Nanomechanical subsurface characterisation of cellulosic fibres

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Abstract
The mechanical properties of single fibres are highly important in the paper production process to produce and adjust properties for the favoured fields of application. The description of mechanical properties is usually characterised via linearized assumptions and is not resolved locally or spatially in three dimensions. In tensile tests or nanoindentation experiments on cellulosic fibres, only mechanical parameter for the whole fibre, such as elastic modulus or hardness, is usually obtained. To obtain a more detailed mechanical picture of the fibre, it is crucial to determine mechanical properties in depth. To this end, we discuss an atomic force microscopy-based approach to examine stepwise the local stiffness as a function of indentation depth via static force-distance curves. To our knowledge, we are the first authors to apply this method cellulosic fibres. The method was applied to linter fibres (extracted from a finished paper sheet) as well as to natural raw cotton fibres to better understand the influence of the pulp treatment process in paper production on the mechanical properties. Both types of fibres were characterised in dry and wet conditions with respect to alterations in their mechanical properties. The used stepwise analysis method of the force-distance curves allowed subsurface imaging of the fibres. It could be revealed how the walls in the fibre structure protects the fibre against mechanical loading. Via a combined 3D display of the mapped topography and the fitted elastic moduli in z-direction, a spatially resolved mechanical map of the fibre interior near the surface could be established. Additionally, we labelled the fibres with different carbohydrate binding modules tagged with fluorescent proteins to compare the AFM results with fluorescence confocal laser scanning microscopy imaging. Nanomechanical subsurface imaging in combination with fluorescent protein labelling is thus a tool to better understand the mechanical behaviour of cellulosic fibres, which have a complex, hierarchical structure.

Supplementary Information
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1 Introduction

Paper, as a high-tech material made from cellulose, has promising applications in areas such as electronics, sensor technology, microfluidics and medicine [7, 16, 23, 26, 32, 53]. Cellulose is a natural material, is abundant and renewable, and is the most important raw material in the papermaking industry. During papermaking, the natural structure of fibres can be mechanically or chemically altered, particularly at the fibre surface. To better understand how these alterations may affect the mechanical stability or water uptake of the fibres, it is essential to investigate the mechanical fibre properties at and beneath the surface. To tailor paper to advanced applications, the impact of fibre or pulp treatment before the paper-making process on the structure and mechanical properties of cellulose fibres must be understood. In particular, the fibre surface is relevant in technology because its composition and roughness determine the fibre–fibre bond strength and because it is exposed to the environment on the paper sheet surface [50, 52, 60]. The properties of the bulk, however, are of similar relevance because they determine, for example, how well fluids such as water can be absorbed and how the fibre swells in a humid environment. To better understand how cellulose fibres swell and thus change their mechanical stabilities and how this process is related to the pulp treatment process of the papermaking procedure, it is essential to characterise bulk and surface properties close to the interface between the fibre and the surrounding atmosphere.

Cellulosic fibres have a hierarchical structure. By forming larger networks of cellulose molecules, highly oriented linearized polymer chains can form a unit cell. This unit cell is surrounded by unordered areas. With the arrangement of oriented (crystalline) and unordered (amorphous) areas, fibril structures are developed. The arranged microfibrils have a diameter of a few nanometres and are a few micrometres in length. By aggregation of microfibrils, macrofibrils with a diameter of 60–400 nm and a length of a few millimetres are formed (H.P. Fink 1990). These fibrils, together with hemicellulose, lignin, pectin or waxes, form the cell walls of plant-based fibres. The formed fibril structures possess a hierarchical wall structure. A cotton fibre has a central, hollow lumen. Around the lumen, a secondary wall with layers S2 and S1 is formed. This wall is surrounded by the primary wall (P). The mature fibre is enclosed by the cuticle (C), a waxy protection layer, which is a few molecules thick. Before processing the fibre, the cuticle
must be removed. The cellulose fibrils in the P wall are arranged in a disordered network around the fibre axis. The wall thickness of P is 0.1–0.2 µm, and it contains pectin, hemicelluloses and cellulose microfibrils and small amounts of cutin/wax and proteins. During the paper production process, this wall is often milled off. For the fibre or pulp treatment process, it is essential to remove C and a small amount of P, as the fibres are pressed in a wet state to bond to each other and thus form a paper sheet. Because of the missing C layer and intact P wall, the water molecules can intrude into the fibre network and break the hydrogen bonds between the cellulose molecules, which leads to softening of the fibres, which are maintained through the insolubility of cellulose [9, 22, 31, 34]. The thickness of the S1 layer is 0.1–0.2 µm, and it contains small portions of pectin and hemicelluloses and high portions of cellulose. The fibrils orient in a predominant direction and lie parallel at an angle of 20° (microfibril angle MFA) to the lumen. The most relevant layer for paper production is the S2 layer. A switch in the fibril direction is observable in the transition region from S2 to S1. The fibrils here lie side by side in a predominant direction oriented along the fibre axis. With a thickness of 1–5 µm, the S2 wall represents 90% of the fibre mass and therefore determines the mechanical properties of the fibre. The S3 layer separates the lumen from S2 and is 0.1–0.2 µm thick. The angle of the fibrils is 45° to the fibre axis [M.Dochia 2012; Mohamed A.L. 2015; [56]. The changing and spiralled arrangement of the fibrils results in high inherent robustness of the fibres [40, 46, 56].

