Chapter 1

The Nanostructures of Native Celluloses, Their Transformations upon Isolation, and Their Implications for Production of Nanocelluloses

Rajai H. Atalla,[*,1,2] Rowan S. Atalla,[1] and Umesh P. Agarwal[3]

[1]Cellulose Sciences International, 510 Charmany Drive, Ste. 259, Madison, Wisconsin 53719, United States
[2]University of Wisconsin, Madison, Wisconsin 53706, United States
[3]Forest Products Laboratory, USDA Forest Service, 1 Gifford Pinchot Drive, Madison, Wisconsin 53726, United States
[*]E-mail: rhatalla@wisc.edu.

Native celluloses in plant cell walls occur in a variety of highly periodic fibrillar forms that have curvature and varying degrees of twist about their longitudinal axes. Though X-ray measurements reveal diffraction patterns, the celluloses are not crystalline in the traditional sense. The diffraction patterns rather are a consequence of the high degree of spatial periodicity inherent in the fibrils and in their hierarchic organization at different levels in the cell walls. Upon dehydration and exposure to elevated temperatures, the fibrils tend to collapse into linearly parallel structures that are not unlike traditional crystals. However, in alleviating the inherent twist of the fibrils during formation of linear domains, less tightly ordered terminal points are formed at the connection between adjacent linear domains. These domains are less tightly aggregated than the linear domains between which they occur, and are therefore more readily accessible to hydrolytic agents than the tightly aggregated linear domains between which they occur. Hydrolysis at the less tightly aggregated points transforms the tightly aggregated linear domains between them into nanocellulose particles. In consequence nanocelluloses isolated from different native forms, are likely to have structures that are as much a function of the isolation history as they are

Agarwal et al.; Nanocelluloses: Their Preparation, Properties, and Applications
ACS Symposium Series; American Chemical Society: Washington, DC, 2017.

of the native source. We present data reflecting the effects of different thermal histories on the states of aggregation of cellulose. We also present data that confirm a previously proposed model, which we believe to be representative of nanoscale transformations of higher plant celluloses that occur upon dehydration and exposure to elevated temperatures. We anticipate that they provide a basis for understanding observations made upon preparation of nanocelluloses from higher plant tissues.

Introduction

Nanocelluloses are unique among nanoscale substances in that they can only be prepared from isolated native celluloses, which are primary structural constituents in hierarchically organized biological tissues. The molecular chemical structure of celluloses has been accepted as the homopolymer of β-(1→4) linked anhydroglucose units since the early 1930s (*1*). However, the patterns of aggregation of cellulose molecules in the hierarchic structures of native biological tissues remain the subject of controversy. Early structural studies led to the evolution of paradigms that paralleled those that are useful in studies of synthetic polymers, and these have been helpful in advancing the technologies of traditional cellulose based industries. These include among others, cotton and bast fiber textiles, pulp and paper, and the cellophane and rayon industries.

An important factor in the evolution of paradigms regarding crystallinity of cellulose was that X-ray diffractometry was widely applied in characterizing celluloses at a time when electron microscopy was in its infancy. This led to adoption of the fringed micelle model for the nanostructure of celluloses and it was presented in many of the classics of plant science (*2*, *3*). The fringed micelle model implies that cellulose fibrils, in their native state, can be represented by alternating domains one of which is nanocrystalline while the other is disordered. Applicability of this model was further encouraged in the pulp and paper industry by observations of a leveling off degree of polymerization (LODP) when most pulps were subjected to dilute acid hydrolysis.

The early structural studies were influenced by observation of diffraction patterns from a variety of isolated celluloses as well as cellulose containing substances. This led to the introduction of the notion that celluloses were crystalline in their native state. With the development of new investigative methods, such as electron and atomic force microscopies and new spectroscopic methods that are sensitive to variations in nanoscale molecular organization, it became clear that native cellulose fibrils are more complex substructures within hierarchically organized biological tissues. These structures are highly periodic and thus capable of producing diffraction patterns. However, both electron and atomic force microscopies and newer computational modeling methods have revealed that the highly ordered fibrillar cellulosic substructures have curvature and an inherent twist. Furthermore, the periodicity of the twist appears to be inverse to the diameter of the fibrillary substructures. The departures from linear

translational symmetry suggest departures from classical definitions of crystalline order.

It is important to note that the curvature and periodic twist do not prevent the formation of diffraction patterns, because the organization of the monomeric entities remains highly periodic. However, the curvature of the lattice structures results in broadening of the diffraction patterns. Historically, the broadening has been interpreted in terms variations in the parameters of the fringed micellar structure in a manner not unlike concepts developed to describe synthetic polymers. Thus, the broadening was assumed to reflect the occurrence of amorphous domains. This led to the micellar model of celluloses noted above, embraced in the fifth and sixth decade of the past century. This primitive model of native celluloses remains in use in some of the plant science literature and in applied research related to the technologies of established cellulose based industries.

The occurrence of curvature of the fibrils, the twist and its periodicity noted above, have led Atalla et al. (*4*) to propose that they are the key to formation of nanocrystalline domains upon exposure to elevated temperatures. Elevated temperatures are always used during separation of most celluloses from the other constituents of the biological tissues within which they are embedded. The formation of these domains is accompanied by development of small linkage regions within which the cellulosic molecules are not as tightly packed and thus are much more susceptible to penetration by hydrolytic agents. The proposal was *presented as a speculative one* at the time because experimental observation of nanoscale phenomenology of celluloses had not yet advanced to a level that allowed direct validation.

In the present report, we first outline some key observations regarding native celluloses. We then review recent experimental observations of celluloses that are isolated with minimal disruption of the native state and that therefore retain both the curvature and the periodicity. We will then describe in summary experiments that provide evidence of alteration of the native state during treatments similar to those carried out in the course of most typical procedures for isolation of native celluloses from their native sources. We will also present evidence that supports the proposal more directly demonstrating that exposure to elevated temperatures are prerequisites to the formation of nanocelluloses from plant feed stocks. Finally, we will present again the model that we believe to be the best representation of the manner in which native celluloses are transformed into nanocrystalline domains that can then be hydrolyzed to result in the formation of nanocrystalline celluloses (CNC).

