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Topographical characterization and surface force spectroscopy of the photochemical lignin model compound

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Abstract

By combining the results from atomic force microscopy (AFM) and environmental scanning electron microscopy (ESEM), herein we investigate properties of photochemical lignin model compounds. We provide evidence that photochemical lignin forms random, probably non-functional structures. The topography of such structures is explored using ESEM. Non-functionality of such structures is proved by AFM and atomic force spectroscopy experiments wherein the photochemical lignin functionalized tip is approached to the substrate covered with photochemical lignin. There was no evidence of existence of any kind of host–guest interaction during the approach/retraction experiments. These results provide evidence for our previously stated hypothesis that photochemical lignin polymerization may be one of the degrading effects of UV radiation to the plant cell.

Keywords: Lignin model compounds; Photochemistry; Atomic force microscopy; Environmental scanning electron microscopy; Intermolecular forces; Supramolecular chemistry

1. Introduction

Lignin is one of the main structural polymers in the cell wall of any higher plant. As such, it is known that lignin is responsible for the mechanical stress protection of plant cells, as well as for providing support for the plant against gravity [1]. Lignin is a highly cross-linked polymer created by enzymatically-catalyzed polymerization of different phenyl-propanoid units, such as conferyl, *p*-coumaryl, and synapyl alcohols. Lignin is intertwined and covalently bound with molecules of cellulose, hemi-cellulose, supramolecular structures such as fibers and lamellae and other con-

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stituents of the cell plant wall membranes. Thereby, it has been impossible up to now to isolate lignin in its unaltered form [2].

Due to the above-stated facts, until today, even more than 100 years since lignin discovery, its structure is still unresolved [3]. Most of our understanding of lignin structures comes from spectroscopic and microscopic studies. Some of the landmark publications in lignin chemistry are in-situ data about orientation of phenyl rings in the plant cell walls [4], obtained by Raman spectroscopy, and lignin shape based on high-resolution electron microscopy studies of extracted lignin [5].

To obtain more insight into the lignin molecular shape and form of its supramolecular structure, it is necessary to be able to do in-vitro experiments on unmodified polymers. It is virtually impossible to extract unmodified lignin from the plant cell, as severe conditions connected with lignin isolations such as during the NaOH based kraft pulping [6], inevitably leads to lignin structural modification. Thereby for many fundamental studies of lignin molecular and supramolecular structures in-vitro synthesized lignin model compounds are better suited then extracted polymer. One of the most common lignin model compounds is enzymatically polymerized poly-coniferyl alcohol [7], well known as a DHP polymer. DHP has been successfully used in many experiments as one of the best-known lignin substitutes. In our recent works [8–12] we have presented our novel findings about organization of the DHP molecule and its supramolecular structures, based on scanning tunneling microscopy (STM) [8,9], environmental scanning electron microscopy (ESEM) [10–12] and atomic force microscopy (AFM) [12]. Therein was shown that DHP polymer tends to organize itself into the semi-regular spherulitic assemblies, which then organize to supramolecular structures which resemble pores, channels, chains and other geometrical structures which could have possible physiological significance. Using the AFM force scan spectroscopy [12] we have shown that enzymatically synthesized lignin model compound macromolecules express complex pattern of host-guest interactions which may have certain physiological roles. All DHP lignin structures express fractal dimensionality [8,9], which could be explained in terms of lignin semi-random structure, as the lignin is mostly a cross-linked natural polymer. Existence of such structures, lead us to hypothesize that lignin may have, in addition for being a building block of plant tissues, some significant physiological roles in the plant cell, due to its ability to organize into such regular assemblies such as hexagonal lattices, chains and pore-like structures [11].

It has been known for a while that lignin monomers are photosensitive and photoactive materials [13,14]. Besides an enzymatic mechanism of synthesis, a photochemical polymerization of monomers to lignin, induced by UV radiation, was shown [15] (and ref. cited therein). Also it is well known that the lignin macromolecule is photo-degradable, [16,17], a process that is significant both from an ecological point of view and a practical one in studying preservation of wood and for possible lignin applications as a fuel sources [18].