The influence of humid air or water on the mechanical properties of natural fibres is an important topic to be addressed in the application of cellulose-based materials. The impact of relative humidity on the elastic modulus or strength has been investigated by authors like [20, 49, 55]. With an AFM-based indentation method, it was possible to check the mechanical properties of wet cellulose and also estimate the Young’s modulus, was in the kPa range [27]. Also, nano mechanical mapping by AFM indicated a decrease of the DMT modulus in a humid environment [1]. Additionally, the bending behaviour of humid cellulose fibres could be shown by [2]. Additionally, the viscoelastic properties of pulp fibres could be investigated with AFM. The fibres exhibited a decrease in the elastic moduli by a factor of 100 after water immersion, and the viscosity decreased by at least three orders of magnitude [13]. The breaking load of a single fibre depending on the RH could be shown by [30].

Also, numerical studies on mechanical properties of single fibres to whole paper sheets were executed. [33] showed the humidity influence of the mechanics of paper materials by using experimental AFM data to develop an numerical model on the fibre network scale.

In [57] a review of the recent challenges in computational modelling of paper is introduced. The effect of hygroelastic properties of wood fibres was modelled by [43].

How moisture is adsorbed in cotton fibres and how the dynamics behave in a paper sheet was studied by [11]. They showed that the hysteresis is dependent on the cellulose content of the fibres. [8] experimentally studied the kinetics of the cellulose deposition in developing cotton fibres.

To date, tensile tests have characterised the tensile strength or elastic modulus of cellulosic fibres. An experimental approach to determine the longitudinal elastic modulus has been described by [47]. Theoretical works, e.g., [39] and [54], established that the MFA in the S2 layer is the determining factor for the strength of the fibre. Soon, it was established that the MFA in the S2 layer is the factor that determines the strength of the fibre. A small MFA angle leads to a high longitudinal elastic modulus [42]. In addition, [3] found that the mechanical properties depend on the orientation of the microfibrils in the S2 layer. Likewise, [58] determined that the more closely the microfibrils are longitudinally aligned with each other in the S2 layer, the more tensile force could be applied. However, the transverse elastic modulus depends, according to Bergander and Salmen, less on the S2 layer than on S1 and S3 [5]. Nanoindentation was introduced as a method to investigate the different layers in the fibre. [62] determined the Young’s modulus and the hardness of wood fibres via nanoindentation. [21] expected in their nanoindentation experiments that the Young’s modulus of the S2 layer should be higher than the modulus of the other layers.

Sensitive nanoindentation can be performed by atomic force microscopy (AFM), which even makes it possible to map mechanical properties on the surface [18, 48]. A straightforward approach to obtain a picture of the landscape of local mechanical properties is “force-volume mapping”. In this approach, the force is measured via the cantilever deflection, which leads to force-distance curves. [51] extended this method by introducing the “stiffness tomography” method, where they evaluated static force-distance curves in segments to show the stiffness differences along the indentation path. Thus, it is possible to estimate the Young’s modulus of the sample at a desired indentation spot for various indentation depths. Previously released studies have shown that this approach can be applied to soft materials such as cells [59], polymers [14], bacteria [35], graphene oxide [15] or collagen fibrils [37].

In the following, we discuss how AFM-based nanoindentation can be used to probe the near-surface bulk of cellulose fibres under varying environmental conditions (relative humidity). Variations in the local nanomechanical
properties could be established and related to recovery from the hydrated state.

2 Materials and Methods

2.1 Fibres

Processed cotton linter fibres were extracted manually from a linter paper sheet. The sheet was produced according to DIN 54,358 and ISO 5269/2 (Rapid-Köthen process). As these fibres were already processed, they are referred to as PF in the following. Unprocessed cotton linter fibres were manually extracted from a dried natural cotton boll. These fibres were raw and unprocessed and will be referred to as UPFs. The fibres were fixed on both ends on glass substrates.

For hydration, fibres were stored in deionised water and allowed to swell for 45 min. It can be assumed that the water progressed into the cotton linter test stripes in seconds [10, 29]. Typically the free swelling time for a whole pulp is 70 min [45]. For single cellulose fibres, however, 45 min are enough to reach equilibrium [38]. We performed the measurements in the wet state as described elsewhere [1]. After incubation the residual water droplet was removed before the measurement, to avoid further swelling. Overall, nine regions of interest (nine fibres) of every fibre type and condition were investigated.

