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Irreversible transformations of native celluloses, upon exposure to elevated temperatures

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ABSTRACT

Current research, basic and applied, assumes that observed recalcitrance of celluloses is an inherent characteristic associated with their state of aggregation in their native state; it is thought that processes of isolation remove other components of plant cell walls leaving the celluloses unchanged, even though elevated temperatures are routinely used during isolation. Since temperature elevation is known to influence the structures of all polymers, it is important to explore its influence on the character of isolated celluloses, almost always assumed to be still in their native state. Deuterium exchange is a measure of accessibility of reactive sites in celluloses. We report significant reduction in accessibility to deuterium and other probe molecules for celluloses isolated at ambient temperature and then exposed to elevated temperatures. Our results indicate that native celluloses, which are highly ordered biological structures, are irreversibly transformed and develop polymeric semi-crystalline character upon isolation at elevated temperatures.

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1. Introduction

In the context of national priorities assigned to production of cellulosic biofuels, barriers to conversion of cellulose to glucose have been examined (DOE, 2006; Himmel et al., 2007). Current efforts to develop biofuels from lignocellulosic feedstocks, including agricultural residues and short rotation crops focus on recalcitrance of cellulose assumed inherent to its native state. And in modern plant science it is common to regard isolation as removing other components of plant cell walls, leaving celluloses unchanged. Observed recalcitrance is attributed to an inherent micro-crystallinity limiting accessibility to hydrolytic reagents.

The questions motivating the present study were twofold. The first is whether the isolated celluloses are indeed in their native state and whether the processes of isolation using elevated temperatures modify them in a manner that must be taken into consideration when we seek to understand the function of cellulose *in planta* within the context of biological systems, particularly higher plants. The processes of biogenesis are diverse and in most instances the cellulose is intricately interwoven with other cell wall constituents including other cell wall polysaccharides and lignin. The second question is whether the natural recalcitrance of cellulose, which is part of the evolutionary adaptive response of living plant systems for defense against enzymatic attack by polysaccharide hydrolases produced by pathogens and herbivores, is further multiplied by the exposure to elevated temperatures during isolation. In summary then the questions are whether the types of nanoscale order traditionally attributed to isolated celluloses limit the range of questions that need to be asked in order to fully understand the biogenesis and roles of celluloses in higher plants and whether these processes of isolation make celluloses structurally different and more recalcitrant than they are in their native states.

The states of aggregation usually attributed to celluloses have been by and large rooted in early investigations using X-ray diffraction and borrows from paradigms developed for synthetic semicrystalline polymers. It has been incorporated in models for structures of plant cell walls since the post WW II era (Frey-Wyssling, 1976; Jones, 1971; Preston, 1974; Tonessen & Ellefsen, 1971) most of which predated modern electron microscopy. The models arose from investigations of celluloses isolated from plant tissues and purified at elevated temperatures. Wood celluloses are isolated through pulping, whereas those that are relatively pure in their native state are de-waxed by boiling in caustic solutions. The fundamental question then is the degree to

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which their nanoscale organization implicit in the accepted models approximates the native state.

2. Background

It is important to inquire whether celluloses in their native states, where they constitute highly ordered structural tissues intimately mixed with other constituents of cell walls in living organisms, have the same type of nanoscale organization as the semicrystalline nanodomains usually observed in semicrystalline synthetic polymers. In order to establish a basis for further discussion of the issues in question as well as our findings, it is useful to review current understanding of the crystallinity usually attributed to semicrystalline synthetic polymers because the procedures for published structural studies on cellulose have much in common with procedures used in investigations of structures of synthetic polymers in general.

At the outset we note that we are quite aware that celluloses in many native forms produce diffraction patterns when exposed to collimated beams of X-rays, electrons or neutrons. This has been known since the earliest studies of the effects of kiln drying on wood. However, the question arising for us is whether the structure of cellulose in its native state in higher plants, wherein it is blended with other cell wall constituents, including other cell wall polysaccharides as well as lignins and extractives can be understood as a crystalline material in the same sense as is the case with single crystals of smaller molecules. There has been much discussion of the structures of celluloses on the basis of crystallographic studies incorporating the single crystal paradigm (Frey-Wyssling, 1976; Gardner & Blackwell, 1974; Jones, 1971; Nishiyama, Langan, & Chanzy, 2002; Nishiyama, Sugiyama, Chanzy, & Langan, 2003; Preston, 1974; Sarko & Muggli, 1974; Tonessen & Ellefsen, 1971), however we view those discussions as not relevant to our questions. Our rationale is based on the fact that single crystals are usually defined as continuous homogeneous phases that at equilibrium are governed by thermodynamic criteria such as for example the phase rule. Furthermore, their states of aggregation are associated with intensive thermodynamic variable. For example all crystalline materials have a fixed melting point under ambient conditions and it is frequently used as a measure of their purity.

