# Molecular Organization in the Native State of Wood Cell Walls: Studies of Nanoscale Structure and its Development

Final Report

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## DISCLAIMER

## SUMMARY

Our progress has been in three major areas. With respect to cell wall biogenesis we have developed a theory concerning the formation of lignin in which the regulation of structure is attributed to the hemicelluloses; they are viewed as templates for the assembly of lignin. The key supporting evidence is derived from the symmetry of annual rings in trees free of reaction wood. This symmetry is interpreted to point to genetic encoding as the dominant factor in the pattern of interunit linkages in lignin. More recently, we have explored further the implications of annual ring symmetries within the contexts of systems and information theory and theories of organization of hierarchic structures. This has led us to propose a unifying model for cell wall biogenesis that comprehends cell wall polysaccharides as well as lignin. The model is based on examining the implications of symmetries and of hierarchic relationships between different levels of structure, with respect to synchrony and coordination of the stages of formation of the individual constituents. It also addresses questions concerning the regulation of genetically encoded information.

The second area where significant progress can be reported is that of application of the Raman microprobe to studies of structural variations over microdomains within cell walls. The advance in this area has facilitated our long sought goal of relating structural information at the nanoscale level to biological function. The advance arises from availability a new generation instrument that is two orders of magnitude more sensitive than its predecessors; it has made possible acquisition of spectra much more rapidly and at much lower laser power levels for excitation. In illustration of the capability of this new system, we can now acquire spectra of individual thickenings on differentiating cells of *Zinnia elegans* within 10 seconds, and this has enabled us to detect variability in the degree of polymerization of the lignin among the thickenings of a single cell. In addition, it allows us to carry out much more extensive spectral mapping within cell walls so that we can explore the degree to which the processes of biogenesis are autonomous within different domains within the walls of the same cell.

We have also made progress in the difficult task of understanding the organization of lignin in the native state. We have examined the solid state $^{13}C$ NMR spectra of tissues enriched in $^{13}C$ and detected differences that can be associated with the different stages of growth. We have investigated the pattern of interunit linkages in synthetic lignin analogs prepared using procedures that simulate different aspects of the chemical micro-environment in which lignin is polymerized; these have provided closer approximations to milled wood lignin (MWL) than prior preparations. And we have continued our exploration of the ordering of lignin within the polysaccharide matrix through investigations of electron transport processes and the coupling of the electronic states of species associated with the polysaccharides. We have found that the electron transport processes we first demonstrated through detection of photoconductivity in wood can play a role in facilitating the action of laccases on wood. We have also the explored the capacity of the cell wall polysaccharides to organize adsorbed species in a manner that allows coupling of their molecular orbitals sufficient to create pathways for electron

transport. Our first exploration of this phenomenon is in the context of couplings between polyiodide ions where we found that although the basic covalent structure was one of tri-iodide and penta-iodide, the electronic structure is coupled over domains that include three poliodide ions. Though the characterization of the polyiodide system was initially carried in the context of the complex with amylose, we have detected the same ordering effect in associations with xylans, demonstrating that they are capable of inducing long range order among associated species.

## OVERVIEW

For some time now, we have been seeking to establish the relationship between cell wall structures at the nanoscale level (domains of 1 to 10nm) and the biological functions that determine these structures. This would help us to understand the basis for the variations in structure that we observe and allow us to relate structure at the nanoscale level to structures at higher levels of organization. It would also provide a basis for interpreting the structures that we observe as part of the record of the events that unfold during morphogenesis. Within this perspective, the progress during the most recent program period represents important breakthroughs. We have developed a conceptual framework that allows us to explore the relationships between variations in structure and variations in conditions that influence biogenesis. We also have acquired an instrument system that allows the generation of structural information within a time frame that allows us to address questions of biological function. The first two sections of this report will describe our progress in these two arenas.