2.2 Atomic Force Microscopy

A NanoWizard II atomic force microscope (JPK InstrumentsAG, Berlin, Germany) was used to record maps of force–distance curves of the PF and UPF. Two types of cantilevers were used. The first type was the RTESPA-525 (Bruker, Santa Barbara, USA) with a high spring constant (HSC) of 162 N/m, a 15° opening angle only at the tip end and a radius of 30 nm. The second type was the ISC-125 C40-R (Team Nanotec, Villingen-Schwenningen, Germany) with a high aspect ratio (HAR), an opening angle of 10° for 3 µm, a radius of 10 nm and a spring constant of 30 N/m. The deflection sensitivity of the cantilever (invOLS) was calibrated by performing a force-distance curve on a hard sapphire surface. Both tip radii were taken as stated by the producers and reviewed by scanning electron microscopy. Each map had an image size of 10 µm × 10 µm with 128 × 128 data points. Force curve measurements were carried out at a tip approach speed of 20 µm/s and a force setpoint of 3000 nN. Thus, 128 profiles in xz- and in yz directions for the analysis of $E_{\text{dark}}(z)$ are created (9 spots per fibre type = 9 × 128x128 profiles).

2.3 Force-Volume Mapping

Performing force-distance curves allows not only investigation of the surface nanomechanics but also probing of the mechanical properties near the surface. To map sub-surface properties, i.e., to obtain a three-dimensional characterisation of the mechanical properties, the force-distance was analysed stepwise (30 nm) after the contact point $d_0$. Local slopes were interpolated stepwise.

![Fig. 1 Principle of the stepwise analysis of a force-distance curve. The local fits are indicated in red. The indentation depth is calculated as the distance from the contact point](image)

![Fig. 2 SE images of the tip geometry for a the HSC cantilever and b the HAR cantilever. SE images of cross sections for UPF in (c) and (e) and for PF in (d) and (f)](image)
to obtain estimates of the effective local elastic modulus as a function of depth $E_{lok}(z)$ (see Fig. 1). Extracted force-distance curves from the measurements are shown for every cantilever type, every fibre type and every environment condition in the figures S1-S4.

Before analysing the local slopes, the force-distance curves had to be processed. The noise was removed by a Savitzky-Golay filter. The slope of the baseline was corrected with a linear fit function to the initial flat region. The initial offset and the inclination (tilting) of the curves were corrected. The contact point was identified as the first point in the repulsive regime (positive slope). More precisely, the first contact point was identified as the point where the force exceeded that of the preceding point by at least 10 times the standard deviation of the baseline signal. The position of the tip over the height $z$ was calculated from the deflection of the cantilever, which transformed the force-z-piezo data to a force-distance or force-indentation curve, respectively.

From a two-dimensional map of force-distance curves, various parameters were calculated. The topography was calculated from the contact points. The penetration depth reached at the setpoint (maximum force) yielded the maximal indentation. To obtain mechanical information at intermediate depths, the data were interpolated in a stepwise manner in the approach curve. Thus, for the intermediate penetration depths, local fits were calculated, which served as estimates of the effective elastic modulus assuming Hertzian contact mechanics [28] as

$$F = \frac{4}{3} E^* \cdot R^\frac{3}{2} \cdot \delta^\frac{3}{2}$$  \hspace{1cm} (1)

Here, $F$ (unit nN) displays the applied force, $E^*$ (unit Pa) the effective modulus (unit Pa), $R$ (unit nm) the tip radius and $\delta$ the indentation (unit nm).

To obtain good spatial and depth resolution, the AFM cantilever was selected that structures beneath the surface could be probed. This was accomplished via two different approaches: (i) by using hard cantilevers with a high spring constant (HSC) but conventional tips or (ii) by using softer cantilevers equipped with extremely sharp tips with a high aspect ratio (HAR). By using the HSC cantilever, a sufficiently large loading force can be applied that the tip probes the structure inside the fibres. In contrast, with the HAR cantilever, a large local pressure can be obtained at moderate loading forces. Both tip geometries are displayed in Fig. 2 with SE images. The data were processed by using MATLAB code. The two-dimensional images were generated by using Gwyddion [44], and three-dimensional images were created by using Blender [12].

### 2.4 Scanning Electron Microscopy

The individual cantilever tip shapes were analysed by a scanning electron microscope (SEM) (MIRA3, TESCAN, Brno, Czech Republic) using secondary electron (SE) imaging mode at an acceleration voltage of 10 kV. Both tip geometries are displayed in Fig. 2 with SE images. Furthermore, cross sections for each fibre type (UPF and PF) were recorded via SEM. They are shown in Fig. 2 c-f).

