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ZL-DHP lignin model compound at the air–water interface

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Abstract

In this paper we present our surface chemistry studies of enzymatically polymerized, poly-coniferyl alcohol lignin model compound (dehydrogenate polymer a.k.a. ZL-DHP) at the air–water interface. Using the $CHCl_3$/MeOH (5:1 v/v) spreading solvent, we found an average molecular area of ZL-DHP of approximately 1200 Å^2. The monolayer expresses a high compressibility with a collapsed area of 500 Å^2 and collapsed surface pressure of 28 mN m^{-1}. In the range of applied surface pressures, ZL-DHP polymer have no phase changes, as shown by the very high linearity ($R=0.994$) of absorbance vs. surface pressure cure. There was no symmetry transitions observed as shown by absence of shifts of absorption peak maximums. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Lignin is a highly cross-linked, amphiphillic biopolymer formed from different phenyl-propanoid units, connected mostly by various ester bonds. It is the second most abundant polymer compound on the earth [1]. Lignin is, together with cellulose, the main constituent of the higher plant cell wall. While it is more than 150 years since lignin discovery, its role and structure still remain largely unknown [2]. There is a common belief that lignin only play mechanistic support roles in the plant cell. Knowledge of the physical and chemical properties and organization of lignin is of great importance to several sciences and technologies, including but not limited to molecular biopolymer sciences, molecular and cell biology, botany, wood chemistry and technology. One thing that is known for sure, from structural biology studies of the plant cells is that the lignin are located within the structures of trachea, lamellae and secondary cell wall membranes. As this molecule is highly interlinked and cross-linked with cellulose and other constituents of cell wall and other structures, its isolation in unaltered form is impossible to achieve. Therefore, lignin model

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compounds are used in most studies of lignin, as best available in vitro substitutes.

In plant cell, lignin is synthesized through the highly orchestrated process of enzymatic synthesis involving peroxidase enzymes and monolignon compounds such as *p*-coumaryl and coniferyl alcohol [2]. Such synthesis produces highly cross-linked polymer, with at least 11 different types of bonds formed between monolignon units [3]. Until today, a plethora of work has been done to reveal details on all mechanism steps of enzymatic lignin synthesis, wherein the mystery of existence [4] or non-existence [5] of templating protein involved in synthesis is not yet resolved. Since the early 1950s [6], lignin-like macromolecular compounds are synthesized in vitro, using the enzymatically catalyzed free-radical reaction involving peroxidase and monolignons such as *p*-coumaryl and coniferyl alcohols, the most common one being dehydrogenate poly-coniferil alcohol also known as ZL-DHP polymer.

As lignin is mostly found in the interfacial structures of the outer cell wall, it is right to expect that most realistic biomimetic experiments will be to study its behavior at the air–water interface using the Langmuir monolayer technique. It is a well-known fact that most materials, especially amphiphillic ones behave completely different at interfaces than in bulk, due to the surface free energy and associated phenomena. The Langmuir monolayer technique is unique for studies of amphiphillic molecules, as it allows us to follow the change of the molecular properties in respect to their two-dimensional organization by limiting free molecular area, starting from gaseous phase, through liquid and condensed, and up to the crystalline phase. While the main parameter followed in the Langmuir monolayer study is the surface pressure–molecular area isotherm, there is a lot of information that could be retrieved, if in parallel with compression we are just following other properties, such as surface potential, UV-Vis spectra, and Brewster angle microscopic topography. In the case of the lignin, Langmuir monolayer studies are extremely important, as plant cells during their life cycles go through significant changes in osmotic pressure, physiologically represented as a tugor, and through a dramatic change in size (shrinkage/expansion). These effects could be mechanistically modeled using the Langmuir monolayer compression and thereby associated studies.

In this paper we explore interfacial properties of the lignin model compound based on its most common monolignon—coniferyl alcohol. We have prepared enzymatically polymerized poly-coniferyl alcohol, i.e. lignin model compound synthesized when monolignon, hydrogen peroxide and enzyme are mixed at the beginning of the reaction, which is well known as the dehydrogenate polymer or ZL-DHP. The questions that we are trying to answer by this work is to obtain a clear, stable and repeatable Π-A isotherm of lignin model compound, and if so, to explore the change of lignin's physico-chemical properties with compression and influence of polymer composition on its properties. Most significantly, herein we provid an answer if there is a phase transition occurring during the lignin monolayer compression, and what changes to expect in spectral data, and its possible physiological significances.