The goal of this study is to explore topography and interactions between individual molecules and supramolecular assemblies of photochemical lignin model compounds using ESEM for the topological investigation, and AFM running in the spectroscopy mode for measuring intermolecular forces. Based on our previous investigation, we know that photochemical lignin [10] have an amorphous, random appearance, while enzymatic one have ordered structures. Here we will be able to answer the question if topographically less ordered photochemical lignin can have any repetitive pattern of host–guest interactions and recognitions, and to compare it with our previously discovered pattern of enzymatic lignin [12]. Such interactions are crucial for the biological active role of any molecules.

2. Materials and methods

2.1. Photochemical lignin model compound synthesis and preparation

Photochemical lignin was synthesized by irradiation for 2 h of 2×10^{-2} M solution of coniferyl alcohol in 5×10^{-2} M phosphate buffer at pH 7.6, using a 120 W low-pressure mercury lamp. Three

different experimental designs were performed for photochemical lignin synthesis. (1) The starting solution was placed in a quartz vial during irradiation. For the purpose of exploring the topography of lignin deposits on cellulose, a drop of lignin water suspension was placed on the surface of a cellulose diacetate film. (2) The starting solution was poured on a cellulose film placed on the bottom of a Petri dish. (3) The conditions were as in (2), but irradiation was performed through a 35-μm cellulose diacetate film placed on the top of the dish, in order to exclude short-wavelength UV radiation (below 290 nm) and in this manner simulating the part of UV radiation spectrum coming to the Earth's surface. In all procedures the cellulose support with deposited lignin suspension was dried at room temperature. More details about photochemical lignin synthesis may be found in Radotic et al. [15].

2.2. AFM cantilever functionalization with lignin

Photochemical lignin model compound water suspension was sonicated for 5 min in order to homogenize it. Unsharpened, Si_3N cantilever tips (Thermomicroscopes, MLCT-AUHW, Sunnyvale, CA) were immersed in a lignin suspension during 12 h at 37 °C. Afterwards, the cantilevers were rinsed and dried at 37 °C. No additional treatment has been performed, as a strong adhesion between the tip surface and lignin occurs, as proved by ESEM imaging of the cantilever as per method described in Micic et al. [21].

2.3. AFM microscopy

All force measurements were done using the custom made atomic force microscope [19], running in the force scan mode. Cantilevers were calibrated by thermal fluctuation analysis and had a spring constant of 0.010 N m^{-1}. Measurements have been done in the neutral phosphate buffer solution (pH 7.0), in order to reduce the charging effect artifacts.

2.4. ESEM microscopy

Visualization of photochemical lignin model compound structures on the cellulose substrates was performed using the FeiCo Phillips-Electroscan FEG XL-30-ESEM field emission gun environmental scanning electron microscope running in the so-called environmental, wet mode [20]. It has been demonstrated previously that thin film and colloidal topography can be successfully examined using the field emission SEM [21] and ESEM [8–12,22–26] without alternation of its surface. The conditions for lignin imaging were following: atmosphere 1 torr of water vapor pressure as imaging gas and electron beam of 10 kV.

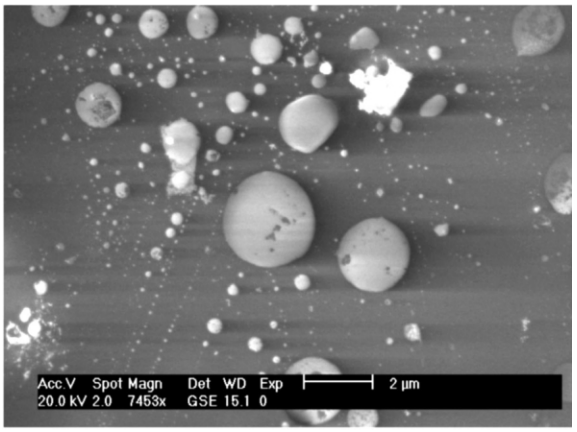

Fig. 1. ESEM micrography of the large patched spherulites of directly-irradiated photochemical lignin model compound (magnification: 7453×; water vapor pressure: 0.9 torr).