Polymeric materials in contrast are not homogeneous at the nanoscale level and their states of aggregation are determined as much by past history as by their molecular composition. This is well known in many synthetic polymers such as polyolefins and polyesters for which the melting points measured by dilatometry depend on the temperatures to which they have been cooled after exposure to temperatures that are sufficient to melt them completely.

Our view is that to advance understanding of the nature of native celluloses in living plants, it is important to explore their states of aggregation in the native state as well as the degree to which they may be transformed by manipulations similar to those routinely used during their isolation for further characterization. It is well known that even though cellulose cannot be heated to melting, the states of aggregation of isolated celluloses respond to elevated temperatures as for example in the preparation of cellulose IV by heating cellulose II in glycerol at 250 °C (Ellefsen & Tonnesen, 1971).

It is important to note here that all prior structural studies, carried out on a wide variety of celluloses, examined celluloses that had been isolated at elevated temperatures. We believe that isolation procedures are very likely to have altered the states of aggregation of celluloses at the nanoscale relative to what they are in their native state in the living plant while there have been some past studies of the influence of temperature on structures of celluloses, all have started with celluloses initially isolated at elevated temperatures, and usually explored effects of further exposure to elevated temperatures usually above 100 °C (Atalla & Nagel, 1974; Atalla & Whitmore, 1978; Atalla, Ellis, & Schroeder, 1984; Hofstetter & Hinterstoisser, 2006; Suchy, Kontturi, & Vuorinen, 2010); thus they began with celluloses already isolated from living plants and transformed in the process of isolation.

We describe in brief the preparative procedures used in some of the citations regarding the effects of temperature. In Atalla and Nagel (1974) cellulose dissolved in phosphoric acid was regenerated into glycerol at 165 °C and recognized to be cellulose I, later identified as the pure I_β form. In Atalla and Whitmore (1978), cellulose isolated from loblolly pine at 70 °C was post treated at 100 °C, 125 °C, 150 °C, and 175 °C. In Atalla et al. (1984), amorphous cellulose regenerated from an anhydrous organic solvent into an anhydrous regeneration medium was post treated at different temperatures in aqueous media; it is well to note that at temperatures in excess of 200 °C this procedure resulted in celluloses similar to those produced in the work of Atalla and Nagel. In Hofstetter and Hinterstoisser (2006) and Suchy et al. (2010), the starting materials were commercial pulps, usually exposed during isolation and purification to temperatures above 180 °C.

Published investigations of crystallinity in celluloses, based on the single crystal paradigm noted above have been consistently carried out with samples prepared by classical methods at elevated temperatures (Gardner & Blackwell, 1974; Nishiyama et al., 2002; Nishiyama, Kim, et al., 2003; Sarko & Muggli, 1974). These structures do not reflect native states in higher plants but rather states of aggregation modified by the particular isolation procedures, all of which involved extended exposure to temperatures above 100 °C.

3. Objectives

As noted above, the primary objective of our work was to explore effects of elevating the temperature of samples of cellulose that were in the first instance isolated at ambient conditions and then exposed to elevated temperature. We present data for cellulose isolated by delignification at ambient temperature and pressure and evidence for significant decline in its accessibility to deuterium exchange and other probe molecules upon mild exposure to elevated temperatures. We have observed a parallel decline in susceptibility to saccharification by fungal enzymes to be reported elsewhere. We believe that recalcitrance observed in isolated celluloses is significantly enhanced well beyond that inherent in native states of celluloses.

The nature of the native states is central to advancing understanding of cell walls in plant science as well as to the many ongoing research programs, both basic and applied, directed at the production of cellulosic biofuels.

Questions regarding native states are reinforced by recent theoretical analyses pointing to an inherent tendency for cellulose molecules to develop a right handed twist when they aggregate into nanofibrils at ambient temperatures (Matthews et al., 2006). These analyses of course confirm findings of electron microscopic observations (Haigler, 1991).

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 vast 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 (Terashima, Fukushima, He, & Takabe, 1993).

The process of isolation at ambient conditions required 8 weeks and was a variation on a procedure by Thompson and Kaustinen

(1964) for isolation of hemicelluloses from softwood species. Delignified wood was extracted with aqueous 4% NaOH for 72 h to remove hemicelluloses then filtered and washed.

