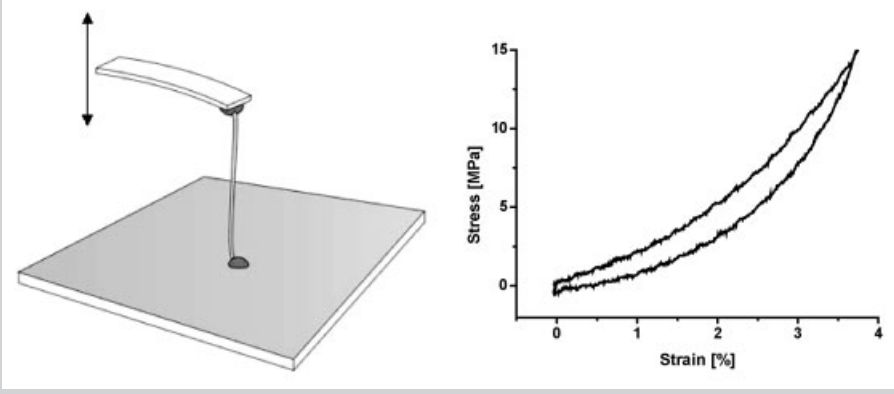
Summary: A novel method based on AFM was used to attach individual collagen fibrils between a glass surface and the AFM tip, to allow force spectroscopy studies of these. The fibrils were deposited on glass substrates that are partly coated with Teflon AF®. A modified AFM tip was used to accurately deposit epoxy glue droplets on either end of the collagen fibril that cross the glass-Teflon AF® interface, as to such attach it with one end to the glass and the other end to the AFM tip. Single collagen fibrils have been mechanically tested in ambient conditions and were found to behave reversibly up to stresses of 90 MPa. Within this regime a Young's modulus of 2–7 GPa was obtained. In aqueous media, the collagen fibrils could be tested reversibly up to about 15 MPa, revealing Young's moduli ranging from 0.2 to at most 0.8 GPa.



Micromechanical Testing of Individual Collagen Fibrils[a]

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Received: March 16, 2006; Revised: June 2, 2006; Accepted: June 8, 2006; DOI: 10.1002/mabi.200600063

Keywords: atomic force microscopy (AFM); biofibers; collagen fibrils; force spectroscopy; mechanical properties

Introduction

Collagen fibrils are the major constituent of several vertebrate tissues, such as vasculature, skin, lungs, cartilage, bone and connective tissue.[1] Collagen is largely responsible for the mechanical and elastic properties of these tissues. Knowledge of the mechanical and elastic properties of the collagen fibril is the key to understand the structural and functional mechanisms of these biocomposites on the microscopic level. Furthermore, the mechanical properties and, in a next step, the ability to change them using cross linking strategies is extremely important in the development of new materials based on biological polymers for the application in for example heart valves.

At the lowest hierarchical level the structure of these fibrils consists of collagen molecules. Each collagen molecule is made of three peptide chains that form a triple helical structure. Five triple helices organize into a microfibril. These microfibrils in turn aggregate both in lateral and longitudinal directions, to form fibrils. The collagen fibril

[a] Supporting information for this article is available at the bottom of the article's abstract page, which can be accessed from the journal's homepage at http://www.mbs-journal.de, or from the author.

has a diameter of 100–500 nm and a length up to the millimeter range.[2] In the next step of the hierarchy multiple fibrils make up the collagen fiber.[3] Although the general picture of the structure of the collagen fibril is clear, there are still parts which are not completely understood. Despite extensive research on its mechanical properties on the macro-scale over more than four decades, it is still not possible to explain these from the underlying structure.

A number of force spectroscopy studies on different collagen structures are reported ranging from single collagen monomers to larger substructures of the collageneous tissue.[4–6] Only recently, mechanical measurements on human type I collagen fibrils are reported.[7] In this study individual fibrils were attached non-covalently to the glass surface and the AFM tip. During the stretching numerous discontinuities and a plateau were observed indicating major reorganization at forces in the 1.5 to 4.5 nN range. From the relaxation part of the cycle a Young's modulus of 32 MPa was obtained.