Native Celluloses

Here we first review evidence regarding the native state with a focus on microfibrils. Perhaps the most informative observations from a quantitative perspective are those of Haigler regarding celluloses formed by the bacterium *Acetobacter xylinum* (*5*) and those of Hanley *et al*. regarding the alga *Micrasterias denticulata* (*6*). In both instances a long period helical form is observed.

In Haigler's pioneering work on the structures of bacterial celluloses from *Acetobacter xylinum* and their response to different perturbations of their growth environment (*5*) an example of which is shown in Figure 1, a curvature of the fibrils and a period of about 600 nm was consistently observed. The period of about 600 nm for celluloses formed by *Acetobacter xylinum* has also been reported by Hirai *et al.* (*7*).

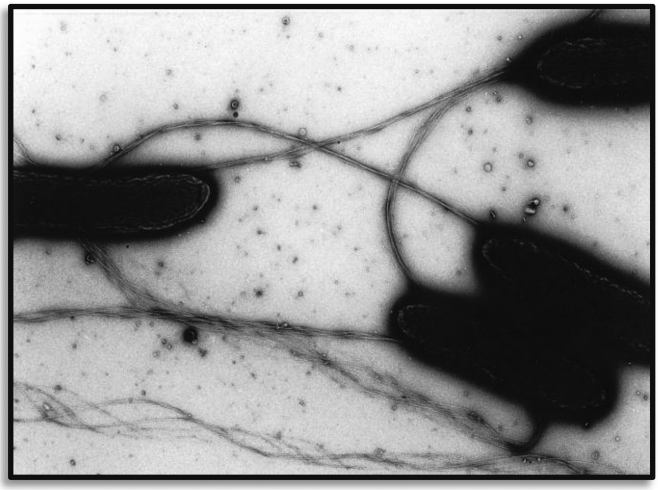

Figure 1. Cellulose formed by Acetobacter xylinum, which normally is formed as a ribbon, is revealed to consist of fibrillar substructures, when it is grown in a medium that has components that can inhibit aggregation of the constituent subfibrils. Each of the subfibrils is an aggregate of cellulose molecular chains. The image reveals the inherent tendency of subfibrils to develop curvature and a long period twist. Adapted from (5). Copyright 1991, with permission of Taylor and Francis Group.

In the other landmark study, Hanley *et al.* (*6*) investigated microfibrils of the alga *Micrasterias denticulate.* It was of particular interest because its fibrils have a rectangular cross section. A micrograph of *Micrasterias denticulata* provides an excellent demonstration of the regularity of the periodicity. It is shown in Figure 2, where the period of about 1200 nm is evident. Panel A shows the micrograph of the fibril as it appeared on a substrate for examination by electron microscopy, while panel B provides the researchers' rationalization of the appearance of linear segments connected by highly deformed segments within which the 180° turning occurs. The linear segments are about 600 nm each, so that a helical twist of 180° occurs over 600 nm, and two linear segments totaling about 1200 nm corresponding to the full period wherein a complete turn of 360° occurs.

A key point to be emphasized here is that the long period of the helical biological structure appears to depend on lateral dimensions. The lateral dimensions of *Micrasterias denticulata* fibrils are approximately 10 x 20 nm, while the lateral dimensions of bacterial cellulose microfibrils are approximately 6 to 7 nm. By extrapolation, it would seem to follow that the period of nanofibrils

in higher plant cell walls, which have lateral dimensions of the order of 3 to 5 nm, would be less than 600 nm.

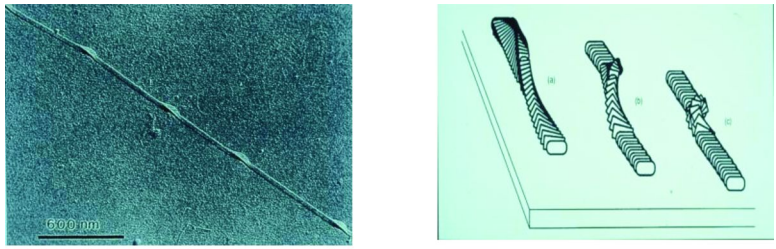

Figure 2. A fibril of Micrasterias denticulata as observed in panel A, and the rationalization of the appearance of the dehydrated sample (B) proposed by the authors. Adapted from (6). Copyright 1997 with permission of Springer Science.

The speculation regarding the period of higher plant fibrils has been confirmed by the recent comprehensive molecular modeling studies carried out for hydrated aggregates of cellulose molecules (*8*). It is also confirmed by Atomic Force Microscopic images of maize parenchyma cell walls shown in Figure 3.

Here one sees that the fibrils appear to vary in width as indicated at the arrows. The variation in width is indicative of an ellipsoidal cross-section as suggested by the ellipsoidal polyhedral model proposed by Ding and Himmel (*9*). The separation of the narrower sections along an individual fibril appears to be of the order of 200 to 250 nm. The lateral dimensions of the ellipsoidal polyhedron are approximately 3 x 5 nm.

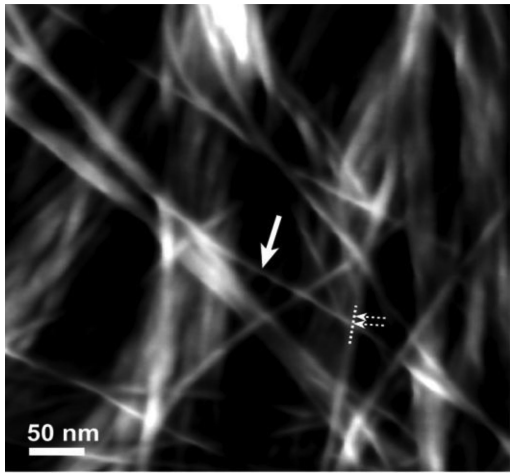

Figure 3. An AFM image of the surface of maize parenchyma cell. The large arrow shows a single microfibril at its narrow point. The two smaller arrows show where it is atop another microfiber. Measurement of the elevation provides an approximate dimension. Adapted from (9). Copyright 2007 with permission of American Assn for Advancement of Science.