4. Experimental program

4.1. Delignification

We focused on samples of cellulose isolated from quaking aspen (*P. tremuloides*) sapwood. As noted above the choice of sapwood was because it is the most freshly formed part of the wood. The majority of cellulose isolated will be from secondary walls. It has been shown that the sequence of formation of secondary walls in wood begins with deposition of cellulose microfibrils followed immediately by release of hemicelluloses that adsorb on the surface of microfibrils and become the interface with lignin, which is polymerized within the hemicelluloses matrix (Terashima et al., 1993). Thus the procedure used was intended to remove both lignin and hemicelluloses as gently as possible with the least possible perturbation of the cellulose in its native state.

The choice of aspen was in part because it is one of the species contemplated as a plantation source of biomass for fuels, and in part because it allowed the preparation of a significant amount of morphologically uniform fibers.

A sample of fresh sapwood was obtained from the Forest Products Laboratory, where it was also Wiley-milled to 40 mesh in a laboratory mill at ambient temperature. The raw, milled wood was delignified at room temperature (21 °C) using sodium chlorite (Fluka Analytical Grade) and acetic acid (Aldrich ACS Reagent Grade 97%+). The delignification took place over a period of 8 weeks, with weekly addition of sodium chlorite and acetic acid.

The procedure began with 20 g of Wiley-milled wood chips, on a dry basis. They were placed in a 1 L Erlenmeyer flask and 800 mL of water was added. The first chemical addition was 36 g of sodium chlorite. The pH was then adjusted to 4 with acetic acid. The temperature was maintained the ambient temperature of 21 °C. The Erlenmeyer flask was capped with a small upturned beaker to allow excess chlorine dioxide fumes to escape. The flask had a magnetic stirrer inserted and operated at 300 rpm. The addition of the same amount of sodium chlorite was repeated once a week, followed by adjustment of the pH to the value of pH 4 with acetic acid. Adequacy of this frequency of addition was indicated by the constant canary yellow color of the dispersion indicating that the solution was saturated with chlorine dioxide, the active oxidizing agent. All of these processes were of course carried out in an enclosed laboratory fume hood.

Upon completion of delignification, the delignified wood was first washed thoroughly to a neutral pH then extracted with an aqueous solution of 4% NaOH for 72 h at the ambient temperature of 21 °C to remove soluble hemicelluloses. This also was done within a 1 L Erlenmeyer flask at ambient temperature with agitation at 300 rpm. It was then filtered and washed with water to neutrality.

4.2. Exposure to elevated temperatures

Two samples of the resulting fiber were placed in water to prevent drying. This is an important point as in some of our prior work the fibers delignified at room temperature were allowed to dry in air prior to further study because it has generally been assumed that drying in air does not affect the states of aggregation. In the present study, the samples were not allowed to dry at any time. One of the samples was then placed in a 500 mL beaker with a magnetic stirring at 300 rpm and heated up to 100 °C within approximately 10 min. It was held at the boiling temperature for 30 min, and then allowed to cool to room temperature and kept wet; the other was simply kept wet. Those samples were used in the Raman spectral studies.

In a later experiment, a sample isolated at room temperature was heated for 2 h at 120 °C in a pressure cooker in a 1% solution of NaOH to simulate the much more common conditions used for purification and to approximate the low temperature end of pulping conditions usually used in preparation of many commercial samples of celluloses from cotton or wood pulps.

5. Raman spectral studies

Completion of the removal of lignin and hemicelluloses was confirmed on the basis of Raman spectra of the samples over the range between 200 and 1700 cm^{-1} presented in Fig. 1.