Based on our AFM force spectroscopy research [7], we propose a hypothesis that the lignin structure consists of hollow spherical supramolecular assemblies. One of our goals for this monolayer work will be to see if we can observe such lignin assemblies in a two-dimensional system such as monolayer to identify its fusion with an increase of surface pressure, and, to comment on its structure and the orientation of lignin in living plant cell wall membrane systems.

2. Materials and methods

2.1. Enzymatic lignin model compound (ZL-DHP) synthesis

Fifthy milliliter solutions of $5 \cdot 10^{-3}$ M coniferyl alcohol, $5 \cdot 10^{-3}$ M *p*-coumaryl alcohol, and respective mixtures of 0.75/0.25, 0.50/0.50 and 0.25/0.75 by weight/weight (w/w) parts of coniferyl and *p*-coumaryl alcohol's at approximate concentrations of $5 \cdot 10^{-3}$ M were prepared in aqueous phosphate buffer, pH 7.6. A solution of $5 \cdot 10^{-3}$ 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 Chem. Corp., NY) has been prepared. Lignin was polymerized in solution by mixing enzyme and H_2O_2 solution with alcohol solutions, in the same beaker at room temperature, 25 °C. The reaction took 48 h to complete. Molecular weights were determined using the GPC. Dehydrogenate polymers (DHPs) were recovered from suspension using the freeze-drying technique. More details about lignin DHP synthesis can be found in ref. [8].

2.2. Preparation of monolayer spreading solutions

DHP polymer, prepared as described above, was dissolved in HPLC grade solvent mixture, consisting of $CHCl_3$ and CH_3OH in 5:1 volumetric ratio. Concentration was calculated for 10^{-4} M of polymer, based on an estimated average macromolecule molecular weight of 50000 Da, as obtained by gel permeation chromatography (GPC).

2.3. Langmuir monolayer experiments

All surface chemistry experiments were conducted in a clean room class 1000, at constant temperature of 20.0 ± 0.5 °C. For the purpose of obtaining Langmuir monolayer isotherms, a KSV mini-trough (KSV Instruments Ltd, Helsinki, Finland) with a spreading area of 225 cm^2 was used. Surface pressure was monitored with trough electronic balance based on paper Wilhemly plate pressure detector with a sensitivity of 0.02 m Nm^{-1}. Compression of the monolayer was performed using the two symmetrically movable barriers with a computer-controlled step motor. Used spread volume was 15 μl, and the average compression speed was 5 A^2 mol^{-1} min^{-1}. Sub phase in all experiments was de-ionized ultra-pure water, with the following physico-chemical properties: specific resistance: 18 MΩ cm; surface tension 72.6 mN m^{-1} at 25 °C. To avoid possible lignin photochemical alteration experiments were performed in a dark environment.

Surface potential was measured in situ with a capacitance based system (Kelvin probe). The vibrating plate was placed 1 mm above the surface of the monolayer air–water interface and this was immersed in the subphase at the back of the moving barriers. As a reference, signal from clean subphase was taken as arbitrary zero.

KSV though has a quartz window at the bottom for the purpose of spectroscopic investigations. We have performed in situ UV-Vis spectroscopy on the monolayers as a function of surface pressure using the HP 8452A diode array spectrophotometer (Hewlett Packard) mounted on a sliding rail surrounding the through. UV-VIS spectra, in the range between 200–450 nm were acquired with every 2 mN/m increase of surface pressure, for all polymers. Spectra were later processed using the Origin v. 4.0 software, and maximum absorbance was plotted against the surface pressure to examine if phase transitions occur during the compression or expansion of monolayer.

