

# Investigation of Lignin Deposition on Cellulose During Hydrothermal Pretreatment, Its Effect on Cellulose Hydrolysis, and Underlying Mechanisms

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**ABSTRACT:** In dilute acid pretreatment of lignocellulosic biomass, lignin has been shown to form droplets that deposit on the cellulose surface and retard enzymatic digestion of cellulose (Donohoe et al., 2008; Selig et al., 2007). However, studies of this nature are limited for hydrothermal pretreatment, with the result that the corresponding mechanisms that inhibit cellulosic enzymes are not well understood. In this study, scanning electron microscope (SEM) and wet chemical analysis of solids formed by hydrothermal pretreatment of a mixture of Avicel cellulose and poplar wood showed that lignin droplets from poplar wood relocated onto the Avicel surface. In addition, nuclear magnetic resonance (NMR) showed higher S/G ratios in deposited lignin than the initial lignin in poplar wood. Furthermore, the lignin droplets deposited on Avicel significantly impeded cellulose hydrolysis. A series of tests confirmed that blockage of the cellulose surface by lignin droplets was the main cause of cellulase inhibition. The results give new insights into the fate of lignin in hydrothermal pretreatment and its effects on enzymatic hydrolysis.

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**KEYWORDS:** hydrothermal pretreatment; lignin droplets; deposition; enzymatic hydrolysis; inhibition mechanisms

## Introduction

In order to overcome the recalcitrance of lignocellulosic biomass for large scale, low cost biofuels production, pretreatment is a critical prerequisite to achieve high sugar yields from plant cell wall deconstruction by enzymes and microorganisms (Lynd et al., 1991; Wyman, 1994, 2007). The role of pretreatment is to disrupt and/or remove lignin and hemicellulose, the major plant cell wall structural polymers that protect cellulose microfibrils, to create high cellulose accessibility that facilitates enzymatic saccharification (Kumar et al., 2009; Mosier et al., 2005). In light of this, several leading pretreatments have been developed, most of which involve high temperatures and pressures, mostly with addition of chemicals such as acids and bases (Lloyd and Wyman, 2005). But high temperature pretreatments also result in formation of various inhibitory intermediates and byproducts that can slow down subsequent enzymatic hydrolysis. For example, xylooligosaccharides, important hemicellulose hydrolysis intermediates, were shown to strongly inhibit cellulose hydrolysis (Kumar and Wyman, 2009b; Qing et al., 2010). However, the highly hydrophobic, complex, and heterogeneous nature of lignin still limits understanding of its role in enzymatic hydrolysis.

By tracking lignin release pattern in batch reactor and flowthrough reactor at similar pretreatment condition, lignin appeared to cycle between the solid and liquid phase through depolymerization and repolymerization, resulting in both morphological and structural changes (Liu and Wyman, 2003; McKenzie, 2012; Yang and Wyman, 2004). In dilute acid pretreatment of corn stover or purified lignin in the presence of filter paper, for example, lignin was inferred to coalesce on cell walls and migrate into the bulk liquid phase above the

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lignin glass transition temperature, which resulted in a lignin deposition in form of droplets back on the cell wall surface that negatively impact cellulose hydrolysis (Donohoe et al., 2008; Selig et al., 2007). In addition, pseudo-lignin, which is caused by degradation of carbohydrates like xylan, was also found to form on cellulose surface and retard enzymatic hydrolysis (Kumar et al., 2013). All these results indicate that understanding the effects of lignin relocation or deposition is as important as lignin removal in improving cellulose digestibility. It has been generally agreed that lignin reduces enzyme efficiency in two possible ways (Berlin et al., 2005; Kumar and Wyman, 2010; Mansfield et al., 1999; Yang and Wyman, 2008): enzyme binds nonspecifically to lignin (nonspecific binding mechanism) and lignin acts as a physical barrier that blocks enzyme access to the cellulose surface (surface blockage mechanism). However, such studies are still limited, and the mechanism of deposited lignin droplets formed during hydrothermal pretreatment in inhibiting the subsequent enzymatic hydrolysis is not yet clear.

In this study, lignin deposited Avicel (LDA) was prepared by batch hydrothermal pretreatment of Avicel PH-101 cellulose mixed with poplar wood as a lignin source. Avicel cellulose without poplar was also pretreated at similar conditions as a control. Lignin droplets deposited on LDA were characterized by SEM, wet chemistry, and  $^{13}\text{C}$ - $^1\text{H}$  HSQC NMR. Enzymatic hydrolysis at different protein loadings was performed on both the control Avicel and LDA to investigate inhibition patterns to cellulase. In addition, isolated lignin deposited Avicel (iLDA) was prepared by mixing lignin chemically extracted from poplar with Avicel to confirm the enzymatic inhibition pattern of LDA. Two possible mechanisms for such inhibition, non-specific binding and surface blockage, were evaluated; and surface blockage of enzyme to cellulose was proposed to be main mechanism responsible for the inhibition pattern of enzymatic hydrolysis of cellulose by deposited lignin droplets.