At the same time, we have continued our effort to advance our understanding of structure at the nanoscale level, particularly in the native state of woody tissue. Here also we have made significant progress that is not unrelated to our formulation of the conceptual framework for biogenesis of the cell wall. We have continued to explore the coherence of order in lignin that is responsible for the existence of electron transport pathways within the structure of its native state. We have developed evidence of the effect of the matrix on the structure of lignin. And we have investigated the capability of the matrix to induce coherence of order among adsorbed species by exploring the coupling of electronic states of polyiodide ions associated with the polysaccharides. The final section of the report will be devoted our efforts in this area.

## Models of Biogenesis

**I.** Our first effort in this area was based on a proposal we presented at the beginning of the current program period. Its basic premise was that the hemicelluloses are instrumental in organizing the structure of lignin by functioning as templates for its assembly. They act by providing sites for association of monomers and oligomers of lignin, thus, establishing the spatial relationships between them prior to polymerization by free radical coupling or propagation reactions. The basic concepts were first presented at the Division of Energy Biosciences Workshop on the Plant Cell Wall in 1994. Its first

public presentation was at the 8th International Symposium on Wood and Pulping Chemistry in Helsinki in 1995, appearing in the Proceedings of that symposium as:

**Cellulose and the Hemicelluloses: Patterns for Cell Wall architecture and the Assembly of Lignin**, by R.H.Atalla.

The conclusive evidence in support of this proposal was developed from analysis of the implication of symmetries in the annual rings of trees that are free of reaction wood. Such symmetries are interpreted as indicating regulation of lignin structure by genetically encoded information rather than by microenvironment. The model and the rationale for its adoption are summarized in the following and included in a chapter in the peer-reviewed proceedings of the symposium on the "Biosynthesis of Lignin and Lignans", edited by Lewis and Sarkanen. The chapter is entitled:

**Cellulose and the Hemicelluloses: Patterns for the Assembly of Lignin,** by R.H. Atalla.

The results of our most recent studies, point to significant coupling between the tertiary structures of all three major constituents of the cell wall in native woody tissue. It is clear that the hemicelluloses are intimately involved in the aggregation of cellulose, and the hemicelluloses, together with cellulose, provide the pattern for assembly of the lignin. Our studies of the organization of lignin in native woody tissue, including Raman microprobe spectra and measurements of photoconductivity and fluorescence, leave little question that the level of order in lignin is well beyond that expected from random free radical coupling, in the absence of a stronger organizing influence. We believe that a dominant organizing influence is provided by strong associative interactions between lignin precursors and the polysaccharide matrix, and that the hemicelluloses, in particular play a central role in selective binding of specific lignin precursors. In this context the hemicelluloses are viewed as carriers of an important component of the information needed for the organization of lignin. The binding of the lignin precursors prior to their polymerization is expected to be one of the fundamental organizing influences that determine the primary structure of lignin. The precursors that are expected to participate in this include, in addition to the monolignols, a number of oligomers of lignin, which are formed by membrane bound enzymes. Our proposal concerning the role of the hemicelluloses in the assembly of the cell wall suggests, for the first time, direct pathways for genotypic control of the assembly of lignin. Thus, variation in the structures of the hemicelluloses, the biosynthesis of which is controlled within the golgi apparatus, is expected to result in corresponding systematic variations in the structure of the associated lignin. In consequence, it would appear that any effort to modify the assembly of lignin requires an orchestrated modification of the pathways for biosynthesis of the hemicelluloses, together with any programmed changes in the phenylpropanoid pathways for synthesis of mono- and oligolignols. Finally, our emerging view of the complexity and dynamic character of lignification in the developing cell wall matrix leads us to the view that the wall of the developing cell is a very active participant in both intracellular and extracellular processes.

**II.** More recently, we have extended our effort to relate structure to biological function by further exploration of the implications of annual ring symmetries within the contexts of systems theory, information theory and theories of organization of hierarchic structures. The primary purpose of this effort is to establish whether organization of a biological system at one level can provide information concerning organization at another level. Though the analogy is not an exact one, it is not unlike what is routinely accomplished in statistical thermodynamics when information about molecular level interactions is derived from the properties of macroscopic systems; an example would be the interpretation of the immiscibility of liquid phases in terms of different associative properties at the molecular level. Our analysis of annual ring symmetries has led us to propose a unifying model for cell wall biogenesis that comprehends cell wall polysaccharides as well as lignin. The model is based on examining the implications of symmetries and of hierarchic relationships between different levels of structure with respect to synchrony and coordination of the stages of formation of the individual constituents. It also addresses questions concerning the aggregative properties of the constituents as they progress through the different stages of the biosynthetic pathways and issues of regulation of genetically encoded information.

