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Helical assembly of cellulose elementary fibrils in virgin spruce S₁ layer

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ABSTRACT. The ultrastructural organization of cellulose elementary fibrils (EF) in wood cell wall is considered to be the prime factor regulating the material characteristics of wood in micro to macro levels and the conversion of delignified wood fibers into various products. Specifically, the complex assembly of EFs in wood cell wall limits its swellability, solubility and reactivity *e.g.*, in dissolution of cellulose for regeneration of textile fibers, fibril separation for the manufacture of nanocellulose, and enzymatic hydrolysis of cellulose into sugars for their subsequent fermentation to various products, like ethanol for future fossil fuels replacement. Here cryo-transmission electron tomography was applied on ultrathin wood sections to reveal the EF assembly in the native cell wall. The resolution of these tomograms was then further enhanced by computational means. Direct visualization of EFs shows that they are both curved and bundled. Remarkably, EFs are observed to be often assembled into helical bundles similar to steel wire rope, a structural feature that must have a significant impact on the swelling, accessibility and solubility of woody biomass for its conversion into the aforementioned value added products.

KEYWORDS. cellulose microfibril, computational modelling, conifer, transmission electron tomography, wood cell wall

INTRODUCTION

Wood is a porous natural material composed of tissues of differentiated cells, especially cellulosic fibers, glued together by a cementing agent, lignin. The xylem is highly organized in several hierarchic levels from the tree, down to molecular level and it is these hierarchical differences that are of particular importance when selecting raw materials for wood based industries. Cellulose - the principal component of wood cell wall - is a linear chain of β -D-glucopyranose units linked together by 1,4-bonds and these cellulose chains assemble into elementary fibrils¹ (EFs, also known as microfibrils²) to form the skeleton of wood cell walls.

The cell wall is built up of several layers, namely: middle lamellae, primary wall (P), and the S₁, S₂ and S₃ layers of the secondary wall. These layers differ from each other with respect to their structure and chemical composition and thus play different roles in the processing of woody biomass. Due to the complex hierarchical structures of cellulose fibers, a heterogeneous swelling and dissolution can be observed along the fibers in various solvents.³ The most spectacular effect of this heterogeneous swelling is the ballooning phenomenon, in which swelling takes place in specific regions giving a bead-like appearance along the fibers (see micrograph in **Figure 1a**). The outer layers of the cell wall, P and S₁, play an important role in the unusual swelling behavior of wood fibers – it has been stated that the former forms collars surrounding the balloons, while the later forms the membrane of the balloons as it slowly dissolves.⁴ However, the mode of swelling and dissolution of cellulose depends on the solvent system, *e.g.*, the water content in mixtures of *N*-methylmorpholine-*N*-oxide (NMMO) and water.⁵ The dissolution of the primary wall is inefficient even in good cellulose solvent systems and the reason for the restricted solubility of the swollen S₁ layer remains unknown.⁶ Thus, understanding the structural difference between the cell wall layers could provide a better insight on this important issue. Also many other studies have suggested that the fiber ultrastructure along with solvent quality has the most impact on the swelling and

dissolution process^{3,4} although so far, the ultrastructure of the wood cell wall outer layers has not been studied in detail due to their small width.

Figure 1. Swelling and dissolution of cellulose in solvents. (a) The ballooning of wood fibers occurs along the fibers prior to dissolution in most cellulose solvents – it has been stated that the primary wall restricts the swelling by forming a collar surrounding the fiber, while the S_1 layer forms membrane of the balloons.⁴ Micrograph reprinted with permission. © 2015 Singh P, Duarte H, Alves L, Antunes F, Le Moigne N, Dormanns J, Duchemin B, Staiger MP, Medronho B.

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<http://dx.doi.org/10.5772/61402> (Pictures were taken by NLM at CEMEF - MINES ParisTech). (b)

A speculative description on restricted solubility of cellulose. Generally, cellulose elementary fibrils (EF) are dissolved in cellulose solvents, but the intact helical bundles of EFs in wood cell wall would not support this scheme directly. These bundles may need additional treatments e.g., enzymatic hydrolysis to lower the degree of polymerization (DP) before their dissolution in solvents – a recent study showed that cellulase treatments of pulps significantly increased their alkali solubility.⁸

The organization of EFs and matrix materials (lignin and hemicelluloses) has long been the subject of investigation due to its importance in various applications of wood. Nevertheless, while aggregates of EFs and matrix materials have been previously reported in chemically or physically altered cell walls,⁹ little information is available on this topic in the native cell wall as the compact arrangement of the cell wall polymers poses significant challenges to the observation of their ultrastructural assembly in native wood. As a result, aggregates of EFs and the associated matrix materials are usually investigated using a range of techniques (like atomic force microscopy, transmission electron microscopy, X-ray methods) on less lignified immature cells, delignified fibers or decayed wood as the substrate.¹⁰⁻¹² Likewise, non-plant sources are also often preferred to study the structure and properties of cellulose.¹³

