

# A Study of Lignin Formation at the Molecular Level by Scanning Tunneling Microscopy

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**ABSTRACT** A scanning tunneling microscope (STM) was used to observe the temporal formation and organization of dehydrogenative polymer (DHP) synthesized from coniferyl alcohol. The images obtained elucidate this structure for the first time. The structure of DHP, as seen from STM images, shows long-range order. It appears that DHP consists of building units or modules assembled into larger assemblies called supermodules. Supermodules are interconnected into the overall lattice-like polymer structure with or without spherical regions. One module consists of about 20 monomers, while the supermodule contains about 500 monomers. Calculated molecular weights for modules and supermodules agree with DHP molecular weight distribution peaks. Samples prepared at two different pH values, 6.4 and 7.6, have the same characteristics. The results presented demonstrate that the process of lignification, even in *in vitro* conditions, is highly ordered, and as such contribute to our understanding of the structure of lignin, a significant constitutive and functional element of cell walls.

## INTRODUCTION

Lignin is a widely distributed cell wall polymer in the plant kingdom, and its structure and function have been extensively studied by various methods. Experimental evidence has shown that lignin is formed through a free-radical polymerization of phenolic alcohols (coniferyl, synapyl, and *p*-coumaryl alcohols) catalyzed by different peroxidases (Nozu, 1967; Wayman and Obiaga, 1974; Lewis and Yamamoto, 1990). However, much remains unknown about lignin formation, structure, and bonding environment in the cell wall, because of the great heterogeneity of polymers at the subcellular level and lack of method for isolation of lignin in unaltered form. *In vitro* experiments of the polymerization of phenolic alcohols in the presence of peroxidase (Freudenberg et al., 1950, 1951; Freudenberg and Harkin, 1960; Freudenberg and Toribio, 1969; Higuchi et al., 1971; Wayman and Obiaga, 1974; Hwang, 1982; Lewis et al., 1987) could further our knowledge about these processes. Comparative studies of artificial and *in situ* lignin show that artificial lignin is not identical to the native form, and that bonding differences exist between the two forms (Lewis et al., 1988). In addition, it is thought that the formation of synthetic dehydrogenative polymer (DHP) is an uncontrolled process which cannot mimic properly the finely orchestrated polymerization of lignin in the cell wall and, consequently, synthetic DHP preparations should have a random structure, contrary to ordered lignin *in situ* (Lewis and Yamamoto, 1990).

Here we report the results of our study of the temporal formation and organization of DHP formed from coniferyl

alcohol (CA) catalyzed by horseradish peroxidase (HRP) observed with a scanning tunneling microscope (STM). The images obtained present for the first time the structure of this biological polymer and its modular spatial organization.

## MATERIALS AND METHODS

DHP was synthesized according to the procedures of Freudenberg (1956), Nozu (1967), and Wayman and Obiaga (1974). The reaction mixture contained  $5 \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$  CA,  $5 \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$   $\text{H}_2\text{O}_2$ , and  $2.5 \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$  HRP in  $50 \text{ mmol} \cdot \text{dm}^{-3}$  phosphate buffer. The stock solutions of CA,  $\text{H}_2\text{O}_2$ , and HRP were prepared in phosphate buffer, pH 6.4 or 7.6. The reaction mixture was prepared by simultaneous addition of  $\text{H}_2\text{O}_2$  and CA solutions to HRP solution. The final volume of the reaction mixture was 5 ml. After mixing, the solution was shaken constantly for 48 h. We examined polymers prepared at pH values of 6.4 and 7.6. The samples for the microscope were taken after 2 h or 2 days of mixing. For observation of intermediate species in the course of polymerization reaction, a drop of reaction mixture was transferred to substrate 2 h after the start of reaction and evaporated in a vacuum at  $5^\circ\text{C}$ . Pure enzyme and CA were used as

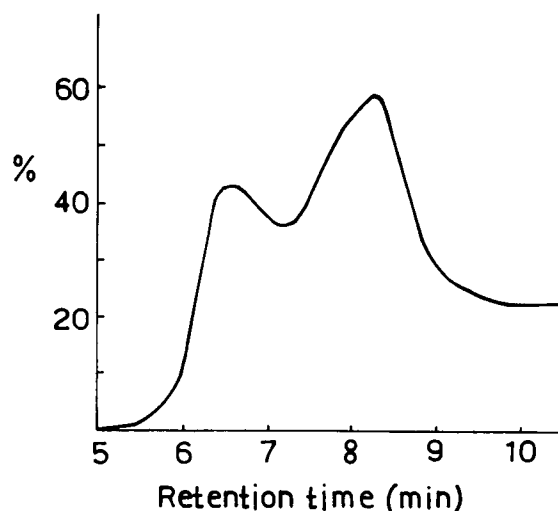


FIGURE 1 Molecular weight distribution of DHP from coniferyl alcohol.

