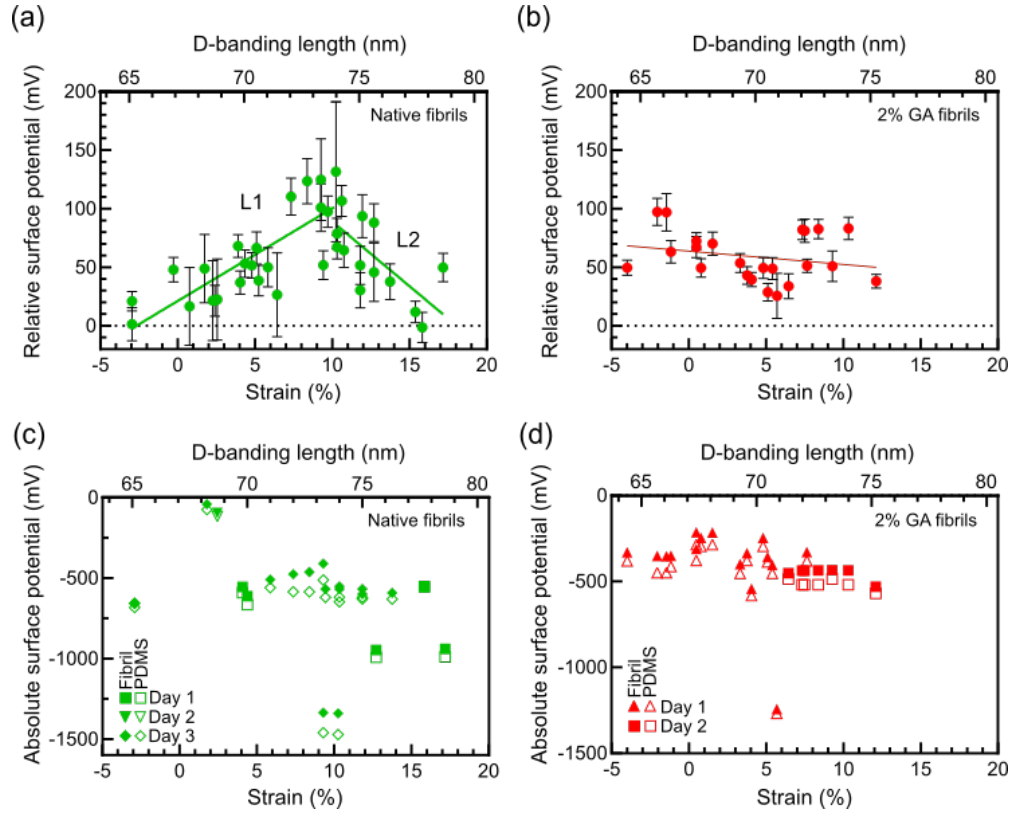


Figure 7.4: The average relative surface potential of native (a) and 2% GA artificially cross-linked fibrils (b) are compared. All measurements were performed in air under ambient conditions using the same type of AFM tip. Each data point represents an individual fibril. The uncertainty of the D-banding length measurement is ± 1.4 nm (not shown here). (a) L1 = linear regression for strains up to 10% (slope = 7.91 ± 1.34 mV; $R^2 = 0.6254$; p-value < 0.0001), L2 = linear regression for strains between 10% and 17% (slope = -11.10 ± 3.47 mV; $R^2 = 0.4577$; p-value < 0.0001). (b) Linear regression on whole dataset (slope = -1.14 ± 1.10 mV; $R^2 = 0.0530$; p-value = 0.29). Error bars represent the rms-error of the potential of single fibrils. The average absolute surface potential of a native fibril sample (c) and a sample with GA treated fibrils (d). The vertical error bars are too small to be shown.

