Bidirectional tensile testing cell for in situ small angle X-ray scattering investigations of soft tissue

F. Schmid a,b, G. Sommer b,c, M. Rappolt a, P. Regitnig d, G.A. Holzapfel b,c, P. Laggner a, H. Amenitsch a,∗

a Austrian Academy of Sciences, Institute of Biophysics and X-ray Structure Research, Graz, Austria
b Graz University of Technology, Institute for Structural Analysis – Computational Biomechanics, Graz, Austria
c Royal Institute of Technology, Department of Solid Mechanics, Stockholm, Sweden
d Medical University Graz, Institute of Pathology, Graz, Austria

Available online 17 February 2006

Abstract

An X-ray cell for the acquisition of one-dimensional mechanical force–displacement diagrams of soft tissue samples has been developed. The combination of X-ray and mechanical data allows new insights into the coupling of the macroscopic behavior and nanoscopic structural changes during the application of load. Two linear μ-translation stages are used to symmetrically – i.e. bidirectionally – stretch the sample. A video-extensometer is installed to record geometrical changes of the sample during measurement which allows to determine true stresses and strains. The sample can be immersed in a liquid and heated. We demonstrate the advantages over common unidirectional tensile testing devices without length measurement systems by comparing different mechanical and diffraction data sets of human arterial tissue.

© 2006 Elsevier B.V. All rights reserved.

PACS: 61.10.Eq; 87.14.−g; 81.70.Bt; 87.19.Rr

Keywords: Tensile testing; X-ray cell; Soft tissue; Biomechanics; Small angle X-ray diffraction; Small angle X-ray scattering; SAXD; SAXS; Artery; Collagen; Fibre diffraction

1. Introduction

Nowadays there is a growing interest in composite materials, both in basic and applied research. The mechanical properties of composites combine the advantages of different materials and can be tailored to specific needs. Examples of artificially produced reinforcement materials include silicon-carbide-fibers and assemblies of carbon-nanotubes [1]. Biological examples are hierarchically structured fibers such as cellulose and collagen (for a review see [2]), which are found in an abundance of biological tissues.

Collagen fibers are used in varying amounts in a large number of tissues to provide mechanical strength. Their concentration, distribution and orientation greatly influence the response of the tissue to applied mechanical load. This coupling of the nanostructure to macroscopic observations has been investigated in great detail [3–7]. Further, understanding the mechanics of animal soft tissue is an essential requirement for the design of numerical models to simulate physiological tissue behavior, interaction with other materials and to design tissues with similar properties.

Our ultimate goal is mathematical modeling of the mechanical behavior of soft tissue with particular attention to medical intervention. For example in balloon angioplasty [8], where physiologically extreme loads are applied to human blood vessels to enlarge their diameter. A balloon is inserted into an area of diminished diameter of the artery – vulgo referred to as a “calcification” – and
inflated. After deflation the restored vessel diameter enables proper bloodflow and hence the supply of tissue with oxygen. Even though this is a common procedure worldwide, the mechanical processes during the treatment are not well understood. We aim to increase the safety and success of this medical intervention. We hope to reduce the number of fatal incidences during the dilatation and to optimize the long term stability of the created lumen by none invasive analysis and computer simulation prior to intervention.

A common way to understand the constitutive relationship between stress and strain of a material under uniaxial load is the comparison of stress–strain curves. However, this issue is complex for composite materials, where the constitutive equations have to describe different materials as well as their interaction. Stress is deduced from force, while strain is calculated from elongation. Therefore, the basis for a stress–strain relationship is a force–elongation curve recorded with a tensile testing device.

As described in an earlier work [9], geometrical changes necessary for the calculation of true stress and strain (see Fig. 1) can be recorded using optical techniques like a video-extensometer or laser speckles [10]. Contactless techniques are of crucial importance when dealing with extremely delicate samples, since any mechanical interference would falsify the measured geometrical deformations and the determination of the load. Additionally, our setup allows tensile tests of various tissue samples simultaneously with the acquisition of Small angle X-ray diffraction (SAXD) images at a synchrotron light source. In this manner macroscopic mechanical properties can be correlated with nanoscopic structural changes.

The present work illustrates our latest developments for a tensile testing device. Stretching is now done with two motors instead of only one. The center of the sample is fixed with respect to the direct X-ray beam at all times, i.e. during stretch the beam does no longer scan axially over a sample area (see Fig. 2). This avoids artifacts arising from possible tissue inhomogeneities, when neighboring tissue areas contain different amounts of scattering matter.

Further we demonstrate the importance to measure the true cross section and sample length during the stretch to retrieve correct stress and strain data, respectively. In contrast, the approach to use the initial cross section and clamp distance leads to erroneous results.