## Materials and Methods

### Materials

Pure cellulose, Avicel PH-101 (Lot No. 1094627) was purchased from FMC Corporation (Philadelphia, PA), 1,4-dioxane (J.T. Baker, Lot No. K06622) was purchased from Avantor Performance Materials, Inc. (Phillipsburg, NJ), and bovine serum albumin (BSA, 98% purity, Batch No. 078K0730) was purchased from Sigma-Aldrich (St. Louis, MO). Debarked poplar (*Populus trichocarpa*) was provided by the National Renewable Energy Laboratory (NREL) in Golden, CO and then knife milled (Wiley Laboratory Mill Model 4, Arthur H. Thomas Company, Philadelphia, PA) to pass through a 20-mesh screen ( $<0.85$  mm). As determined by following the NREL two-step strong acid hydrolysis procedure, the poplar was found to contain 25.1% lignin, 42.4% glucan, and 18.2% xylan.

Lignin was also extracted from poplar according to reported methods (Chang et al., 1975; Holtman

et al., 2004). Briefly, the poplar was refluxed with ethanol: toluene (1:2, v/v) for 24 h in a Soxhlet apparatus to remove extractives, followed by washing with water and air drying. The extractives-free dry poplar was placed in a porcelain ball mill jar, along with porcelain grinding media and ground in a rotary ball mill for 120 h under an inert (nitrogen) atmosphere. The ground poplar powder was then extracted with *p*-dioxane: water (96:4, v/v) under stirring at room temperature for 48 h in the dark. The extracted mixture was centrifuged and the supernatant was collected, roto-evaporated, and freeze dried; the crude ball milled lignin was collected and purified according to literature (Pu et al., 2009).

### LDA and iLDA Preparation

Knife milled poplar was first sieved using USA Standard Testing Sieves (Fisher Scientific Company, Pittsburg, PA) to isolate  $-20/+40$  mesh (425–850  $\mu\text{m}$ ) particle-size fractions that were then washed with room temperature deionized (DI) water to remove fines and dust and dried in a conventional oven at 65°C (Model No. 6520, Thermo electron corporation, Marietta, Ohio). Then, 60 g of this poplar wood and 20 g of Avicel cellulose were hydrothermally pretreated in a 1 L high pressure, mechanically stirred Parr reactor (Model No. 236HC, Parr Instrument, Moline IL) at 200°C for 15 min with a solid loading of 10 wt%. After pretreatment, this lignin deposited Avicel (LDA) was separated from poplar particles using a 100 mesh screen (150  $\mu\text{m}$ , vendor), washed with room temperature DI water, and filtered until the moisture dropped to about 50%.

Meanwhile, isolated lignin deposited Avicel (iLDA) was also prepared using isolated lignin chemically extracted from poplar wood. In brief, 11.5 mg of isolated lignin was first dissolved in a 11 mL mixture of 1,4-dioxane and water (10:1 v/v) in a 50 mL glass vial with screw cap. 0.5 g Avicel PH-101 was then added, and the mixture was kept in an incubation shaker (Multitron Infors-HT, ATR Biotech, Laurel, MD) at 30°C for 3 h. The slurry was then transferred to an aluminum weighing dish and allowed to dry overnight in a fume hood. For comparison, Avicel control samples were also prepared without poplar wood and lignin adding at otherwise the same conditions as LDA and iLDA. The moisture content of Avicel cellulose control, LDA, and iLDA was determined by an automatic infrared moisture analyzer (Model No. HB43-S, Mettler-Toledo, Inc., Columbus, OH).

### LDA and iLDA Characterizations

Prior to compositional analysis, the solids were dried at 105°C overnight in conventional oven (EW-52501-03, Cole-Parmer Instrument Company, Vernon Hills, IL). The glucan, xylan, and acid-insoluble lignin (Klason lignin) contents in LDA were measured by NREL standard biomass analysis procedures (Sluiter et al., 2008). Sugar concentrations were determined with a Waters Alliance e2695 HPLC outfitted with a 2414 refractive index (RI) detector (Waters

Corporation, Milford, MA) and a BioRad Aminex HPX-87H column (Bio-Rad Life Science, Hercules, CA).

For SEM, samples were first coated with gold for 1 min under a 20  $\mu$ A current by a Sputter Coater Cressington 108 Auto. Then, SEM images were developed using XL30 FEG SEM (Philips, address) at 5 kV accelerating voltage.