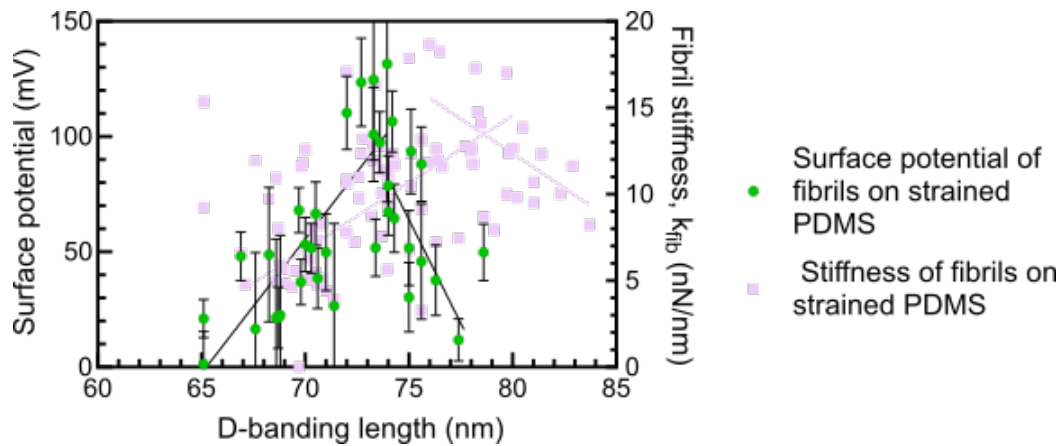


Figure 7.5: Comparison of the surface potential and stiffness of collagen fibrils as a function of their individual D-banding lengths. The surface potential data and the stiffness data were obtained from fibrils from the same rat tail tendon but each property was investigated on different samples.

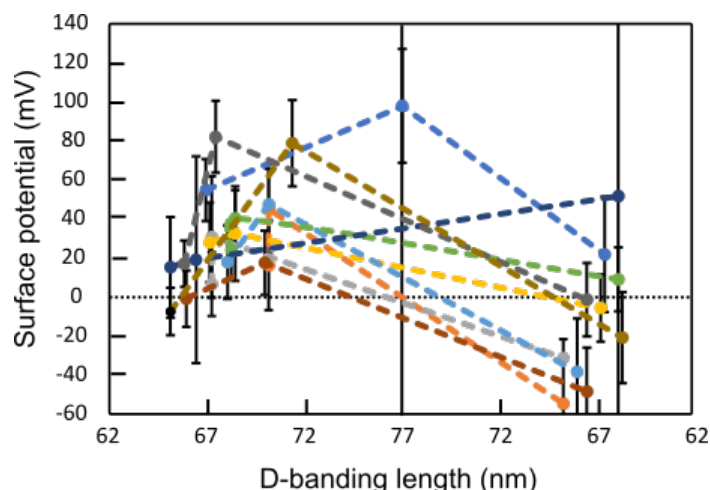


Figure 7.6: Tracking the surface potential of native, individual fibrils. Each color represents a specific fibril. The lines should be read from left to right and represent the straining paths that fibrils were subjected to. The lines connect three data points for each fibril, before straining (left data point), strained (middle data point) and after having been released (right data point). Note: The horizontal axis is mirrored at 77 nm, the highest D-banding length that was observed, in order to make the graph clearer. Error bars represent the rms-error of the potential of single fibrils

In order to investigate the reversibility of the effect, native fibrils were tracked individually on a film that was stretched and then released again (Figure 7.6). For each fibril, the actual strain was determined together with its surface potential before straining, in the strained state, and after releasing the PDMS film from strain. All fibrils showed an increase in surface potential when strained. When the fibrils were released from strain, the surface potential decreased again for all but one fibril. Furthermore, for every fibril except one, the potential after release is lower than before straining.

In the context of inorganic, solid materials (e.g. metals), the surface potential measured in KFM is generally interpreted as a contact potential difference between two different materials [175]. Here, however, such a concept is not applicable. The local surface potential of a protein structure corresponds to a representation of the local surface charge density [131]. In proteins, the local surface potential is caused by ionizable, chemical groups. In particular, the local surface potential depends on the presence of amine and carboxy groups from amino-acids exposed to water accessible inside the structure. At pH7, amine groups are predominantly positively charged whereas carboxy groups are predominantly negatively charged, both, due to their respective propensity to accept or donate a proton. This proton exchange occurs between the ionizable groups and water molecules in the surrounding environment. Hence, the local ratio of accessible amine versus carboxy

groups on a surface determines the overall net charge of that locality [131][176][177].

