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Wood Extractives Promote Cellulase Activity on Cellulosic Substrates

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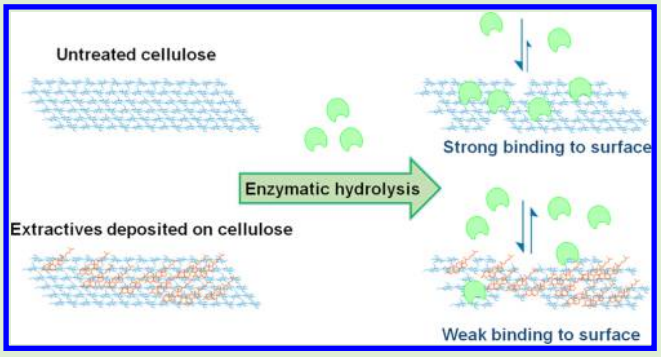
ABSTRACT: Deposition of hydrophobic wood extractives and representative model compounds, on the surface of cellulose prior to enzymatic hydrolysis was found to either enhance or inhibit the action of cellulase enzymes. The effect of these compounds was correlated with their chemical structure, which may in part explain the differential effects observed between softwood and hardwood extractives. Specifically, the addition of sterol, enhanced enzymatic hydrolysis of microcrystalline cellulose by 54%, whereas the addition of a triglyceride could inhibit the hydrolysis by 49%. The effects of the different extractives' could be explained by considering their Hansen solubility parameters. The amphiphilic and/or hydrophobic character of model extractives was found to be the variable that affected the deposition of extractives on cellulose surfaces and the eventual adsorption of cellulolytic enzymes on it. The observed beneficial effects of extractives are likely related to a reduction in the irreversible binding of the enzymes on the cellulose surface.



1. INTRODUCTION

Low molecular weight terpenes, polyphenolic and hydrocarbon compounds, commonly denoted as extractives, may contribute up to several percent of the dry mass of wood. Despite their relatively minor abundance, the presence of extractives is known to contribute to undesirable effects during the wood pulping and papermaking processes.[1] These extractives also influence the enzymatic hydrolysis of biomass, which is a key step in biofuels production processes, although their effects have drawn little attention. Specific wood extractives have structural elements similar to nonionic surfactants,[1] which are known to increase the effectiveness of cellulolytic enzymatic used in cellulose hydrolysis.[2,3] Similarly, Feng et al., (2013)[4] have recently reported that saponins (glycosylated extractive like compounds) enhance the cellulolytic enzymatic degradation of biomass.

Recovery of extractives from the bioethanol process for subsequent production of chemicals has been proposed as an additional source of revenue.[1,5,6] Using the biomass extractives to boost the enzymatic saccharification process may also be a commercially attractive idea. However, given the mixed results on the impact of extractives reported in the literature there is a need to understand the underlying mechanisms on how extractives can impact enzymatic activity.

A cursory examination of the common wood extractives suggests that their hydrophobic character offers very limited potential for interaction with the more hydrophilic cellulose surface. Nevertheless, deposition of colloidal extractives (i.e., pitch) from aqueous dispersions onto the cellulose surfaces is known to take place.[7] The deposition of oleic acid on cellulose has been modeled using computational approaches and interactions have been found. This modeling work found specific interactions between oleic acid and the chemically inequivalent faces of the cellulose crystal.[8] Similar modeling and experimental work found interactions between aromatic compounds and cellulose.[9] These kind of detailed analyses of the interactions between wood extractives and cellulose surfaces have not been undertaken.

Group contribution approaches provide a semiquantitative means to examine the interactions between solvents, or solvents and solutes.[10,11] One common methodology is the Hansen solubility parameter (HSP), which provides an empirical but quantitative method for expressing the hydrophobicity/hydrophilicity of different compounds (solvents or solutes), and can be used to predict interactions such as adsorption.[11,12] Specifically, this approach to understanding surface phenomena (e.g., adsorption) has been used successfully for coatings and pigments.[11] Even without a means to predict some specific solution properties, e.g., zeta potential or pH effects, the HSP may be useful in understanding the interactions between cellulolytic enzymes and cellulose in the presence of hydrophobic compounds such as extractives. To the best of our knowledge the HSP methodology has not been applied to complex enzymatic systems, and the HSPs for enzymes are not available.