For NMR, the deposited lignin from LDA was isolated with a dioxane/water (96:4, v/v) mixture with stirring for overnight. The mixture was filtered, and the filtrate was roto-evaporated and freeze-dried. The freeze dried lignin was dissolved in deuterated dimethyl sulfoxide for NMR analysis. NMR experiments were performed using a Bruker Avance-400 spectrometer operating at a frequency of 100.59 MHz for  $^{13}\text{C}$  (Hallac et al., 2010; Pu et al., 2009). Conditions for the two-dimensional heteronuclear single quantum coherence (HSQC) analysis were as follows: 13-ppm spectra width in F2 ( $^1\text{H}$ ) dimension with 1,024 data points (95.9-ms acquisition time), 210-ppm spectra width in F1 ( $^{13}\text{C}$ ) dimension with 256 data points (6.1-ms acquisition time); a 0.5-s pulse delay; and a  $^1J_{\text{C-H}}$  of 145 Hz and 380 scans. A Shigemi NMR tube (Shigemi, Inc., Allison Park, PA) was used for the deposited poplar lignin. The central solvent peak ( $\delta_{\text{C}}$  39.5 ppm;  $\delta_{\text{H}}$  2.5 ppm) was used for chemical shift calibration. NMR data was processed using TopSpin 2.1 (Bruker BioSpin) and MestreNova (Mestre Labs) software packages.

### Enzymatic Hydrolysis

Enzymatic hydrolysis was performed according to NREL standard biomass analysis procedures (Selig et al., 2008), using 1 wt% glucan loading in a 0.05 M citrate buffer (pH 4.8) with 0.2 g/L sodium azide at 50°C and 200 rpm. All hydrolysis experiments were run in triplicates in 25 mL Erlenmeyer flasks with 15 mL total volume in a temperature controlled incubation shaker (Multitron Infors-HT, ATR Biotech). Accellerase<sup>®</sup> 1500 (Lot No.4901131618, BCA protein content—86 mg/mL) cellulase and Accellerase<sup>®</sup> XY (Lot No.1681198062, BCA protein content—51 mg/mL) xylanase were from DuPont<sup>™</sup> Genencor<sup>®</sup> (Science, Palo Alto, CA). For LDA and the control Avicel cellulose hydrolysis, a low (30 mg total protein/g glucan) and high (120 mg total protein/g glucan) total protein loadings were applied at 1:1 protein mass ratio of cellulase to xylanase, respectively. BSA blocking experiments were performed by adding BSA (10 g/L) to the hydrolysis slurry at room temperature 30 min prior (Berlin et al., 2005; Rollin et al., 2011) to adding enzymes (30 mg enzyme protein per gram of glucan at a 1:1 protein mass ratio of cellulase to xylanase). For iLDA and control hydrolysis, 20 mg of enzyme protein per gram of glucan was added at a 3:1 ratio of cellulase to xylanase. To analyze sugar release, about 1 mL samples were collected in 2 mL microcentrifuge tubes at selected time points and centrifuged at 14,600 rpm for 3 min. The liquid hydrolyzate samples along with appropriate calibrations standards were run on a Waters HPLC as discussed previously to determine the sugar concentrations. Glucose yield (GY) reflects the amount of glucose released out of available sugar in raw biomass.

Relative inhibition was calculated as:

$$\text{Relative inhibition (\%)} = \frac{\text{GY (control)} - \text{GY (sample)}}{\text{GY (control)}} \times 100\%$$

where GY(sample) is GY of LDA or iLDA, and GY(control) is GY of the corresponding Avicel control.

### Ultraviolet (UV) Absorbance

iLDA hydrolyzates collected at different time points were centrifuged at 12,000 rpm in 2 mL microcentrifuge filter tubes (Cat No. 24137, Grace) for 5 min. The liquid samples were diluted by 100 times using DI water, and UV absorbance was measured on a SpectraMax M5e UV-Vis spectrophotometer (Molecular Devices, Sunnyvale, CA) at 240 nm. The corresponding hydrolyzates of Avicel control at each hydrolysis time point were also centrifuged and diluted using the method above as a background blank. Relative UV absorbance was calculated as:

$$\text{Relative UV absorbance} = \frac{\text{sample absorbance } (t) - \text{absorbance of control blank } (t)}{\text{sample absorbance } (t = 1) - \text{absorbance of control blank } (t = 1)}$$