In a more recent study Agarwal *et al.* have reported a number of observations of native wood that are not consistent with occurrence of crystalline domains within constitutive celluloses (*10*).

Before proceeding to discuss the effects of elevated temperatures and dehydration of native celluloses it is helpful to consider why the observations reported above negate the proposal that celluloses in their native state possess a crystallinity in the classical sense. It is helpful here to consider the illustrations in Figure 4. In panel A, we see scale representations of fibrils of different celluloses if their nanocrystalline domains were typical crystalline domains in the classical sense. In panel B, we see a qualitative representation of how these domains would appear if they possessed the inherent twist that has been shown above to occur in native cellulose microfibrils. For convenience, the period of the twist has been set at 1200 nm for all irrespective of their lateral dimensions.

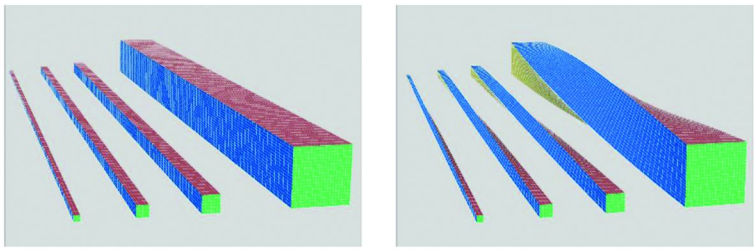

Figure 4. Geometric representation of nanofibrils that are 2x2, 4x4, 6x6, and 20x20 nm in cross-section. In panel A they are shown as they would be if translational symmetry in three directions prevailed in a Cartesian space. In panel B, they are shown as possessing a long period of 1200 nm as implicit in Figure 2, although for simplicity the length is limited to 300 nm corresponding to a 90° twist. (see color insert)

The key point to be made here is that both structures represented in panels A and B would give rise to a diffraction patterns because of their high degrees of periodicity. The central difference is that diffraction patterns from structures represented by panel A can definitely be interpreted in terms of the methods of classical crystallography. The coordinates of the atoms in such a lattice can clearly be described in terms of the linear axes of a Cartesian space and as such, they can be used to define atomic coordinates within a unit cell. The whole crystal can then be defined in terms of translation symmetry along three linear axes. Such linear translational symmetry is the prerequisite for construction of a reciprocal lattice that can allow the application of the method of classical crystallography to determine a crystal structure.

On the other hand, the structures represented in panel B lack linear axes and thus cannot be defined on the basis of linear translations of the unit cell. Thus, they cannot be interpreted using the methods of classical crystallography as has been common in published crystallographic structures. Published

crystallographic structures of celluloses, typically derived from diffraction patterns from microfibrils with very long periods, *are at best approximations* and often must rely on questionable assumptions because, as in all polymer crystallographic analyses (*11*), the data typically correspond to only 20% to 25% of the amount necessary for determination of atomic coordinates of a definitive structure. Thus, the *traditional attributes of crystalline substances cannot be associated with cellulose*.

Transformations during Isolation

Most studies described in the vast literature on cellulose science and technology have been based on celluloses isolated from plant tissues of different sources. In all but very few cases, the purification of the celluloses involved exposure to elevated temperatures. The vast majority that are used in commerce have been isolated at elevated temperatures. And because it has generally been assumed that the crystallinity is preexisting in the native state, the possibility of transformation during isolation has received very little attention. *This can no longer remain the case in the context of nanocellulose science, particularly when the sources of nanocelluloses are complex biological structures.*

We present here the results of two studies that leave little question that exposure to elevated temperatures as well as dehydration result in significant changes in the nanoscale states of aggregation of native celluloses during isolation. The first, is based on a study of wood delignified at ambient temperature, and the decline in its accessibility to deuterium exchange upon exposure to elevated temperatures and upon air drying at ambient temperature. The second is based on a demonstration that nanocellulose cannot be prepared from wood unless it is first exposed to elevated temperatures for an extended period.

Elevated Temperatures and Transformations in Accessibility

While there have been numerous studies (*12–14*) related to the aggregation of cellulose fibrils upon hydrothermal treatments observations are not directly related to nanoscale architecture of the celluloses except in a speculative manner.

We present in summary the results of a previously published assessment of the effects of elevated temperatures on celluloses initially isolated at ambient temperature (*15*). Additional past studies of temperature effects had started with celluloses isolated at elevated temperatures and assessed effects of further exposure to elevated temperatures usually well above 100° C (*16–20*); thus, they began with celluloses already transformed in the process of isolation.

Our study was based on cellulose isolated by delignification at ambient temperature and pressure, with the control sample never allowed to be dehydrated. The data provided evidence indicating significant decline in its accessibility to deuterium exchange and other probe molecules upon mild exposure to elevated temperatures and upon drying at ambient temperature. We present here only the results of the deuterium exchange observations.

The key departure of our experiments from prior work is that the celluloses we investigated were isolated entirely at room temperature. Quaking aspen (*Populus tremuloides*) sapwood was delignified at room temperature (21° C). The choice of sapwood was because it is freshly formed and the clear majority of cellulose would be from the secondary walls, the formation of which begins with deposition of cellulose microfibrils followed by hemicelluloses that are adsorbed on the surface and become the matrix within which lignin is polymerized (*21*).

The process required 8 weeks and was a variation on a procedure by Thompson and Kaustinen (*22*). The delignified wood was extracted with aqueous 4% NaOH for 72 hours to remove hemicelluloses then filtered and washed.

Fibers consisting of whole cell walls were kept in water at all times. One sample was heated to 100° C, held at that temperature for 30 minutes, then allowed to cool. Another was rinsed in acetone to remove water held by capillary action between fibers and within lumens then allowed to dry in air at ambient temperature.