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control and observed in parallel with samples obtained from the reaction mixture to exclude artifacts. The final DHP structure was observed 2 days after the start of reaction. The milky white suspension of DHP was centrifuged at 5000 rpm. The precipitate was resuspended in bidistilled water and reprecipitated twice. Finally, the precipitate was resuspended again in bidistilled water. One drop of diluted suspension was deposited onto the substrates for STM imaging and evaporated in a vacuum at 5°C. Two types of substrates were used. Highly oriented pyrolytic graphite (HOPG) plates were commercially obtained from Digital Instruments Inc. (Santa Barbara, CA). The gold plates were made by vacuum deposition of metal in a custom-made device.

The STM images were obtained using a commercially available model of the microscope (Nanoscope II, Digital Instruments). All STM images were recorded under ambient conditions using a Pt/Ir tip which was mechanically sharpened.

Molecular weight distribution of DHP was determined using a gel permeation chromatograph (Shimadzu LC-GA) consisting of: refractive index detector (RID-6A), computer unit (C-R4A chromatopac), and column G2500H8 (Supelco). Tetrahydrofuran was used as the mobile phase. The column used was not calibrated for molecular weight determination. The samples of DHP for liquid chromatography were prepared by washing out precipitate of DHP, as described above, and drying in a vacuum at 5°C to yield the fluffy cream-colored polymer, which was subsequently dissolved in tetrahydrofuran.

## RESULTS AND DISCUSSION

There is considerable disagreement over the size of lignin macromolecules, a wide range of molecular weights being reported (Lewis and Yamamoto, 1990). Fig. 1 shows bimodal molecular weight distribution of DHP with high molecular weight peak at retention time 8.4 min and low molecular weight peak at retention time 6.6 min. The molecular weight distribution curve is almost identical to that obtained by Wayman and Obiaga (1974) using the same procedure of DHP synthesis. These authors have also reported bimodal molecular weight distribution for isolated lignins (Wayman and Obiaga, 1974). Through their extensive study of lignin formation and degradation, they found that high and low molecular weight peaks corresponded to  $M_w = 80,000$ – $100,000$  and  $M_w = 3,000$ – $4,000$ , respectively. Since the low molecular weight peak was formed first, and subsequently decreased with a simultaneous increase in the high molecular weight peak, the authors suggested that the lignin macromolecule is an assembly of subassemblies which consist of about 20 CA monomers. They called them modules and we will adopt that name throughout this paper. Low molecular weight component in bimodal molecular weight distribution of DHP was assigned by the authors to these modules. It was suggested (Wayman and Obiaga, 1974) that modules formed in the first step of polymerization were combined to form a macromolecular assembly consisting of about 500 CA mono-

mers in a subsequent step, and the high molecular weight peak in molecular weight distribution of DHP was assigned to these assemblies (Wayman and Obiaga, 1974), which we named supermodules.

The goal of this study was to observe the lignin structure, its spatial ordering at highest possible resolution and, if feasible, to observe intermediates. The image in Fig. 2 *a* and a detail of it in Fig. 2 *b* were obtained 2 h after the start of the reaction. It appeared that, at a very early stage of synthesis, the components of the system had not yet formed the ordered structure of DHP. The STM images with molecular resolution (Fig. 2, *a* and *b*) revealed individual aromatic monomers and many dimers and small oligomers. The generally accepted pathway of lignin synthesis starts from CA monomer from which a free radical is produced by hydrogen abstraction. The free radical, which has several resonance forms (Elder and Worley, 1984; Hwang, 1985), attacks at the  $\beta$  position of the propenyl group of a CA monomer to form different dimer radical combinations. Subsequently, it undergoes various dehydrogenation, oxidation, hydration, and condensation reactions in which a variety of functional groups are formed on the aromatic ring and in the side chain. STM images displayed in Fig. 2, *a* and *b*, show clearly visible aromatic rings, while the structure of aliphatic substituents remains unresolved, since under the conditions used, the current through the aromatic ring is generally higher than that through aliphatic parts. Therefore, exact structural assignment to reaction intermediates was not possible. Individual aromatic monomers observed could be CA molecules or different species derived from it including radicals. The structure of some dimers and oligomers, seen in Fig. 2 *b*, could be correlated with predicted intermediates. The structural pattern shown in the inset of Fig. 2 *b* and proposed by Hwang (1985) on the basis of LCAO-MO calculations as one of the possible intermediates of lignin formation is compatible with the STM image in Fig. 2 *b*.