3. Results and discussion

The photochemical lignin model compound adhered well to the cellulose surface in both cases, as deposited after synthesis onto the cellulose support or synthesized in its presence. On the large lignin spherulites on Fig. 1, a rough surface with numerous patches and holes can be observed, which may be a consequence of UV radiation damage to the polymer structure. Such irregular patched structure may be also generated due to non-uniform polymerization of assembled Photochemical lignin has rather amorphous structure (Fig. 2), with occasional irregular spherulites (Fig. 3). As in the previous case with photochemical lignin deposited on other surfaces, there is virtually no regular agglomeration on the cellulose sub-

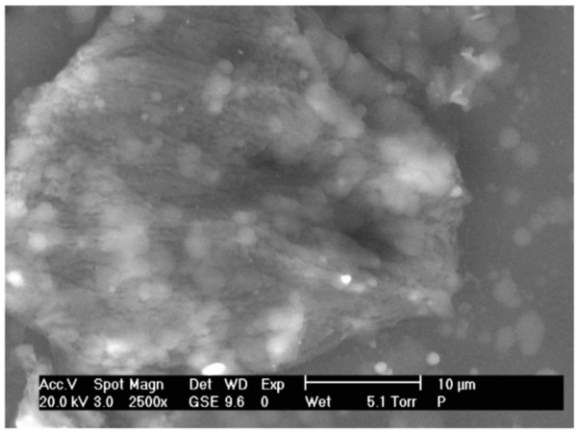

Fig. 2. ESEM micrography of the amorphous agglomerate of photochemical lignin model compound (magnification: 7453×; water vapor pressure: 5.1 torr).

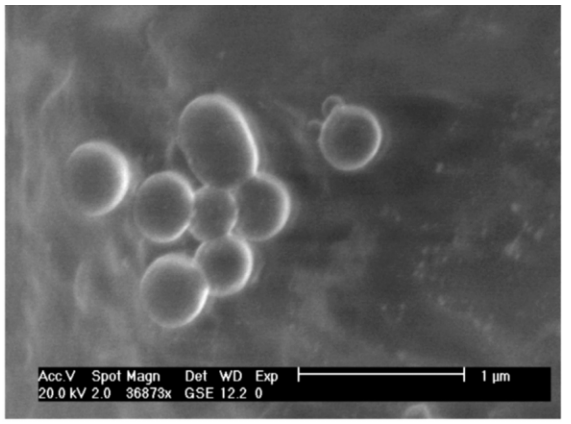

Fig. 3. ESEM micrography of the irregular spherulitic structures of photochemical lignin model compound (magnification: 36 873×; water vapor pressure: 1.0 torr).

strate. The sizes of agglomerates are very diverse, with diameters ranging from 2 μm to 50–80 μm.

Observations of both amorphous structures and rough globules could be explained in terms of the uncontrolled fusion of several globules into larger amorphous structures. This is due to UV irradiation, which induces two parallel processes: polymerization and depolymerization of formed polymer with formation of small fragments that can interconnect in different ways. Also, UV radiation can induce other mechanisms of polymerization not present in the case of enzymatic polymerization, such as photo-addition and photocyclization of double bonds in monomers, which can lead to different overall properties of the photochemical modified/polymerized lignin macromolecule. Exposure to UV irradiation will induce formation of covalent bonds between several adjoining macromolecules, which is impossible in the case of enzymatically-polymerized lignin. Finally, the enzyme during the polymerization process probably plays a templating role, dictating the shape of the macromolecules. In the case of photo-polymerization there are no templating effects, and the final product is a highly stochastic macromolecule.

However, occasional formation of spherulites and spherulite-like structures in the case of photopolymerization occurs and many of the amorphous structure actually are composed of spherulitic structures fused together, which could be observed on the images with higher electron beam intensity, as presented in Fig. 4, with 20 a kV imaging beam. Formation of spherulitic structures in the case of a photopolymer is of different origin than in the case of an enzymatic one. While enzymatic polymer shape is determined by both enzyme catalysis and a templating effect, spherical shapes in the case of photopolymerized lignin could be described partially in terms of the thermodynamics of such process.

