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Study of thin films of kraft lignin and two DHPs by means of single-molecule force spectroscopy (SMFS)

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Abstract

The adhesion of single and associated lignin chains to a substrate has been studied by means of single-molecule force spectroscopy (SMFS). Softwood kraft lignin (KL) and two lignin polymer models (dehydrogenation polymers, DHPs) based on coniferyl alcohol (DHP_{c.alc.}) and coniferinaldehyde (DHP_{c.ald.}) were in focus. The desorption force from the “silicon nitride SMFS tip” for the KL was significantly greater than that of the DHPs. The higher desorption force was interpreted as being due to the interaction of carboxyl groups through hydrogen bonding with the tip as well as to the less compact polymeric layer at the interface. The distribution of the extended chain lengths was determined, and self-association of lignin chains was observed. For both KL and the DHP_{c.ald.}, chains were extended significantly beyond the limit that would be expected for polymers with the corresponding degree of polymerization. The α -carbon on the DHP_{c.alc.} has a strong intramolecular hydrogen bonding interaction with the adjacent aryl ether, which inhibits the possibility of the ether to participate in intermolecular hydrogen bonding with nearby lignin chains. Thus, the self-association for KL and DHP_{c.ald.} was found to be dominated by intermolecular hydrogen bonding with carboxylic groups and aryl ether functionalities.

Keywords: adhesion; atomic force microscopy (AFM); dehydrogenation polymer (DHP); kraft lignin; lignin; polymer association; poor solvent; surface forces.

Introduction

Lignin is a naturally occurring macromolecule found in the cell wall of the stems of the plant kingdom. It is the second or third most abundant biopolymer after cellulose. Lignin is a branched polymer consisting of substituted phenylpropanes connected in about 10 different ways (Freudenberg and Neish 1968; Sarkanen and Ludwig 1971; Hon and Shiraishi 1991; Sjöström 1993; Terashima et al. 2009). The substituents and bonding connections are highly dependent upon the source, and significant variation can even occur between trees of the same species. Thus, understanding the structure at the molecular level is a challenge, particularly because available techniques for its isolation inevitably alter the chemistry in terms of both degree of polymerization as well as the introduction of non-native chemical groups (Vanholme et al. 2008).

The noncovalent interaction of lignin with itself or with the carbohydrates (hemicelluloses and cellulose) in the cell wall is still not well understood although several authors have contributed to this topic (Lawoko et al. 2005; Salmén and Burgert 2009; Stevanic and Salmen 2009; Li et al. 2011). Molecular modeling seems also promising with this regard. Besombes et al. (2004) demonstrated the interaction between adjacent hydroxyl and methoxy groups via hydrogen bonding by studying dimeric lignin model compounds with the guaiacyl type β -O-4 units. These interactions are relatively weak and do not confer rigidity to the dimer. The intermolecular hydrogen bonding with solvent molecules was found to be dominating as shown by the nuclear magnetic resonance (NMR) measurements.

Kubo and Kadla (2005) studied the hydrogen bonds of lignin model compounds by FTIR spectroscopy: (1) Aliphatic hydroxyl groups (OH_{aliph.}) formed stronger hydrogen bonds than the phenolic hydroxyl (OH_{phen.}) groups. (2) The hydrogen bonding between monomeric models was significantly weaker than between the biphenyl models. This study confirmed the presence of intramolecular hydrogen bonding between adjacent hydroxyl and methoxy groups. The quoted authors also studied kraft lignins (KLs) from softwood (SW) and hardwood (HW) and demonstrated that the extent of the noncovalent interactions was greater for HW (containing a lot of syringyl type units).