Fig. 2, *c*–*h*, shows STM images of DHP samples obtained after 2 days of polymerization. They show a well-ordered array of structural motifs which repeat regularly in two dimensions in almost all cases, with a repeating unit of 5–20 nm. Fig. 3 shows STM images of DHP on the gold substrate. The primary goal of these experiments is to exclude any possible artifacts caused by graphite substrate. Overall features and dimensions of the polymer on HOPG and gold substrate are similar but with lower resolution and less pronounced ordering on gold substrate. The difference of images on two substrates could be explained in terms of more convenient interaction of  $\pi$ -electrons of aromatic constituents of

FIGURE 2 STM images of temporal formation and organization of DHP deposited onto HOPG substrate. (a) Two hours after mixing the components, ordered structure of DHP is not yet visible. CA molecules (arrow) are observable.  $U = 199.9$  mV,  $I_T = 0.50$  nA. (b) Detail from (a). CA molecules, 1.5 nm in diameter, are indicated by arrows.  $U = 199$  mV,  $I_T = 0.41$  nA. *Inset*: one of possible intermediates of lignin formation, proposed by Hwang, compatible with encircled pattern on the image. (c) DHP 2 days after mixing components, with the spherical regions.  $U = 20.1$  mV,  $I_T = 0.50$  nA. (d) The same as (c), seen at another place of the surface. Supermodules, 5 nm in diameter, are indicated by arrows.  $U = 20$  mV,  $I_T = 0.50$  nA. (e) Three-dimensional image of DHP presenting the layer of DHP with spherical regions.  $U = 20.1$  mV,  $I_T = 0.50$  nA. (f) DHP 2 days after mixing the components, without spherical regions.  $U = 180.1$  mV,  $I_T = 0.46$  nA. (g) The same as (f), showing another region of the sample. Supermodules, 5 nm in diameter, are indicated by arrows.  $U = 180.1$  mV,  $I_T = 0.60$  nA. (h) Three-dimensional image of the layer of DHP without spherical regions.  $U = 180.1$  mV,  $I_T = 0.46$  nA.

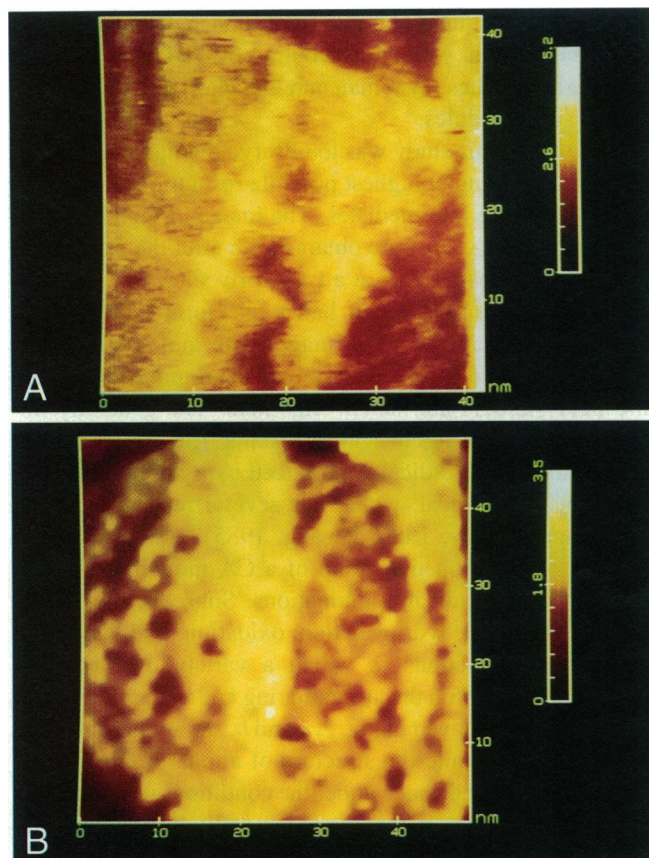


FIGURE 3 STM images of DHP deposited onto the gold substrate 2 days after mixing the components. (a)  $U = 166.6$  mV,  $I_T = 0.83$  nA. (b) Lattice-like structure of DHP.  $U = 200$  mV,  $I_T = 1.2$  nA.

the thin planar polymer with corresponding  $\pi$ -electrons of HOPG. This interaction caused better adherence of the polymer on a graphite surface than on a "rough" polycrystalline gold surface. The structural motifs of DHP observed on both substrates repeated from one sample to another, as well as from one site to another within one sample. This provides evidence that the observed structure is the STM image of DHP polymer and not a substrate artifact.