As described in chapter 1, mammalian type I collagen contains a Glycine amino acid for every three amino acids and a large amount of Proline and Hydroxyproline. These three amino acids are uncharged and, thus, do not contribute to the surface potential of the structure. The only amino acids that can be positively charged are Arginine and Lysine, whereas the only amino acids that can be negatively charged are Aspartic acid and Glutamic acid. These amino acids only occur in small numbers in the collagen sequence, but correspond to the ionizable amino acids which determine the overall, net surface potential [163] of the collageneous structure. It should be noted that KFM measurements cannot readily be performed in water [178]. However, even KFM measurements in air are indicative of the surface charge that biological or organic structures have in water. It has been shown in an earlier work that the surface potential values obtained in air of surfaces exposing either amine or carboxy groups correlate with the net charge that was expected to be obtained for these same surfaces in water [131]. This is due to the fact that, in air and under ambient conditions, there is always a significant water layer present, which ensures the supply/removal of protons. In collagen fibrils, these tightly-bound water layers are not only present on the surface but also probably inside the fibrils as it is known that they can take up a significant amount of water when immersed (see section 1.2.3) [55].

The increase and subsequent decrease of the surface potential of native collagen fibrils (Figure 7.4 a) is not a piezoelectric effect as the potential curve is not linear, and the strains and potentials involved are far too large. The behavior observed can only be explained by a structural rearrangement of the collagen molecules inside the fibrils when strained, e.g., molecular unfolding or sliding of different molecules against each other. This rearrangement seems to affect the number of accessible, ionizable groups on or near the surface. However, it is unclear why fibrils first become more positive and then more negative again with increasing strain (Figure 7.4 a). In fact, a change to a more positive potential can mean, both, an increased exposure of positively ionizable residues and/or a decreased exposure of negatively ionizable residues. On the other hand, a change to a more negative potential can mean the reversed processes. In collagen, there is no signifi-

cant imbalance of the number or location of charged residues of either polarity anywhere in the amino acid sequences of the constituent molecules [163]. That is, if molecules exposing positive and negative residues with equal probability were to rearrange themselves randomly the surface potential should not change significantly.

The fact that GA-treated fibrils do not show any change (Figure 7.4 b), could explain the direction of the surface potential change towards positive values in native fibrils (Figure 7.4 a). GA is a strong, non-enzymatic protein crosslinker which produces Advanced Glycation End products (AGEs). As detailed in chapter 1, AGEs are the result of a non-enzymatic, uncontrolled reaction between proteins and reducing sugars or, more generally, carbonyl-exposing compounds such as GA. As described in section 1.2.2 the attack point for crosslinking on the protein is usually a positive residue, and more particularly an exposed Lysine group. When crosslinked, the overall concentration of Lysine is reduced, thereby reducing the positive contribution to the overall charge [128][163]. It is conceivable that, upon straining some of the naturally present enzymatic crosslinks in native fibrils break and thereby shift the overall charge towards a more positive value again. This does, however, not explain why fibrils become negative again with strains greater than 10% (Figure 7.6 a). Other factors such as the presence of streaming potentials inside the fibrils could also play a role. Future investigations to elucidate the exact, molecular mechanism that leads to the behavior in figure 7.4 a will need to focus on crosslinks and AGEs in collagen fibrils undergoing strain. Mass spectrometry and/or fluorescence measurements could quantify the crosslink content on unstrained vs. strained fibrils [179][180][181][182]. This could be coupled with titration experiments using different pH as well as electrolyte ions of different valency and size, which is often helpful in assessing the nature of ionizable residues.

While the overall molecular mechanism that could explain Figure 7.4 a cannot be ascertained from KFM measurements alone, the interpretation of a structural rearrangement is corroborated by the works on collagen stiffness presented in chapter 6. Also, as shown in Chapter 4, the D-banding height decreases when fibrils are stretched. Furthermore, there is evidence that the gap region is more disordered than the overlap region [149],

suggesting an unfolding of molecules preferentially in the gap region. As a more positive potential in the gap region of unstrained fibrils was also observed [131], it can be hypothesized that the structural rearrangement happens predominantly in the gap region and reveals more, initially buried, residues from it, leading to a greater contribution from this positively charged region to the overall fibril potential. However, again, this does not explain why the potential becomes more negative again when stretching further (Figure 7.4 a).