**Toward a More Comprehensive Model for the Biogenesis of Plant Cell Walls,** R.H. Atalla

Studies of molecular organization in wood cell walls, point to significant coupling between the tertiary structures of all three major constituents of the wall in native tissue. It is clear that the hemicelluloses are intimately involved in the aggregation of cellulose, and the hemicelluloses, together with cellulose, have a significant influence on the structure of lignin. Yet these patterns of correlation in organization at the nanoscale level are not readily interpreted within the framework of paradigms associated with the biosynthesis of the constituents of plant cell walls. The work presented here represents an effort to develop a unifying model for cell wall biogenesis. The search for such a model has been guided by two considerations related to the spatial organization of the processes of biogenesis. The first is the likelihood that that the processes of biosynthesis are distributed in space, with early phases unfolding in the intracellular environment and later ones occurring in the extracellular domains adjacent to the plasma membrane. The second consideration is associated with the synchrony of molecular biosynthetic processes that is prerequisite for the morphological symmetry that is evident at higher levels of structural organization. It is proposed that the hemicelluloses play a central role in facilitating the regulation of molecular architecture that is implicit in the patterns of symmetry at higher levels of organization.

## Raman Micro-spectroscopic Studies

In our earliest studies using the Raman microprobe, we were using a system based on a scanning double monochromator with a photomultiplier detector; acquisition time per spectrum was of the order of 3 to 6 hours. Our second-generation instrument used a diode array detector attached to a triple monochromator. The diode array improved the

efficiency of data acquisition by the triple monochromator reduced the throughput of the spectrometer; acquisition times were reduced to 30 to 40 minutes. In both instances the laser excitation was with an argon ion laser at about 300 milliwatt (mw) output. With these systems it was possible to demonstrate the type of information that can be derived about cell wall structures, but the possibility of acquiring structural data at a rate that would allow explorations of biological function remained limited. The major advance alluded to above has been the development of an instrument that was designed from the outset to for rapid acquisition of spectra. It relies on a holographic notch filter to eliminate the Raleigh scattering of the exciting line, and uses a wide aperture single monochromator to disperse the spectrum. The system first became available in 1996. We have now tested its capabilities and acquired and installed such a system in our laboratory. The possibility of addressing questions of biological function arises because we are now acquiring the spectra using only a 20 mw HeNe laser line for excitation and the spectra in the region of interest can be acquired in 10 to 30 seconds. It is best illustrated with the results of a preliminary investigation of cells of *Zinnia elegans* carried out in collaboration with Dr. Candace Haigler and her associates. The results were presented by Dr. Haigler at the Vancouver meeting of the American Society of Plant Physiologists in august 1997.

**Raman Microprobe Characterization of Lignification in the Secondary Thickenings of Differentiating Tracheary Elements from Cultures of *Zinnia elegans*.** By R.H. Atalla, C.H. Haigler, J.G. Taylor, L.M. Main, and V.C. Tirumalai.

Recent advances in the application of Raman microspectroscopy to plant tissue has made possible observations of the progress of lignification and of cellulose orientation in the secondary thickenings of differentiating tracheary elements in cultures of *Zinnia elegans*. The spectra are dominated by the bands characteristic of lignin, with only minor contributions from the features associated with cellulose. In the spectra of lignin, it is possible to monitor the progress of the lignification by comparing the intensities of the bands at 1600 $cm^{-1}$ and at 1657 $cm^{-1}$. The former is associated with the aromatic ring stretching vibration and the latter is due to the a,b C=C bond on propane side chains that do not participate in interunit linkages. Variation of the relative intensities of the two bands point to differences in the degree of polymerization of the phenyl propane units between different thickenings on the same cell, and, occasionally, at different points on the same thickening. The two dominant bands in the spectrum of cellulose are very weak. Nevertheless, since both are very sensitive to the relative orientations of the electric vector of the exciting laser and the molecular chain axis, they can provide an indication of the orientation of the cellulose chains relative to the thickenings. In all of the spectra observed, the intensities of these bands confirm orientation of the cellulose chains parallel to the direction of thickenings, which has previously been reported on the basis of observations of birefringence.