2. Materials and methods

2.1. Stress and strain

Stress–strain diagrams are essential for the comparison of the tensile behavior of materials. They are derived from force and elongation, respectively, which are then normalized to the geometry of the sample. Force is usually normalized by dividing it by the sample cross section perpendicular to the direction of the force. The corresponding stress \( \sigma \) is defined as \( \sigma = \frac{F}{A} \) with \( F \) being the force applied to stretch the sample and \( A \) the area of the cross section. Assuming constant volume of the sample during elongation, the diameter of the sample must shrink and with it the cross section. Due to the holding clamps, soft tissue changes from a cuboid shape to a bone shape upon elongation, broader at the ends than in the middle. Further from the clamps, the cross section diminishes significantly to stay rather constant over a certain length in the center of a sufficiently long and homogenous sample. This behavior is shown in Fig. 1.

Accordingly, also elongation depends on the position on the sample. Strain \( \varepsilon \) is calculated by dividing the current sample elongation \( L - L_0 \) by the initial sample length \( L_0 \), which results in strain \( \varepsilon = \frac{L - L_0}{L_0} \).

Recording the geometrical changes of the shape, however, turns out to be a non-trivial task, particularly when
it comes to small sample sizes, small forces and the presence of sample environments that do not allow contact measurement of the change of lengths.

The approach of using the initial cross section and the current clamp distance to calculate stress and strain, respectively, underestimates the true values. Depending on the geometrical deformation, also qualitative deviations from the true stress/strain curve can result. However, this constant cross section method might be applicable to compare homogenous samples of same dimensions.

For reasons of mechanical stress homogeneity, in our studies we use a small part in the center of a sample to measure and calculate true stress and strain. Only the part of the sample defined by the markers (see Fig. 1) is considered as quasi incompressible, i.e. Carew and colleagues [11] the soft tissue studied in this work can be considered as quasi incompressible, i.e. \( A = \frac{F}{\sigma} \). Thus, the true stress is given by \( \sigma = \frac{F}{V} \).

2.2. Bidirectional stretch of the sample

A unidirectional tensile stretching setup as described in [9] is perfectly suitable for homogenous samples. However, homogeneity cannot always be taken for granted in the case of biological material, like the soft tissue used in this study. If one side of the sample is fixed, every point of the sample is moving towards the end of the sample that is pulled away. Normally this does not cause problems for the macroscopic observation of the image with the video-extensometer, which is only following the markers. However, if very large extensions are needed – such as in rupture experiments – it is important to keep the sample centered in the X-ray window, as only a centered sample enables the maximum stretching distance. As soon as one marker on the sample moves outside the X-ray window, the video-extensometer camera cannot detect it any more and the extension measurement fails.

In addition, in unidirectional stretching devices problems might arise with the X-ray images (compare Fig. 2). Since the X-ray beam is fixed in its position, a number of different areas of the tissue are passing through the beam during the measurement. If these parts of the tissue contain different amounts of diffracting material they change the diffraction pattern, which may yield to ambiguous results and correct interpretation becomes problematic.

Therefore, a second translation stage was installed to circumvent these two problems. First, this enables centered mounting of the sample to use the full size of the X-ray windows. Second, the illuminated tissue portion should stay the same unless the tissue is extremely inhomogeneous. A loss in scattered intensity should be observed, since a certain amount of tissue is constantly removed from the beam area due to the thinning of the material.

2.3. Sample stage and mechanical measurements

A schematic drawing and a photography of the sample stage, which is designed specifically for uniaxial tensile experiments, are shown in Fig. 3. The sample is mounted between two clamps inside the sample container. Both the clamps can be moved independently by motorized linear translation stages M-126 (Physikinstrumente (PI), Karlsruhe, Germany). These allow a minimum incremental motion of 0.1 \( \mu \text{m} \) and a maximum velocity of 1.5 mm/s. A 25 N load cell (Type F1/25N, class 1 according to DIN 51220, Messphysik, Fürstenfeld, Austria) is inserted between the clamp and the upper translation stage for force measurement.

The main part of the sample stage is a U-shaped stainless steel bar. Plexiglas plates are attached on the front and back of it with Neoprene sealings in between.

An axially adjustable X-ray window covered with a 10 \( \mu \text{m} \) thin poly-ethylene-terephthalate film (Kalle GmbH, Wiesbaden, Germany) is mounted on either plate. This allows minimization of absorption and scattering arising from the solution, whereas the film itself was found to give negligible contribution to the measured signal.