### 2.5 Preparation of Recombinant His- and Fluorescence-Tagged Carbohydrate-Binding Proteins (CBMs)

The DNA sequences of CBM3a [6, 19, 61] (“semi-crystalline” cellulose), CBM1Cel6a (crystalline cellulose) [6, 19] and CBM77 [63] (pectin) were taken from the CAZy database and linked DNA or protein databases (GenBank, UniProt, PDB). The evaluated sequences of Clostridium thermocellum (CBM3a; CCV01464.1, 4JO5), Ruminococcus flavefaciens (CBM77; WP_009983557, 5FU5) and Trichoderma reesi (CBM1Cel6a; AAA34210.1, P07987) were then ordered as gene fragments (IDT, Coralville, USA) or 1:500 (CBM3a-smURFP) or 1:500 (CBM3a-smURFP) or 1:500 (CBM3a-smURFP)

For fibre labelling, CBMs were diluted 1:1000 (CBM77-mclover3, CBM1Cel6a-mKOK) or 1:500 (CBM3a-smURFP) in ddH2O to a final concentration of ~40 µg/ml and both types of fibres were incubated in it for 5 - 20 min each (PF 5 min, UPF 20 min). UPF fibres were preincubated for 30 min with a 0.02% solution of Tween 20 in ddH2O to remove or permeate the cuticula of cotton fibres.

According to our research, the fibre structure does not change due to the natural process of swelling. SE images show no differences between dry, swollen and re-dried...
fibres. This is especially true for cotton linters fibres, which have been altered by paper production anyway (lack of cuticle and primary wall). It is also true that untreated cotton fibres hardly absorb any water, due to a water-repellent substances in the cuticular and primary wall. Surfactants such as Tween 20 which we have used here, have direct effects on cuticular permeability that are not completely understood. However, studies show no change in the cellulose fibre structure.

Our studies show that CBMs are able to penetrate the cell wall after a short time, but not on the complete thickness. The cell wall acts like a molecular sieve. Dyes like Calcofluor white or P4B are able to penetrate the complete cell wall. Cotton linters fibres in particular can also be marked from the inside, as closed systems are no longer present due to paper production. Therefore, the large lumen of the fibres can be seen particularly well here. In principle, a longer incubation time always allows a greater penetration depth. This can be seen very well in untreated cotton fibres. It can also be seen that with completely closed intact cells (fibres) the CBMs do not succeed in entering the lumen. However, micro-damages occurring during preparation are sufficient to obtain the labelling on the inside as well.

2.6 3D Fluorescence CLSM Measurements

Confocal xyz series of CBM3a-mClover3-, CBM1Cel6a-mKOk- and CBM3a-smURFP-labelled fibres were recorded with a Leica TCS SP8 confocal system (Leica Microsystems GmbH, Mannheim, Germany) using an HyD detector with an HCX PL APO 63 × NA 1.2 W CORR CS2 objective and the normal scanner system at 512 × 512 pixels in the 12-bit mode. Z-sections were set at a system-optimised value of 0.36 µm or a custom value of 0.2 µm per section. Sections were obtained using an appropriate laser for excitation and a small range of emission, 10 to 15 nm around the emission maximum (mClover3 ex. 488 nm, em. 505–525 nm; mKOk ex. 561 nm, em. 570–590 nm; smURFP ex.635 nm, em. 660–690 nm). Fluorescence channels were obtained in sequential frame detection mode to avoid cross talk. The position of the images in the 3D stack are the middle of each fibre.
3 Results and discussion

From the force curve data, a surface topography map was calculated. In addition, maps of the local stiffness at various indentation depths were generated. The combined 3D representation of the topography with the corresponding local stiffness is shown in Fig. 3. The topography is given in a yellow colour scale as 3D representation. The colour code in z-direction encodes the local stiffness beneath. In Fig. 3b) the top image shows the minimized Fig. 3a) to illustrate that the image at the bottom is a cross section through the same fibre with the corresponding local stiffness maps of $E_{lok}(z)$ is shown in the xz- and yz-directions at this slice of the mapped fibre.

Figure 3a) shows an overview of the PF surface with its local stiffness maps. A cross section through the fibre with the corresponding local stiffness maps of $E_{lok}(z)$ is shown in the xz- and yz-directions in Fig. 3b). The parameter for the local stiffness as a function of the depth beneath the surface $E_{lok}(z)$ is meaningful in regions where the fibre topography could be mapped with sufficient resolution. “Sufficient resolution” here means a feature size of 12.8 pixels/µm.

In Fig. 4, an overview of cross sections of $E_{lok}(z)$ in the xz-direction in the PF and UPF mapped with the different cantilever types are shown.