Moreover, the information on wood ultrastructure given by different two-dimensional imaging techniques may vary depending on the methods of study.^{14, 15} In contrast, use of transmission electron tomography, an established technique for high-resolution three-dimensional (3D) imaging, is able to provide comprehensive information on wood cell wall ultrastructure.^{10, 16} Mathematical modelling has also become a powerful, complementary analytical technique to study the structure and behavior of cellulose in various environments.^{16, 17} In a recent study, applying transmission electron microscopy on ultrathin wood sections, we observed that EFs form an out-of-plane angle with respect to the longitudinal cell axis.¹⁸ Here, following our previous work, transmission electron tomography and mathematical modelling have been applied to observe nanoscale geometries and assembly of EFs in native cell walls. Thus, this work can be viewed as a deeper analysis of wood cell wall ultrastructure employing few more cutting-edge techniques.

EXPERIMENTAL SECTION

Sample preparation. In order to extract high-resolution information on the tracheid wall, a disk of Norway spruce wood was collected at a height of ~1.3 m from a ca. 40 years old tree originating from Ruotsinkylä in Southern Finland. Cubes ($3 \times 5 \times 10 \text{ mm}^3$) of latewood were prepared without embedding in resin before sectioning. Ultrathin sections of ~100 or 150 nm were cut from transverse and radial longitudinal wood surfaces (**Figure 2**) at cryogenic temperature (-40 °C) with a diamond knife on a Leica EM FC7 ultramicrotome. A fuller description of sectioning can be found in Reza *et al.*¹⁸ Grids with sections were post-stained for 30 min with 1% aqueous KMnO_4 to selectively stain for lignin followed by drying at room temperature.

Figure 2. Schematic diagram of a piece of wood shows different wood surfaces. Sectioning was performed on the transverse and radial longitudinal surfaces.

Acquiring tilt series. Nine sets of single-axis tilt series of transverse and radial longitudinal sections were acquired from -63° to +63° at 3° angular increment using SerialEM¹⁹ software at a pixel size of ~0.45 nm (unbinned) or ~0.9 nm (binned 2x). A TEM image is included in

Supplementary Figure 1 showing the location of tomography. Micrographs were recorded with a Gatan Ultrascan 4000 CCD camera on a cryo-TEM (JEOL JEM-3200FSC) at an accelerating

voltage of 300 kV. The images were taken in bright-field mode and using zero loss energy filtering (Omega type) with a slit width of 20 eV. Low-dose mode of the acquisition software was used during the data collection. Specimen temperature was maintained at -187 °C during imaging.

Tomogram assembly and visualization. Tilt series were aligned by tracking 25-35 gold markers (~15 nm) with IMOD software package.²⁰ Tomograms were reconstructed from the tilt series using the Simultaneous Iterative Reconstruction Technique (SIRT) within IMOD and with 10 iterations. Finally, tomographic volumes were visualized with volume viewer plugin of ImageJ.²¹ Gaussian filtering within UCSF-Chimera was applied to reduce the noise to some extent.²² In order to avoid the effect of sectioning on wood structure²³ tomographic slices were captured from the middle part of the tomograms.

Computational modelling. Tomographic subvolumes were imported and displayed in Matlab R2015a (The Mathworks, USA) using functions adapted from the PEET software package.²⁴ In total 81 subvolumes, of which 57 represented S₁ layer areas with EF bundles, were selected for mathematical analysis. The tomographic density in these subvolumes was fitted with a geometric model for the individual EFs, using an algorithm based on the one by Ciesielski *et al.*¹⁶ and modified to make it suitable for native wood. In such model, which is fully explained in the Supporting Information, EFs are approximated by parametric space curves of the form:

$$\mathbf{h}(t) \equiv \begin{cases} x = c_1 \sin(\omega t + \varphi) + P_x(t) \\ y = c_2 \cos(\omega t + \varphi) + P_y(t) \\ z = t \end{cases} \quad (1)$$

where $\mathbf{h}(t)$ is an xyz triple that defines the point of a space curve $\mathbf{h}$ at the parameter value of t . The trigonometric terms in the equation describe a helical geometry with x and y amplitudes c_1 and c_2 , respectively, angular frequency ω and phase shift φ . The terms $P_x(t)$ and $P_y(t)$ are polynomials that confer the flexibility to bend and adapt to the fibril structure observed in the particular tomographic

