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Visualization of Artificial Lignin Supramolecular Structures

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Summary: In this paper we are presenting the results of our environmental scanning electron microscopy (ESEM) investigation of the lignin model compound—enzymatically polymerized coniferyl alcohol, also known as dehydrogenate polymer (DHP). The goals of this study were to visualize the supramolecular organization of DHP polymer on various substrates, namely graphite, mica, and glass, and to explore the influence of substrate surface properties and associated collective phenomena on the lignin self-assembled supramolecular structure. Based on results obtained with ESEM, combined with previously published results based on scanning tunneling microscopy (STM) and electron spin resonance (ESR) technique, we looked at lignin structure ranging from a monomer on a fraction of nanometer scale to a large aggregate on a fraction of millimeter scale, therefore using six orders of magnitude range of size. Herein, we are presenting evidence that there are at least four different levels of the supramolecular structure of lignin, and that its supramolecular organization is well dependent on the substrate surface characteristics, such as hydrophobicity, delocalized orbitals, and surface-free energy.

Key words: lignin, biopolymers, dehydrogenate polymer, supramolecular structures, environmental scanning electron microscopy

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Introduction

Lignin, together with cellulose and hemicellulose, is a main structural polymer in the cell wall of any higher plant that is responsible for providing mechanical strength and mechanical stress protection for the plant cell. After the cellulose, lignin is the second most abundant polymer occurring in nature. Its synthesis in the cell originates from phenolic alcohols (coniferyl, p-coumaryl, synapyl) and proceeds through a well-orchestrated enzyme-catalyzed process governed by cell wall peroxidases, which is still not completely understood. Lignin is a highly cross-linked polymer intertwined with hemicellulose and crosslinked with cellulose lamella (Lewis and Yamamoto 1990). However, lignin's structure and morphology are not exactly described, because it has never been isolated in an unaltered form from the plant cell wall. Difficulties in its isolation arose due to lignin-covalent cross linkage with cellulose, so any separation of lignin from cellulose irrecoverably alters its structure. There were several experimental and theoretical attempts to visualize lignin's molecular shape. For example, Rezanowich *et al.* (1964) observed a spherical form of isolated lignin fragments. Nearly spherical lignin particles were observed in lignocellulose from beechwood (Kosikova *et al.* 1978). In a theoretical model of lignin synthesis, Jurasek (1995) predicted an irregular shape of lignin macromolecules growing freely without interference from the other molecules (hemicellulose and cellulose), and spherical shape in the proximity of cellulose or hemicellulose layers. Since naturally occurring lignin cannot be isolated in unaltered form, enzymatically polymerized dehydrogenate polymer (DHP) of coniferyl alcohol, obtained in vitro, is used as the best model compound for the purpose of structural studies of lignin (Lewis *et al.* 1988). Using scanning tunneling microscopy (STM) images of DHP, combined with gel permeation chromatography (GPC) measurements (Radotic *et al.* 1994), it was shown that lignin has a well-ordered structural organization with fractal dimensionality, even if it is synthesized *in vitro*. It was found that the lignin polymer is built of modules containing about 20 monomers, which are further polymerized and cross-linked into the supermodules containing about 500 monomers.

In the study herein presented, we are discussing the results of environmental scanning electron microscopy

(ESEM) imaging of the DHP lignin model compound deposited as a self-assembled layer on three different substrates, with different hydrophobic/hydrophilic properties, in order to investigate the influence of these surface properties on the lignin supramolecular organization. As suitable substrates, we selected graphite, which is highly hydrophobic, mica substrate, which expresses hydrophilic properties, and glass which has a highly hydrophilic surface. The goals of this study were (1) to obtain ESEM images of synthetic lignin; (2) to get insight into lignin's higher order of supramolecular organization, and (3) to explore the influence of substrate surface properties, mostly of the hydrophobic and hydrophilic characteristics of the surface, on the form and organization of lignin supramolecular structures. Those facts will provide insight into the ordering of lignin at the mesostructural level and into the type of interactions that are holding lignin macromolecules together in the living cell, as well as into the possible mechanism of mediation and alternation of lignin organization, with alternation of the hydrophilic properties of hemicellulose and other layers in cell wall/membranes structures.