In Fig. 4a), the depth profiles of $E_{lok}(z)$ in the xz-direction of a PF mapped with a HSC cantilever in a) and with a HAR cantilever in b) are shown. Both depth profiles exhibited a hard layer, shown in in yellow. The hard layer on the top exhibits a value of 95 ± 15 MPa. A sharp transition between a hard layer (yellow) and softer material (red and blue) can be identified. Thus, a stiffness gradient from the fibre surface to the softer interior was observable. $E_{lok}(z)$ was calculated for each data point, for a total of 128 profiles. All of them showed the same behaviour with the hard layer at the surface and sharp transition to the softer layer beneath. To verify the trend of $E_{lok}(z)$ in the xz- and yz-directions, further profiles are displayed in Fig. 3b), where the topography and the profiles of $E_{lok}(z)$ are shown for another cross section. Additionally, here, a hard layer (yellow) can be identified above the softer layers (red and blue). Thus, it is reasonable to assume that the PF is covered by a hard layer (or several hard layers that could not be resolved). Beneath this top layer, $E_{lok}(z)$ decreases with further indentation depth. Cross sections of the $E_{lok}(z)$ of PF are shown in Fig. 5b). Note that we investigated nine spots of every fibre type. The cross sections in Fig. 4 are representative cross sections only. As UPF and PF are natural occurring fibres it is not expected them to own the same roughness at the surface.

However, in the fibre or pulp treatment process, the P wall is usually milled off. Thus, it has to be excluded that the hard layer on top in the PF presents the S2 layer. To verify this, force-volume mapping was applied to the UPFs. The UPFs were directly extracted from a natural cotton boll and therefore raw and unprocessed, which means that all layers should be intact. In Fig. 4c), a depth profile of $E_{lok}(z)$ in the xz-direction mapped with an HSC cantilever and in d) mapped with a HAR cantilever are shown.

Fig. 5 Depth profiles of this Elok(2) of (a) a UPF, (b) a PF and (c) a combined graph of UPF and PF. In (c), the UPF profiles are plotted in red, and the PF profiles are plotted in blue. The suggested corresponding wall layers are indicated with different colours. The C layer is highlighted in dark blue, the P wall in blue, the S1 layer in light blue and the S2 layer in grey. The error bars are ±30% of the values. The three profiles in (a) and (b) were extracted from cross sections with a HSC cantilever. The profiles in (c) were extracted with both cantilever types. The maximum indentation in z-direction was reached for PF with 1233 nm and for UPF 485 nm. Thus, we did not consider that the hollow lumen affects the measured the Elok(2)values.

Fig. 6: 3D images of confocal fluorescence microscopy imaging. The fibres are labelled with CBM77 in green (binds to pectin), with CBM3a in cyan (binds to “semi-crystalline” cellulose) and with CBM-1Cel6a in red (binds to crystalline cellulose). (a) UPF fibre and (b) PF fibre.
Fig. 4c) and d), it can be seen that the UPF possessed a hard layer (yellow) on the fibre surface with softer layers beneath (colour coded in red and blue). The $E_{\text{lo}(z)}$ in the $xz$-direction decayed with increasing indentation depth. Compared to the PF, the UPF exhibits a harder layer on the surface at 1 GPa. The indentation depth in all measurements was sufficient to reach the S2 layer depth, i.e., at least 300 nm. However, it was observed that the indentation depth in the UPFs was not as high as that in the PFs. Cross sections of the $E_{\text{lo}(z)}$ of UPF are shown in Fig. 5a). The recorded profiles in Fig. 5a) and b) are recorded at different regions of interest on different fibres.

We interpret the trend of the depth profile of UPF as follows: In UPF, all layers should be intact. Therefore, the hard layer (yellow) on the top most likely represents the $E_{\text{lo}(z)}$ of C with incrustations (eventually with a slight crosstalk caused by P). Layer C is a waxy layer containing cutin, waxes and cell wall polysaccharides, which is assumed to be harder than the layer beneath it, which is embossed by the fibril structure of cellulose. The slightly softer layer beneath (red) might directly represent the P wall. In comparison to the C layer, the $E_{\text{lo}(z)}$ in the P wall is reduced because it contains additives to waxes, such as lignin, minerals and cellulose fibrils. Compared to the S layers, which are predominantly constructed of cellulose fibrils, the P wall with waxes, lignin or minerals is therefore harder. At the indentation depth, where S1 and S2 should be present, $E_{\text{lo}(z)}$ decayed noticeably.

Regarding the question of what happens to the fibres when they are processed into paper fibres, we propose the following: In the PF, a maximum average value of $E_{\text{lo}(z)}$ 87 ± 16 MPa was measured (hard layer on the top). In UPF, this $E_{\text{lo}(z)}$ value is reached at an indentation depth of 235 ± 58 nm. As the depth of S2 should be 300–500 nm, this result suggests that in PF, C was completely removed during the paper production process. Additionally, the P and S1 layers were slightly milled off in PF, and the lignin content decreased. However, the data indicate that the P and S1 layers were not completely removed during paper production, as the $E_{\text{lo}(z)}$ of the S2 layer was not reached in the first 200 nm. Since the P wall has a thickness of 100–200 nm, as does the S1 layer, it can be assumed that both layers could still be partially intact.