Here, we describe experiments in which we used an atomic force microscope on top of an inverted optical microscope to attach individual collagen fibrils between the glass surface and the AFM tip using epoxy glue droplets. Furthermore, we do show that this method can be used to obtain reproducibly information on the mechanical properties of the fibrils as they reside in an aqueous buffer.

Experimental Part

Materials

Concentrated sulfuric acid (ca. 96 wt.-%), hydrogen peroxide (ca. 30 wt.-%) and concentrated hydrochloric acid (ca. 37 wt.-%) were obtained from Merck, Darmstadt, Germany. Acetone and toluene (AR stabilized) were obtained from Biosolve, Valkenswaard, the Netherlands. Phosphate buffered saline solution (PBS, B. Braun, Melsungen, Germany) at pH 7.4 containing 140×10^{-3} M NaCl, 13×10^{-3} M Na_2HPO_4 and 2.5×10^{-3} M NaH_2PO_4 was used as received. Teflon AF® 1601S (6 wt.-% solution in Fluorinert® FC-75) was obtained from Dupont, Wilmington, DE, USA. AFM images were analyzed using the program SPIP 1.9212, details at www.image-met.com. The Mitutoyo ID-C112B micrometer was obtained from Mitutoyo, Veenendaal, the Netherlands.

Preparation of Teflon AF®-Coated Glass Surface

Glass discs (diameter: 15 mm, thickness: 0.3 mm, Knittel Gläser, Braunschweig, Germany) were immersed in a mixture of 70 vol.-% of sulfuric acid and 30 vol.-% of hydrogen peroxide. After this, the discs were washed five times in demineralized water (10 min each), three times in acetone (5 min each) and three times in toluene (3 min each). After drying at 130 °C for 14 h, the glass discs were partly coated by dipping them into a Teflon AF® 1601S solution.

Deposition of Collagen Fibrils Onto the Glass Discs

Bovine Achilles tendon collagen type I (3.1 g, Sigma-Aldrich, Steinheim, Germany) was swollen in hydrochloric acid (333 ml, 0.01 M) for 14 h at 0 °C. The resulting slurry was shred for 10 min at 0 °C at 11 000 rpm using a Braun MR 500 HC blender (Braun, Kronberg, Germany). The resulting collagen dispersion was filtered through a 74 μm filter (Belco 200 mesh, Vineland, NJ, USA). The filtrate, a dispersion of mainly collagen fibrils was diluted 150 times using phosphate buffered saline solution (PBS). The partly Teflon AF®-coated glass discs were incubated for 10 min in the diluted collagen dispersion. The surfaces were washed in PBS (10 min) and three times in demineralized water (10 min each) and subsequently dried for 14 h at ambient conditions.

Attachment of the Collagen Fibril Between the Tip and the Surface

The two components of Araldite glue (Araldite Precision, Bostik Findley Ltd., Staffordshire, UK) were intensively mixed for at least 15 min using a Teflon® spoon before it was deposited and spread out onto a standard microscope glass. The AFM head with a triangular shaped cantilever (coated sharp cantilevers MSCT AUHW, multilever type F, spring constant $k = 0.5\ \mathrm{N \cdot m^{-1}}$, Veeco, Cambridge, UK) was positioned on top of this microscope glass and the tip was lowered once in order to dip into the glue layer (Figure 1A).

Collagen fibrils with one end on the glass surface and the other end on the Teflon AF®-coated part were selected using the inverted microscope. Optical microscopy and AFM imaging was used to characterize the collagen fibril over its entire length, to ensure its structure is uniform. Having the selected fibril in the field of view, the AFM head was positioned on top of the collagen fibril (Figure 1B). In this configuration the optical microscope allows accurate positioning of the AFM tip just above the fibril. First a glue droplet (30–50 μm) was deposited on the end of the fibril that was on the glass surface (Figure 1C). Second, the AFM tip was moved towards the other end of the fibril (above the Teflon AF®) where a second glue droplet was deposited (10–20 μm in size, Figure 1D). If needed, an additional dip into the glue layer was performed in between these two steps.