Solid polymer samples shown in Figs. 2, *c-h*, and 3 are the final product of polymerization reaction which involves many different intermediate steps discussed above. There are a number of proposed reaction pathways of lignin synthesis with a similar overall mechanism. Hwang (1985), using LCAO-MO methods, proposed the model in which one building unit is a chain consisting of 17 aromatic rings connected by different aliphatic functional groups. The chain is linear with few branches. On chemical grounds, Adler (1961) proposed a formula for lignin which consists of a combination of 12 structurally varied coniferyl monomers joined to each other by carbon-carbon bonds and ether bridges forming a branched structure. Another structural unit of lignin, consisting of combination of 18 coniferyl monomers, was proposed by Freudenberg (1965). In all of these models, it is assumed that sequences of phenylpropyl monomers are statistically ordered to account for the variety of natural lignins.

Using the formula for calculation of molecular weights in crystallography (Stout and Jensen, 1968) and assuming that one structural unit (motif) from STM images contains one formula unit, we obtained, for the estimated polymer density of  $1.1 \text{ g}\cdot\text{cm}^{-3}$ , an approximate molecular weight of  $85,000 \pm 25,000$  for the structural unit of  $5 \pm 0.5$  nm in diameter. Deviations are the average of several measurements. This would mean that one structural unit is composed of  $\sim 500$  monomers of CA. The structural motifs seen in Fig. 2, *c-h*, would represent the supermodules (Wayman and Obiaga, 1974) or their multiplets connected by intermolecular forces in the solid lignin polymer. The substructure of supermodules is not well resolved, but entities of  $\sim 2$  nm in diameter could be identified in Fig. 3. From the size of these subunits ( $\sim 2 \pm 0.5$  nm diameter), the calculated approximate molecular weight is  $6000 \pm 4000$ . This would mean that one subunit is made up of  $\sim 20$  monomers. These values are close to values found for modules (Wayman and Obiaga, 1974). The rather large deviations from calculated average values of molecular weights are a consequence of the uncertainty of the module diameter ( $\pm 0.5$  nm) introduced into the calculation. More precise determination of the diameter was not possible due to low resolution of images, probably caused by dynamic disorder of the polymer. However, the calculated molecular weights are in good agreement with those obtained experimentally (Wayman and Obiaga, 1974).

Our results conform to the following mechanism. Globular modules consisting of  $\sim 20$  monomers are formed first. These subsequently polymerize to form supermodules, which in turn aggregate to yield a solid DHP polymer. The bonds between modules in a supermodule are weaker than the bonds between monomers within modules. However, they are of covalent type, since both modules and supermodules exist as distinct molecular species in solution and are quite stable. On the other hand, supermodules are interconnected by intermolecular forces within their aggregates. These aggregates exist only within the solid lignin and cannot be detected in solution as independent molecular species. The bonds between supermodules are split in solution.

It was not possible to obtain atomically resolved STM images of the polymer, indicating that a considerable degree of dynamic disorder exists. However, it could be observed that modules and supermodules have a globular structure (Figs. 2, *c-h*, and 3), contrary to the linear form predicted by Hwang (1985). It is evident from STM images that modules and supermodules combine into the overall structure of DHP polymer in two ways: forming spherical regions regularly arranged within a polymer (Fig. 1, *c* and *d*) or without them (Fig. 1, *f* and *g*). This variation was not pH-dependent.

As mentioned above, it is thought that DHP, or synthetic lignin, is formed through a random polymerization of monolignols (Lewis and Yamamoto, 1990). Consequently, some authors have concluded that lignification in situ is also a random process. The results of some studies on lignin in situ (Hatfield et al., 1987a,b) support this opinion. On the other hand, other authors argue that the formation of lignin in

growing plants is a carefully orchestrated process under spatial and temporal control (Lewis and Yamamoto, 1990). A model involving identical repeating units in spruce lignin was described (Forss and Fremer, 1983) but lacked convincing experimental evidence. There is some evidence from Raman spectroscopy studies for the ordering of aromatic rings in cell walls (Atalla and Agarwal, 1985; Agarwal and Atalla, 1986). Our results provide proof that peroxidase-catalyzed polymerization of CA in the presence of H<sub>2</sub>O<sub>2</sub> is not a random statistical process, even under in vitro conditions, but rather an ordered process.

In conclusion, our results provide direct proof for the modular structure of lignin, previously predicted by other authors. The most important result of our work is evidence demonstrating that the process of lignification in in vitro conditions is a highly ordered and well-orchestrated process.

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