volume. The algorithm optimizes the parameters of equation 1 to place the space curves in the regions with highest relative density. The optimization was carried out in two phases: first, a Particle Swarm Optimizer²⁵ scanned the whole subvolume to find the best location of the initial curve, whose shape parameters were manually set to reasonable values; second, the exact EF geometry was obtained by optimizing the remaining parameters with a simplex method.²⁶ Once an optimal space curve was found, the tomographic density within 6 nm of the curve was removed from the dataset, a new curve was added and the whole process was repeated until a suitable number of curves were placed. In order to verify the consistency of results, most of the considered subvolumes overlapped each other and fits that did not match in the overlapping region were discarded.

RESULTS AND DISCUSSION

In the transmission electron micrographs, cellulose elementary fibrils (EF) are visualized by virtue of the associated matrix materials since cellulose is left unstained by the staining chemicals. A mantle of hemicelluloses may be present at the periphery of EFs as KMnO_4 does not fully stain hemicelluloses.²⁷

In this study, the structure and organization of EFs in Norway spruce wood cell wall is visualized in high-resolution transmission electron tomograms. 3D tomograms of the tracheid S_1 layer show that EF structures run parallel to each other and are transversely oriented (**Figure 3b**). An almost rectangular EF angle with respect to the tracheid longitudinal axis was observed in the S_1 layer corresponding to a previous study on the same tree species.²⁸ Frequently, bundles of EFs are visible in the tomograms (**Figure 4b**), although the exact arrangement of fibrils in the bundles is difficult to visualize without further analysis because of the compact association of cell wall components.

The tomographic slices obtained from the secondary wall clearly show the fibrillar structure of the wood cell wall, however the modelling of such a structure via manual segmentation methods²⁰ is a challenge due to the dense association of EFs and matrix materials. To address this challenge, the tomographic density was fitted with the geometric model described in the previous section, which is capable of extracting the unique, nanoscale geometry of individual fibrils in the tomograms and provide a more quantitative structural description. **Figures 3 and 4** show the application of such method to tomographic subvolumes extracted from various locations within the cell wall. In the S_1 layer regions containing fibrous structures (**Figure 3b,d** and **Supplementary Figure 2a**), EFs run parallel to each other, while a denser arrangement can be seen in the fibril bundle region (**Figure 4b,c**). The observed nanoscale geometries of the EFs in native cell walls can be described as curved and bundled over the length scales used here.

Figure 3. Tomography of ultrathin wood sections followed by fitting of the subvolumes with parametric space curves show nanostructural assembly of cellulose elementary fibrils (EF). (a) A schematic of tracheids depicting cell wall layering; CML compound middle lamellae (combined primary wall and middle lamella). (b) Tomographic slice of the transverse section, where fibrous structure can be seen in the secondary wall. The color bar represents the tomographic density. Scale bar is 50 nm. (c) A tomographic slice through a subvolume extracted from b (white box) containing fibrous structure. (d) The resultant fitted space curves show the nanoscale geometry of EFs; plot units are nm.

In some regions, such as the one presented in **Figure 4** and **Supplementary Figure 2b-f**, helical bundles of EFs were observed where several twisted EFs are coiled up whilst embedded in the matrix materials. Remarkably, all the bundles showed a right-handed helical twist, which is in line with previous theoretical and experimental studies.²⁹⁻³² Helical structures are ubiquitous in nature, especially in plantae. Scientists have also made many synthetic efforts to mimic this fascinating morphology of the helices because of their unique shape and material properties, *e.g.* elasticity. An analogy for the helical EF bundles would be steel wire rope, where individual helical strands assemble into helical bundles and rope. Cellulose EFs, helically oriented in the wood cell wall, are also thought to have helicoidal twisting along their longitudinal direction (presented later in this article and also speculated in previous papers³³). EF bundles were observed in the S₂ layer of the secondary wall in several investigations^{7, 10, 15} - a positive correlation between the size of bundles and degree of lignification was reported.³⁴ In addition the presence of hemicelluloses has also been reported in the EF structures.³⁵ The role of matrix materials (*i.e.*, lignin and hemicelluloses) in EF aggregation in pulps has been studied extensively.^{36, 37}