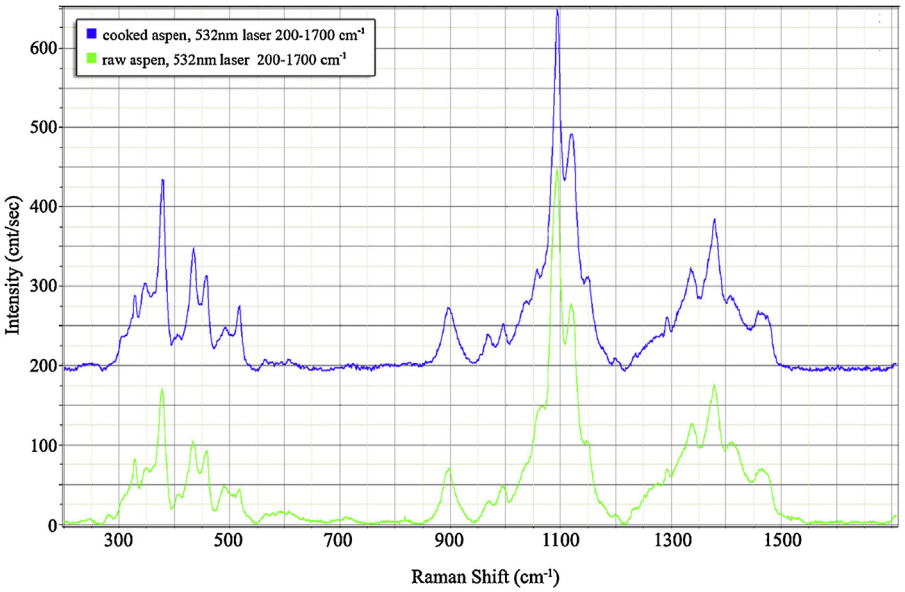

Fig. 1. The Raman spectra in the 200–1700 cm^{-1} region for the room temperature delignified pulp, termed raw, in green and the one exposed to 120 °C for 2 h, termed cooked, in blue. Both spectra were acquired with 532 nm laser excitation.

All Raman spectra were acquired with an Xplora Raman Microspectrometer manufactured by the Horiba Jobin-Yvon division. It is equipped for laser excitation at 532 nm and 785 nm. The samples in this series were all rinsed in and dried from acetone to avoid effects of interfiber adhesion on the spectra; when pulp fibers are dried from water surface tension effects cause the interfiber adhesion that is the key step in papermaking. The fibers were then pressed into pellets for acquisition of the spectra. The spectra in Fig. 1 were acquired with the 100× objective, using the 532 nm laser excitation line.

A number of features of these spectra are noteworthy. First is the absence of the symmetric ring stretching band at 1600 cm^{-1} that is characteristic of most lignins, indicating complete removal of the lignin. Next is absence of any bands that could be associated with the carboxyl groups of methyl glucuronic acid residues that are attached as side groups to angiosperm xylans; they are usually a composite of complex bands that overlap in the region between 1500 and 1700 cm^{-1}.

Comparison of the two spectra is also instructive. The bands in the spectrum of the cooked sample appear more resolved, reflecting the ordered coalescence of cellulose molecules within fibrils. Yet another interesting feature is the HCH bending vibration of the methylene group on C6 of the anhydroglucose units. For the raw sample the corresponding band is spread between 1445 and 1480 cm^{-1} suggesting considerable variation in the orientation of the primary hydroxyl group vis-a-vis the pyranose ring. In the spectrum of the cooked sample the band appears to begin developing a distribution between 1455 and 1475 cm^{-1}, the two characteristic frequencies observed in the spectra of highly crystalline samples such as those derived from the alga *Valonia ventricosa* and the tunicate celluloses.

Fibers consisting of whole cell walls were kept in water at all times. Some samples were heated to 100 °C or 120 °C, and allowed to cool. Another was kept wet, and yet another was rinsed in acetone to remove water held by capillary action between fibers and within lumens then allowed to dry in air.

The next stage was exchange of hydrogen on cellulosic hydroxyl groups with deuterium. Deuterium exchange in combination with vibrational spectroscopy to probe accessibility of hydroxyl groups has a long history (Mann, 1971). Deuterium exchange has also been used in studies of neutron scattering though all involved samples previously exposed to elevated temperatures (Nishiyama, Langan, & Chanzy, 2002; Nishiyama, Kim, et al., 2003; Nishiyama, Sugiyama, et al., 2003). Our goal was to establish whether cellulose underwent a significant modification of nanoscale character upon dehydration during isolation.

Samples of raw pulp, pulp cooked at 100 °C and raw pulp air dried from acetone were immersed in D_2O and 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 was repeated three times for a total of four exchanges with fresh D_2O, each for a minimum of 24 h 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. The spectra in the CH stretching (2800–3100 cm^{-1}) and OH stretching (3150–3750 cm^{-1}) regions were recorded using 532 nm laser excitation. The methine CH stretching band at 2900 cm^{-1} is used as an internal standard.

6. Results and discussion

The spectra of the samples subjected to D_2O exchange are shown in Fig. 2. The dramatic differences in accessibility of OH groups resulting from elevated temperatures are immediately obvious. 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. The OD stretching region of the spectra is not shown because it is not possible to discriminate between Raman scattering from OD groups within the structure of the celluloses and Raman scattering from D_2O within the lumens of the fibers or held between the fibers by capillary action.