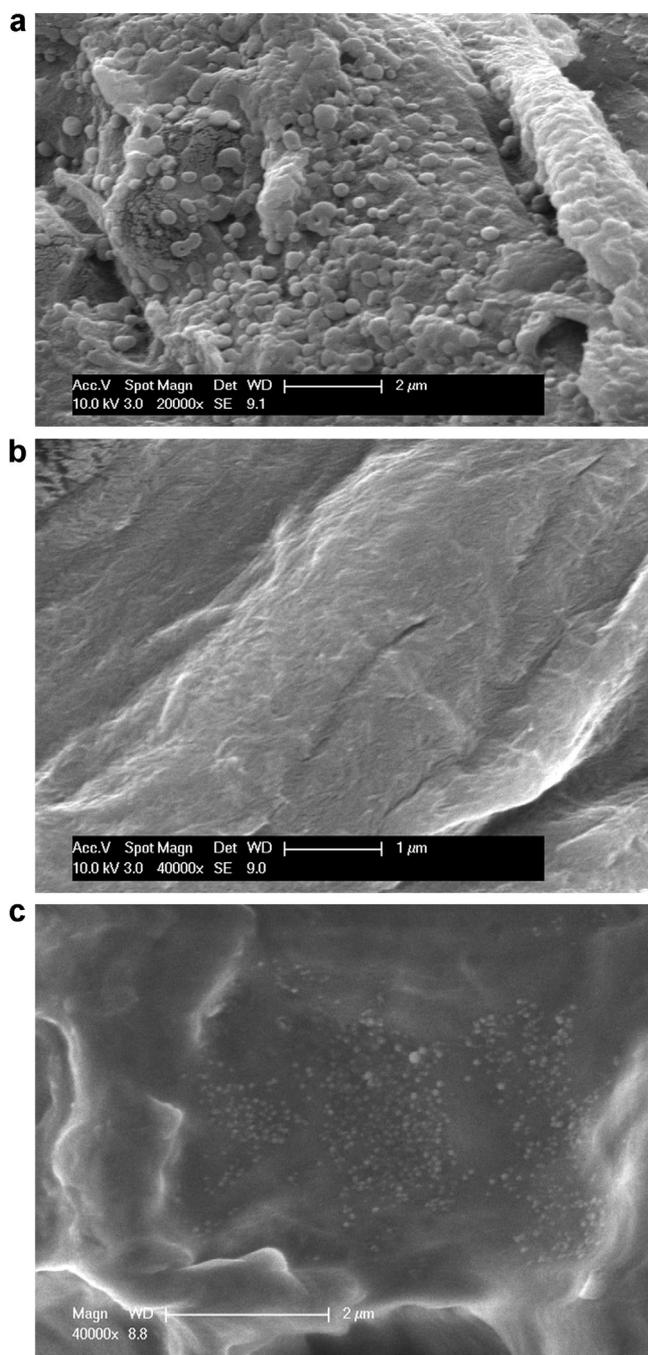
where  $t$  is the hydrolysis time with a unit of hour.

## Results and Discussion

### LDA and iLDA Characterization

The compositional analysis results show that LDA contained approximately  $2.3 \pm 0.1\%$  acid insoluble lignin,  $90.0 \pm 2.3\%$  glucan, and  $1.2 \pm 0.2\%$  xylose equivalents, while its Avicel control sample contained about  $93.5 \pm 3.1\%$  glucan. SEM images presented in Figure 1 clearly show that numerous droplets were deposited on LDA while the surface of the Avicel control was very smooth. Compositional analysis by wet chemistry and SEM imaging confirmed that lignin droplets were deposited from poplar wood onto Avicel cellulose at the hydrothermal condition tested, although minor amounts of xylan was also measured for the LDA sample. These results are consistent with several previous studies that observed and identified lignin droplets on various biomass, including corn stover, switchgrass, wheat straw, and *Tamarix ramosissima*, following hydrothermal or dilute acid pretreatments (Donohoe et al., 2008; Kristensen et al., 2008; Pingali et al., 2010; Selig et al., 2007; Xiao et al., 2011). Lignin droplets deposited on iLDA sample were characterized by SEM only, with the image also shown in Figure 1. The size of lignin droplets on iLDA was relatively small compared to that found on LDA.

Figure 2 shows aromatic and aliphatic regions of 2D  $^{13}\text{C}$ - $^1\text{H}$  HSQC spectra for the lignin sample isolated from



**Figure 1.** SEM images of (a) lignin deposited Avicel (LDA; magnification 20k $\times$ ), (b) Avicel control (magnification 40k $\times$ ), and isolated lignin deposited Avicel (iLDA; magnification 40k $\times$ ; iLDA).

LDA. The NMR spectra of lignin sample isolated from LDA showed typical poplar lignin structural features, further confirming that lignin was migrated from poplar wood and then deposited on Avicel during hydrothermal pretreatment. The aromatic region of HSQC spectrum showed prominent correlation signals for lignin syringyl (S) and guaiacyl (G) units along with p-hydroxyphenyl benzoate (PB) substructure.