The next stage was exchange of hydrogen on cellulosic hydroxy groups with deuterium. Deuterium exchange in combination with vibrational spectroscopy to probe accessibility of hydroxy groups has a long history (*23*). Our goal was to establish whether cellulose underwent a degree of denaturation upon dehydration during isolation.

A sample of pulp held at room temperature, a pulp cooked at 100° C and one air dried from acetone were immersed in D_2O, centrifuged at 4000 rpm, then placed in an incubator, with agitation at 800 rpm and room temperature, and held overnight to insure diffusion of D_2O into lumens and secondary walls. Next day the D_2O was separated through filtration, fresh D_2O added, and the procedure repeated three times for a total of four exchanges with fresh D_2O, each for a minimum of 24 hours at 800 rpm.

Samples were separated through filtration formed into pellets and to avoid exchange with atmospheric moisture, each pellet was immediately mounted on a slide and covered with D_2O and a cover slip. Slides were mounted on the stage of an Xplora Raman microscope. Spectra in the CH stretching (2800 cm^{-1} to 3100cm^{-1}) and OH stretching (3150 cm^{-1} to 3750 cm^{-1}) regions were recorded using 532 nm laser excitation and are shown in Figure 5. The methine CH stretching band at 2900 cm^{-1} is used as an internal standard.

Though the shapes of OH bands are very similar, the total number of inaccessible OH groups in the cooked sample appears approximately three times those in the sample held at ambient temperature.

As the sample boiled in water was never allowed to stand without water or D_2O, the decline in accessibility to D_2O is clear evidence of an irreversible change. The cellulose is no longer in its native form. Investigations of celluloses as they enter into life processes of plants can no longer ignore the reality of such irreversible changes; the natural hydration of biological macromolecules in living systems has been altered irreversibly in these celluloses.

In addition to the effect of temperature elevation, Figure 5 shows the effects of dehydration at room temperature, a matter ignored in most laboratory preparations. The center (red) spectrum is that of room temperature delignified pulp dried in air at room temperature. The change in this spectrum when compared to that of the never-dried sample (blue) indicates that air drying also reduces accessibility to

reagents. This sample had been rinsed in acetone prior to dehydration. Had it been dried directly from water the reduction of accessible hydroxy groups would have been greater. This spectrum monitors interlamellar and intermicrofibrillar adhesion within individual fibers at the nanoscale level while avoiding interfiber adhesion at the microscale; the latter phenomenon is at the heart of papermaking as it is the key to cohesion of sheets of paper made from pulp fibers.

The bands at approximately 2950 cm^{-1} and 2970 cm^{-1} are assigned to the antisymmetric CH stretching vibrations of the methylene group on C6. This observation is consistent with occurrence of two different bands in the HCH bending region at approximately 1455 cm^{-1} and 1475 cm^{-1}. These confirm occurrence of two nonequivalent orientations of methylene groups *vis a vis* the pyranose rings (*24*, *25*). Spectra in the region between 200 and 1700 cm^{-1} are presented in (*15*).

It is to be noted here that the spectrum of the never dried and never heated sample also reflects the presence of hydroxy groups that are inaccessible to exchange. This observation is consistent with evidence presented by Agarwal et al. (*10*) suggesting the presence of domains within native cellulose fibrils that are inaccessible to exchange while other adjacent ones are accessible.

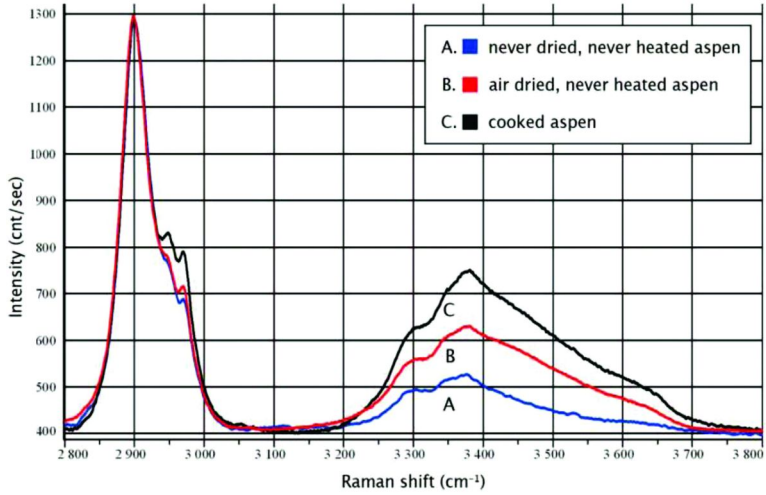

Figure 5. Raman spectra in the CH and OH stretching regions from fibers isolated at room temperature (A), from fibers that were first air dried (B) and from fibers boiled for 30 minutes (C). All spectra are recorded for the fibers immersed in D_2O. Adapted from (15). Copyright 2014 Elsevier. (see color insert)

Raman spectral studies similar to those based on deuterium exchange were also carried out using ethylene glycol as a probe molecule. It was observed again that the sample never exposed to elevated temperatures was more deeply penetrated than the one exposed to an elevated temperature.

In addition to the Raman spectral studies, the change in accessibility upon exposure to elevated temperature was tested by application of Graff's C-Stain (*26*). This stain contains polyiodide anions that can form charge transfer complexes with

ordered (1,4)-linked glucans to produce a dark blue color most often observed with starch (*27*, *28*). Upon application of the stain to the room temperature sample it produced a dark blue color. The sample exposed to an elevated temperature, in this instance 120° C in water, remained yellowish gray. This indicates that the room temperature sample of cellulose was accessible to I_{13}^- and I_{15}^- anions, whereas the sample heated to 120° C was not.

Taken together the explorations of native celluloses with probe molecules and with Graff's C-Stain, point to tighter coalescence of the nanofibrils upon exposure to elevated temperatures or dehydration. It is likely that the hydration of nanofibrils is significantly diminished upon first exposure to elevated temperature, resulting in tighter aggregation and crystallization of the cellulose substructures/molecules. We regard this transformation as a form of denaturation of celluloses.