The thin films technique – obtained from well-defined lignin polymer samples – is also suited for studying non-covalent interactions (Constantino et al. 1996, 1998, 2000; Micic et al. 2000, 2001a,b, 2004; Pasquini et al. 2002, 2005; Norgren et al. 2006, 2007; Notley and Norgren 2006, 2008; Tammelin et al. 2006). Several authors studied the physico-chemical properties of KL at an interface based on smooth, well-characterized films (Norgren et al. 2006, 2007; Notley

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and Norgren 2006, 2008). In particular, the interaction of aqueous solvent with lignin was in focus as a function of solution conditions including pH and ionic strength by monitoring the amount of water within a lignin film (Norgren et al. 2007). There was no significant water uptake into the thin film at pH < 9. This was interpreted as the lignin chains experiencing poor solvent conditions at the interface and within the bulk. As the pH was increased, the lignin film began to swell, and at pH > 10.5, the lignin became completely soluble. These observations are in agreement with other studies focusing on the solubility of KL (Norgren and Lindstrom 2000). In summary, at elevated pH levels, the polymer-solvent interactions dominate over the polymer-polymer interactions.

Single-molecule force spectroscopy (SMFS) (Rief et al. 1997; Chatellier et al. 1998) is an efficient way for studying interaction of surfaces and interfaces. SMFS is a special form of atomic force microscopy (AFM), the tip of which is in a specific physisorption interaction with the material. The goal of the present study is to contribute to the knowledge about lignin-lignin and lignin-solvent interactions by studying model lignin films with SMFS. The films will be prepared from a KL and two different dehydrogenation polymers (DHPs).

Materials and methods

KL

The KL was isolated from the black liquor of pulping of *Picea abies* (SW) as was the case in the studies of Norgren et al. (2006), Notley and Norgren (2007), Norgren et al. (2007), Notley and Norgren (2008, 2010). Typically, SW lignin consists of guaiacyl propan units (>95%), which is the results of the dehydrogenative polymerization of coniferyl alcohol. Most of the linkages are of the type β -O-4 bonds. The mean molecular weight (MW) of the KL (3 kDa) was determined according to Norgren et al. (2006) from ^1H pulsed field gradient NMR self-diffusion measurements (Bruker Avance DPX 250 MHz spectrometer, Bruker BioSpin, Germany).

Preparation of DHPs

The DHPs were prepared with the monolignols coniferaldehyde (leading to DHP_{c.alc.}) and coniferyl alcohol (leading to DHP_{c.alc.}) according to Holmgren et al. (2009). One gram of monolignols was dissolved in 10-ml acetone and then mixed with horseradish peroxidase type VI (5 mg, 1450 U) and 190 ml of 50 mM K_2HPO_4 buffer (pH 6.5) ("bulk polymerization" mode). A solution of hydrogen peroxide (74 mM) in 200 ml of phosphate buffer was dropped at 10 ml h^{-1} into the monolignol solution. After 24 h, the reaction was stopped, and the suspended polymerisate was centrifuged at 10,000 rpm and the residue was washed with deionized water two to three times. The material was suspended finally in water to be freeze-dried. Low MW compounds were extracted for 1 h with 50 ml of CH_2Cl_2 .

The MW of the DHP_{c.alc.} was about 4500 Da (Mw/Mn 1.4) and that of DHP_{c.alc.} about 2700 Da (Mw/Mn 1.4) (data obtained by using size exclusion chromatography according to Holmgren et al. 2009, who also provided the ^{13}C NMR data of these samples).

Lignin phosphorylation

Lignin phosphorylation was performed according to Argyropoulos et al. (1993), Argyropoulos (1994), Granata and Argyropoulos (1995), Sun and Argyropoulos (1995), and Guerra et al. (2006). Shortly, a solvent mixture (solution A) of dried pyridine and CD_3Cl mixed in a 1.6/1 v/v ratio was prepared, and molecular sieve (3 Å) was added to minimize the moisture. In a 10-ml flask, 110 mg of cyclohexanol and 55 mg of chromium(III)acetylacetonate, serving as an internal standard and as a relaxation reagent, were accurately weighted and diluted to the mark with solution A to create solution B. The lignin samples (40 mg) were accurately weighted into 2-ml volumetric flasks, and 820 μl of solution A was added to each sample. The flasks were sealed with Teflon-lined screw caps and dissolved by thorough mixing for 72 h. Thereafter, the lignin samples were phosphorylated by an addition of 80 μl of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, and the flasks were sealed and shaken. In a final step, 100 μl of solution B was added, and the flasks were sealed and shaken once again.