The temperature of the tissue bath is controlled by an external water bath that can be adjusted with a precision of 0.1 °C in the range of 0–90 °C (Unistat CC, Huber, Offenburg, Germany). Two copper blocks are attached to either side of the U-shaped steel bar of the container for heat transfer.

A video-extensometer ME-NG (Messphysik, Fürstenfeld, Austria) is used to determine online the change of length and width of the area defined by the markers in the center of the sample (see Figs. 4 and 5). The working principle of a video-extensometer is to recognize significant changes in contrast in an image that is acquired by a camera. We used the sample edges to measure lateral contraction and the artificial markers for longitudinal elongation. A coldlight source with an optical fiber light guide is used to ensure a contrast rich image. In the present setup, a 75 mm lens with a 2x converter and a digital camera (FLEA HBW-CS, Point Grey Research, Vancouver, BC, Canada) connected to the FireWire port of a 2.4 GHz AMD computer is used. This allows theoretical data read-out frequencies of more than 200 Hz (limited by the frame rate of the camera) and a resolution better than 1 \( \mu \text{m} \).

Fig. 4 shows a top view scheme of the complete setup. The X-ray beam impinges on the sample and the diffracted image (dashed line) is recorded with a CCD camera. The camera for the video-extensometer is mounted perpendicular to the beam. It is facing a mirror which reflects a visible image of the sample during measurement. To minimize aberration errors, the mirror is set at an angle as close as possible to 45° with respect to the X-ray beam. Before each measurement the video-extensometer is calibrated to the sample geometry. The whole setup is PC controlled using specifically designed programs in LabView (National Instruments, Austin, TX, USA).
2.4. X-ray measurements

All diffraction patterns were recorded at the Austrian SAXS beamline at ELETTRA, Trieste [12] using a two-dimensional image intensified CCD detector (Model CV 12, Photonic Science Ltd., Millham, UK). The sample to detector distance was set to 1.22 m to cover the corresponding s-range (s = 2sin(\(\Theta\))/\(\lambda\), with \(\Theta\) as Bragg angle, and \(\lambda\) as X-ray wavelength) of interest from about 1/380 Å\(^{-1}\) to 1/27 Å\(^{-1}\) at an X-ray energy of 8 keV. The beamsize was 0.5 mm in axial and 1.5 mm in transversal sample direction. The angular calibration and the beam center was determined with silver behenate [13]. For this the programs Fit2D [14,15] and FibreFix from CCP13 [16] were used.

During the time course of the mechanical stretch and release the diffraction patterns were taken continuously with an integration time of typically 4 s, which varied depending on the scattering power of the samples.

2.5. Sample preparation and conditioning

Arteries used in this study were taken during autopsy and frozen immediately. The use of the human material was approved by the Ethics Committee, Medical University Graz, Austria. Before preparation, the arteries were thawed and stored in a 0.9% physiological saline solution.
The artery was cut open along the length axis and dissected into the three major arterial layers, the adventitia, media and intima, being the outer, middle and inner layer, respectively. Strips in longitudinal and circumferential direction were cut from these tissue sheets (for prepared strip samples see, for example, Fig. 3 in [17] and Fig. 5).

For better clamping, pieces of sandpaper were glued to the end of the samples. Further, two black markers (see Fig. 5) were glued perpendicular to the length axis at a distance of a few millimeters in the middle of the sample with cyanoacrylate adhesive gel (Henkel, Vienna, Austria). These markers serve as reference for the video-extensometer which measures the distance of the markers.

Human arteries show a non-linear stress–strain diagram. However, a number of cycles are needed to eliminate the stress softening typical of soft tissues before a nearly reproducible curve is reached. These load–unload cycles, which are done before the tests, are called “pre-conditioning”. We found three cycles to be adequate. We have used a constant crosshead speed of <0.05 mm/s. Elongation was adapted to each sample and then kept the same for all the tests on the sample.

During measurements the samples were immersed in a calcium-free buffered 0.9% physiological saline solution at a temperature of 37 ± 0.1 °C. After experimental testing the samples were stored in alcohol for histological examinations.

3. Performance

A typical force–elongation diagram recorded with our cell is shown in Fig. 6. As can be seen in panel (A), the sample tests are linear clamp-displacement driven stretch–release cycles. The exponential increase of force with increasing displacement is typical for fiber reinforced soft tissues, such as the arteries tested here, and is attributed to the fiber structure and the fiber–matrix interaction [4]. Force–elongation as well as stress–strain curves of soft tissue often consist of three major regions: a rather flat “toe” region, where no significant increase in stress is observed, followed by the onset of stress in the “heel” region. Lastly, stress increases rather linearly in the so-called “lock” region [5,18]. In aortic tissue, this sequence of behaviour was attributed to first a straightening of the fiber bundles, then a rotation of the bundles towards the tensile axis and finally the extension of the fibers themselves [9].