The compact arrangement of the principal lignocellulose components (cellulose, hemicelluloses and lignin) poses a serious technical challenge to their accessibility and thus various approaches have been presented in an attempt to enhance the reachability to some extent.³⁸ Nevertheless, the presence of helical bundles in native cell wall, outlined in this paper, will underline the need to further tailor the existing processes in order to extract cell wall materials more efficiently for value added products. The presence of helical bundles in the outer layers of the cell wall must hinder the dissolution of cellulose fibers in the solvents as shown in **Figure 1b**. In order to overcome this difficulty cellulose fibers may require additional pretreatments, such as enzymatic hydrolysis, to reduce the degree of polymerization (DP) of cellulose prior to its dissolution. It has been reported that cellulose with lower DP dissolves faster in a particular solvent than the cellulose fibers with

higher DP.³ A recent study by Kamppuri *et al.*⁸ showed that cellulase treatments of pulps diminished the ballooning phenomenon and significantly increased the alkali solubility of cellulose. Nevertheless, the ultrastructural origin of the balloon membrane and the collar shown in **Figure 1a** still remains unclear.³⁹

Mathematical analysis of the tomographic subvolumes provided quantitative data on the helicoidal twisting of individual EFs. A mean period of about 321 (± 8) nm was observed between the helical twists in the EFs outside the bundles, which was, however, 371 (± 55) nm for fibrils forming helical bundles. This means that an EF shows a 90° twist over ~80-90 nm interval depending on where it resides. The observed periodicity of the helices seemingly corresponds to the range shown in the micrographs of cellulose nanofibrils (CNF) from various sources.²⁹ Nevertheless, these results need to be corroborated by fitting the space curves over larger subvolumes, a calculation that requires improvements to the algorithm. The convergence of the algorithm within the tomographic subvolumes is visualized in **Supplementary Videos**. The nearest neighbor distance in the subvolumes was also calculated for the fitted curves. The average spacing (11 ± 1 nm) between the parallel EFs is almost same in different subvolumes, but is significantly different than those reported for heavily pre-treated (thermochemical) corn stover.¹⁶

Figure 4. Tomography of ultrathin wood sections followed by fitting of the subvolumes with parametric space curves show nanostructural assembly of cellulose elementary fibrils (EF). (a) A schematic of the tracheids that depicts cell wall layering; CML compound middle lamellae. (b) A tomographic slice shows the arrangement of EF bundles in the S_1 transverse section. The color bar represents the tomographic density. Scale bar is 50 nm. (c) A tomographic slice through the subvolume of a bundle extracted from b (white box). (d,e) The resultant fitted space curves are shown in two different angles below the tomographic slice where a helical bundle of tightly arranged EFs can be seen. (f) Same subvolume without tomographic density shows the geometry of EFs; plot units are nm.

Further investigation of the formation mechanism of such helical bundles would be required to understand the supramolecular arrangement of the cellulose in relation to the other cell wall components. It is known that cellulose chains are synthesized in the plasma membrane by cellulose synthase (CesA) complexes, so called *rosette*, before their arrangement into EFs. Cortical microtubules are assumed to guide the deposition of EFs in the plasma membrane although the exact mechanism still remains unclear.⁴⁰ The findings of this research initiate a number of new

questions as to the formation of helical bundles in the developing cell wall that include: – Is there a rosette of rosettes involved in helical bundle formation? How do these rosettes synchronize? How do cortical microtubules guide such a number of CesA complexes?

CONCLUSIONS

To summarize, transmission electron tomography combined with mathematical modelling of nanoscale geometry of cellulose elementary fibrils (EF) showed the detailed structure and orientation of EFs in the S₁ layer. The orientation of EFs in this layer was not always the same. Direct visualization of EFs showed that they are bundled and not straight. Moreover, EFs were observed to be often assembled into helical bundles like steel wire ropes, a structural feature that is believed to have significant impact on the swelling, accessibility and solubility of woody biomass for its conversion into the value added products. The connection between the directly observed, ultrastructural assembly of EFs and recalcitrance in biomass processing will be further informed and validated by analyzing tomographic volumes acquired from processed (biochemically, chemically and mechanically) wood fibers. Much work remains in order to achieve complete understanding of the structural difference between different layers in native cell wall, which will be obtained by applying these analytical methods to rest of the cell walls, especially thick S₂ layer. Nevertheless, the ultrastructural assembly of cellulose in one of the outer layers presented in this work constitutes a step towards a better understanding of the biomass recalcitrance during fiber deconstruction process.

Supporting Information

This material contains low magnification TEM micrograph of spruce transverse section, mathematical details of the fitting algorithm and calculation of Nearest-Neighbor distances, fitted

subvolumes, and videos showing the convergence of the fitting for the subvolumes in Figures 3 and 4. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

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Author Contributions

M.R. planned the experiment, performed sectioning and staining, acquired tilt series, reconstructed the tilt series, analyzed the tomograms and wrote the preliminary manuscript. C.B. performed the mathematical modelling and analysis. J.R. and T.V. supervised the work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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