Preparation of lignin films

The lignin films were prepared according to Norgren et al. (2006). A 1% solution of lignin in 1 M NH_4OH was spin coated onto oxidized silicon wafer. The wafer was first cleaned in NaOH solution, then rinsed with Milli-Q water, and rinsed again with EtOH before being blown dry under N_2 . Finally, a mild water plasma treatment was undertaken to ensure that the surface was clean and completely wetting before the deposition of the lignin. The spinning parameters were 1500 rpm for 60 s, yielding smooth, continuous, and uniform films. The films were rinsed with Milli-Q water immediately after deposition, blown dry with N_2 , and stored in a desiccator before SMSF.

Characterization of lignin films

The lignin films were characterized in terms of their thickness, surface roughness, and surface energy. The thickness was measured by ellipsometry. The instrument used was a high-resolution phase modulated ellipsometer (Beaglehole Instruments, New Zealand). At least two different films and three different places on each film were measured. The surface roughness was characterized by tapping mode AFM imaging under ambient air conditions. A multimode AFM (Veeco, USA) was used to scan the surfaces in the noncontact mode with a TESP silicon dioxide cantilever (Veeco). Scan sizes of $1 \times 1 \mu\text{m}$ up to $10 \times 10 \mu\text{m}$ were performed on various areas of the film to ensure that the surfaces were smooth and continuous. The surface roughness was determined from the height images over an area of $1 \mu\text{m}^2$. At least five images were taken for each film, and the roughness presented is the average of these images.

^{31}P NMR analysis Instrument

Bruker Avance 500 MHz NMR spectrometer (Bruker BioSpin) according to Granata and Argyropoulos (1995), Sun and Argyropoulos (1995), and Guerra et al. (2006) was used. NMR precision tubes (5 mm) (Glasser AB, Sweden) were applied. Spectrometer settings were as follows: sweep width, 6.6 kHz; pulse angle, 45° ; inverse gated decoupling; relaxation delay time of 25 s between successive pulses. The chemical shifts reported are relative to the reaction product of water with the phosphorylation reagents at 132.2 ppm. In the processing of the spectra for the quantitative determinations, an exponential window function with a line broadening of 3 Hz was used.

AFM single-molecule stretching technique Instrument

Multimode AFM (Veeco) equipped with a silicon nitride cantilever (Veeco) of spring constant 0.06 N m^{-1} was used; calibration was based on the thermal noise method (Hutter and Bechhoefer 1993). For each of the solution conditions tested, a minimum of 100 force-extension curves were measured with the data presented here representative for each condition. A schematic representation of the desorption of a chain from the surface is shown in Figure 1. The single-molecule stretching experiments were performed in NaCl solutions with a concentration of 1 mM with pH adjusted to 8.0. All solutions were prepared in Milli-Q water and used within 24 h.

Results and discussion

Before beginning the discussion, the model nature of the materials used in this study needs to be recalled. A KL is heavily denatured during the course of the cooking process and subsequent isolation. Moreover, the structure of DHPs are very different to native lignins, especially if the DHP preparation was done according to the “bulk polymerization” mode and at the relatively high pH of 6.5, as in this paper, rather than the “end-wise polymerization” mode at pH 4–5 (Sarkanen and Ludwig 1971; Landucci et al. 1998; Sipilä et al. 1998). Thus, the SMSF results of native lignin may be different.

The surfaces of the lignin thin films were imaged by AFM. Figure 2 shows tapping mode height images of the KL surface as well as those of the two DHPs prepared through spin coating of the sample solution onto an oxidized silicon wafer. As readily visible, the DHP films are significantly smoother than the KL film. The roughness calculated from the images for the DHP surfaces is in the range of 0.3–0.4 nm, whereas the surface roughness of the KL is in the range of 1.1 nm. This difference is likely related to the solution properties of the samples, which influence the formation of aggregates. Furthermore, the branched nature and greater MW of the KL compared with the DHPs will influence the film-forming ability after deposition by spin coating.