3. Results and discussion

An interfacial property of lignin have been the object of scientific inquiry in the last 7 decades. Freudenberg and Braun [9] has done the first work of spreading extracted lignin derivatives which had a molecular size of 20 Å^2, and concluded that lignin is at least 20 Å thick and has a three-dimensional structure. Following research by Wedekind and Katz [10] it was shown that lignin monolayer has been approximately 10 Å thick, with lignin average molecular size in the area of 80 Å^2. In this early study, acetylated lignin fraction from spruce was used. A much larger molecular area of 318 Å^2 and with a monolayer thickness of 16 Å was reported by Loughborough and Stamm [11], who were working with maple lignin extracted using methanol as a solvent.

However, these early studies suffered from several problems. At first, it was not possible to purify and characterize accurate lignin fractions. Also, the surface pressure measurement techniques at this time were not as accurate as today so these results needed to be taken with certain precautions. The first modern surface chemistry work on milled wood lignin, dioxane lignin, and craft lignin on air–water interface was done in 1970 by Luner and Kemp [12]. Besides surface pressure-area isotherms, they have also analyzed surface potential and calculated dipole moments for above-mentioned lignins. Interesting findings were that lignin forms monotonous gel-like film with very low compressibility, lower than for most polymers spread on the air–water interface. The surface chemistry results depend on the method of lignin extraction. The average molecular area found was from 17.1 to 10.0 Å^2 per molecule. Low electric dipole moment was observed in all extracted lignins, with the lowest one for the Kraft lignin. They have observed for the first time an irreversibility in terms of the surface pressure-area per molecule hysteresis.

Luner and Roseman [13] extended their work on a larger number of model compounds and also studied the thermal dependence on the surface pressure-area isotherms. The most significant finding was that lignin express anomalous isotherm behavior at a temperature of 37 °C, and higher, due to the disruption of hydrogen bonding, and for the first time the monolayer of lignin model DHP compound obtained by polymerization of coniferyl alcohol with lactase enzyme was characterized. It was found that such DHP polymers have a limited molecular area of 18.4 Å^2 per molecule, higher surface potential compared to other studies on lignin, higher collapse surface pressure, and lower hysteresis energy i.e. 1.85 cal. Gilardi and Cass [14] studied the colloidal behavior of solvent-extracted spruce lignin. Their most significant finding was that the pH has an effect on the apparent molecular weight and size of aggregations, whereas a decrease in pH induced an increase in molecular weight and size of lignin supramolecular aggregates. Using the Langmuir techniques with a fraction of MW 70000 Da they found a limiting area per lignin macromolecule in the range of 190 nm^2, and a collapsed surface pressure at 45.5 mN m^{-1}.

Oliveira and associates published several articles about the preparation of Langmuir–Blodgett film of lignin [15–18]. They presented for the first time formation of a L–B film of HPLC-GPC lignin fractions with different molecular weights, and have explored the influence of the molecular weight on the molecular area. The number of layers deposited against the film thickness (60 Å per layer) was reported [15]. For the first time composite L–B films consisting of lignin and cadmium stearate have been prepared for surface-potential, FTIR, UV-Vis and XRD measurements. UV-Vis results presented therein, suffered from very poor resolution. Combining the above-mentioned techniques they concluded that both lignin and cadmium stearate were present in domains, and that the spatial arrangement and shape of cadmium stearate domains were influenced by the incorporation of lignin domains. Some groups recently reported the observation of experimental artifacts in measuring the surface pressure of lignin [17], concluding that the pressure area isotherm and its hysteresis is deeply influenced by different forces experienced by Wilhelmy plate pressure sensor, depending on the compression speed and position of the Wilhelmy plate, as well as the size and configuration of the through. Topographical characterization, seeing the AFM microscopy, of L–B film of sugar cane baggasse lignin and cadmium stearate has been reported by O.N. Oliveria Jr and co-workers [18], finding that composite lignin–cadmium stearate mixed L–B film express smoother surface compared with pure lignin or pure cadmium stearate L–B film. The film roughness increased with the number of deposited layers in L–B film. Visualization of immiscibility of cadmium stearate with lignin in the form of the formation of distinct separate domains of cadmium–stearate which could be easily distinguished from the topography.