The HSP provides a framework for estimating interactions but experimental verification is required. In this work we focused on understanding the adsorption of cellulase enzymes

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Table 1. Calculated Solubility Parameters, RED Numbers and Affinity Parameters for the Compounds That Were Used in This Study[a]

Compound	δ_D (MPa$^{1/2}$)	δ_P (MPa$^{1/2}$)	δ_H (MPa$^{1/2}$)	δ_T (MPa$^{1/2}$)	$RED_{W\text{-}A}$	$RED_{C\text{-}A}$	$RED_{E\text{-}A}$	θ
Cholesterol	19.0	3.0	13.6	23.6	1.07	1.24	0.94	0.92
Cholestane triol	17.3	3.1	33.4	37.7	1.36	1.76	1.56	0.50[b]
Myrtenol	17.5	5.9	12.4	22.3	0.87	1.14	1.06	0.72
Deoxycholic acid	19.5	2.9	24.6	31.6	1.17	1.26	0.87	1.07[b]
Cholestene	19.2	5.2	5.8	20.7	1.12	1.59	1.32	0.58
Docosanol	16.1	5.1	12.9	21.3	0.88	1.26	1.25	0.55
Linoleic acid	15.9	0.3	9.1	18.3	1.19	1.75	1.60	0.42
Cholesteryl palmitate	17.5	5.7	5.8	19.3	1.04	1.60	1.45	0.48
Abietic acid	17.6	1.2	5.9	18.6	1.24	1.81	1.57	0.45
Pinene	16.9	4.1	5.3	18.2	1.11	1.72	1.58	0.43
Docosane	15.4	3.5	6.0	16.8	1.10	1.79	1.72	0.36
Triolein	16.0	1.7	5.2	16.9	1.21	1.89	1.74	0.38
					RED_W	RED_C	RED_E	
Water	15.1	20.4	16.5	30.3	-	0.63	1.01	-
Cellulose	20.3	16.3	18.7	32.1	0.63	-	0.66	-
Enzymes (Zein model)	22.4	9.8	19.4	31.2	1.01	0.66	-	-

[a]HSP values for water, cellulose, and enzymes from Hansen (2007).[11] $RED_{W\text{-}A}$ = RED between compound and water. $RED_{C\text{-}A}$ = RED between compound and cellulose. $RED_{E\text{-}A}$ = RED between compound and enzyme (Zein protein used as model). [b]Compound was excluded from the plot presented in Figure 4 due to uncertainties in HSP values. - = Value not applicable. The calculated values were in general agreement with the respective literature values, while values for certain individual compounds may differ significantly from the experimental HSP.

on a series of wood extractive model compounds. Quartz crystal microgravimetry (QCM) with dissipation monitoring (QCM-D) is commonly used to study adsorption/desorption processes for low molecular weight solutes and enzymes, and hydrolytic reactions on cellulose thin films.[13−16] QCM is an excellent tool for observing and quantifying the initial stages of enzyme adsorption that cannot be emulated by macroscopic scale experiments that involve biomass hydrolysis.

The primary goal of this work was to document and rationalize our observations of the interactions between wood extractive model compounds, cellulase enzymes and cellulose substrates. This involves a series of complex surface phenomena. Model compounds were used to simplify and control the interactions in these complex systems. The secondary goal was to establish a better understanding that may offer some insights on the practical impact of extractives on biomass pretreatment and hydrolysis systems. The HSP framework was used to explain the obtained data using macroscopic scale hydrolysis and QCM. Furthermore, the HSP framework was used to quantify the different structural characteristics of extractive/cellulose/cellulose systems and to relate these differences to the observed beneficial/inhibitory effects on cellulose hydrolysis. Overall, the models and the analytical methods applied are aimed at improving the enzymatic saccharification technologies for lignocellulosic biomass in the presence of readily available additives.