Materials and Methods

Synthesis

A solution containing $5 \cdot 10^{-3}$ M coniferyl alcohol, $5 \cdot 10^{-3}$ M H_2O_2, and $2.5 \cdot 10^{-8}$ M horseradish peroxidase, in $5 \cdot 10^{-2}$ M phosphate buffer pH 7.6 (all reagents Fluka Chemical Corp, Ronkonkoma, N.Y., USA), was conditioned for 48 h at room temperature. Polymer was precipitated as a nonsoluble fraction with yellowish-white coloration.

Imaging

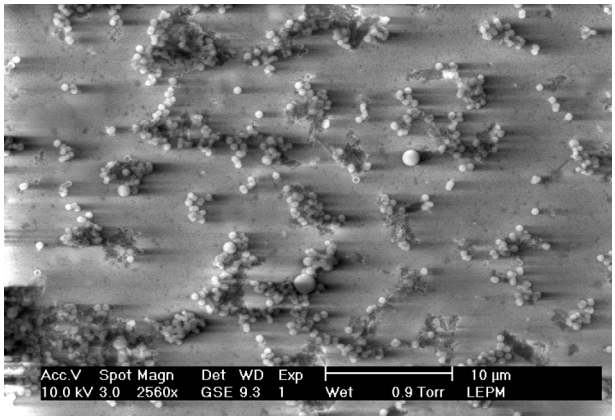
Samples for ESEM imaging were prepared from water suspension of the lignin DHP polymer. The water suspension of lignin was prepared by sonification, using an ultrasound bath for 60 s. A drop of suspension was placed on a highly oriented pyrolytic graphite (HOPG) substrate (Advanced Ceramics Corporation, Cleveland, Ohio, USA), a mica substrate, and a clean glass microscopic slide. All substrates had been previously glued with double-sided carbon tape to the ESEM sample holder. The substrates, with deposited films, were then placed in the chamber of a FeiCo-Phillips-Electroscan FEG XL-30-ESEM field emission gun ESEM. Previously, it has been shown that field emission gun SEM and ESEM could be successfully used for investigating the surface morphology of self-assembled and Langmuir Blodgett films (Micic *et al.* 1999, Neves *et al.* 1999, Sui *et al.* 2000) without applying additional sample treatment, such as coating, thus avoiding the introduction of artifacts. All imaging was performed in wet mode, with 1 Torr of H_2O as an imaging gas. The imaging signal was gaseous secondary electron (GSE) signal. For the purpose of this study, we used the wide field GSE detector, without an external pressure limiting aperture.

Results and Discussion

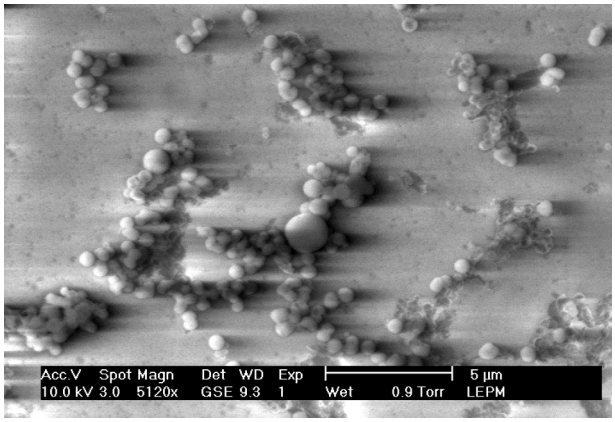
Using ESEM, we have obtained good quality images of deposited polymer on the substrates. The best images from an esthetic and technical point of view have been acquired on mica, followed by glass and graphite. Such change in image quality could be described in terms of the charging effects. It is most likely that mica, glass, and lignin possess similar dielectric properties, which cause a minimal difference in image contrasts and less charging effects. These facts result in good quality images, without charging artifacts. On the other hand, in the case of lignin deposited on the HOPG surface, we have a situation where a highly conductive surface is covered with lignin, which is an electrical insulator. In such case, we are not able to manage charging artifacts (bright stripes over the image) successfully. While these artifacts led to poorer image quality of lignin on graphite than the images of lignin on mica and glass, they did not significantly reduce the amount of information that could be obtained from the images. On all substrates, lignin is presented in the globular form, and those macromolecular globules are usually associated into the larger clusters that can form even larger superstructures. The observed size of the lignin globular structures ranges from 10–1000 nm. Most of those structures are expressed within a narrow size distribution, with the maximum at about 400 nm, which can be considered as the average globule size. Figure 1 shows lignin globules on mica that have well-defined spherical shapes. As it can be seen from Figure 1, most of the globules are monodispersed in terms of their diameter size of 400 nm. If we assume that an average globule of 400 nm represents a single macromolecule, and that the entire space inside the globule is uniformly filled with monomers, a simple calculation shows that such a macromolecule would contain 10^8 phenylpropanoid monomer units and its molecular mass would be 10^{11}. This value is calculated for an assumed polymer density of 1.3 g/cm^3. However, the experimentally determined molecular mass of the DHP lignin model compound is on the order of magnitude of 10^5 (Radotic *et al.* 1994, Wayman *et al.* 1974), which is six orders of magnitudes less than previously indicated in the calculated value based on the average size of the observed globules. We strongly believe that this discrepancy can be explained in terms of the modular structure of the globule. The envelope of the globule that we see is only the first layer of supermodules assembled in a sphere. Supermodules are held together with strong intermolecular forces, probably by hydrogen bonds. The interior of the globule is filled with smaller spherical layers of self-assembled supermodules. This means that the globule has an "onion-like" structure; it does not necessarily mean that the globule is completely filled with spherical assemblies. Most probably, solvent and/or air occupy