The other study that supported reduction of accessibility to water upon high temperature treatment was based on the decline in the increase of intensity of the 1380 cm^{-1} Raman band in the OH-to-OD exchanged sample of wood cellulose that was heated to 170° C. As shown in Table 1, the thermal treatment resulted in a 47% decline in accessibility to water.

Table 1. Accessibility of wood cellulose to water

Sample	*% Increase in intensity at 1380 cm^{-1}*
Never-dried wood cellulose	75.0*
Wood cellulose heated to 170 °C	39.9**
% Decline	46.8

* Ref. (*10*). ** Agarwal (*29*).

We note here that the above two studies, though they clearly reflect denaturation of the cellulose, they do not represent direct evidence regarding the formation of crystalline nancellulosic domains. Rather they are consistent with their formation.

Studies of Cellulose Nanocrystals Formation from Wood

Two other investigations of the role of elevated temperatures in nucleation of crystalline nanodomains in wood were undertaken. Here samples of wood were subjected to the processes for preparation of cellulosic nanocrystalline domains both directly, without any prior processing to isolate the cellulose, as well as after the wood had been held at elevated temperatures similar to those used in most industrial processes. This was carried out with both softwoods and hardwoods (*10*). Here we report only a result on samples of poplar.

Two portions of the Wiley-milled wood were subjected to the standard hydrolytic protocol for preparation of nanocrystalline celluloses described in greater detail elsewhere (*10*). They involve exposure to 64% H_2SO_4 at 45° C for 90 minutes. The first sample was subjected to the protocol without additional

pretreatment. The second had its temperature increased up to 170° C in a Parr reactor over a 90 minutes' period, then it was held at that temperature for an additional 60 minutes.

The finding was that *no part of the wood survived the hydrolytic process* when the Wiley-milled wood was hydrolyzed without any pretreatment, while in the case of the heat-treated wood, *a crystalline nanocellulose very similar to that usually formed from kraft pulps was formed.*

In Figure 6 we compare a Raman spectrum of the cellulose nanocrystals (CNC) prepared from the heat-treated wood with a Raman spectrum of a CNC prepared from a kraft pulp. It is to be noted that the Raman band at 93 cm^{-1}, which has been shown to be typical of highly crystalline celluloses (*10*) is at least as prominent in the spectrum of the CNC sample prepared from the poplar exposed to elevated temperature as it is in the spectrum of the CNC sample prepared from the bleached kraft pulp. Correlation of the 93 cm^{-1} band in Raman spectroscopy with crystallinity data previously obtained using the 380-Raman method (*29*) is shown in Figure 7. For the calibration set samples, the (I_{93}/I_{1096}) ratio and 380-Raman crystallinities were correlated in Figure 7. An excellent correlation was obtained ($R^2 = 0.9968$).

We would note also that the investigation reported in (*10*) included a similar experiment on wood samples delignified at 70° C and then subjected to the protocol for conversion to CNC, both with and without prior exposure to elevated temperatures similar to those described above. Here again only the samples that had been exposed to 60 minutes at 170° C resulted in the formation of CNCs.

The results of this study leave little question that exposure to elevated temperatures, 120° C or higher, is a prerequisite for nucleation of nanocrystalline domains in cellulosic fibrils in higher plant tissues. The exposure to such elevated temperatures denatures the cellulose, that is, it is irreversibly transformed into a form wherein there are domains that are sufficiently more tightly aggregated and crystalline that their accessibility to hydrolytic agents is dramatically reduced.

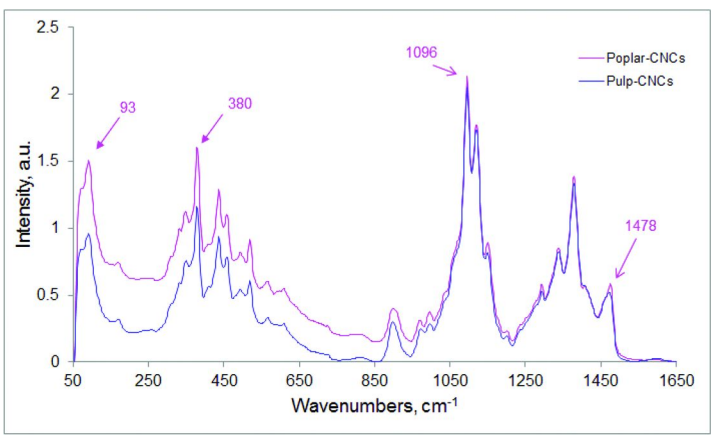

Figure 6. Comparison of Raman spectra of CNCs prepared from kraft pulp and from Wiley-milled poplar sapwood after heat treatment at 170° C for 60 minutes. (see color insert)

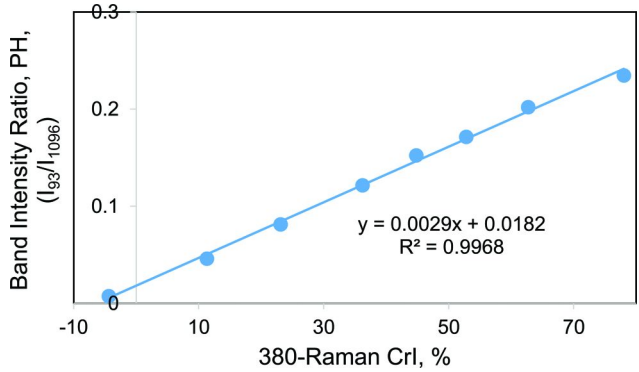

Figure 7. Correlation of (I_{93}/I_{1096}) ratio to 380-Raman method estimated crystallinities for the calibration set samples (30).

We note here that it has been argued in a frequently cited publication (*31*) that the disordered regions that are susceptible to hydrolysis are preexisting domains in ramie fibers. The outline of the experimental protocol described in the subject study clearly indicates that the sample of ramie fiber was obtained from a fiber merchant. It is unfortunate that in investigations of the structural aspects of native celluloses, many have started with commercially available fiber samples. This has been true of common cellulosic fibers such as cotton, ramie and other fibers from the bast family. It is not widely recognized that commercial processing always begins with boiling the native tissue in caustic solutions at elevated temperatures under pressure, in order to remove other polysaccharides as well as traces of other cell wall constituents that co-exist with the cellulose in plant cell walls.