It should be noted that even within the sample isolated at room temperature there were domains that remained inaccessible to D_2O

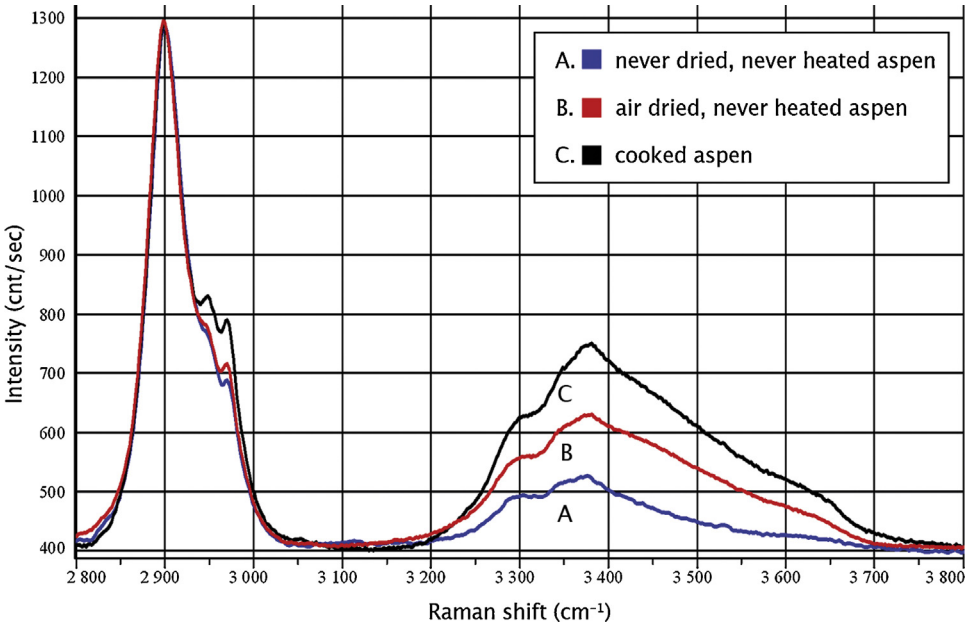

Fig. 2. Raman spectra in the CH and OH stretching regions from fibers subjected to exchange deuterium exchange with D_2O. (A) Fibers isolated at room temperature; (B) fibers air dried at room temperature; (C) fibers boiled for 30 min. All spectra are recorded for the fiber pellets immersed in D_2O.

reflecting a not insignificant level of tight aggregation in the native state likely associated with the evolutionary adaptive response intended to make the tissue less susceptible to the enzymatic attack agents of pathogens and herbivores.

Since 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, Fig. 2 shows 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 hydroxyl groups would have been greater. This spectrum monitors inter-lamellar and inter-microfibrillar 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 phenomenon of hornification arising from drying of pulp fibers has been known for generations in the context of papermaking craft and technology, and it is very likely a consequence of similar processes at the molecular level, it is not often addressed in discussions of preparative procedures for the isolation of celluloses from higher plants.

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 (Wiley & Atalla, 1987, 1989).

Raman spectral studies similar to those based on deuterium exchange were 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.

The challenge in using ethylene glycol as a probe molecule is that when pulp fibers are immersed in a liquid that has any polarity, given sufficient time, it can penetrate the structures of the primary and secondary walls to varying degrees, but it can also be retained within the lumens of fibers and between them by capillary action. Therefore, prior to preparing pellets for spectral examination they must be rinsed in a manner that allows removal of ethylene glycol both from the lumens and from the surfaces and domains of capillary action between the pulp fibers. However, the rinsing must be done in a manner that does not extract the ethylene glycol that has penetrated into the walls. After many trials, the approach finally adopted for acquiring the Raman spectra in Fig. 3 involved immersion of the samples in ethylene glycol for a period of six weeks to allow full penetration into the walls. This was followed by rinsing in acetone for a period of 90 s. The spectra were acquired with the 10× objective using the 785 nm laser excitation line.

The key observation in Fig. 3 is that the amount of ethylene glycol retained within the cell walls of the raw sample is considerably greater than the amount retained in the cell walls of the cooked sample. While it can be noted from a number of features that are characteristic of the spectrum of ethylene glycol it is most obvious in connection with the most intense band in the spectrum of ethylene glycol at approximately 865 cm^{-1}. The observation is facilitated by the fact that it is easily distinguishable from the band in the spectrum of cellulose at 898 cm^{-1}.