some part of the interior of individual globules. This assumption is supported by several pieces of evidence. Open shell structures, such as the one observed in Figures 2 and 3, strongly support the hypothesis that the sphere is actually a thin envelope composed of only several layers of supermodules, acting as layer building blocks. An interesting observation is that globules are easily elongated into an ellipsoidal shape by interactions with HOPG carbon and glass surfaces. This can be observed on all figures of lignin deposited on those two surfaces, most significantly in Figures 4d, 5, 6, and 7c. Such elongation, induced by surface effects, is evidence of the existence of inside-globule subdomains with increased mobility, and associated empty-solvent or gas-filled space. Electron spin resonance (ESR) measurements (Lindberg *et al.* 1975, Tormala *et al.* 1975) support such theory, as they reveal domains of increased mobility in lignin polymers. Such domains are held together via intermolecular bonds, probably covalent bonds. Based on the above facts, we believe that the discrepancy between the measured molecular mass and the size of the observed globules could be described by the next fact: in solution, the intermolecular bonds that hold the domain into globular modules are broken, and the measured molecular mass is of an individual compact domain, herein referred to as supermodule, that is on the order of magnitude of 10^5. From an average diameter of 400 nm per globule and an average diameter of 10 nm per supermodule, one can calculate that one globular, self-assembled structure consists of 16,000 supermodules. This calculation holds for a single layer structure.

There is a slight discrepancy between previously published STM results (Radotic *et al.* 1994) and herein presented ESEM images of fragments of a globule surfaces. While STM images reveal the modular structure of the globule, ESEM images show a smooth surface of a globule. Obviously, the resolution of ESEM is not enough to reveal the subunits of a globule. This can be explained in part by different procedures of preparation and states of the sample in STM versus ESEM imaging. Samples for STM are dried up in vacuo, while the ESEM images are taken under natural, "wet," atmospheric conditions, without any drying. In the case of ESEM, the surface of the globule may



(a)



(b)

FIG. 1 Environmental scanning electron microscopy image of enzymatically polymerized lignin deposited on mica surface. Well-defined spherical shape of globules can be observed (a) at magnification of 2560 ×, (b) at magnification of 5120 ×, wet mode, 0.9 Torr H_2O.

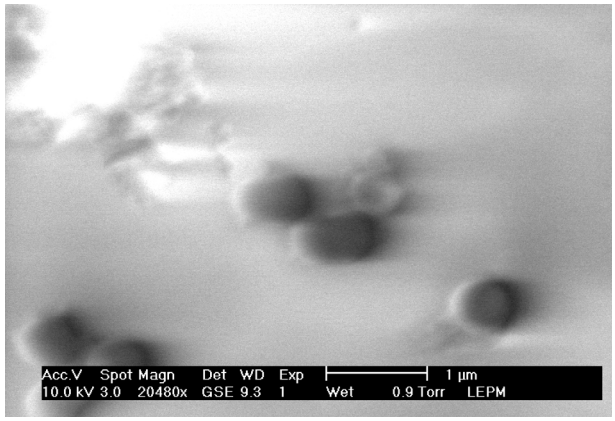


FIG. 2 Environmental scanning electron microscopy image of enzymatic lignin deposited on mica surface, presenting a proof for the "open shell structure" hypothesis (magnification of 20480 ×, wet mode, 0.9 Torr H_2O).