While common laboratory procedures for purifying native celluloses typically rely on boiling in 1% NaOH under nitrogen for about 12 hours, in commercial practice higher temperatures at elevated pressures are usually used to accelerate the process.

These preparative procedures induce the same types of tight aggregation of cellulose nanodomains as are suggested by our own investigation outlined above. It is for this reason that we believe that studies of celluloses in the context of plant science directed at understanding the native state, can no longer assume that the published crystallographic structures, which are based on diffractometric studies of isolated celluloses, can be the basis for understanding the native state of celluloses in plant cell walls.

Mechanism of Formation of Crystalline Nanodomains

It is clear from the information reported in many chapters in this symposium that some nanocrystalline domains of celluloses can survive the severe hydrolytic conditions prerequisite for the preparation of CNCs. These conditions are capable of hydrolyzing the linkages between the anhydropyranose units that occur in domains that are accessible while not disrupting others that remain within the CNCs produced. Much of the evidence compels the conclusion that when the

CNCs are formed from higher plant native celluloses, the history of the source material must include significant exposure to elevated temperatures.

Yet the studies of accessibility based on deuterium exchange described above, also point to the existence of domains that are inaccessible to hydrolytic agents even in samples that have never been exposed to elevated temperatures. These domains are much smaller than those that become inaccessible after exposure to elevated temperatures, but there is little question that they do occur in the native state.

In order to reconcile these observations one must conclude that a limited subset of the hydroxy groups that occur in the native state are within domains that are tight enough to exclude deuterium exchange even prior to exposure to any elevated temperatures during isolation. On this basis it is plausible to argue that the most elementary subfibrillar structures may be sufficiently tightly aggregated to exclude deuterium exchange. They may be aggregates from synthase complexes that are directly adjacent to each other such that the fibrils can aggregate immediately after formation. In some of the pioneering work of Haigler on bacterial celluloses (*5*), wherein she was examining substructures kept apart by modifying agents, she did observe substructures that appeared to be 1.5 nm in diameter. In other plants the most elementary substructures may be up to 2 or 3 nm in diameter. These most basic of substructures can be thought to be the primary building elements of the hierarchically organized lamella of plant cell walls, with the hierarchic organization specific to the particular tissue and species.

It is anticipated that in the native state these most elementary of substructures are likely to be hydrated at their surfaces to facilitate their movement relative to each other as the plant tissue is subjected to deformation of any form. But it must be postulated that their interiors, though very limited in size, are likely not accessible to deuterium exchange. Only such a postulate can explain the inaccessible hydroxy groups that remain inaccessible in sample A in Figure 5. And it is plausible that exposure to elevated temperatures, or to other forms of dehydration, can diminish the degree of surface hydration and result in enlargement of the tightly aggregated domains that are inaccessible to deuterium exchange.

In the example shown in Figure 5, the sample exposed to elevated temperature had the highest degree of inaccessibility, suggesting a higher degree of aggregation. However, it is also clear the sample simply dried at room temperature also declined in accessibility, though to a lesser degree.

In an effort to provide a visual representation of what we believe to be occurring, we have developed Figure 8 to depict the mechanism we believe may be unfolding to create the nanodomains that can become CNCs. We emphasize again here that our model has been developed for the transformation of cellulosic fibrils in higher plants upon exposure to elevated temperatures.

In Figure 8, **A** is intended to be representative of the native state. The scaling is rather arbitrary. We have assumed an elementary fibril that is about 4 nm in diameter, and we have depicted a bundle of nine such fibrils associated together as they would be if they were produced by adjacent synthases on the plasma membrane. The long period of the twist has been borrowed from the observations of a 1200 nm period for the *Micrasterias denticulate* in the work by Professor Gray and his associates (*6*). To be sure the period of the twist is likely to be

shorter, but this was chosen for ease of depiction. It should be noted that here again only 300 nm of length are depicted, so that the total twist represented is only 90°.

The subsequent clusters, **A′**, **A″** and **A‴** are intended to depict progressive degrees of aggregation. **A′** depicts 35% aggregation, **A″** depicts 65% aggregation and **A‴** depicts 85% aggregation. 85% was chosen as the upper limit because by many classical measures of crystallinity in kraft pulps it is thought to be the degree of crystallinity.

While it is not as obvious in **A′** and **A″**, it becomes obvious in **A‴** that the type of aggregation into linear domains, wherein the chains are parallel, does result in the occurrence of periodic domains wherein the chains cannot be very tightly packed and are instead twisted. We believe these domains are the most likely sites of action of hydrolytic agents used in the production of CNCs.

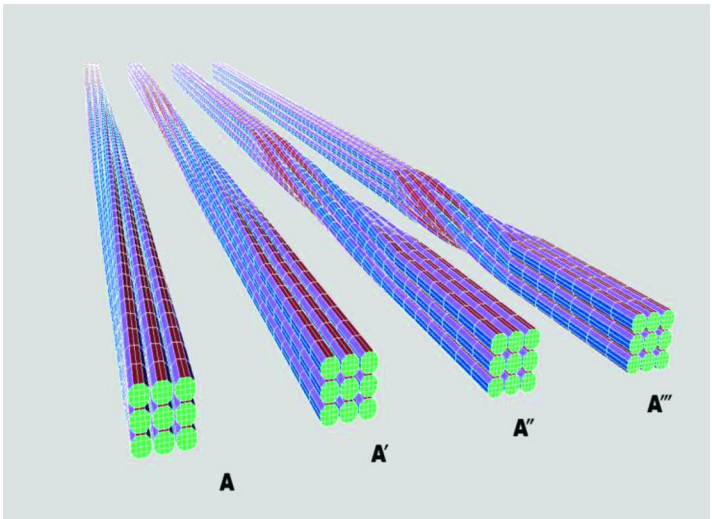

*Figure 8. Clusters of adjacent fibrils at different levels of aggregation in a native cellulose. As in Figure 4, the diameter of the most elementary fibril has been scaled to be 4 nm. In A, the fibrils are depicted as having a long-term twist with a period of 1200 nm though only 300 nm are depicted. In addition, it is assumed that the aggregate of 9 fibrils also has a twist with a period of 1200 nm. These are arbitrary but helpful in the visual depiction. As noted in the text, **A′**, **A″** and **A‴** represent increasing degrees of aggregation. Adapted from (15). Copyright 2014 Elsevier. (see color insert)*