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 (Isenberg, 1967). The stain contains iodine dissolved in a solution of KI, together with significant amounts of $CaCl_2$, $AlCl_3$, and $ZnCl_2$; all of which can interact with β(1,4)-linked polysaccharides to facilitate association with the I_{13}^- and I_{15}^- poly anions responsible for the blue coloration when they form charge transfer complexes with the polysaccharides not unlike the complex that is formed with amylose in starch (Yu & Atalla, 2005; Yu, Houtman, & Atalla, 1996).

Upon application of the C-stain to the RT sample it produced a dark blue color. The sample exposed to an elevated temperature, in

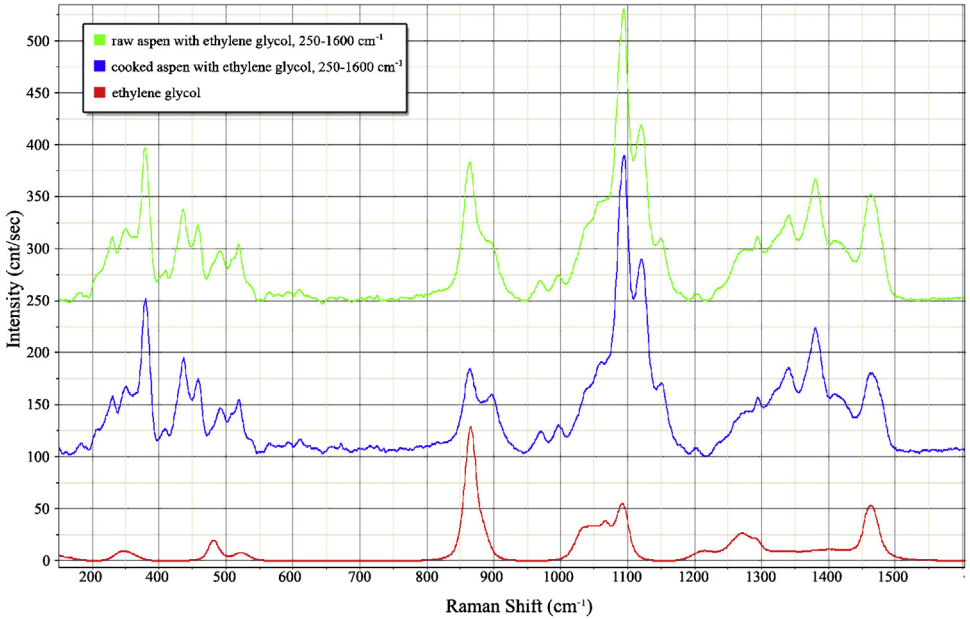

Fig. 3. Raman spectra of samples identical to those used for (A) and (C) in Fig. 2, after immersion in ethylene glycol for six weeks, and rinsing with acetone for 90 s. The raw is shown in green and the cooked shown in blue. The spectrum in red is that of pure ethylene glycol.