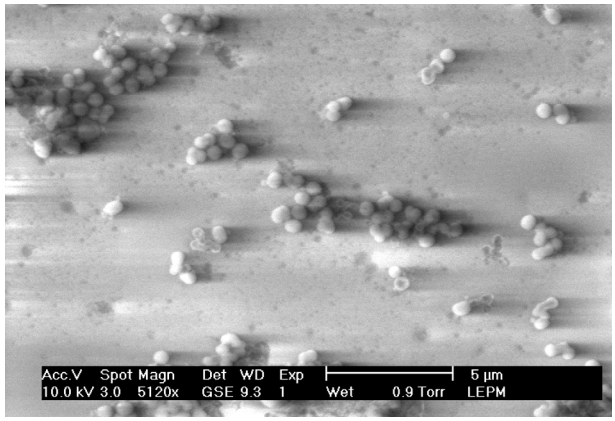


FIG. 3 Double and triple chain-like clusters, observed in the environmental scanning electron microscopy image of enzymatic lignin deposited on mica surface (magnification of 5120 × wet mode, 0.9 Torr H_2O).

be covered by a water layer that smoothens the surface. Other possibilities are that we are observing an "average surface," whereby averaging is induced by the vibrational motion of a spherical superstructure. Energy for such movements-vibrations of the globule substructure came from absorption and thermal release of energy from the scanning electron beam. Almost all of the globules on mica, and many individual globules on HOPG carbon and glass, express perfect spherical shape (Figs. 1, 4c,e). This is in agreement with transmission electron microscopy observations of isolated lignin fragments from plant cells which also showed the spherical shape (Kosikova *et al.* 1978, Rezanowich *et al.* 1964). Electron micrographs of the middle lamella (Donaldson 1994) suggest that lignin is deposited