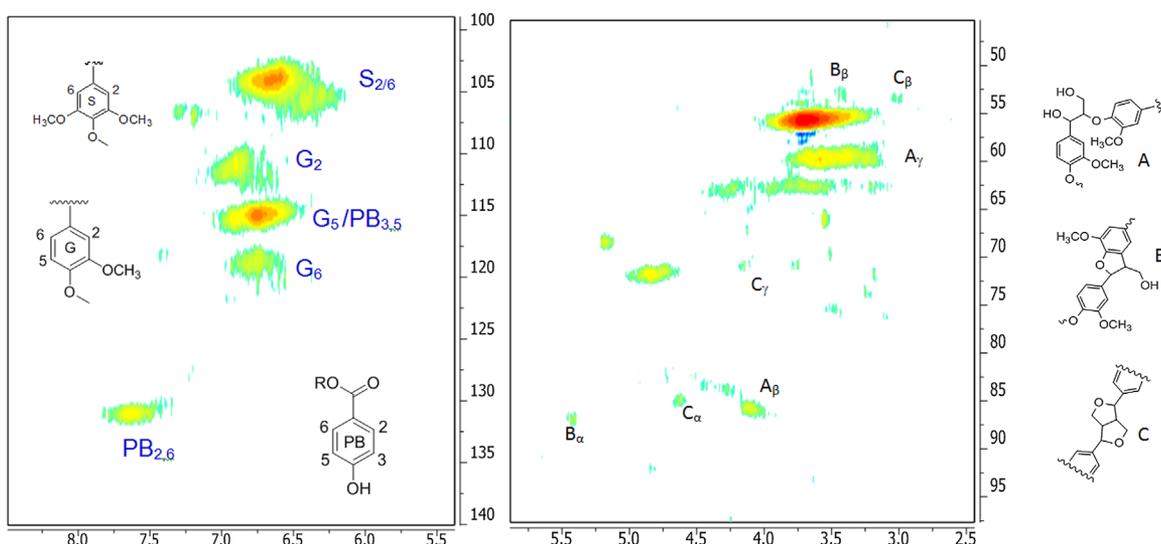
The aliphatic region of HSQC spectrum showed that the signals for  $\beta$ -O-4 substructure (A) were well resolved for  $C_{\alpha}/H_{\alpha}$ ,  $C_{\beta}/H_{\beta}$ , and  $C_{\gamma}/H_{\gamma}$  correlations. The presence of phenylcoumaran substructures (B) was confirmed by C-H correlations for  $\alpha$ -,  $\beta$ -, and  $\gamma$ -C positions centered around  $\delta_C/\delta_H$  86.8/5.43, 53.1/3.42, and 62.8/3.74 ppm, respectively. The lignin resinol subunit (C) was also evidenced by its C/H correlations around  $\delta_C/\delta_H$  84.9/4.66 ( $C_{\alpha}/H_{\alpha}$ ), 53.5/3.04 ( $C_{\beta}/H_{\beta}$ ), and 71.1/4.15 ( $C_{\gamma}/H_{\gamma}$ ) ppm. The presence of phenylcoumaran and resinol subunits in lignin isolated from LDA demonstrated that such structures were also dissolved during hydrothermal pretreatment and deposited onto Avicel cellulose. The HSQC analysis also revealed that the signal of  $\beta$ -O-4 substructure which was linked to syringyl units was stronger than that linking with guaiacyl units, suggesting the relative higher abundance of syringyl units in the deposited lignin. One explanation is that the lignin syringyl units were more prone to cleavage/acidic degradation (Samuel et al., 2011) in hydrothermal pretreatment, thereby being preferably dissolved and relocated onto the Avicel surface.