MATERIALS AND METHODS

1.1. Materials. The cellulosic substrate used for the bulk hydrolyses was microcrystalline cellulose (MCC; Avicel PH-105) purchased from FMC BioPolymer (USA). Wood extractives were isolated from steam exploded (200 °C; 6 min) Norwegian Pine and Birch sawdust (BioOil AS, Norway)[17] by Soxhlet extraction with dichloromethane for 24 h. The crude extractives were then fractionated using an acetone-pentane partitioning procedure. Lignin oligomers were first precipitated by adjusting the acetone to pentane ratio to 2:1. A second precipitation containing lower molecular weight lignin fragments and phenolic compounds was generated using a 1:10 acetone:pentane mixture. The most hydrophobic extractive fraction was recovered from the remaining supernatant by evaporation. Each precipitation step was repeated three times to ensure complete fractionation.

The model compounds for specific classes of wood extractives,[1] and their homologues (detailed structures provided in Figure 2) were abietic acid (Alpha Aesar, 75%), 5-α-cholestane-3-β,5,6-β-triol (Aldrich, purity unspecified), cholesterol (MP Biomedicals, 99%), cholesteryl palmitate (Aldrich, 98%), delta-5-cholestene (Aldrich, purity unspecified), 7-deoxycholic acid sodium salt (Aldrich, purity unspecified), docosane (Aldrich, 99%), 1-docosanol (Aldrich, 98%), linoleic acid (Aldrich, 99%), 1R-(−)-myrtenol (Aldrich, 95%), DL-α-pinene (Aldrich, 99%), and triolein (Aldrich, 99%).

All solvents used were purchased from Fisher scientific and were of HPLC grade or higher. The cellulolytic enzyme cocktail used was Ctec2, obtained from Novozymes (USA), with determined activity of 107 FPU/mL.

1.2. Deposition of Extractives and Enzymatic Hydrolysis. A 0.5 g portion of dried MCC (25 °C, overnight) was weighed into a 25 mL crimp seal bottle. The desired quantity of extractives or model compounds (wt % in comparison to cellulose specified in the text) was dissolved in 3 mL of acetone, or if the model compound was not soluble in acetone a mixture of diethyl ether and methanol (3:1) was used. The extractive solution was then mixed with the cellulose and the mixture was let to stand for 1 h. The solvent was then evaporated at atmospheric pressure from a paper covered bottle over a period of 18 h, while continuously agitating the mixture in an orbital shaker. Complete solvent removal was ensured by keeping the samples under high vacuum over a period of 4 h at 25 °C.

Enzymatic hydrolyses were carried in acetate buffer (pH 4.9) at a 5 wt % consistency of the treated cellulose. The enzymes were added as dilute buffer solutions and the reaction was allowed to take place in an orbital shaker at 50 °C. The reactions were quenched by cooling the mixture to approximately 10 °C. Enzyme dosages and hydrolysis times

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are specified in the text. An aliquot sample was withdrawn from the liquor, filtered through 0.22 μm nylon filter and subjected to HPLC sugar analysis. Glucose concentrations were determined using an Agilent 1200 HPLC system equipped with a Shodex SP0810 8*300 mm column, using Milli-Q eluent at 0.5 mL/min flow rate at a temperature of 80 °C. Calibrations were carried out with six standard solutions of glucose ranging in concentration from 0.1 to 20 mg/mL. All analyses were carried out in duplicates or triplicates.

1.3. Hansen Solubility Parameters and Affinity Calculations. The HSP of model compounds were calculated by the group contribution method adapted from Stefanis and Panayiotou (2008, 2012).[18,19] The contributions of the carbon backbones and secondary ring structures were calculated using the tabulated values of hydrophobic compounds, except for quaternary carbons where general values were used. The contributions of polar groups and double bonds, as well as other secondary contributions, were calculated using general tabulated values. The tabulated values of the group contributions are provided elsewhere.[19]

Literature values[11] were used for water, cellulose and Zein protein (used to simulate the proteins present in the cellulase, as this is the only protein at the moment with experimentally determined HSP). All calculated and literature values can be found in Table 1.