With respect to the two sets of results above, in relation to Figure 8, we believe that the celluloses subjected air drying or to heating followed by deuterium exchange would fall in the domains between **A′** and **A″** and though more tightly aggregated than the undehydrated sample, they would not result in the formation of CNCs if subjected to the usual preparative protocols. On the other hand, we both of the samples shown in Figure 6, that is the CNC from kraft pulp and the CNC

from the wood heated to 170° C, are very likely the consequence of aggregation at the level shown in **A‴**.

In an effort to depict more clearly how we believe the disordered domains are formed we have added Figure 9 wherein only four fibrils are shown together. It becomes clear how an ordered linearly parallel domain can arise from compacting fibrils that in their native state are highly aligned as if in the most elementary thread of a rope designed to carry a significant load with very little opportunity for shear stress to develop when the thread is subjected to a tensile load. From a classical mechanical perspective, the arrangement depicted as A can carry a tensile load with little development of shear stress, while the arrangement depicted as **A′** is expected to have shear stresses develop in the tightly aggregated linear parallel parts of the structure.

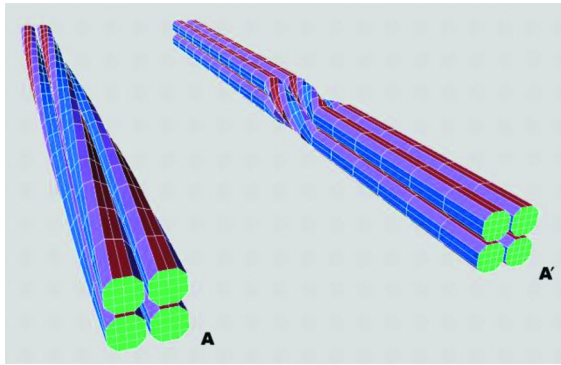

*Figure 9. Depiction of four adjacent elementary fibrils, **A**, as they are envisioned to be in the native state in the context of our proposed model and, **A′**, the consequence of their coming together in much tighter aggregation as a result of dehydration. This may be the result of dehydration upon drying or upon exposure to elevated temperatures. (see color insert)*

It is our view that native celluloses in higher plants are highly hydrated at the surfaces for lubrication so they can respond to mechanical stress in a manner similar to the strands of a steel cable.

In this context, it is important to note also that the changes in the interaction of water with celluloses cannot be attributed solely to the eccentricities of cellulose, although its structure does impart to it some unique behavioral characteristics. But water also has some unusual characteristics that change as temperatures are elevated. At temperatures up to 40° to 45° C it occurs as short lived ice-like clusters of four or five water molecules with lifetimes in the 10^{-9} to 10^{-10} second range. However, at temperatures above 70° C it is predominantly monomolecular. This may be an important factor in decline of its capacity to hydrate cellulose at elevated temperatures. Very few living systems can survive such elevated temperatures.

Concluding Remarks

We have summarized above some observations of changes in the accessibility of celluloses during their isolation. They appear to be in two categories. One is the result of dehydration after separation from other cell wall constituents even at relatively mild temperatures. The other, seems to be specific to isolation at elevated temperatures, typically used in commercial processes.

Our findings suggest that simple dehydration, while resulting in reduction in accessibility to deuterium exchange, does not seem to alter the nanoscale state of aggregation sufficiently to create domains resistant to the hydrolytic processes that occur during the production of CNCs.

On the other hand, isolation at elevated temperatures, particularly for extended periods, does result in a much tighter aggregation that makes nanoscale semicrystalline domains inaccessible to the hydrolytic agents. Simultaneously, as a consequence of the inherent curvature and twist in the native fibrils, small domains of loosely aggregated cellulose chains are created. These latter domains appear to be the loci of action of hydrolytic agents. The resulting breaks leave behind the highly ordered domains that are the CNCs.

We have presented a graphical representation of the manner in which we believe that the native celluloses are transformed during the different processes. The images we have created are of course hypothetical. But we believe they capture the subtle differences in the manners in which native celluloses can be transformed during isolation. Our observations also reflect the sensitivity of the transformation to the combination of stages selected for isolation of celluloses from their native sources.

Acknowledgments

We wish to acknowledge partial support of this work by the USDA Forest Service Forest Products Laboratory and Cellulose Sciences International; the BioEnergy Science Center (BESC) funded by the DOE Office of Biological and Environmental Research (BER) under the Genomes to Life (GTL) program; the DOE Office of Science ASCR SciDAC program; and the Wisconsin Energy Independence Fund for a grant for purchase of the Xplora Raman Micro spectrometer.

References

1. Purves, C. B. Historical Survey. In *Cellulose and Cellulose Derivatives*, Part 1; Ott, E., Spurlin, H. M., Graflin, M. W., Eds.; Wiley: New York, 1954; pp 29−53.
2. Preston, R. D. *The Physical Biology of Plant Cell Walls*; Chapman and Hall: London, 1974.
3. Frey-Wyssling, A. *The Plant Cell Wall*; Gebruder Borntrager: Berlin, 1976.
4. Atalla, R. H., Brady, J. W., Mathews, J. F., Ding, S.-Y., Himmel, M. E. Structure of Plant Cell Walls. In *Biomass Recalcitrance: Deconstructing the*

Plant Cell Wall for Bioenergy; Himmel, M. E., Ed.; Blackwell Publishing, 2008; pp 188−212.