For small strains, the effect of strain on the surface potential seems to be reversible (Figure 7.6). When strained, the potential increases, but after being released from strain, the surface potential decreases again. In fact, it decreases to values more negative than the values obtained before straining the fibrils. It is unclear why this happens but it is conceivable that repeated loading/unloading cycles cause repeated unfolding/refolding of disordered molecule domains, and that, after a few cycles, these domains adopt a more steady molecular arrangement. The observation that this steady-state is more negative after one cycle could simply be a coincidence of our particular sample and would need to be investigated more systematically in future works, e.g., by using samples from different sources and performing a greater number of cycles to “condition” the samples into steady-state.

Native fibrils show a pronounced electrical response, going through a maximum of the surface potential at about 10% strain (Figure 7.4 a). The change of approximately 100 mV from 0% to 10% strain (L1) is very high for a biological material, especially as it is caused exclusively by mechanical deformation, that is, a purely physical process. For comparison, the change of the surface potential of collagen fibrils upon glycation, that is, a chemical reaction, is only about 10-15 mV [128][163]. Such a large alteration of the surface charge of a biomolecular structure would certainly have significant consequences on the interaction with other structures, molecules and on the distribution of ions in the surrounding liquid or in the ECM. As described in chapter 1, there is experimental evidence to show that protein adhesion and affinity to surfaces can be altered by the charge of the surfaces [183]. This could ultimately affect cell adhesion with significant conse-

quences on processes such as development and wound healing [184]. Our observation that GA-treated fibrils did not exhibit any change in surface charge, despite being able to undergo strains of up to 10% (Figure 7.4 b), is critical for tissue-engineering applications. Artificial and targeted crosslinking, whether by GA exposure or by other methods, is a common and widely accepted method to adjust the mechanical properties of the tissue scaffolds to the desired level. As the charge vs. strain relationship of crosslinked fibrils is different from that of unmodified ones, this will have to be taken into account in scaffold design and, possibly, require further, chemical treatment of the scaffold in order to reconstitute the electromechanical properties of native fibrils.

7.4 Conclusions

Native and glycated type I collagen fibrils were quasistatically strained by up to 17%. Native fibrils showed a pronounced alteration of the surface potential and, hence, surface charge. Glycated fibrils however, did not show such an alteration. Such a change in charge is likely to have an effect on the interaction with adhesion proteins and, thus, ultimately on cell adhesion. The exact, molecular mechanisms that lead to charge alteration are not yet known in detail, but the fact that glycated fibrils do not show charge alteration points towards the involvement of Lysine residues and the possible breaking/reformation of crosslinks. While future works would need to focus on those crosslinks and how they are affected by strain, applications such as tissue-engineering would need to take into account surface charge more than before in the design of collagen-based scaffolds and, most importantly, investigate if and how cell adhesion is affected. As mentioned earlier, it would be impossible to measure the surface potential of fibrils used in collagen-based scaffolds under fully hydrated conditions. However, studies have shown that qualitatively, changes in surface potential due to strain or prior exposure to different buffers would be representative of the trends that one would expect to obtain in liquid. However, as of today, KFM measurements cannot provide quantitative surface potential values for immersed surfaces.