Interactions between the different components were calculated using eq 1. The strength of the interactions, favorable or unfavorable, between any two components were calculated using the relative energy distance (RED) numbers eq 2, where R_0 is an empirical radius of a sphere for favorable interactions.[11] The strength of the interactions for the ternary hydrolysis system of extractive model, cellulase enzyme, and cellulose can be described by a dimensionless parameter denoted as the affinity parameter (θ) (eq 3).

$$R_a^2 = 4(\delta_{D1} - \delta_{D2})^2 + (\delta_{P1} - \delta_{P2})^2 + (\delta_{H1} - \delta_{H2})^2 \quad (1)$$

$$\mathrm{RED} = \frac{R_a}{R_0} \quad (2)$$

$$\theta = \frac{\mathrm{RED}_{A-W}}{\mathrm{RED}_{A-C} * \mathrm{RED}_{A-E}} \quad (3)$$

1.4. Quartz Crystal Microgravimetry. A general description of the methods and instrumentation used can be found in the following literature citations.[13,16,20] Spin coating of extractive model compound films was carried out from acetone solution (1.0 mg/mL) of corresponding compound. Gold sensors (Q-sense, Gothenburg, Sweden) were pretreated as described by Song et al. (2015).[16] Spin-coating was carried using a coater (Laurell Technologies model WS-400A-6NPP) spinning at 3000 rpm for 20 s. The spinning sensor was initially cleaned with pure acetone, then the extractive solution was casted, and then dried with one additional spinning cycle. Films were stored in a desiccator prior analysis.

Adsorption of enzymes onto extractive films was carried out using a model E4 instrument from Q-sense (Gothenburg, Sweden). The temperature during the experiment was 25 °C, and the solvent reservoir was kept at a temperature of 5 °C higher than the analysis chambers using an external heater. A lower temperature in comparison to the actual hydrolysis conditions was used to prevent gas formation within the QCM instrument. Prior to injecting the enzyme solutions (10mFPU/mL), the films were allowed to stabilize under 0.1 mL/min flow of buffer solution. Once films showed stable baseline, the enzyme solution was injected using the same flow rate. After 20 min, buffer was used to rinse off reversibly bound enzymes. Mass gain on films was determined using the Sauerbrey equation as described by Rodahl (1995).[20]

2. RESULTS AND DISCUSSION

2.1. Influence of Isolated Wood Extractives on Enzymatic Hydrolysis of Cellulose. During initial experiments, the effects of various wood extractives on sugar release, an indirect measure of enzyme activity, were studied on bulk systems. These extractive fractions isolated from each species were deposited on microcrystalline cellulose, and sugar release was measured after 72 h. Figure 1 clearly shows that the extractive fractions affected the sugar release of enzymatic hydrolysis in different ways. The phenolic extractives fraction isolated from Pine wood resulted in a 9% reduction when compared to the untreated control, whereas the phenolic fraction isolated from Birch wood increased the glucose yield by 7%. These phenolic fractions are composed mainly of lignin oligomers created by the steam explosion pretreatment. It is to be noted, however, that there are significant differences in their specific chemical structures recovered from hardwood and softwoods.[17] Differences in enzyme inhibition behavior for mono- and dimethoxylated phenols have been documented in the literature[21]

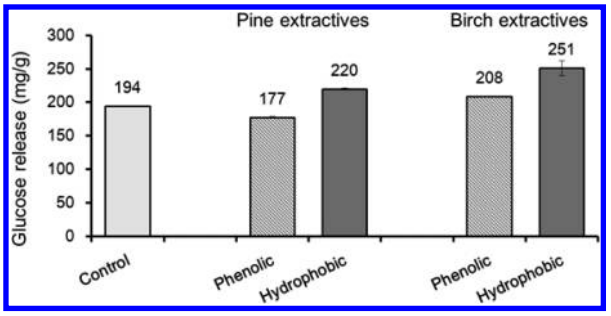

Figure 1. Glucose release from microcrystalline cellulose after deposition of 3 wt % of wood extractives. Hydrolysis was carried out using a 2 FPU/g enzyme loading and a 72 h incubation time. (Triplicate measurements, although error bars are small and not apparent for all systems.)

By contrast, the effect of the relatively hydrophobic extractives fraction is somewhat surprising. The results presented in Figure 1 show that the hydrophobic extractive fractions from both wood species enhances sugar release. Up to 29% relative increase was apparent for Birch. The natural extractive compounds found in these wood species are known to differ in composition, and this may explain the observed differences. It is also important to note that these extractives have been further modified by the initial steam explosion process.[17] These initial results were replicated several times and found to be highly reproducible. Additional studies with representative model compounds were carried out to better understand these results.