5. Haigler, C. H. Relationship between Polymerization and Crystallization in Microfibril Biogenesis. In *Biosynthesis and Biodegradation of Cellulose*; Haigler, C. H., Weimer, P. J., Eds.; Marcel Dekker: New York, 1991; pp 99−124.
6. Hanley, S. J.; Revol, J.-F.; Godbout, L.; Grey, D. G. Atomic force microscopy and transmission electron microscopy of cellulose from *Micrasterias denticulate*: Evidence for a chiral helical microfibril twist. *Cellulose* **1997**, *4*, 209–220.
7. Hirai, A.; Tsuji, M.; Horii, F. Helical sense of ribbon assemblies and splayed microfibrils of bacterial cellulose. *Sen'i Gakkaishi* **1998**, *54*, 506–510.
8. Matthews, J. F.; Skopec, C. E.; Mason, P. E.; Zuccato, P.; Torget, R. W.; Sugiyama, J.; Himmel, M. E.; Brady, J. W. Computer simulation studies of microcrystalline cellulose I. *Carbohydr. Res.* **2006**, *341*, 138–152.
9. Himmel, M. E.; Ding, S.-Y.; Johnson, K. Y; Adney, W. S.; Nimlos, M. R.; Brady, J. W.; Foust, T. D. Biomass recalcitrance: Engineering plants and enzymes for biofuels production. *Science* **2007**, *315*, 804–807.
10. Agarwal, U. P.; Ralph, S. A.; Reiner, R. S.; Baez, C. Probing crystallinity of never-dried wood cellulose with Raman spectroscopy. *Cellulose* **2016**, *23*, 125–144.
11. Kakudo, M., Kasai. N., Procedure for Structural Analysis of X-ray Data. In *X-ray Diffraction by Polymers*; Elsevier: New York, 1972, pp 285−286.
12. Larsson, P. T.; Hult, E.-L.; Wickholm, K.; Pettersson, E.; Iversen, T. CP/MAS ^{13}C-NMR spectroscopy applied to structure and interaction studies on cellulose I. *Solid State Nucl. Magn. Reson.* **1999**, *15*, 31–41.
13. Duchesne, I.; Hult, E-L.; Molin, U.; Daniel, G.; Iversen, T.; Lennholm, H. The influence of hemicellulose on fibril aggregation of kraft pulp fibres as revealed by FE-SEM and CP/MAS 13C-NMR. *Cellulose* **2001**, *8*, 103–111.
14. Fahlén, J.; Salmén, L. Pore and matrix distribution in the fiber wall revealed by atomic force microscopy and image analysis. *Biomacromolecules* **2005**, *6*, 433–438.
15. Atalla, R. S.; Crowley, M. F.; Himmel, M. E.; Atalla, R. H. Irreversible transformations of native celluloses, upon exposure to elevated temperatures. *Carbohydr. Polym.* **2014**, *100*, 2–8.
16. Atalla, R. H.; Nagel, S. C. Cellulose: Its regeneration in the native lattice. *Science* **1974**, *185*, 522–523.
17. Atalla, R. H.; Whitmore, R. E. The influence of elevated temperatures on structure in the isolation of native cellulose. *J. Poly. Sci.: Pol. Lett. Ed.* **1978**, *16*, 601–605.
18. Atalla, R. H.; Ellis, J. D.; Schroeder, L. R. Some effects of elevated temperatures on the structure of cellulose and its transformation. *J. Wood Chem. Technol.* **1984**, *4*, 465–482.
19. Hofstetter, K.; Hinterstoisser, B.; Salmen, L. Moisture uptake in native cellulose – The roles of different hydrogen bonds: a dynamic FT-IR study using deuterium exchange. *Cellulose* **2006**, *13*, 131–145.

20. Suchy, M.; Kontturi, E.; Vuorinen, T. Impact of drying on wood ultrastructure: Similarities in cell wall alteration between native wood and isolated wood-based fibers. *Biomacromolecules* **2010**, *11*, 2161–2168.
21. Terashima, N., Fukushima, K., He, L.-F., Takabe, K., Comprehensive Model of the Lignified Plant Cell Wall. In *Forage Cell Wall Structure and Digestibility*; Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; American Society of Agronomy: Madison, WI, 1993: pp 247−270.
22. Thompson, N. S.; Kaustinen, O. A. Some chemical and physical properties of pulps prepared by mild oxidative action. *Tappi* **1964**, *47*, 157–162.
23. Mann, J., Deuteration and Tritiation. In *Cellulose and Cellulose Derivatives, Part. IV*; Bikales, N. M., Segal, L.; Eds.; Wiley-Interscience: New York, 1971; pp 89−116.
24. Wiley, J. H.; Atalla, R. H. Band assignments in the Raman spectra of celluloses. *Carbohydr. Res.* **1987**, *160*, 113–129.
25. Wiley, J., Atalla, R. H. Raman Spectra of Celluloses. In *The Structures of Cellulose*; Atalla, R. H., Ed.; ACS Syposium Series 340; American Chemical Society: Washington, DC, 1989; pp 151_168.
26. Isenberg, I. H. *Pulp and Paper Microscopy*; Institute of Paper Chemistry: Appleton, WI, 1967; p 232. http://smartech.gatech.edu/handle/1853/11673 (accessed 8/28/2017.
27. Yu, X.; Houtman, C.; Atalla, R. H. The complex of amylose and iodine. *Carbohydr. Res.* **1996**, *292*, 129–141.
28. Yu, X.; Atalla, R. H. The complex of xylan and iodine: the induction and detection of nanoscale order. *Carbohydr. Res.* **2005**, *340*, 981–988.
29. Agarwal, U. P. Unpublished results.
30. Agarwal, U. P.; Ralph, S. A.; Reiner, R. S. Cellulose I crystallinity determination using FT-Raman spectroscopy: Univariate and multivariate methods. *Cellulose* **2010**, *17*, 721–733.
31. Nishiyama, Y.; Kim, U.-J.; Kim, D.-Y.; Katsumata, K. S.; May, R. P.; Langan, P. Periodic disorder along ramie cellulose microfibrils. *Biomacromolecules* **2003**, *4*, 1013–1017(2003).