Previously, the chemical functionalities of KL and DHPs investigated here were characterized by FTIR and NMR spectroscopy (Holmgren et al. 2009; Notley and Norgren 2010). For quantifying the proportion of functional groups capable of H bonding, the samples were analyzed by ^{31}P NMR after phosphorylation. This procedure allows a determination of the

Figure 1 Schematic representation of the interacting area during the withdrawal of a polymer chain under poor solvent conditions. As the surfaces are separated, monomer units are sequentially desorbed from the surface; the energetic cost is the same for each monomer, hence giving rise to the constant force plateau.

amounts of different hydroxyl groups present (Table 1). The KL and the DHP_{c.ald.} sample both contain significantly greater amounts of total OH_{phen} groups than the DHP_{c.alc.}, however, fewer OH_{aliph} groups. In addition, the KL has carboxylic groups, which are absent in both DHPs.

The stretching of single chains of lignin from the different DHPs was investigated by SMFS. A typical force-distance curve on separation of the Si_3N_4 AFM tip after contacting a lignin film interacting in aqueous solution is presented in Figure 3. Although the tip is notionally Si_3N_4 , the surface readily oxidizes to give SiO_2 (Senden 2001). Thus, it is likely that the lignin chains interact with the tip through H bonding. Three constant force plateaus are observed, each approximately an integer multiple of the lowest force, indicating that multiple chains are extended into the solution. The plateau depth is defined as the difference between the average magnitude of the force along the plateau region to the zero force condition of the baseline at very large separation. The form of the data in Figure 3, that is, the constant force plateau profile, is characteristic of lignin chains pulled into a poor solvent. By investigating these adhesion events statistically in terms of the magnitude of the depth of the force plateau as well as the detachment distance, a great deal of information about the polymer properties may be derived.

Single-molecule stretching studies were performed on cellulose and hemicelluloses (Morris et al. 2004; Radtchenko et al. 2005; Notley 2009). Only a few polymer chains are pulled away from the surface, with each chain causing an “event”, which can be observed in the force-distance curve. Typically, the shape of the events in the force-distance curve gives an indication of the solubility of the polymer in the solvent into which it is being stretched (Chatellier et al. 1998;

Figure 2 Tapping mode AFM height images of KL film (a), DHP alcohol (b), and DHP aldehyde (c). $1 \times 1 \mu\text{m}$ with z height contrast of 5 nm.

Table 1 Hydroxyl content (mmol g⁻¹) in lignin samples determined by quantitative ³¹P NMR analysis (single point measurements).

Sample	Various phenolic groups (phen.)			Total OH _{aliph}	COOH groups
	G-units+demeth. phen.	Cond. phen.	Total		
KL	2.7	1.0	3.7	1.9	0.48
DHP _{c.alc.}	0.8	0.8	1.6	3.1	NA
DHP _{c.ald.}	4.7	NA	4.7	1.2	NA

KL, kraft lignin; DHP_{c.alc.}, DHP prepared from coniferyl alcohol; DHP_{c.ald.}, DHP prepared from coniferyl aldehyde.

Senden et al. 1998). A good solvent gives rise to “Langevin” type interaction curves that may be described as an increasing, nonlinear adhesion force with separation before desorption back to the baseline. The increasing adhesion is due to the decrease in entropy as the polymer chain is stretched in such a way that all bonds are in the *trans* configuration. These curves may be fit by means of either a freely jointed chain or worm-like chain model to yield the contour length of the polymer as well as the persistence length. A poor solvent interaction is characterized by a constant force plateau as a function of separation of the probe from the surface. If more than one chain is pulled away from the surface, then a plateau is observed for each chain, with the magnitude of the plateau corresponding to an integer multiple of the lowest force. Micic et al. (2001a) demonstrated for an enzymatically polymerized DHP: the interaction of the molecules stretched into the aqueous solution have the Langevin characteristic profile of a polymer extended in a good solvent at pH 7.0. This is perhaps counter-intuitive when the solubility of lignin is considered. The results in the present study demonstrate that poor solvent conditions are prevalent as evidenced by the plateau shape extension of chains into the solution. The discrepancy to previous results is most likely due to differences in sample preparation. Here, the lignin has been deposited as a thin film, whereas in the Micic et al. (2001b) study, spherulitic lignin particles were deposited in a fashion,