Other structural parameters determined with our setup are displayed in Fig. 6(B). The time course of the distance of the markers (“length”) and the width of the sample in the central area are measured directly with the video-extensometer (see Section 2.3). Comparing Fig. 6(A) and (B) it can be seen that non-linearity strongly depends on elongation. The visco-elastic behaviour of the tissue is also shown: in panel (A), the stretch and release part are not identical (i.e. not mirrored); in panel (B) at the end of the cycle, when the clamps have returned to their initial position, length and width have not. Sample thickness can be calculated from length and width and allows correction of the measured intensity for variation of the thickness. This is important if the absolute scattering cross section per unit volume (see e.g. [19]) is derived from the measurement.

Furthermore, we would like to demonstrate that the curve of the directly measured force divided by the initial cross section (force/A₀) and clamp distance can vary significantly from the calculated true stress and strain of a particular part of the sample. Not only the absolute values, but also the shape of the curves may differ greatly. This is demonstrated with data of another sample in Fig. 7.
where the clamp strain is very different from the true strain. We believe that the whole sample was not homogeneous in terms of strain, because strain of the total sample is larger than of the central area, which should not be the case in homogenous tissue. The maximum values for true stress and force/$A_0$ are nearly equal. This, however, is coincidence: the curves are generally different, because the cross sectional area diminishes during stretch (compare width and thickness in Fig. 6, Section 2.1).

Fig. 8 shows a typical SAXD image of a sample in stretched state. The diffraction peaks of collagen (type I and III) are indicated with numbers. The dashed line marks the area around the third order peak that was used to fit a Gaussian to the azimuthal intensity distribution (see [9]). The problems that might arise in the diffraction pattern using a unidirectional stretching device are demonstrated in Fig. 9. In the graph the peak intensity of the Gaussian fit is plotted versus time during a stretch–release cycle for two samples. Due to the increased order of the collagen fibers under strain along the tensile axis the peak intensity should follow the course of strain. This is shown by Purslow et al. in [4] for the meridional third and fifth order peaks of rat skin and in Misof et al. [20] for the equatorial diffuse scattering intensity of rattail tendon. The upper panel in Fig. 9 shows a sample with the expected relation. The data was recorded using the bidirectional stage. In the lower panel, however, the curve shows strong intensity fluctuations, yet it is still mirrored. That experiment has been done with the unidirectional stage, the clamp has been moved around 10 mm. The size of the beam of only 0.5 mm in the direction of load and the movement of the sample due to the unidirectional stretch support the assumption that the unexpected changes in signal intensity arise because the beam is scanning across sample regions of varying collagen content. It can be assumed, that an area containing such different amounts of scattering tissue also varies in mechanical properties. Hence, it is particularly important to make sure to probe only one single spot of the sample instead of scanning a continuously changing area.

4. Summary and outlook

Uniaxial stretching devices cause a continuous movement of all the spots of a sample in respect to a fixed point of reference, the X-ray beam for instance (see Fig. 2). In case of tissue inhomogeneities, this might introduce artifacts, as is explained in detail in Section 3 and Fig. 9. A bidirectional stretching device, however, ensures the stability of the position of the center of a sample, hence the X-ray beam is constantly probing the same spot on the sample. A further advantage is the full use of the size of the X-ray window, which is particularly important for experiments where sample stretch is much larger than in normal stretch–release experiments, such as at controlled rupture.

With a bidirectional device a misalignment of sample center and beam is no longer inherent to the system and in normal cases the center of the sample stays practically constant during stretch. However, severe tissue inhomogeneities might still cause central deviation and the initially illuminated sample volume might not remain perfectly aligned with the X-ray beam. Consequently, an auto alignment system shall be installed to correct for this and to keep the sample centered at all times.

The issue that will be addressed in the future is the extension to a second axis while conserving full functionality. A biaxial stretching device using four motors but without force measurement has been described by Liao et al.
The strategy to replace two uniaxial measurements with one biaxial test is important, as two perpendicular uniaxial tests represent only the two extreme cases of two-dimensional stretch.

Acknowledgements

This paper is dedicated posthumously to our honored and very respected colleague and friend C.A.J. Schulze-Bauer. He contributed significantly to the early stage of the project. Financial support for this research was provided by the Austrian Science Foundation (FWF Project No. P17922-N02) and by ELETTRA/European Community under Research Infrastructure Action FP6 (contract: RII3-CT-2004-506008 IA-SFS). The authors gratefully acknowledge the support.

References