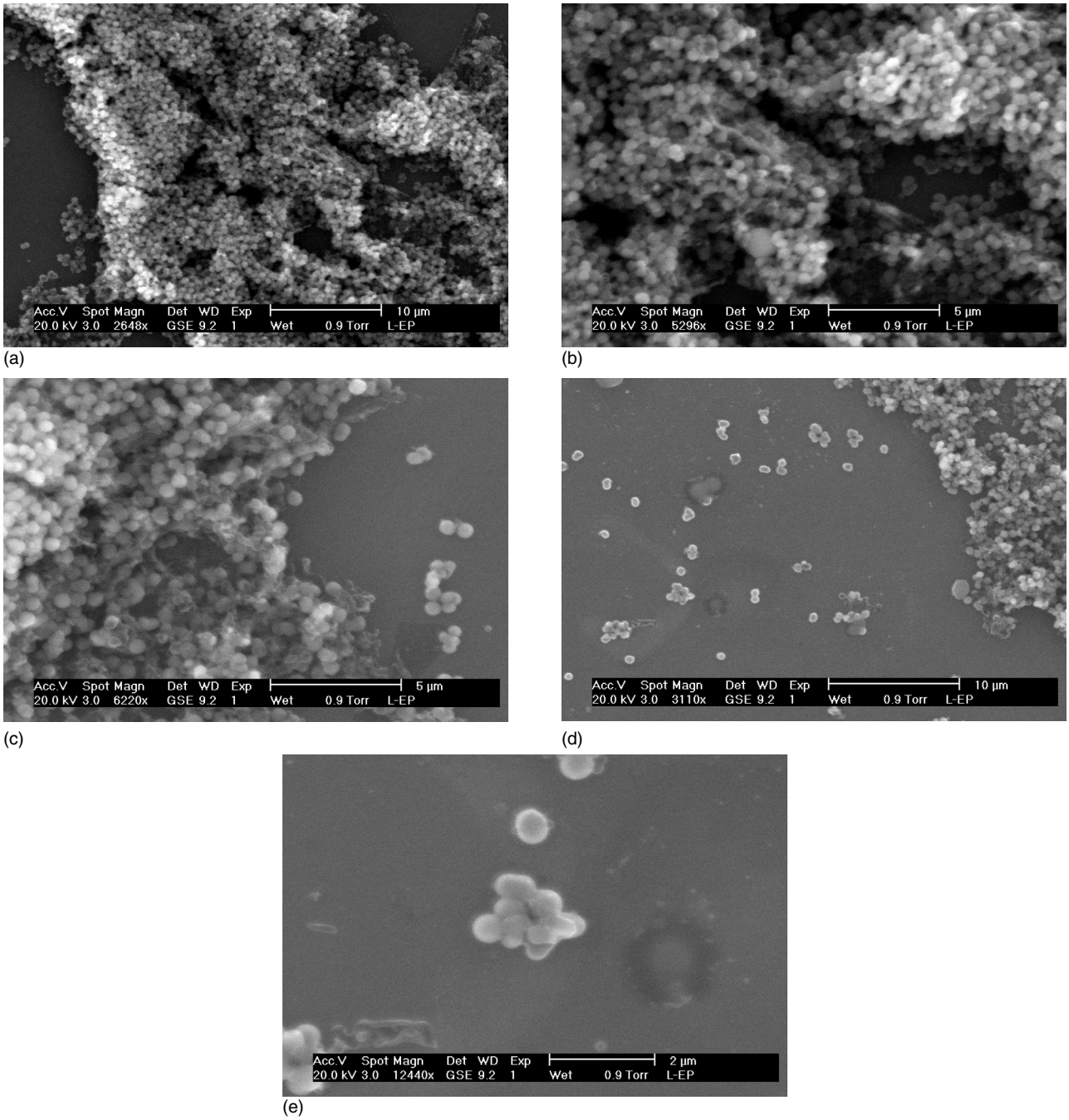


FIG. 4 Environmental scanning electron microscopy image of enzymatic lignin deposited on the HOPG surface. (a–c) Large agglomeration of globules super-clusters, forming "colloidal crystalline state;" magnifications: (a) 2648 ×, (b) 5296 ×, (c) 6220 ×, (d, e) individual clusters of globules, at (d) 3110 × and (e) 12440 × magnification, wet mode, 0.9 Torr H_2O.

in the form of round particles, which later coalesce into a more compact structure, thus again confirming that DHP polymer is an adequate lignin model compound. Such results indicate that lignin, at this level of structural organization, prefers the spherical shape if it is not perturbed by interactions with its surroundings. This fact can be explained in terms of lignin's strong hydrophobic property (Laschimke 1989). Of all possible shapes for solid objects, the sphere has the smallest surface area and hence the spherical globule will have the lowest free energy in contact with water, forcing self-assembling supermodules into the spherical structure.

Environmental scanning electron microscopy images reveal an even higher organization level of lignin polymer, that is, globules assemble in clusters usually containing 2–6 globules. Such association extends further up to compact, well-defined structures, which closely resemble hexagonal crystalline lattices (Figs. 4c, 4d, 5, 6). These clusters have defined geometrical shapes: triangle, square planar, tetrahedral, and hexagonal. Linear aggregates of three globules are seldom observed, while triangular clusters are quite frequently displayed, indicating that this type of aggregation is energetically more stable. Linear chains containing more

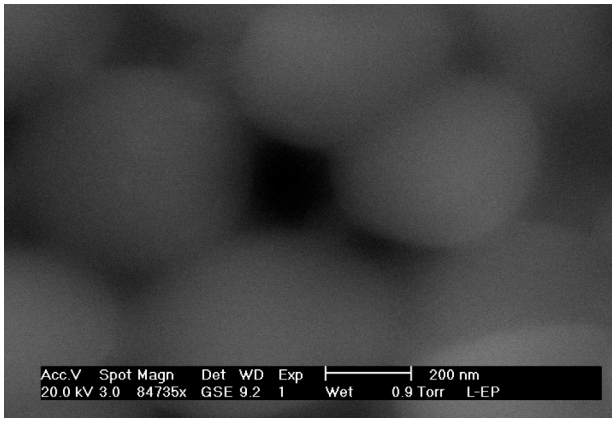


FIG. 5 Environmental scanning electron microscopy image of details of the hexagonal motif of self-assembled globules of lignin on HOPG (magnification: 84,735×, wet mode, 0.9 Torr H_2O).