which permitted a high degree of solvent penetration leading to good solvent conditions.

The distribution of the depth of the force plateau measured by SMSF for the KL as well as for both DHPs is presented in Figure 4. For the KL, there is a dominant peak in the distribution centered at about 85 pN with a smaller secondary peak at 180 pN of approximately half intensity. Apart from these two peaks, which account for over 70% of single molecular stretching events, there are a number of other events that resulted in plateau of variable depth. Interestingly, the distribution of plateau depth for the DHP shows peaks at significantly lower force; in this case, the primary peak corresponding to the pull-out of a single chain is centered at 25 pN with a smaller secondary peak corresponding to two chains pulled out together at 45 pN.

Figure 4 shows that there is a significant difference in the desorption energy for the removal of a DHP chain from the silica surface when compared with the KL. Although the effective radius of the polymer chains could be invoked as the dominant reason for this observation, only polymer-solvent interactions were considered by Haupt et al. (2002) and Thormann et al. (2008). However, according to recent studies, the role of surface-polymer interactions should not be neglected. Sonnenberg et al. (2008) showed a significant variation in the desorption force of poly(acrylic acid) between a gold tip or a silicon nitride coated tip. The authors argued that the lower desorption force for silicon nitride resulted in a shorter extended chain length distribution as the lateral polymer-polymer interactions became increasingly important. The higher desorption force for the KL may be attributed to the greater hydrogen bonding ability of the carboxylic groups to the AFM tip. For the DHPs, this strong noncovalent interaction is absent; however, some hydrogen bonding may be expected to occur between OH_{phen} groups and the surface.

Therefore, the chain length distribution was determined (Figure 5). Obviously, the distribution is dependent upon the degree of polymerisation (DP) and hence the contour length of the lignin chains. In this study, all samples had an MW average between 2 and 5 kDa, which is equivalent to a DP between 15 and 30. The maximum fully extended contour length should be in the region of 20 nm. It must be remembered, though, that the lignin polymers tend to be branched and some cross-linking is possible, which means the fully extended length of the lignin molecules should be reduced. The data in Figure 5 agree qualitatively with the previous work of Sonnenberg et al. (2008). That is, the length distribution of the KL chain, which has the

Figure 3 Typical force plateau measured on separation of the AFM tip from the lignin surfaces in a poor solvent. Plateau depths are approximately integer multiples of the lowest force (~80 pN) in this case.

Figure 4 Plateau depth distribution for the pull-out of single chains of KL. (a) DHP lignin from coniferyl alcohol (b) and DHP lignin from coniferaldehyde (c) into the poor solvent conditions.

greatest desorption force, is much higher than for the DHPs. Interestingly, some KL chains could be extended for many hundreds of nanometers and, in some cases, close to 1000 nm, significantly beyond what may be expected for such short chains.

There are two possible explanations: (1) The MW distribution (MWD) of KL is not monodisperse, and it contains also a few very long polymer molecules. By considering the MWDs published earlier (Norgren et al. 2006), it is reasonable to assume that there are some lignin chains with a significantly greater mass and longer contour length. (2) Another possible explanation is that a network of KL chains is extended away from the interface as the AFM tip is retracted. This assumption is quite reasonable because many studies have suggested a strong self-association mechanism between colloidal KL under poor solvent conditions. Norgren et al. (2002) have demonstrated that in the absence of any significant charge on the lignin polymer molecules, that is, at low to neutral pH, dispersion forces