In Fig. 5c), further cross sections of $E_{\text{lo}(z)}$ UPF (in red) and PF (in blue) are shown (150 profiles for UPF and PF each). The suggested corresponding wall layers are indicated with different colours in the graph (see C, P, S1, S2). The C layer is highlighted in dark blue, the P wall in blue, the S1 layer in light blue and the S2 layer in grey. Figure 5c) represents the suggested $E_{\text{lo}(z)}$ trend inside the fibre. The cross sections of $E_{\text{lo}(z)}$ of the PF begin where the $E_{\text{lo}(z)}$ value of the UPF fibre is matched.

To verify our suggested wall structure, we labelled UPF and PF with fluorescence protein-tagged CBMs. We used CBM77 (binds to pectin, shown in green), CBM3a (binds to “semi-crystalline” cellulose (more information in the SI), shown in cyan) and CBM1Cel6a (binds to crystalline cellulose, shown in red). 3D confocal fluorescence microscopy images of UPF and PF are shown in Fig. 6a) and b).

Both fibre types exhibit a green (pectin) outer layer with cyan (semi-crystalline cellulose) parts and a red (crystalline cellulose) fibre interior (see Fig. 6). Intensity cross sections on single confocal planes reveal the order in which the three labelled components are arranged within a fibre wall (Fig. 7). By analysing the peak position of the labels, it is found that for UPF, the order is green, blue, green and red, i.e., pectin, semi-crystalline cellulose, pectin, and crystalline cellulose. The pectin signal is found on the outside and decays towards the fibre interior but is not found on the inside of the fibre wall. The signal for semi-crystalline cellulose, in turn, is found only in the fibre wall’s interior, and the signal for crystalline cellulose is found only inside.
In mature fibres, the P wall contains pectin, hemicelluloses, disordered cellulose fibres and lignin, and the S1 layer of the secondary cell wall has portions of pectin and hemicelluloses in addition to densely packed parallel cellulose microfibrils. Thus, both layers should occur in green but can also occur in blue, as both walls also contain “semicrystalline” cellulose. Therefore, it can be assumed that the green and blue intensity peaks represent the P wall and S1 layer. The crystalline cellulose is marked in red and can be attributed to the S2 layer of the secondary cell wall.

In contrast, in PF, the intensity peaks of the three components are closer together, but their signal distribution is broader. Similar to UPF, the order of the intensity peaks occurring towards the fibre interior is green, blue and red. However, in PF, only one peak is found for the green signal, which is consistent with the milling off of parts of P and S1 during the paper production process.

It was not possible to label the C layer or the different layers P, S1, S2 individually with CBMs. The measured intensities and intensity peaks could therefore also display a mixture of the different layers of walls. Furthermore, the fluorescence CLSM images have a lateral resolution of 250 nm and an axial resolution of 500 nm. Thus, it is not possible to assign the $E_{lok(z)}$ measured with AFM directly to the different wall layers with confocal microscopy. However, the measured order of the occurring intensity peaks and the correlation of the labelled parts with the predicted fibre structure strongly supports our interpretation of the AFM measurements.

In the literature, tensile testing showed that an intact S2 layer is the most important factor for the mechanical properties of the fibres [3, 58]. From nanoindentation experiments, it was inferred that the elastic modulus of the S2 layer should be higher than the elastic modulus of the other walls [21]. Merely, in the work of [5], the transversal elastic modulus was not highly dependent on S2 but on S1 and S3. The presented results in our work indicate that the hardness of the walls/layers and the corresponding meaning of the mechanical properties must be divided in the transverse and longitudinal directions. In tensile tests, the longitudinal elastic modulus of the fibres is determined by pulling at both ends of the fibres. The fibril structure of the S2 layer in the fibre is therefore oriented in the tensile direction. Hence, a higher resistance in the tensile direction is achieved. In contrast to the tensile test, in the force-volume mapping method, the fibre is not pulled at either end, but the stiffness at depth is measured by nanoindentation. Thus, the loading direction is perpendicular to
the orientation of the fibrils of the S2 layer (Fig. 8b). Due to the geometry of the indenters used in conventional nanoindentation experiments, it is not possible to obtain a lateral resolution, as it is by the AFM force-volume mapping method. The difference in the arrangement of the applied force in relation to the fibril orientation in the S2 layer in the fibre is shown in Fig. 8. Our results are in line with [5], who showed that the transversal elastic modulus does not depend on S2. This leads to the interpretation that a cellulose fibre is resistant to tensile forces mainly due to the orientation of the fibril structure in the S2 layer in the tensile direction. The resistance against compression forces is due to a hard layer on top of the surface, i.e., the fibril structure in the P wall.