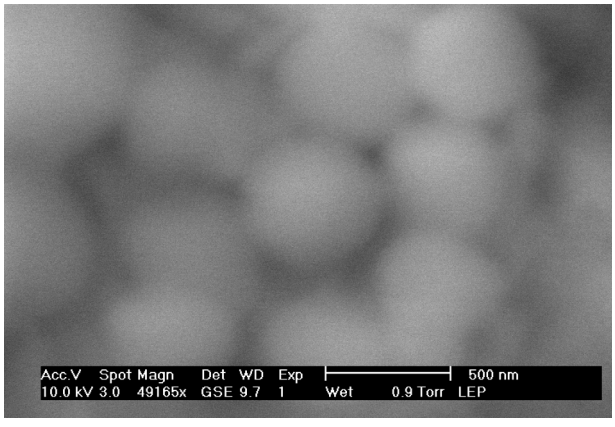
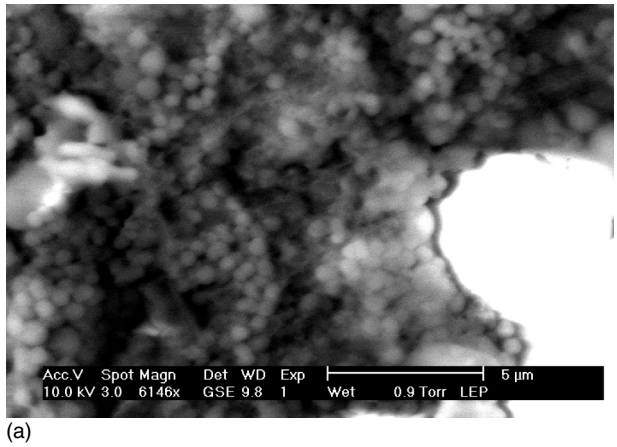
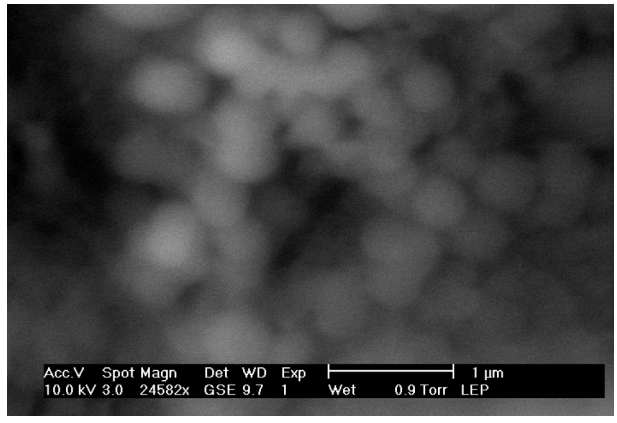


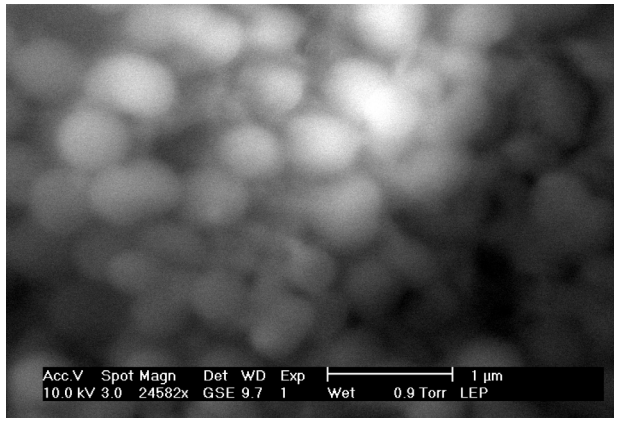
FIG. 6 Environmental scanning electron microscopy image of channel-like, highly self-organized hexagonal structure of enzymatic lignin globules deposited on glass surface (magnification: 49,165 ×, wet mode, 0.9 Torr H_2O).