Surface properties of grass lignin, with binar molecular weight distribution of 3600 and 21000, spread on air–water interface were examined by Baumberger et al. in ref. [19] with special emphasis on the rheology of the film, wherein high relaxation of film is found after the compression is stopped, dilatation modules range from 1.5 to 72 mN m^{-1} and the average molecular area from 20 to 160 Å^2 molecule^{-1}.

Very interesting results of rheological monolayer studies of both ZL-DHP and ZT-DHP lignin model compound synthesized by the enzymatic polymerization of coniferyl alcohol in the anoxic environment in the presence of peroxidase enzyme has been published in 1998 by Cathala et al. [20]. Most significant findings of their study are the great differences in molecular area between ZT-DHP 136 Å^2 molecule^{-1} compared to ZL-DHP 563 A^2 molecule^{-1}, and a collapsed surface pres-

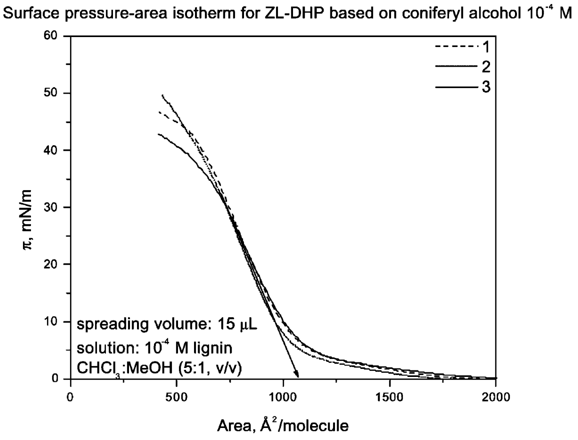

Fig. 1. Surface pressure-area isotherm of enzymatically synthesized lignin model compound (ZL-DHP polymer).

sure of 49.3 and 26.5 mN, respectively. Big differences between molecular area, as calculated by ideal gas equation, and measured area indicate strong presence of intramolecular interactions between lignin macromolecules in monolayer. Both lignin model compounds express expansion after the end of the compression, similar to the extracted lignins. Dilatation modules reported therein are in the range of 29–466 mN m^{-1} for ZT-DHP and 13–270 mN m^{-1} for ZL-DHP. These data indicate that there is a chemical/structural difference between DHP polymers prepared by instantaneous mixing of reactant (ZL) and continuous addition of reactant (ZT) polymers. The most recent paper published about lignin surface properties deals with Langmuir film mixtures of asphaltene with Kraft and lignosulfonate lignin [21], but here we concentrate on the study of pure lignins monolayer properties, their observations are beyond our scope of interests.

Fig. 1 shows surface pressure-area isotherms of lignin model compound. DHP polymer form a very stable monolayer with a high degree of reproducibility of the results, for example curves 1,2,3 which are almost identical, have been taken in time intervals of 30 min. These isotherms by their shape represent the typical isotherms of the polymer monolayer. The monolayer starts forming the liquid expanded state at approximately 1800 Å^2 molecule^{-1}. Limiting molecular area where the film should be most compact is approximately 1200 Å^2 molecule^{-1}. Smooth, virtually linear liquid condensed phase last until the molecular area is decreased to approximately 600 Å^2. The not so clear solid state is characterized by the change in slope and shape of the isotherm, and the collapse suddenly occurs at the surface pressure between 43–49 mN m^{-1}. However, from the shape we can assume that actual growth to the 3rd dimension occurs. Critical molecular area is in the range of 450 Å^2 molecule^{-1}. Change in molecular dimensions for almost 800 Å^2 molecule^{-1} during the monolayer compression could be explained in terms of lignin modular structures, wherein supramolecular structures—association of several lignin macromers held together by non-bonding interactions first provide a rise in the curve at the transition from gaseous to liquid expanded state. At the higher surface pressure, in liquid condensed states, whole film could be construed as one gigantic supramolecular assembly, and the final collapse occurs when we have a formation of multilayer by stacking of lignin macromolecules to a third dimension.

Fig. 2 depicts the surface potential-area isotherms. While we have tried several times, it seems to be impossible to obtain clean, repeatable surface potential-area isotherm. It could be explained again in the modular and random structure of lignin. As lignin macromolecules are three-dimensional structures, and as we are working with the highly redundant polymer, there are no two identical lignin macromolecules. As they have a plethora of functional groups capable of forming hydrogen bonds, thereby there are an infinite number of combinations by which molecules can be oriented at the air–water interface and infinite number of orientations.