In a next step a different cantilever with a higher spring constant (NCH-W Nanosensors, Darmstadt, Germany, $k = 32–62\ \mathrm{N \cdot m^{-1}}$) from which the tip had been removed using a focused ion beam, was positioned into the AFM, replacing the one used for transferring the glue (Figure 1E). After repositioning the AFM cantilever to the collagen fibril to be studied, the end of the cantilever was moved towards the position of the second deposited glue droplet. The cantilever was lowered until a force of about 10 μN was measured and left in this situation for at least 12 h (Figure 1F). Figure 1G is an electron micrograph taken after connecting the end of the fibril with the second glue droplet. The collagen fibril on the surface can be clearly seen, connected to the larger glue droplet on the right and the smaller droplet on the left, that connects it to the AFM cantilever. After this the cantilever was lifted with the end of the collagen fibril attached to it. This was continued until the fibril was completely free from the surface (Figure 1H).

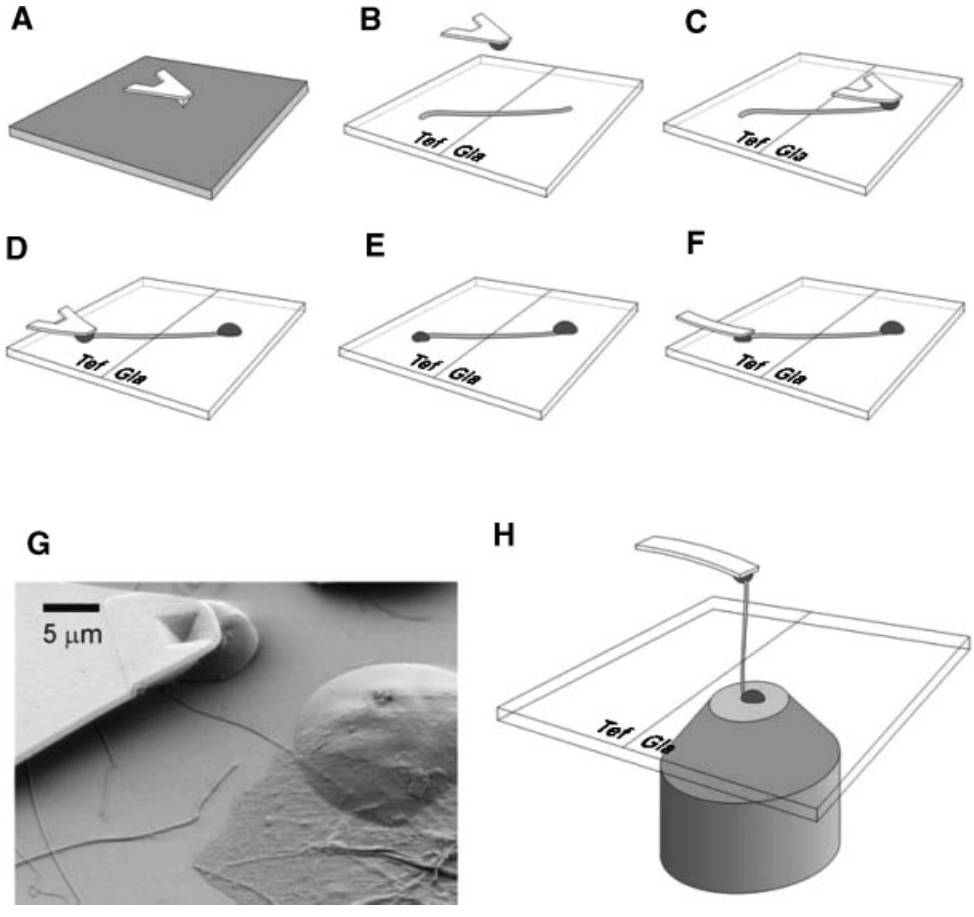

Figure 1. Schematic representation of the procedure followed to fix an individual fibril between the glass surface and the AFM cantilever. (A) A triangularly shaped AFM cantilever was dipped once into a layer of epoxy glue that was spread out onto a microscope glass surface. (B) Using the inverted microscope a fibril that is crossing the boundary between the glass (Gla) and the Teflon AF® (Tef) was selected. (C) The AFM tip with the glue attached was moved down to the end of the fibril on the glass, leaving a droplet on the surface. (D) After this it was lifted up and a droplet of glue was deposited on the fibril end on Teflon AF®. (E) Next the AFM head was removed and the cantilever exchanged for a rectangular shaped one, from which the tip had been removed. (F) The end of the cantilever was now brought into contact with the glue droplet at the fibril end on Teflon AF® and left for at least 12 h. (G) Electron micrograph of the collagen fibril attached with one glue droplet to the surface (down right) and a second droplet to the AFM cantilever (top left). (H) Slowly the cantilever is moved up which releases the fibril from the surface. The set-up is ready for micromechanical testing experiments.

Calculation of the Young's Modulus From the Force Data

The stretching of the collagen fibril with the AFM provides force-distance data. In order to get correct force data, the spring constant of the AFM cantilevers needs to be calibrated. Normally this is deduced from the power spectrum of the movement of the cantilever that can be directly measured. In this case however, the relatively high value of the spring constant made it difficult to accurately obtain the amplitude and therefore the spring constant. The spring constant was determined from the resonance frequency. A more detailed description of this can be found in the Supporting Information. The additional glue droplet that is used for attaching the collagen fibril has no significant effect on the spring constant of the cantilever.