Figure 5 Distribution of chain lengths determined from single-molecule stretching experiments for KL. (a) DHP lignin from coniferyl alcohol (b) and DHP lignin from coniferaldehyde (c) into the poor solvent conditions.

dominate, leading to the formation of large fractal type aggregate structures. These fractal-like aggregates were shown to be up to 1000 nm in one direction, depending on the solution conditions by direct imaging by cryo-transmission electron microscopy (TEM). However, individual KL chains have also been shown to be associated (Lindström 1979; Sarkanen et al. 1982; Bikova et al. 2004; Guerra et al. 2007; Contreras et al. 2008). The longer polymer chain extensions observed only occur when there are multiple plateau features in the force-distance curve as typified in Figure 3. Thus, each part of the lignin aggregate is extended sequentially.

The distribution of bridging chain lengths was also determined by AFM for the DHPs and compared with the KL film in Figure 5. Interestingly, the length distribution of DHPs is significantly different, although both DHP films have similar desorption forces. No coniferyl alcohol-based chains were observed to extend beyond 100 nm, and the majority of

events was centered quite narrowly at about 50 nm. However, the DHP_{c.ald.} chains could be extended to greater lengths at about 70 nm (before detachment with prominent peaks) and to lengths between 110 and 180 nm. The multimodal distribution is somewhat surprising as a Gaussian type distribution should be expected. This is probably related to the size distributions of the primary aggregated lignin chains, with the longer chains representing multiple aggregated states.

The distribution and greatest extension for both DHPs were much less than those for the KL. This is reasonable as a lower desorption force results in a more compact polymer layer due to increased strength of polymer-polymer interactions. Based on the data in Figure 5, it is unlikely that DHP_{c.alc.} forms the same extended network structures as the KL; however, the DHP_{c.ald.} seems to have some self-association effects.

Clearly, there are structural differences beyond the simple MWD of the samples in focus, which determine the length of which the polymer chains can be extended in the solution before the complete desorption by the surfaces. The origin of the self-association of the lignin chains was frequently discussed. Lindström (1979) suggested that intermolecular and intramolecular hydrogen bonding between carboxylic groups is dominant in case of KL, particularly at low pH, where the carboxylic groups are protonated. Furthermore, it was noted that the association through hydrogen bonding between COOH groups and the adjacent ether and OH groups is possible.

Along with the presence of a significant number of COOH groups, KL is also characterized by less benzyl alcohol groups than native lignins (Pouteau et al. 2005). Similarly, the DHP_{c.ald.} lacks significant amounts of OH groups on the α -carbon, whereas they are abundant in DHP_{c.alc.} (Holmgren et al. 2009). This is supported by the ³¹P NMR data (Table 1). The molecular dynamics simulation of Besombes and Mazeau (2004) shows that the strongest intramolecular hydrogen bonding interaction occurs between the benzylic OH and the methoxy group on the adjacent phenylpropane ring. It is reasonable to suggest that this type of interaction is also possible for the DHP_{c.alc.}, which inhibits the aryl ether group interactions with adjacent lignin chains. The observations of chain length distribution in Figure 5, where KL and DHP_{c.ald.} show strong evidence of chain association, support this assumption.

Other potential interactions may also contribute to the observed association of lignin chains under aqueous conditions. Because the solubility of the samples under the aqueous conditions studied here is relatively poor, hydrophobic interactions may also participate in the association mechanisms.

Conclusions

SMFS was suitable to investigate the interaction between lignin chains in aqueous solution. KL typically had a desorption force far greater than the either of the two DHPs studied. This observation can be attributed to the additional hydrogen bonding of carboxyl groups of the KL, which are absent for the DHPs. The extended chain length distribution of the samples evidenced the existence of the chain association for

the KL and DHP_{c.ald.} The lignin chains of these sample types were extended beyond the limits, which could be reasonably expected based on their MWs. However, the DHP_{c.alc.} did not associate because of the strong intramolecular hydrogen bonding in its molecules.

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