In the next experiment, PF and UPF were hydrated to investigate the difference in indentation depth and behaviour of the layered wall structures of the fibres.

In Fig. 9, the cross sections of $E_{lok}(z)$ in the xz-direction of a hydrated PF mapped with an HSC cantilever (a) and with a HAR cantilever (b) are shown. A cross section of a hydrated UPF is displayed in c) mapped with an HSC cantilever and in d) mapped with an HAR cantilever. The hydrated PF exhibited fewer areas with a hard top layer on the fibre surface (yellow). In most parts, the PF showed areas that were attributed to a softer $E_{lok}(z)$ (blue or white). In these volume segments, the water molecules could break hydrogen bonds, which resulted in a softening of the fibre [9, 22, 31, 34]. In only a few areas, the layered structure, observed in the dry state, was visible. Therefore, it is interpreted that in the hydrated state, the layered wall structure of the PF becomes indistinct. The bonding between the fibrils in each layer could be weakened due to the weakening of the H-bonds. Thus, a possible interpretation is that an intact wall structure in each layer cannot be sustained in the wet state. In the hydrated state of the UPF (Fig. 9c and d), a layered structure in the $E_{lok}(z)$ was observable in most parts of the cross section of the fibre. In contrast to the PF, the UPF seemed to preserve the layered wall structure in the hydrated state, which is suggested to be due to the intact C layer and P wall, which prevent the UPF from excessive softening in the hydrated state. The higher maximum value of $E_{lok}(z)$ in the UPF compared to the PF supports this consideration. However, the maximum value of $E_{lok}(z)$ in the hydrated UPF exhibits a lower value than in the dry state. As shown in Fig. 9, the indentation depth of a PF in the hydrated state is higher than that of the UPF.

At next, the indentation depths in the dry state of both fibre types were analysed. The indentation depth in dry and in the wet state of both fibre types were averaged. The average of the indentation depth of the wet state was then set in relation to the dry state to get a measure of the loss in stiffness of the fibre types. In both fibre types, an increase of the normalised indentation in the hydrated condition was observable. The increase in the indentation depth in the wet state compared to the dry state of the PF was $32 \pm 15\%$. In the UPF the indentation depth increased by $13 \pm 9\%$. Thus, the tip could indent into the PF deeper in the hydrated state than into the UPF. This is attributed to the fibre or pulp treatment before the paper-making process. As mentioned before, the C and small amounts of P are removed during the fibre or pulp treatment process in the PF. Therefore, predominantly cellulose-based layers are present. Hence, the normalised indentation in the PF is attributed to the greater softening of the entire cellulose-based fibre. In contrast, in the UPF, the wax-like layer C and the wax-, pectin- and lignin-containing P wall are still intact [25]. With the intact composition and structure in the UPF, the water molecules cannot easily diffuse into the fibre. This can be seen as a natural hydrophobization to prevent massive water uptake into the UPF. Additionally, the standard deviation of the UPF was $\pm 0.05$ lower than that of the PF ($\pm 0.12$). The more uniform behaviour is consistent with an intact layer structure. As all UPFs were extracted from a natural, raw cotton boll, and the fibres had the same unprocessed layer structure. Thus, these measurements were completed with reproduceable samples, unlike the processed PF. In the fibre or pulp treatment process, the PF pass processes, such as beating or pressing, where the original fibre structure is randomly destroyed in each fibre. Thus, the higher standard deviation in the PF originates from statistics in the fibre or pulp treatment before the paper-making process.

To illustrate the variability of the fibre samples, Fig. 9d) shows an example where the stiffer top layer is absent (phenomenon is also present in other profiles). This variability is due to the character of the fibres as a natural product. Differences in the fibres’ properties can be due to the growth process or the extraction and further processing of the fibres from the cotton boll/paper sheet. However, all depth profiles (Fig. 5) are drawn at regions of interest, where the whole layer structure seemed to be intact.

Furthermore, the influence of the cantilever tip system geometry was probed. An HSC and an HAR cantilever were used to investigate the indentation behaviour. The setpoint for both cantilevers was 3000 nN. A higher indentation depth was reached with the HSC cantilever. The difference between the two indentation depths was $18 \pm 6\%$. It was interpreted that with the HAR cantilever, the cantilever could not displace as much volume with its lower spring constant as the HSC cantilever. But, in all measurements, the desired indentation depth was reached.