In order to determine Young's moduli a conversion of the force-distance data into stress-strain data is needed. Before the stretch experiment is started, the AFM tip is moved up until an evident increase in force was detected upon stretching the collagen fibril. The extension at this force (ca. 4 nN) was taken as the contour length of the collagen fibril from which the strain can be calculated. Converting forces into stresses requires a cross-section of the collagen fibril to be determined. Both electron microscopy and AFM imaging were used to determine the diameter of the collagen fibrils being micromechanically tested. In order to assess the effect of flattening due to surface adhesion, diameters were also determined in situations where the collagen fibrils are freely suspended.

Results and Discussion

Using dispersions of collagen type I with a concentration of 20 $\mu g \cdot ml^{-1}$ provided samples in which most collagen fibrils were isolated. Having the inverted microscope allowed visual inspection of the collagen fibrils. They were found to be sufficiently long (100–200 μm) and uniformly shaped (Figure 2A). Collagen fibrils that crossed the Teflon AF®-glass boundary completely, with at least 20 μm of their length on the Teflon AF® layer, and at least 50 μm in total length were selected for force spectroscopy experiments.

Before actually stretching the individual collagen fibril, the atomic force microscope was used to image the selected fibrils as they were deposited onto the surface. Figure 2A presents an optical microscopy image in which several fibrils can be distinguished, of which several cross the border between the glass and the Teflon AF® (vertically in the center). Figure 2B is an AFM image revealing again a collagen fibril crossing the boundary. From this and many other images the Teflon AF® layer was found to be 500 ± 200 nm thick and the transition 6 ± 2 μm wide. The entire collagen fibril was imaged in more detail along its length in order to verify its structural homogeneity and the presence of the characteristic banding pattern of 67 nm, which is the typical D-period for collagen fibrils (Figure 2C).

After this fixation procedure the AFM head and therewith the fibril end was carefully lifted from the surface up to a height of about 100 μm (length of the fibril) above it. This was realized by tilting the entire AFM head, with the manual fine-adjustment spindle in the set-up.[8] A digital micrometer was added to obtain the height and thus the distance between the tip and the surface. The tip was moved vertically until a force of 4 nN was recorded and this was defined as the initial length of the collagen fibril.