### Enzymatic Hydrolysis

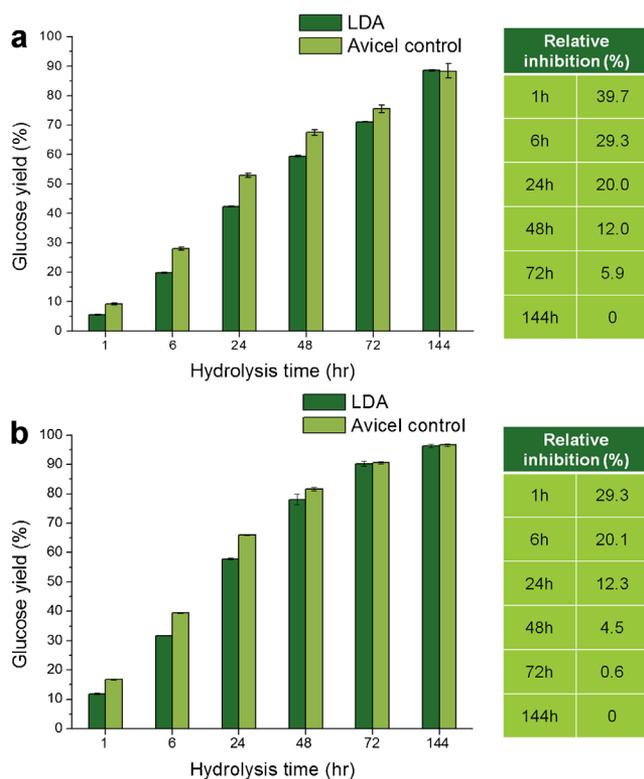
Enzymatic hydrolysis of LDA and the Avicel control were conducted at both low and high protein loadings of 15 mg cellulase + 15 mg xylanase per gram of glucan and 60 mg cellulase + 60 mg xylanase per gram of glucan, respectively. Cellulase was supplemented with xylanase to avoid interferences from residue xylose equivalents that also deposited on LDA. GYs from enzymatic hydrolysis of LDA and the Avicel control shown in Figure 3 indicate that the deposition of lignin droplets did negatively affect enzymatic digestion of cellulose, especially in the early stages of hydrolysis. The relative inhibition for 1 h hydrolysis was 39.7% and 29.3% for low and high enzyme loadings, respectively. More interestingly, inhibition by deposited lignin droplets decreased with hydrolysis time and was relieved when cellulose conversion reached over 80–90%. In order to confirm this finding, iLDA, which was prepared with isolated lignin using the organic solvent method, was also enzymatically hydrolyzed. Although a similar inhibition pattern was observed for iLDA to that for LDA, as shown in Figure 4, the initial relative inhibition appeared to be lower than for LDA. These results demonstrate that the observed slowdown in the enzymatic hydrolysis rate and the drop in inhibition of LDA with extended hydrolysis time were indeed caused by the deposition of lignin droplets.

### Mechanism for Inhibition by Nonspecific Binding

To investigate the key mechanism responsible for enzyme inhibition by deposited lignin droplets, the theoretical amount of enzyme protein that could adsorb on lignin droplets in the LDA sample was first estimated based on the reported maximum cellulase adsorption capacity of lignin (56.8–126.9 mg cellulase protein/g lignin) prepared by leading pretreatments (Kumar and Wyman, 2009a). In this

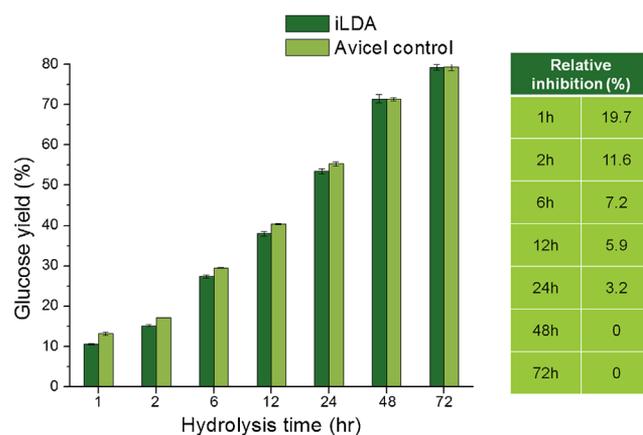


**Figure 2.** Aromatic (left) and aliphatic (right) region of a  $^{13}\text{C}$ - $^1\text{H}$  HSQC spectrum of deposited poplar lignin during hydrothermal pretreatment with Avicel (LDA).



**Figure 3.** Glucose yields from enzymatic hydrolysis of Avicel cellulose control and lignin deposited Avicel (LDA) and percentage relative inhibition at different time points of enzymatic hydrolysis performed at enzyme loadings of (a) 15 mg cellulase + 15 mg xylanase protein/g glucan and (b) 60 mg cellulase + 60 mg xylanase protein/g glucan. Error bars represent standard deviation of three replicates.

study, an upper limit of 127 mg enzyme protein/g lignin was assumed for a calculation based on the 2.3% lignin content in LDA. As shown in Table I, the protein adsorbed by lignin droplets on LDA was estimated to be about 3 mg/g glucan, leading to a protein loss of 10% and 2.5% for low and high enzyme loadings, respectively. However, a 2.5 wt% loss in protein should not result into a 29.3% loss in hydrolysis rate at 1 h for high enzyme loading, suggesting that the nonspecific binding of enzyme on lignin droplets was not the main mechanism retarding enzymatic hydrolysis of LDA.



**Figure 4.** Glucose yields from enzymatic hydrolysis of Avicel cellulose control and isolated lignin deposited Avicel (iLDA) and percentage relative inhibition at different time points of enzymatic hydrolysis for a total protein loading of 15 mg cellulase + 5 mg xylanase/g glucan. Error bars represent standard deviation of three replicates.