Figure S5 shows that the force-volume mapping method does not leave an imprint on the fibre surface in the scanned area with both types of cantilevers in the dry and wet states.
Moreover, considering the shown force-distance curves with the $\delta_2$ and $\delta$ fit in figure S1-S4 the choice of the tip geometry seems to play a role in analysing the measurements. The local fits of $\delta_2$ and $\delta$ (images b), c) for HSC cantilevers and d), e) for HAR cantilevers) are shown in the black curves. The blue points are the recorded indentation points for the applied forces. In every fibre type and dry and wet state the local fits of the HAR cantilever seemed not as accurate as for the HSC cantilever, especially at the contact region between tip and sample surface. There it is visible that the fits in the contact region of the HAR cantilever lead to a higher stiffness. As stated in [17] sharp tips such as the HAR cantilever could lead to a plastic deformation, which could lead to the measurement of a stiff surface layer. As we also detected this stiff layer on the fibre surface with the HSC cantilever, we assume that the hard layer is present on the fibre surface. However, the choice of the cantilever-tip system should be the HSC.

4 Conclusion

We discuss an AFM-based method to assess the intrinsic mechanical properties of cellulosic fibres (processed linters and unprocessed cotton). With the local analysis of recorded static force-distance curves, we were able to measure the local elastic moduli at the fibre surface and 950 nm beneath the surface. This approach was used to investigate how fibre or pulp treatment before paper making affects the mechanical properties of cotton linter fibres at the surface.

In a combined 3D representation, topographic features could be directly related to the local mechanical properties. Dry fibres had a stiffer outer layer (higher $E_{\text{local}}(z)$) on the fibre surface with softer material (lower $E_{\text{local}}(z)$) beneath. The surface of UPF was stiffer than that of the processed fibres. For hydrated fibres, however, differences in the stiffness profile between the processed and unprocessed stiffnesses were found. The maximum indentation depth was much higher for the processed fibres than for the unprocessed fibres. This observation can be explained assuming that during the fibre or pulp treatment process, the C layer and perhaps the P wall were affected in the milling process. Thus, the processed fibres exhibited a lower $E_{\text{local}}(z)$ layer on the fibre surface than the unprocessed fibres where the wall structure was intact. Assuming that intact wax-containing cuticle (C) and primary cell walls (P) serve as protection against intruding water molecules explains why the mechanical properties of the unprocessed fibre were less affected by hydration than those of the linters.

These findings are supplemented by microscopic observations. On the one hand, the data indicate that the P wall is missing in PF, as the external pectin signal is missing but the primary wall would have a high proportion of it. On the other hand, they show that the fibre wall in PF is less clearly layered than in UPF, as the signal intensity peaks are much closer together and at the same time broader than in UPF. Hence, processing not only removes the P wall but also affects the clear stratification and orderly structure of the fibre wall. Compaction, in turn, may result if a structurally challenged wall swells and is then dried during the paper-making process.

To investigate the influence of tip and cantilever properties on the results, data obtained with (relatively soft) cantilevers with high-aspect-ratio tips were compared to data obtained with a high spring constant cantilever with a standard tip. With both approaches, i.e., either using stiff cantilevers or using high aspect ratio tips, an indentation depth of a minimum of 300 nm could be achieved, and the mechanical stiffness was probed as a function of indentation depth. A higher maximum indentation depth was achieved using the hard cantilever HSC.

The subsurface imaging method proved to be a valuable tool for surface near depth-sensitive mapping of the local stiffness of cellulosic fibres. Local, i.e., lateral and vertical variations in the mechanical properties could be investigated and related to features of the fibre wall. Furthermore, the recovering behaviour of the layered wall structure from the hydrated to the dry condition and the potential fatigue could be investigated with the presented AFM-based method.

In the long term, cellulose is the only renewable raw material that is directly available in sufficient quantities and does not have to be produced from other raw materials. Furthermore, plant-based cellulose e.g. from cotton is of high research interest as wood-based fibres increased in price drastically. Thus, the application in the most diverse areas will certainly increase. Possible applications include the use of paper as a construction material especially in packaging, but also in architecture. Above all, however, for applications where papers are already used as so-called wet-strength papers, an understanding of fibre stability is of crucial interest. The chemicals (wet-strength agents) for increasing the wet-strength of paper must be replaced by biodegradable alternatives. A characterisation of the fibre stability is therefore a central component in order to be able to use paper as a sustainable material in the future.

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Authors' contributions JA wrote the manuscript and conducted all AFM measurements and analysis. ML performed the confocal fluorescence microscopy imaging measurements. TK performed the SEM measurements. J-LS prepared the paper sheets and helped with knowledge about paper fibres. TM, MB and RWS planned and supervised the whole project and are responsible for any correspondence. All authors contributed to the writing of the manuscript.

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Data Availability Additional material is available in the supplementary information.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethical approval The manuscript is not submitted elsewhere and is an original research. There are no animal and human studies involved in this research. The authors have no conflicts of interest to declare.

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