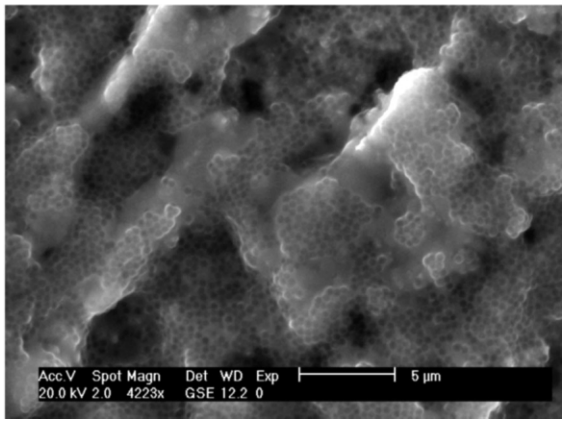

Fig. 4. ESEM micrography of the amorphous structure consisting of fused spherulites (magnification: 4223×; water vapor pressure: 1.0 torr).

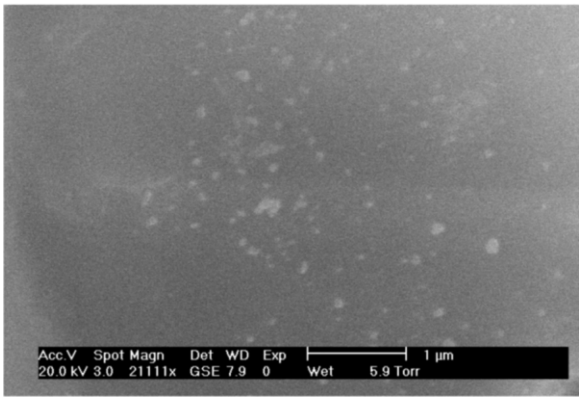

Fig. 5. ESEM micrography of the amorphous structure obtained after coniferyl alcohol irradiation through the cellulose acetate film (magnification: 21 111×; water vapor pressure: 5.1 torr).

The polymer produced by irradiation of a monomer solution through the cellulose filter has a less compact structure (Fig. 5) in comparison with that obtained without the filter, but the irregular sphere-like globules are formed also in this case. Also, it is well known that spehrulitic formations and coils could be expected in the block-copolymer blends. As lignin is a highly cross-linked and redundant polymer, any phenyl-propanoid oligomer can be considered as a monomer, so therefore polymerization is a block-copolymerization. As the lignin molecule has both hydrophilic and hydrophobic moieties, there is a thermodynamic reason to reduce the conformational energy of the molecule, by creating a geometrical object with lowest surface to volume ratio, which is a sphere. In this way, as polymerization is occurring in an aqueous environment, the smallest portions of mostly hydrophobic parts of lignin model compound macromolecules are exposed to the hydrophilic environment and thus the interaction energy minimized. This is essentially the same effect as explored by Laschmike in the case of natural lignin extracts [27]. The reason why it is not a completely spherulitic structure like in the enzymatic lignin is believed to be due to nature of photocross-linking, which occurs at different positions. Photocross-linkage can occur as a cyclobutane ring formation or as an addition reaction. Moreover, it occurs randomly, so the process of forging a perfectly spherical molecule is continuously disrupted.