Based on an idea of the modular structure of lignin, the TEM microscopy results by Falkehag [22], molecular mass distribution by Wayman [23] and Cathalla [24], as well as our results, confirmed that the lignin model compounds based on coniferyl alcohol also expressed modular structures [25,26] and it is to be expected that there should be a phase transition of lignin at the air–water interface, also with an increase in surface pressure there should be a rearrangement of lignin supra-

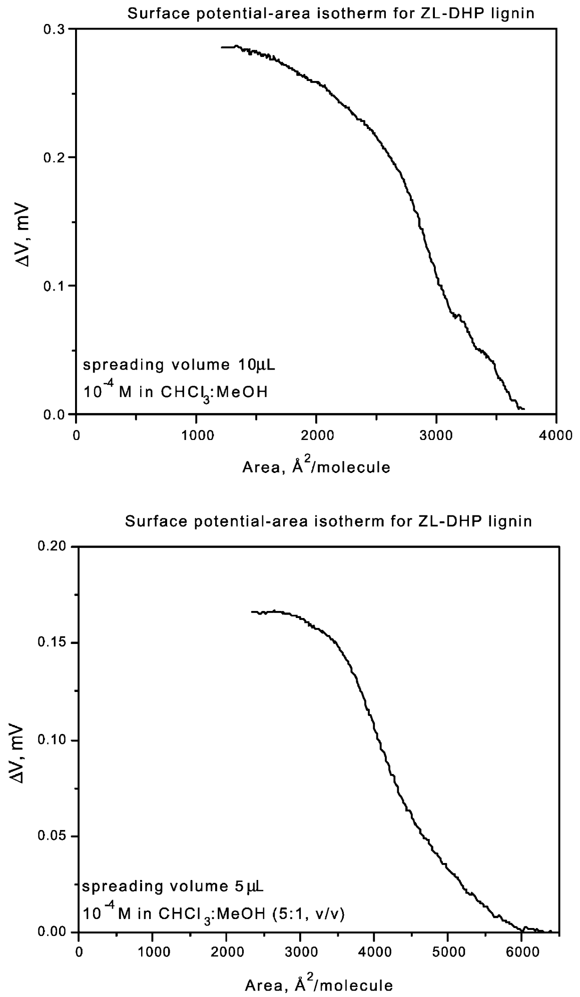

Fig. 2. Surface potential-area isotherm of ZL-DHP lignin model compound.

molecular organization. However, nobody has ever detected such behavior, this is one more reason for us to pursue this study. One of the main differences in our study compared with previous studies is that we used a much more volatile solvent, $CHCl_3$/MeOH spreading the solvent differently to DMSO and THF, which were commonly used in previous studies. In order to examine structural and orientation changes, if any, in gel-kind of monolayer with an increase in surface pressure, we decided to use absorption spectra as a main tool, combined with surface pressure-area isotherm and surface potential-area isotherm. Based on the change in absorption with surface pressure it is possible to estimate if there is a phase transition in the film with compression in the case of existence of non-linear relationship. On the other hand, if change in any of the peak positions occurred during the film compression this will indicate change in symmetry at the molecular level—molecular rearrangement induced by change in conformation or influence of supramolecular assemblies. Using the UV-Vis spectroscopy it will be possible to detect molecular rearrangement in the monolayer at a much higher sensitivity than just looking at the surface pressure-area and/or surface potential-area isotherms.