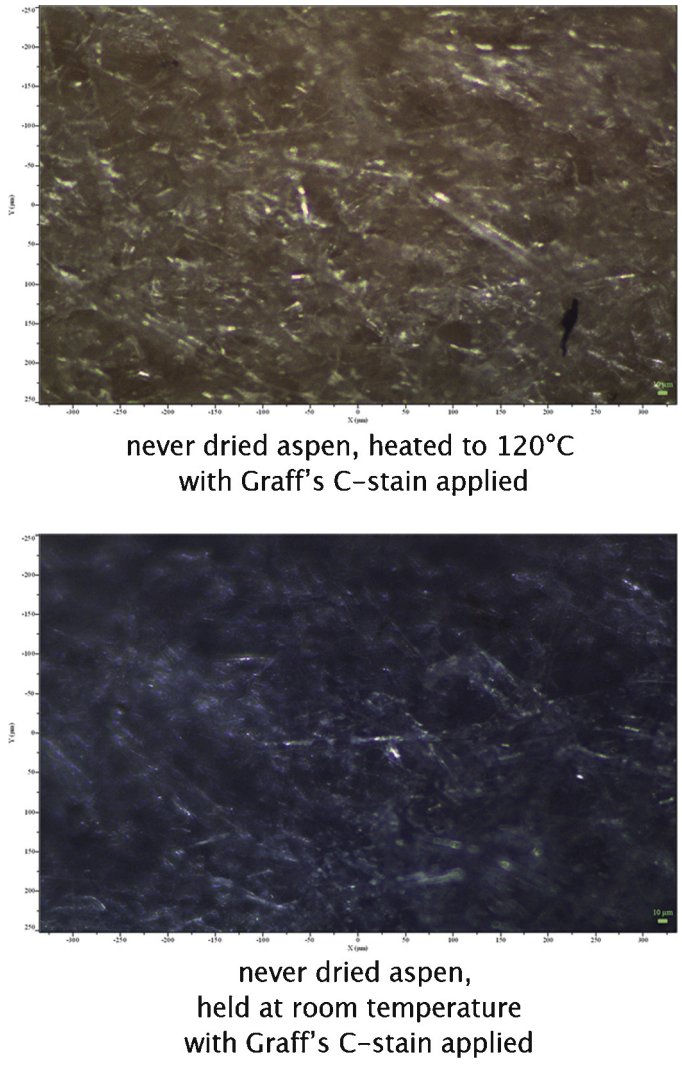

Fig. 4. Fibers stained with the C-stained. The upper are never dried raw aspen fibers, the lower is the never dried after exposure to 120 °C. Both are viewed at 20×.

this instance 120 °C in water, remained yellowish gray. Comparison of the response of the two different samples of cellulose is shown in Fig. 4. It clearly indicates that the RT 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 elevation of temperature. It is likely that the hydration of nanofibrils is significantly diminished upon first exposure to elevated temperature, resulting in diminution of accessible surface area for the action of reagents on the celluloses.

6.1. Ordered biological structure vs. polymeric crystallinity

We have emphasized a distinction between highly ordered biological structures and polymeric crystallinity in the context of discussions of native celluloses. To facilitate visualization of what we believe is to be the result of exposure to elevated temperatures we have developed a speculative model. We assume the occurrence of a mild twist in native fibrils; it is represented in Fig. 5. It begins with acknowledging a tendency to helical character manifested in many electron micrographs of cellulosic nanofibrils from higher plants approximately 4 nm in lateral dimension (Frey-Wyssling, 1976; Haigler, 1991; Hock, 1954; Preston, 1974); this tendency

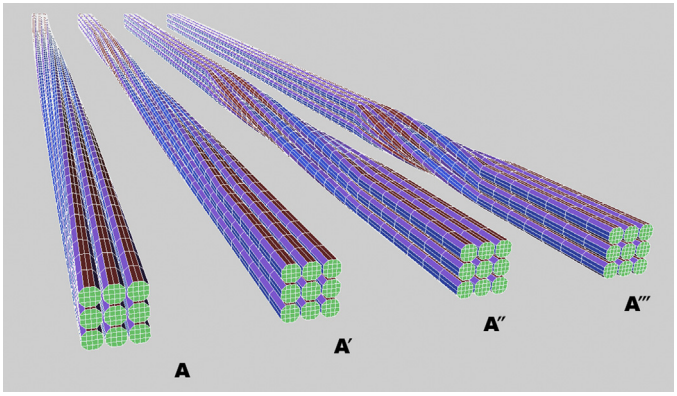

Fig. 5. Qualitative representation of the transformation we believe is unfolding as a result of the dehydration resulting from exposure to elevated temperatures. In A the nanofibrils that are 4 nm in diameter are subjected to a helical twist with a 1200 nm repeat period though only 300 nm are depicted. The secondary aggregate of nine nanofibrils, termed the microfibril, is also depicted as possessing a helical twist similar to those of the nanofibrils, that is, 90° over a period of 300 nm. We believe the uniform pattern of aggregation in A together with the high degree of native hydration represent the most efficient load bearing structure in the secondary walls of plant tissue; it avoids development of shear stresses by allowing slippage of the nanofibrils relative to each other. The progress of coalescence and tighter aggregation resulting from dehydration is depicted in A′, A″ and A‴. They represent increasing levels of coalescence wherein the nanofibrils become parallel in the most tightly aggregated domains and more closely approximate a crystal lattice. The levels of coalescence depicted are 30% in A′, then 65% in A″ and finally 85% in A‴. The degree of crystallinity of pulps isolated at 180°C is commonly found to range between 80% and 90%.

is confirmed by the theoretical analyses (Matthews et al., 2006). Though previous reports of helical periods have ranged from a period of 600 nm to one of 1200 nm, we have found it best to base our speculative model on a repeat of 1200 nm (Revol, Godbout, & Gray, 1997) and depicted a length of 300 nm showing a twist of 90°, representing one quarter of the helix repeat period. We believe that exposure to elevated temperatures results in dehydration of the fibrils and a tighter aggregation of the fibrils in a manner that enhances their parallel alignment.