For the pulling procedure, the AFM piezo tube was used to move up the cantilever over a distance of 2.3 μm, while simultaneously measuring its deflection. Using the calibration of the piezo tube and correcting for its non-linear response and hysteresis, the force versus extension response of collagen fibril was determined. Using the initial length of the fibril and its cross section, a stress-strain curve was calculated (Figure 3). The clearly apparent noise pattern superimposed on the curve is resulting from an interference effect caused by the AFM laser, which could not be eliminated in this set-up.

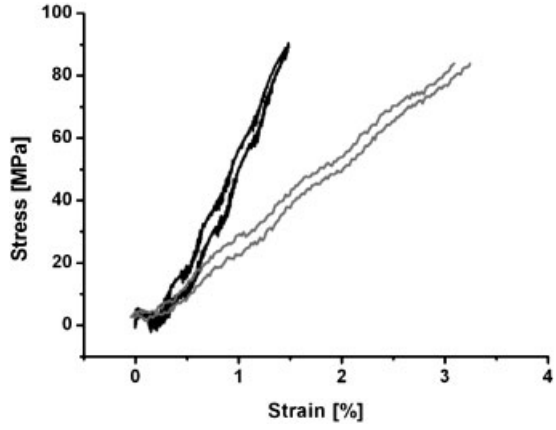

Figure 3. Two examples of stress-strain curves of individual collagen fibrils obtained at ambient conditions (extension rate: 4.6 $\mu m \cdot s^{-1}$).

It was possible to stretch these collagen fibrils up to stress levels of about 90 MPa. Upon applying much higher stresses, subsequently measured stress-strain curves do not overlap anymore. This is attributed to stress-induced permanent deformation of the fibrils. The stress-strain relation appears to be almost perfectly linear, and from the slope a Young's modulus of 5 ± 2 GPa was derived.

After stretching and relaxing these collagen fibrils at ambient conditions, a PBS solution (see legend) was added and the micromechanical testing experiment was repeated. A typical result can be seen in Figure 4. Notable is the increase in diameter of the collagen fibril upon rehydrating the collagen fibril. In a separate experiment tapping mode AFM imaging was used to accurately determine the height of the collagen fibrils at multiple locations along its length in ambient and liquid conditions. A diameter increase of $73 \pm 15\%$ was observed upon rehydration of the fibrils in PBS solution.

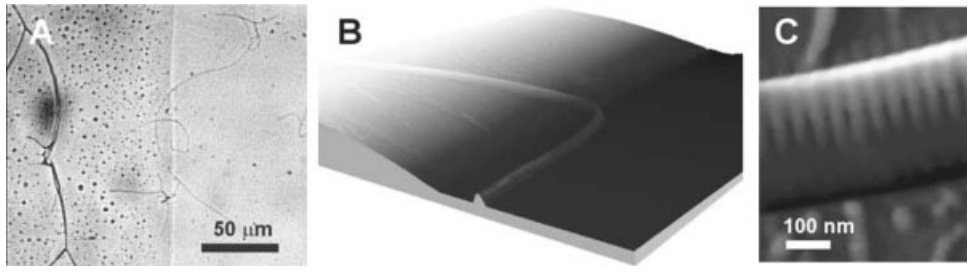

Figure 2. (A) Optical microscopy image of individual collagen fibrils as they are deposited on top of a cover glass partly coated with Teflon AF® (left side). (B) 3D representation of an AFM image of fibril crossing the Teflon AF®-glass boundary. (C) High-resolution AFM image on top of a collagen fibril, revealing the 65-nm D-period very clearly.