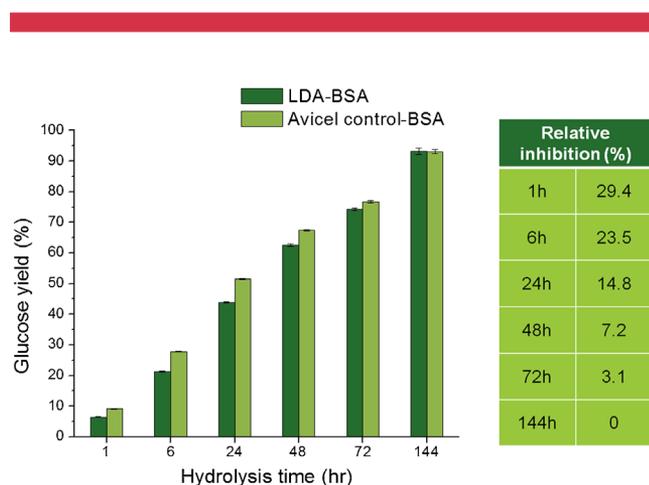
**Table 1.** Estimated maximum protein loss due to nonspecific binding to lignin droplets versus relative inhibition after 1 h of enzymatic hydrolysis.

Protein loading (mg/g glucan)	Estimated maximum protein loss	Percent protein loss	1 h relative inhibition
15 + 15 (cellulase + xylanase)	3.0 mg/g glucan	10.0 wt%	39.7%
60 + 60 (cellulase + xylanase)	3.0 mg/g glucan	2.5 wt%	29.3%

To confirm this deduction, BSA protein was added to LDA slurry prior to adding enzymes to prevent lignin from competitively binding enzymes because BSA was previously shown to irreversibly adsorb onto lignin binding sites without interfering with cellulose hydrolysis (Berlin et al., 2005; Rollin et al., 2011; Zhu et al., 2009). Figure 5 shows results for enzymatic hydrolysis of LDA with BSA blocking. Strong initial inhibition as well as the similar drop in inhibition with LDA hydrolysis without BSA blocking was observed, confirming that nonspecific binding of enzyme to deposited lignin droplets was not the primary inhibition mechanism. Further support is provided by one previous study published by our group, in which the relative inhibition increased with hydrolysis time when a significant amount of enzyme was adsorbed onto lignin (Kumar and Wyman, 2009a), in contrast to the results in this study. Therefore, the results demonstrated that the role of enzyme nonspecific binding to lignin droplets in cellulose hydrolysis inhibition is highly limited.

### Mechanism for Inhibition by Surface Blockage

Evidence reported in the literature suggests that cellulase hydrolyzes cellulose microfibrils layer by layer starting from surface (Igarashi et al., 2011; Zhang et al., 2006), sliding

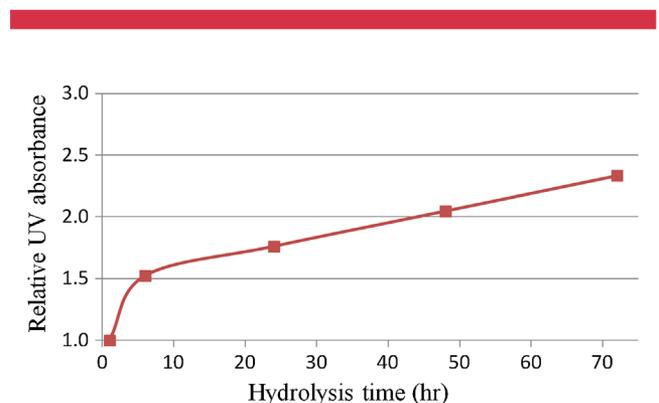


**Figure 5.** Glucose yields from enzymatic hydrolysis of BSA blocked Avicel cellulose control and BSA blocked lignin deposited Avicel (LDA-BSA) and relative inhibition at different time points of enzymatic hydrolysis conducted at a total protein loading of 15 mg cellulase + 15 mg xylanase/g of glucan. Error bars represent standard deviation of three replicates.