From the functionalized atomic force experiment, we can see that there is no recognition observable between two molecules or assemblies of photochemical lignin. In our previous AFM studies of enzymatically polymerized lignin [12] features on the observed approach and retraction curve, with multiple adhesion peaks and events could be partially attributed to orchestrated host–guest interactions between two lignin macromolecules, and to secondary structure alternation of lignin macromolecules or/and macromolecular assemblies. However, in the case of the photochemical lignin model, a compound presented herein, it was not possible to obtain force scans which were as informative. Atomic force scans, shown in Fig. 6 represent only noisy scans where we can recognize only dominance of hydrophobic interactions, which are mostly phenyl rings $\pi-\pi$

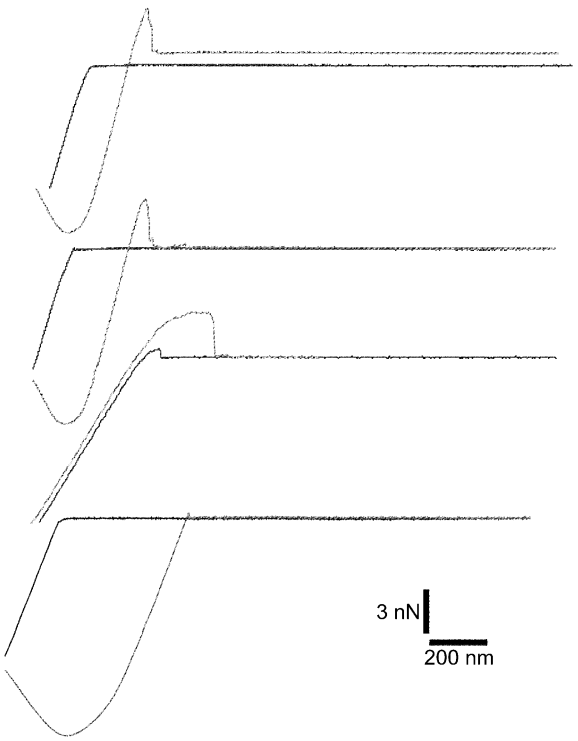

Fig. 6. Atomic force scans — surface spectroscopy of photochemical lignin model compound host–guest interactions.

interactions. An interesting feature is that adhesion strength varies significantly between the force scans, as well as the slope of the curve. Variation of slope indicates that there is a significant difference between the mechanical properties — modulus of elasticity and stiffness of photochemical lignin macromolecules or their assemblies.

There were no events observed which could indicate fusion of structures or any host–guest interactions. It is well known that almost all of the signaling actions in cells are performed via the host–guest interactions. There is no evidence in vivo that lignin is involved in signaling or signal transduction, but the clear observation of host–guest events in the case of AFM force scans with enzymatically polymerized lignin model compound and observed supramolecular assemblies, provide us with reasonable grounds to hypothesize that lignin may have signaling or cytoskeleton modifying functions performed with host–guest interactions. Thereby we can conclude that photochemical lignin, as a more cross-linked and random structure then the enzymatic one cannot perform physiological functions that are related to the host–guest interactions. Recent theoretical studies [28] provide evidence that there is an existence of the host–guest kind of interactions between the lignin and cellulose.

4. Conclusions

The irregular structures of photochemical lignin, unable to form regular motifs, probably could not perform any biological function. Such disordered structures are formed in the absence of horseradish peroxidase enzyme, which could play not only a catalyst but also a templating role in lignin synthesis. Occasionally, photochemical lignin seems to form spherulitic structures, but those structures possess a much wider size distribution than the enzymatic one, and they are not organized into the higher structure. Consequently, if photopolymerization of lignin precursors would occur in the plant cell walls, as a competitive process to enzymatic polymerization, produced structure would be non-functional, and could not form pores, channels and other structures of an ordered and semi-ordered architecture of the cell wall. Moreover, from the functionalized atomic force scans, we see that photochemically polymerized lignin molecules do not express patterns of host–guest recognition as enzymatic lignin model compound does. Lack of the host–guest interactions will make the photochemically polymerized/modified lignin molecule incapable of performing recognition functions, which must have a significant signaling or cytoskeleton reorganizing functions. Photochemical mechanism of lignin synthesis may be possible to occur in the living plant cell, and possible play a significant role in the plant cell wall under conditions of enhanced UV irradiation on the Earth's surface, as a competing process to enzymatic polymerization. The results herein presented provides more evidence that photochemical lignin polymerization may be one of the degrading effects of UV radiation to the plant cell.

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