(a)



(b)



(c)

FIG. 7 Environmental scanning electron microscopy image of enzymatic lignin deposited on glass surface. Hexagonal clusters forming channel-like structures [(a) magnification 6146 ×, (b) magnification 24,582 ×] and spiral structures [(c) magnification 24,582 ×] can be observed (for all images, wet mode, 0.9 Torr H_2O).

than three globules are observed only in a form of double and triple chains (Fig. 3), or aggregated with other structural forms. This finding further supports the above conclusion that single linear chains are energetically unstable and readily aggregate into two- and three-dimensional superstructures. Hexagonal clusters, which sometimes extend spirally in Z direction (Fig. 7c), are interesting structures since they allow the formation of pores and channel-like spaces and cavities (Fig. 7). This type of aggregation is most frequently observed on the HOPG carbon and glass substrates. Large compact aggregates with a crystal-like structure probably belong to the recently proposed new state of matter, called "colloidal crystalline state" (Goltner 1999). Those aggregates contains pores and channel-like spaces (Figs. 5 and 7) that may have biological relevance. The plant cell wall is a well-organized network of polysaccharides, lignin polymers, proteins, enzymes, and so forth, providing firm supporting structure to the cell. The cell wall is not a static structure, but a dynamic metabolic system. Pores and cavities observed in a lignin superstructure may be the space for other macromolecules and active structures, such as different molecular and ion channels, water-conducting pipelines, *tracheas*, and many others.

Inspection of the ESEM images reveals an obvious influence of substrate properties on the formation of lignin superstructures. The most inert substrate is mica. Lignin on mica forms a predominantly planar patterning in a monolayer. The shape of the globules, both individual and in aggregates, is a perfect sphere, indicating that the interaction of lignin with the substrate mica is weak. The strongest influence of the substrate on lignin ordering and packing is observed on graphite, which is in agreement with conclusions from previous STM studies of lignin. It seems that the π-electron system of the graphite strongly interacts with the π-electrons of lignin's phenyl ring in a cooperative manner, favoring observed ordering. It is interesting to note that the ordering of phenyl rings *in situ* is detected by resonance Raman spectroscopy (Atalla and Agarwal 1985). This interaction leads to the elongation of globules into an ellipsoid form. The longer axis of the ellipsoid is always parallel with the substrate surface. Elongation is observed in both aggregated and individual globules, but with smaller effects on the latter. This supports the cooperative mechanism of interaction. The above observation indicates that the interior of the globule may be of different fluidity, as mentioned before. Glass substrate has a stronger influence on lignin aggregation than mica, but the ordering of lignin assemblies is not as well defined as on HOPG, in particular for large assemblies. From the electronic and chemical properties of glass and lignin, one would expect an interaction similar to that between mica and lignin. However, it is stronger and more comparable with the interaction between HOPG carbon and lignin. This can be explained by the fact that the water vapor film formed on glass attracts organic molecules and their clusters by strong adhesion. Such a mechanism may be of great importance in a live plant cell, where a layer of vicinal water could exist on the membrane surfaces and thereby modulate the lignin supramolecular structural organization.

Conclusion

Looking at lignin structure, from a monomer on a fraction of a nanometer scale to a large aggregate on a fraction of a millimeter scale (a range of size of six orders of magnitude), it is possible to say that lignin possesses a property of self ordering, both intrinsic and substrate mediated. Over the six orders of magnitude of the scale range, from nanometer to a fraction of a millimeter, the lignin superstructure could be divided into the four structural levels of ordering. Modules of about 20 monomers form the first. They polymerize into supermodules containing about 500 monomers, which subsequently aggregate into globules. Finally, the globules form clusters and large superstructures. Going from the module through the superstructure, the forces which keep the subunits together weaken. They are covalent for the first two structural levels and intermolecular (hydrogen bonding and Van der Waals interactions) for the two latter supramolecular structures. The shape of the larger assemblies is mediated by the substrate surface properties—both by the electronic-π orbital shape and the hydrophobic/hydrophilic nature of the surface. Such mediation may be important in a live plant cell, as it may lead to the dynamic creation and destruction of required lignin structures of higher order, which may have secondary roles as molecular channels, ion and water channels, or as a matrix for holding other components of the cell wall.

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