unidirectionally as one enzyme molecule moves along one cellulose chain (Igarashi et al., 2011). Thus, according to this mechanism, it appears that when lignin droplets deposit on the cellulose surface, their inhibitory effects on enzymatic hydrolysis arise not only from blocking enzymes from moving along the surface layer but also by preventing accessibility to inner layers. In this context, cellulose surface blockage can be another mechanism responsible for cellulose hydrolysis slowdown by the deposited lignin droplets. However, the drop in inhibition with extended conversion suggests that the physical barrier imposed by these lignin droplets was relieved with more cellulose conversion, leaving a question of how lignin droplets drop off from the cellulose surface? Two previous findings/theories appear to provide support to address this question. One is that a “traffic jam” of enzyme linear movements was observed when there was disturbance on the cellulose surface, resulting in a stop and/or slowdown in enzymatic digestion of cellulose. More interestingly, accumulation of subsequent enzyme molecules was found to lead a “push” that eliminated the obstacle and restarted hydrolysis (Igarashi et al., 2011). The other one is usually termed “enzymatic deinking” as used to recover paper in the pulp and paper industry. In this theory, ink particles are believed to be “peeled-off” with small fibrils which are loosened by enzymatic hydrolysis of the cellulose surface, and the alteration of surface chemistry, such as hydrophobicity, by hydrolysis of adjacent cellulose chains could also facilitate ink detachment from the fiber surface (Bajpai, 1997; Ibarra et al., 2012; Jeffries et al., 1994). Although it is still very difficult to determine which mechanism could account for the results observed in this study, experimental results support the “drop off” of lignin droplets with hydrolysis. As shown in Figure 6, the relative UV absorbance of filtered iLDA hydrolyzate increased significantly with increased hydrolysis time, indicating more lignin droplets were removed from the cellulose surface and moved into bulk liquid phase.

### Hypothesis Explaining Initial Slowdown of Hydrolysis

On the basis of the experimental results in this study and previous reported theories, a hypothesis was developed to



**Figure 6.** Relative UV absorbance at 240 nm of filtered hydrolyzate of isolated lignin deposited Avicel (iLDA) at different hydrolysis times.

explain how deposited lignin droplets retard cellulose enzymatic hydrolysis, especially in the early stages, as schematically represented in Figure 7. This hypothesis suggests that inhibition starts with lignin droplets blocking enzymes that are traveling in line with the blocked area (Fig. 7a). Then a “traffic jam” forms, delaying more enzymes and aggravating inhibition (Fig. 7b). With enzyme accumulation and alteration of surface chemistry by hydrolysis of adjacent cellulose chains, the lignin droplets are either “pushed off” or “peeled off” from the cellulose surface, allowing hydrolysis to continue (Fig. 7c). As more droplets drop off with cellulose conversion, inhibition is reduced with increased hydrolysis time (Fig. 7d). When cellulose conversion is relatively high and all initial surfaces have been hydrolyzed, inhibition virtually stops (Fig. 7e).

## Conclusions

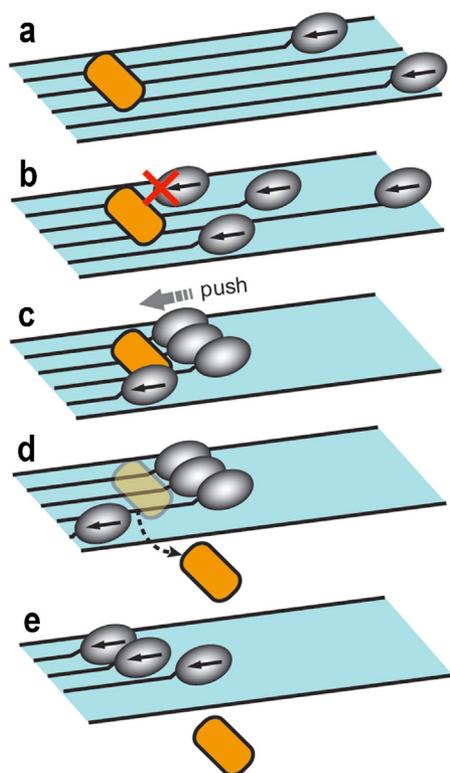
Similar to observations for dilute acid pretreatment reported in previous studies (Selig et al., 2007), lignin migrated out of the poplar wood cell wall and deposited on the Avicel cellulose surface during hydrothermal pretreatment as well. The lignin droplets deposited on the surface of Avicel cellulose retarded enzymatic hydrolysis initially, but inhibition decreased with increased hydrolysis time and was virtually eliminated at high cellulose conversion. Experiment-

tal results demonstrated that nonspecific binding of enzymes to lignin droplets was not the primary mechanism for inhibition. Instead, surface blockage of cellulose by lignin droplets was proposed to be responsible for the inhibition pattern of enzymatic hydrolysis of LDA and iLDA. By comparing experimental results from this study to those from previous studies, the key mechanisms responsible for inhibition of cellulose hydrolysis by lignin, nonspecific binding or surface blockage or both, are believed to depend on the chemical nature and particle size of lignin polymer molecules. Although enzymatic hydrolysis in this study was performed at relatively high enzyme loadings to understand the inhibition mechanism, it is likely that lignin droplets would have an even greater impact on conversion for hydrolysis at low enzyme loadings.

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**Figure 7.** Schematic presentation of possible mechanism of deposited lignin droplets inhibition of enzymatic hydrolysis.

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