Chapter 8

Conclusions

In this thesis I have developed a straining technique capable of stretching (positive strain) or compressing (negative strain) many collagen fibrils in a single experiment and to different strains, thereby expanding the strain range previously achieved for collagen fibrils [105] [134]. Using this technique I examined different ways to measure the mechanical and electrostatic properties of single collagen fibrils, at the nanometer scale using atomic force microscopy and Kelvin-probe force microscopy. By doing this, the main aim was to study the stiffness and the surface potential of individual dehydrated and hydrated collagen fibrils as a function of strain. This work was described in Chapters 3-7. The main conclusions and findings of each chapter are:

- Individual collagen fibrils deposited on soft and flexible polymer such as PDMS can be stretched up to 26% strain or compressed. Fibrils naturally adhere very strongly to the PDMS, and hence stretching the PDMS foil they are on, stretched the fibrils along with it. Strains up to 26% were achieved, which to my knowledge, significantly expended the strain range previously achieved with other tensile tests. As for compression, if fibrils are deposited on a pre-strained piece of PDMS and the PDMS is then released from strain, the fibrils compress along with the PDMS. This stretching technique allows us to study multiple fibrils in parallel on the same substrate, compared to MEMs experiments where only individual fibrils can be studied. Straining or compressing fibrils on PDMS also has the advantage of leaving

the fibrils accessible to AFM imaging and axial loading, KFM probing, but also to cells to study the influence of the fibrils' stiffness or surface potential on cellular behaviour.

- The buckling patterns that result from the compression of individual collagen fibrils can be used to accurately calculate the average Young's modulus of individual native or artificially crosslinked collagen fibrils. This way of obtaining the Young's modulus is much easier than any mechanical loading-based tests such as nanoindentation or tensile tests, as it only requires atomic force microscopy imaging and therefore omits the need for contact model assumptions associated with typical nanoindentation experiments. This technique yields an average Young's modulus and hence, the difference of stiffness between the gap and overlap regions is lost.
- PDMS is roughly 1000 times softer than dehydrated collagen fibrils. When loading single fibrils with loads typically used in nanoindentation experiments (50 nN - 150 nN), the fibrils predominantly bend inside the PDMS substrate and are indented by a much lower extent. A simple Winkler foundation model relying on only three variables (fibrils radius, maximum load applied and the bending depth) can be used to calculate the average Young's modulus of a single collagen fibril. This technique is once again, much easier to use than typical nanoindentation models (Oliver and Phaar model; Hertzian model) because it does not rely on assumptions about the tip-sample contact area which has to be estimated when using typical nanoindentation models to calculate the Young's modulus of individual collagen fibrils. This technique also rids the user of issues linked with the Point and Shoot mode used during nanoindentation discussed in Chapter 5. However, this technique relies on the fact that the fibrils are much stiffer than the underlying substrate which limited my investigations in this chapter to dehydrated fibrils.
- Axial loading of individual collagen fibrils was done to study the stiffness of collagen fibrils as a function strain. It was shown that fibrils first strain stiffen for strains up to roughly 17% and then strain soften for greater strains. It was also shown

that the difference in stiffness between the gap and overlap regions decreases significantly and becomes null in the first 5% of strain. It was hypothesized that a possible molecular mechanism for this stiffening and softening behaviour is:

- 0-5% strain: the disorganised domains of the gap regions are being stretched out rendering the gap region more like the overlap regions from a mechanical stand point.
 - 5-17% strain: Strain-stiffening is due to straining of the inter-molecular crosslinks inside the collagen fibrils.
 - 17% and above: Inter-molecular crosslinks break resulting in an overall softening of the fibrils.
- Kelvin-probe force microscopy was used to track the surface potential of fibrils as a function of strain. It was found that fibrils become more positively charged from 0% to roughly 12% strain and then return to their native surface potential for greater strains. Artificially crosslinked collagen fibrils' surface potential did not change as a function of strain. When releasing strained fibrils from their strain, the surface potential did not return to the native surface potential but instead, fibrils became on average a lot less negatively charged. It was hypothesized that this is due to remodelling of the internal crosslink network. When going from 0% strain to roughly 12% strain and then releasing the fibrils from strain the change in surface potential can be as great as 100 mV. Such a large alteration of the surface charge of a bio-molecular protein would certainly have a significant impact on the interaction with other fibrillar proteins, molecule, cells and on the distribution of ions in the surrounding liquid. Whether such high strains are achieved in natural environments remains to be investigated. However, it is undeniable from my results that the strain-surface potential relationship of native fibrils is much different than the one for crosslinked fibrils. Hence, these findings would have to be taken into account during scaffold design for tissue engineering.

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