2.2. Structure–Effect Relationships As Derived by the Use of Model Compounds. Selected extractive model compounds and their homologues containing varying functional groups were used in order to elucidate structure–function relationships that impact the cellulase enzyme activity.

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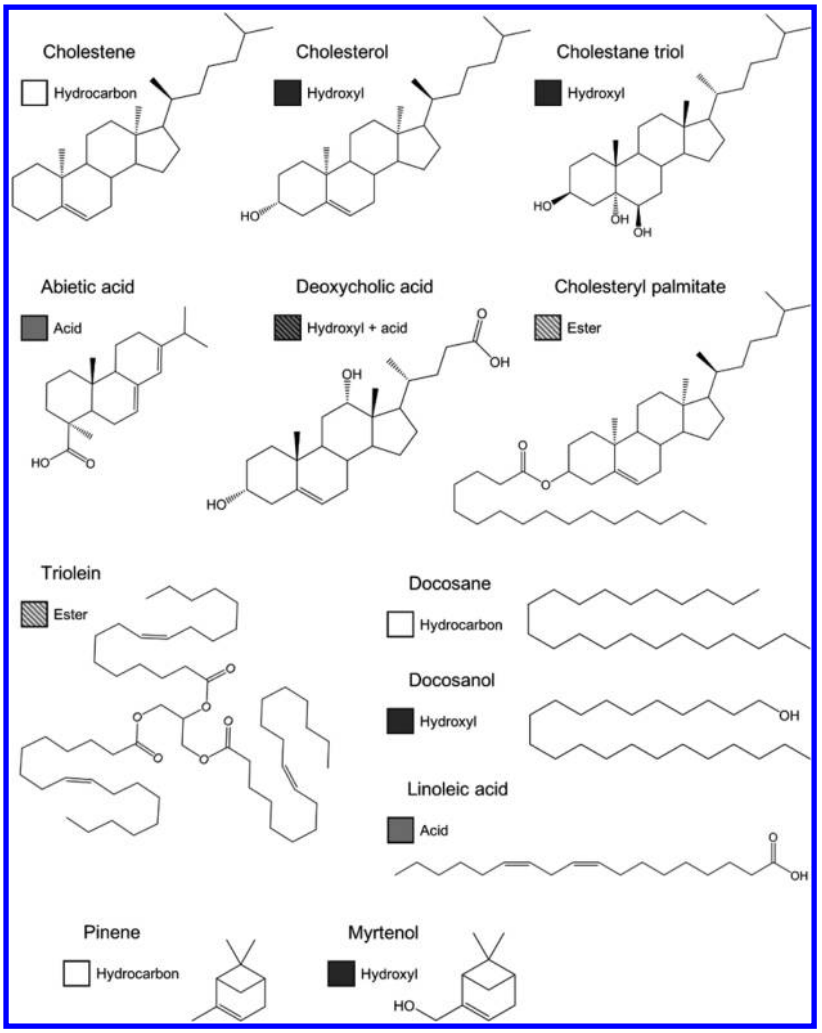

Figure 2. Structures of examined model compounds. The colored squares indicate the codes used to highlight trends related to functional groups within the models and to categorize the data shown in Figure 3.

The complete list of these model compounds, with their molecular structures, is presented in Figure 2. The effect of these model compounds on the enzymatic hydrolysis of MCC, as measured by sugar release, is displayed in Figure 3.

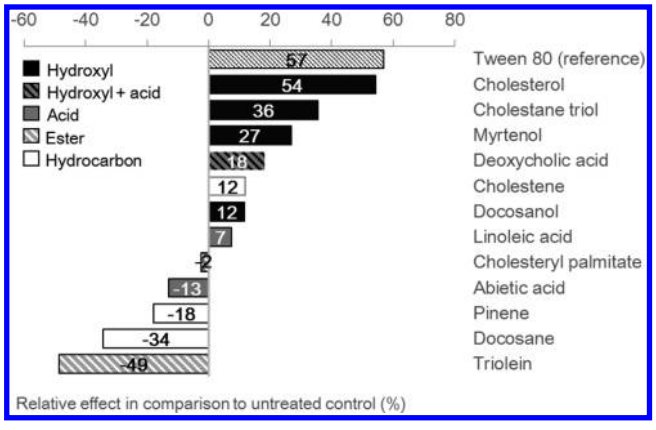

Figure 3. Influence of examined model compounds to the enzymatic hydrolysis of microcrystalline cellulose. The corresponding effect of commercial surfactant Tween 80 is included for comparison. (The main functionalities in these compounds are shown as colored bars, as described in Figure 2.)