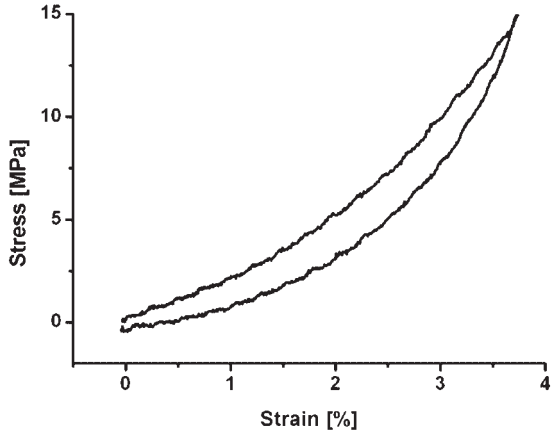

Figure 4. Typical stress-strain curve of a single collagen fibril obtained in PBS solution (140×10^{-3} M NaCl, 13×10^{-3} M Na_2HPO_4, 2.5×10^{-3} M NaH_2PO_4).

The stress-strain curve of the collagen fibril micromechanically tested, while immersed in aqueous media, has a different shape. The slope of the curve, which is equal to the Young's modulus of the collagen fibril, in these conditions varies between 250 and 450 MPa, which is considerably lower than what was found at ambient conditions. Also the maximum level of stress that could be applied before permanent deformation was observed to be lower, namely 20 MPa.

Collagen is the most abundant protein in mammals. The structure and mechanical properties can be studied on different hierarchical levels. In the present study we investigated the collagen fibrils, which are the most ubiquitous structural form of collagen found in biological systems. For the mechanical testing experiments an AFM was selected since it allows the combination of high-resolution imaging and force spectroscopy of individual collagen fibrils. Furthermore, the dynamic range of forces that can be applied and the force resolution fits the requirements for measuring collagen fibrils. The force at break of a single collagen fibril was estimated to be 30 μN.[6] From the stress at break value of non-treated self-assembled collagen fibers (which are composed of collagen fibrils) a stress of break of a single collagen fibril of 200 nm diameter can be calculated. A value of 0.15 μN was deduced. The AFM is capable of measuring these forces.

For attaching the collagen fibril firmly to both, the glass surface and the cantilever, a two-component epoxy glue was used which consists of a bis-epoxide and a tris-amine component that should be thoroughly mixed prior to use. The free amine functional groups of the collagen fibril can participate in the reactions, leading to fixation of the glue. With an expected maximum force of 0.15 μN, the shear stress of the fibril on the glue can be estimated. Assuming a fibril diameter of 100 nm, a 50 μm glue droplet, and half of the outer fibril surface to be in contact with the glue, a shear stress of 0.18 MPa was estimated, which is for lower than the maximum value as specified by the manufacturer. This was further confirmed by visual inspection of the collagen fibril within the glue droplet during the pulling experiments using the inverted microscope. No displacement of the glue droplet and the point where the collagen fibril leaves the glue was observed, leading to the conclusion that the fibril is firmly attached to both the tip and the glass surface, and is not slipping. The Young's modulus of the epoxy glue was reported to be 1.8 GPa, which is in the same order of magnitude of the Young's modulus found for collagen fibrils micromechanically tested at ambient conditions, and much higher than the values reported in aqueous conditions. In order to accurately assess its influence in the stress-strain curve to be recorded, the dimensions of the glue holding the fibril and those of the collagen fibril needs to be taken into account. If the glue droplet is considered to be a cylinder-shaped object of about 10 μm diameter and 5 μm height, and the fibril a rod of 0.2 μm diameter and 100 μm in length, the effect of the compliance of the glue in the strain obtained can be calculated to be less than 0.01% for a collagen fibril stretched at ambient conditions. This can be considered negligible. This leads to the conclusion that the glue has no significant effect on the stress-strain curve obtained.