We show in Fig. 5 two levels of aggregation. The first is the individual nanofibrils that are depicted as 4 nm in diameter; the cross-sections are visualized as octahedral because it would otherwise be difficult to represent the twist. The next level is that of an aggregate of 9 nanofibrils into a microfibril. In Fig. 5 both the individual nanofibrils and the microfibril each begin with a helical form with a 1200 nm period. That is the 4 nm nanofibrils are shown with a 90° twist over a 300 nm interval and the aggregate of 9 nanofibrils in the microfibril is also shown as having a 90° twist over the same interval. We emphasize that these are not intended to be quantitative but rather qualitative and reflect many electron microscopic observations of different plant cell walls including some from woody plants (Hock, 1954). The schematic A is intended to represent the higher level aggregate in the native state. The spaces between the nanofibrils are envisioned as the domains that accommodate hydration of the fibrils.

Our concept of the transformation resulting from exposure to elevated temperatures is depicted in Fig. 5. It is informed in part by the phenomenology of nucleation of crystalline order in linear stereoregular synthetic polymers. As noted above, the schematic A on the left represents the higher level aggregate of nanofibrils that are each approximately 4 nm in diameter with a helical period of 1200 nm, thus for the segment of 300 nm shown, the total twist is 90°. The higher level aggregate of 9 nanofibrils is also twisted by 90° over the same 300 nm segment. Such higher level aggregates are best illustrated in Haigler (1991) where the nanofibrils of bacterial celluloses are shown to assemble into fibrillar ribbons. In nature,

such higher level aggregates likely admit hydration of the surfaces of nanofibrils to allow for "lubrication" of their motion relative to each other. Free movement of the nanofibrils relative to each other within the higher level aggregate is essential to avoid failure due to shear stresses that would otherwise occur upon tensile loading.

Upon exposure to elevated temperatures much above 50 °C, it is anticipated that dehydration will set in causing the fibrils to coalesce with each other more tightly. Schematic A′ represents coalescence at the 30% level and A″ coalescence at the 65% level. The 85% coalescence level is depicted in A‴. The question arises as to which structure represents thermodynamic equilibrium. It is our view that all of the structures represent equilibrium structures because in polymeric systems the equilibrium structure is usually a function of past history. The different structures represent different degrees of coalescence resulting from different thermal histories.

It is our view that the much tighter coalescence of the nanofibrils with each other is the key to the decline of accessibility of the interior of nanofibrils of celluloses upon exposure to elevated temperatures. Such tight coalescence inevitably diminishes the surface area available for reagents to interact with the nanofibrils of native celluloses upon their isolation at elevated temperatures, whether the reagents are chemical modifying agents or as in the instance of our report, probe molecules. It is also our view that the coalescence depicted in A‴ may well be the artifact that led to formulation of the fringed micelle hypothesis so common in models of plant cell wall celluloses.

It is also our view that the periodic disorder reported in (Nishiyama, Kim, et al., 2003) represents precisely the type of twist depicted in A‴ of Fig. 5. The disorder was observed in samples that were "de-waxed ramie fibers purchased from a textile dealer"; commercial de-waxing usually involves boiling in caustic for multiple hours. We regard the disordered domains reported as artifacts of the exposure to elevated temperatures necessary in the de-waxing process. Thus they represent what we view as confirmation of the proposal we put forth in Fig. 5.

7. Conclusions

We believe that our findings point to three key conclusions that are important to all studies of celluloses in plant science and in relation to production of cellulosic biofuels. The first is that a high degree of recalcitrance is not inherent in the nature of native celluloses, and that the encounter of high degrees of recalcitrance in studies of conversion of celluloses represent considerable enhancement of the native recalcitrance as a consequence of the manner in which celluloses have been isolated or biomass pretreated rather than characteristic of the native architecture of celluloses in the source plant biomass.

The second conclusion relevant to all laboratory investigations of celluloses where surface area and accessibility are important is a reaffirmation of the significant degree to which drying from an aqueous medium in air alters their nature irreversibly. Thus drying or dehydration conditions must always be regarded as an important variable.

The third and perhaps most important conclusion is that in their native states celluloses in higher plants are highly ordered biological structures that have states of aggregation that cannot be defined in the context of classical crystallography because they are inherently nonhomogeneous and do not possess the continuity at the nanoscale level that is a prerequisite of distinct phases in the context of classical thermodynamics. As such, their states of aggregation are functions of their biological sources and their histories represented by their processes of isolation.

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