Compounds found in hardwoods, such as sterols and fatty alcohols, clearly enhanced the hydrolysis of cellulose, which is consistent with the results seen for the extractive fractions isolated from the steam exploded wood samples (shown in Figure 1). Model compounds that are representative of softwoods extractives, such as pinene and abietic acid, inhibited the activity of cellulase.

The presence or absence of hydroxyl groups on the extractive model compounds showed a consistent and significant effect. In spite of very different base structures, e.g., linear hydrocarbon compounds, simple ring compounds and multiring compounds, various homologous pairs such as cholesterol-cholestene, myrtenol-pinene, and docosanol-docosane all showed an increase in sugar release with the addition of the hydroxyl group. Notably, the effect was not directly proportional to the number of hydroxyls as seen by the slight decrease in sugar release with the addition of a second and third hydroxyl, e.g., cholesterol versus cholestene triol.

In contrast to the enhancement seen with the addition of hydroxyl groups, other polar groups such as carboxylic acids or esters did not enhance hydrolysis. When esters were added, their hydrocarbon backbone seemed to dominate the influence on the cellulolytic enzymes (see cholesteryl palmitate and triolein). The presence of a single polar carboxylic acid group did not result in an enhancement in the case of abietic acid

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versus cholestene, while linoleic acid induced some positive effects in comparison to docosane. Carboxylic acids and hydroxyls are known to possess different acceptor−donor characteristics in hydrogen bond formation.[19] Ionization of the acids in pH 4.9 buffer solution used during the cellulolytic enzymatic hydrolyses experiments also impacts the strength of the potential interactions between the model compound and both the cellulase and cellulose.

In general the least polar compounds possessing highly saturated hydrocarbon structures showed the greatest inhibiting action on the enzymes. Cholestene, quite unexpectedly showed a minor enhancing effect during the hydrolysis. The multitude of tertiary and quaternary carbons in the cholestene backbone were seen to promote stronger dispersive interactions in comparison to linear hydrocarbons,[19] which may explain such an anomaly. In general, the interaction of the model compounds with the cellulose through hydrogen bonding or dispersion forces seems essential for promoting the hydrolysis-enhancing effects.

2.3. Mechanistic Examination of Cholesterol Induced Hydrolysis Enhancement. In an effort to further understand the mechanism for the enhancement effects, a series of hydrolysis experiments were carried out with varying amounts of cholesterol, hydrolysis time, and enzyme loadings (see Figure 4).

The beneficial effects for depositing cholesterol onto cellulose were seen to increase for up to 3−5 wt %, and thereafter the effect plateaued (Figure 4A). All of the quantities of cholesterol used in these experiments depicted in Figure 4A greatly exceeded the 1.8 μg/mL solubility of cholesterol in water.[22] As such cholesterol was anticipated to be deposited on the cellulose surfaces most likely in the form of aggregates and these aggregates are likely to remain during the hydrolysis experiment. One would expect a "uniform" coating of a hydrophobic material to block enzyme activity and the data with enhanced activity suggests good access of the cellulase enzyme to the cellulose surface. The sparingly soluble nature of cholesterol will limit "dissolution" of the cholesterol in buffer during the hydrolysis experiments.

The results depicted in Figure 4B show glucose release as a function of hydrolysis time, with the common rapid initial release followed by slower, extended release at longer times e.g., 24−96 h. While the two sugar release curves show similar trends there is a notable difference for the cholesterol treated materials (Figure 4B). Even after prolonged hydrolysis periods the sugar release continues to increase, suggesting that the cholesterol has a durable effect on the cellulase activity.