In order to examine if there is any distinct phase or aggregations of macromolecules within the liquid expanded and liquid condensed states, UV-Vis spectra were measured at different surface pressures from 0 to 28 mN m^{-1} at every 2 mN m^{-1}. The reason why spectra were not acquired after the 28 mN m^{-1} is because after the collapsed pressure, the film started growing in a 3rd dimension, i.e. we have a gelation followed by monolayer collapse. From spectra, presented on Fig. 3, we can clearly see that there are no shifts in the position of the absorption bands. No shift means that there is no change in molecular symmetry

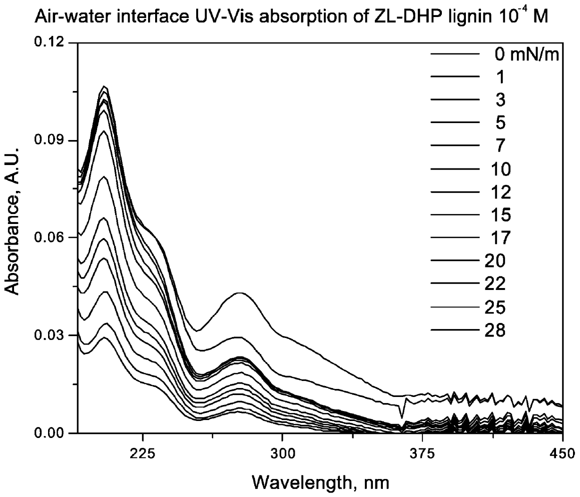

Fig. 3. UV-VIS spectra of ZL-DHP lignin model compound. Spectra has been acquired with the step of 2 mN m^{-1}.

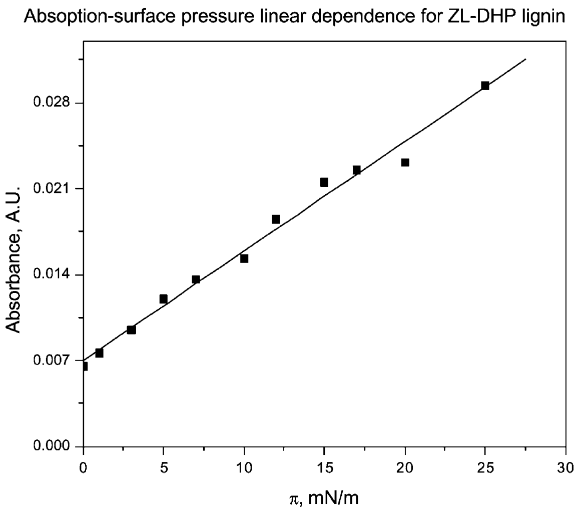

Fig. 4. Linear dependence of optical absorption vs. surface pressure for ZL-DHP lignin model compound.

with monolayer compresson. Moreover, if we follow the change of absorbance with surface pressure, as shown in Fig. 4, we observe a linear increase in absobance against the surface pressure, up to 28 mN m^{-1}. This indicates that there is no phase transition occurring in the lignin monolayer within this surface pressure range.

Such behavior of lignin may have a significant physiological role. As the plant cell life depends on the osmotic pressure, it is of great importance that the membrane structure could expand and contract, as per environmental conditions. This result suggests that the intrinsic property of lignin macromolecule can undergo such tremendous change in size without inducing phase-transition and symmetry changes and its assemblies are the key to osmotic pressure and cell size control mechanism that is developed in the higher plant cells.

4. Conclusion

Stable surface pressure-area isotherm of the poly-coniferyl alcohol based lignin model compound is obtained, using $CHCl_3/MeOH$ (5:1 v/v) solvent system. Surface properties of the lignin model compound monolayer shows that in a wide range of surface pressures, up to 28 mN m^{-1} DHP, polymers have no phase changes, as shown by the very high linearity ($R = 0.994$) of the UV-VIS absorption increases with an increase in surface pressure. The molecular area approximately 1200 $Å^2$ is probably much higher than the molecular area of the in vivo lignin in the cell wall membrane. This molecule expresses a large compressibility, as its collapse occurs at approximately 500 $Å^2$. This is probably due to the enormous number of possible rearrangements of lignin macromolecules in the monolayer in such a way that they can form hydrogen and other non-covalent bonds. It also shows us that the lignin macromolecule is highly flexible due to ester bonds, and that the macromolecule can easily assume many different stable or metastable conformations resulting possibility in reversible dramatic changes in molecular size/volume. An intermolecular non-covalent bonding interaction probably plays a significant role in stabilizing lignin macromolecules in different conformations. Irrepeatability of the surface-potential-area isotherms confirms the above-mentioned hypothesis that it is impossible to have two times the same arrangement of highly random macromolecules.

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