## The Structures of Lignin

The early component of our effort in this area was a continuation of our investigation of the effects of microenvironment on the pattern of interunit linkages in lignin. The work is described in a two part report, published in Holzforschung entitled: **New Preparations of Lignin Polymer Models under Conditions that Approximate Cell Wall Lignification. I. Synthesis of Novel Lignin Polymer Models and their structural Characterization by $^{13}$CNMR. II. Structural Characterization of the Models by Thioacidolysis.** By N. Terashima, R.H. Atalla, S.A. Ralph, L.L. Landucci, C. Lapierre and B. Monties. It established that when the environment created for the polymerization of lignin precursors approximates more closely the conditions in the cell wall matrix, the distribution of interunit linkages increases in similarity to isolated milled wood lignin (MWL). Thus the influence of micro-environment cannot be entirely ignored, but in relation to our earlier discussion of the biogenesis of lignin, it cannot be the primary determinant of structure.

The next component in the cycle of studies of the structure of lignin was an exploration of the possibilities of using $^{13}$C enriched native tissues to investigate the nature of native lignin. This was accomplished by administering enriched coniferin to wheat straw. The coniferin was labeled specifically at the $\alpha$, $\beta$,and $\gamma$ positions of the propane side chain. The work is described in a report in Phytochemistry entitled: **Solid State NMR Spectroscopy of specifically $^{13}$C-Enriched Lignin in Wheat Straw from Coniferin.** By N. Terashima, R.H. Atalla and D.L. VanderHart. Its key finding, from our present perspective, is the difference in the patterns of interunit linkages between the more mature upper part and the less mature lower part of the internode regions investigated. It also confirms differences between the spectra of protolignin and milled wood lignin.

The third area of study is focused on the spatial organization of lignin. That is, the coherence of order that appears to be the key to the electronic properties of the native state. As we have reported previously, we have observed a photoconductive effect in woody tissue that cannot be explained unless their is sufficient coherence in the organization of the monomers and oligomers of lignin to allow coupling of the molecular orbitals of adjacent aromatic centers. These coupled electronic states would then provide the pathways for electron transport. Our interpretation has been confirmed by studies of the action of laccases on woody tissue that cannot be accounted for without allowance for electron transport pathways in the native tissue.

In addition to our continuing to explore the coherence of order in native woody tissue, we have turned our attention to investigating the capacity of the structural polysaccharides to induce coherence in molecular species adsorbed within their structure. It has long been know that, under appropriate conditions, wood cell wall polysaccharides can form complexes with the iodine-iodide system that are quite similar to the iodine starch complex. We have demonstrated that xylans can indeed form a such a complex. In order to establish a basis for interpretation of our observations we sought first to understand the

starch iodine-iodide system. We found that a pattern of electronic coupling similar to the one we have proposed for lignin does indeed occur in this system. And its behavior is similar in many respects to the xylan-iodine-iodide system, suggesting that the β 1,4 linked polysaccharides can induce coherence among the molecules of adsorbed species. The work on the starch system is described in a report, published in Carbohydrate Research entitled: **The Complex of Amylose and Iodine.** By X. Yu, C. Houtman and R.H. Atalla. Its key finding is that while the covalent structures that occur in this complex are the tri-iodide and the penta-iodide ions, the electronic spectra are determined by coupled states involving three such ions. This observation indicates coherence of order over domains containing as many as 15 iodine atoms. Such coherence is, of course, not surprising in the iodine-amylose complex where the iodine atoms are organized within the helical structure of the amylose. But that similar coherence occurs in the iodine-xylan complex points to the occurrence of linear ordered domains even in this amorphous structure.