The collagen fibrils were tested both at ambient conditions as well as immersed in aqueous media, and revealed a quite different mechanical and elastic behavior. At ambient conditions only a few stress-strain curves were measured and these appeared to be almost perfectly linear, revealing a Young's modulus in the order of 2 to 7 GPa. This is very much in agreement with moduli determined for rat tail tendons[9,10] and self-assembled fibers[11] as reported in literature. When immersed in PBS solution, the stress-strain behavior changed dramatically. The collagen presented in Figure 4 appears to have a 0.2 GPa modulus at strains up to 1% and a 0.5 GPa modulus at higher strains. Other stretching experiments in buffer conditions revealed similar results having Young's moduli in the range of 0.2 to at most 0.6 GPa at higher strain values. The shape somewhat resembles that of curves as obtained by Gutsmann et al.,[5] which could be accurately described using an exponential function. Elasticity of materials is usually modeled using simple Hookean springs. Puxkandl et al. introduced a Voight Kelvin mechanical model consisting of a parallel arrangement of an elastic and viscous component.[12] This model leads to an exponential stress-strain curve as observed in our measurements.

Recently, force spectroscopy experiments on human type I single collagen fibrils have been reported, which were non-covalently attached to an AFM tip while being immersed in PBS solution.[7] When collagen fibrils were loaded to a force of up to 4.5 nN at an extension of 3 000 nm (equivalent to a stress of 15 MPa and a strain of 4.5) distinct rupture patterns with an average elongation of 22 nm were observed in the loading curve. The relaxation profile reveals a plateau at a force level of 300 pN. Within the time scale of

the experiment of 1–5 s the fibril has regained its structural equilibrium and can be loaded and unloaded again leading to the same force-extension curve. In our experiments we pulled individual collagen fibrils and found a stress of 15 MPa already at strains of only a few percent. Furthermore, the stress-strain curve does not clearly reveal numerous rupture events as the fibril is being stretched. The laser interference present in the system did cause some additional noise in the measured data that might have obscured the 23 nm rupture events. Graham et al. found a value for the Young's modulus of only 32 MPa, which is an order of magnitude lower than what was measured in this study.

There are some differences in the collagen sample used for the experiment described in Graham et al. and in this paper. But even if the presence of cross-links and an inaccurate determination of the fibril's diameter were anticipated, it is not enough to explain the 10-fold difference in Young's modulus. Therefore we believe that in the experiments reported by Graham et al., the collagen fibrils were not firmly fixed to the AFM and/or the substrate as in this study, causing the collagen fibril to be peeled off in a stepwise manner from the surface. This stepwise peeling off explains the apparent lower Young's modulus and might possibly also explain the rupture events that were observed in their study.

Conclusion

In this present paper, we described in detail a new procedure to micromechanically test individual collagen fibrils, which form the most ubiquitous form of collagen in biological systems. The set-up used consisted of an AFM positioned on top of an inverted microscope. This allowed extensive visual inspection as well as high-resolution imaging of the collagen fibrils prior to the force spectroscopy experiment. The fibrils were deposited onto a glass surface that was partially covered with Teflon AF®. This allowed fixing the fibrils to the glass substrate on one end and to the AFM cantilever on the other end.

When micromechanically tested in ambient conditions an almost linear stress-strain curve was obtained from which a Young's modulus of 5 ± 2 GPa was derived. When the collagen fibrils were micromechanically tested in PBS solution, a different stress-strain curve was obtained. The curve was not linear but better approximated with an exponential function. The Young's moduli found ranged from 0.2 GPa at short extensions up to 0.5 GPa at strains up to 4%.

The method described here allows for stretching collagen fibrils up to maximum strains of typically only a few percent, which is sufficient to determine the Young's modulus. In our future work we intend to implement a larger piezo tube in order to allow stretching of the collagen fibrils to large strains. This provides more detailed information such as yield strength, tensile strength, hysteresis and the strain at break of individual collagen fibrils.

Acknowledgements: Medtronic Bakken Research Centre is acknowledged for their financial support to the research leading to these results. *Mark Smithers* (MESA+ Institute for Nanotechnology, University of Twente) is acknowledged for performing SEM analysis of the collagen deposited surfaces and *Frans Segerink* (Optical Techniques Group, Faculty of Science and Technology, University of Twente) is acknowledged for the removal of the tips of the AFM cantilevers using the focused ion beam (FIB).

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