Other work has shown that cellulase enzymes interact with the cellulose surface through a series of adsorption/desorption steps, and that over time the cellulase can become irreversibly bound to the cellulose substrate.[23] The durable effects of cholesterol addition at longer times suggests that the cholesterol may be limiting the irreversible binding of the cellulase enzyme and allowing for more effective enzyme activity over an extended period. It can be seen for both curves in Figure 4B that the reactions did not reach a constant release rate, which would be expected without any inhibition effects. The decline in the initially higher hydrolysis rate can be interpreted by slow inhibition kinetics[24,25] with cholesterol apparently able to reduce this inhibition. Substrate depletion and specific reactivity of different cellulose morphologies also influence the observed progress curves, but do not explain the effects of cholesterol.

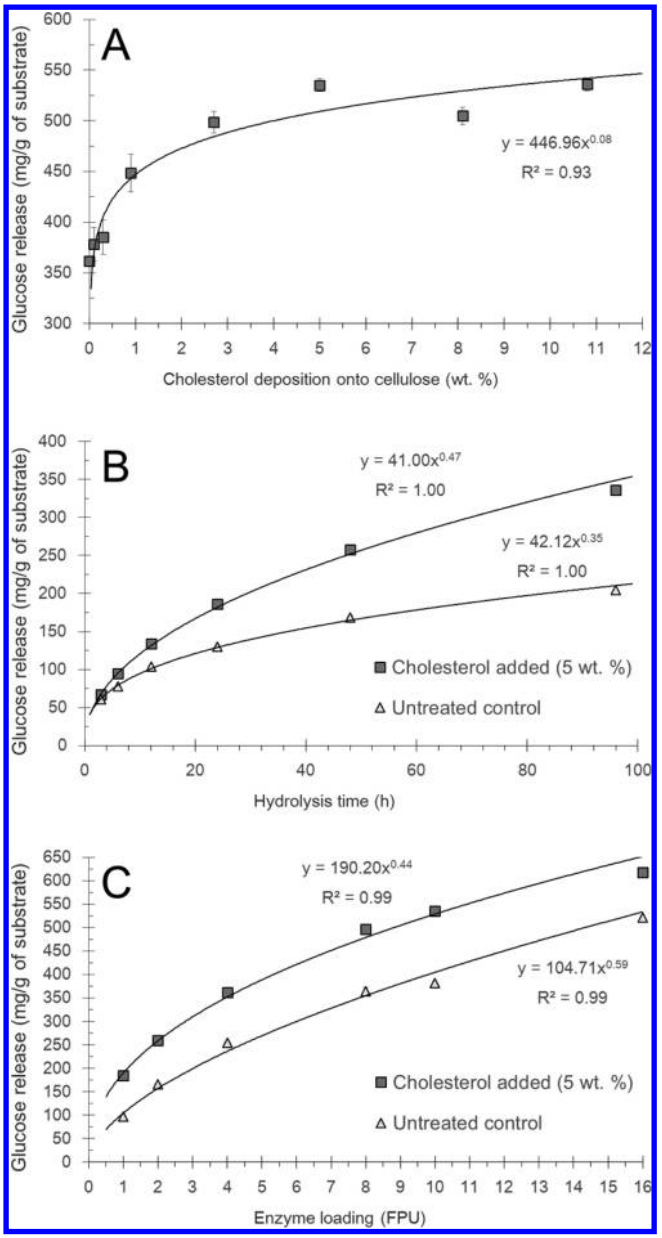

Figure 4. Enzymatic hydrolysis of microcrystalline cellulose in the presence of cholesterol, under various conditions. (A) 72 h hydrolysis using 10 FPU/g of enzymes ranging from 0−10.8 wt % of deposited cholesterol. (B) Hydrolysis with 2 FPU/g of enzymes and 5 wt % cholesterol with reaction times between 3−96 h. (C) 72 h hydrolysis with 5 wt % of deposited cholesterol, using enzyme loading ranging from 1−16 FPU/g. (While error bars are used in the tabulation of these data, they may not be visible due to the plotting scale used.)

Different types of enzymes interact with binding inhibitors to different extends.[21] In the context of this study, the extractive effects may also vary between enzyme types. In the absence of further data pertaining to specific enzyme related mechanisms, it can only be hypothesized that β-glucosidases (active in solution) may be less affected due to low extractive concentrations in the hydrolysates. Alternatively, endoglucanases and cellobiohydrolases (active on surfaces) are more likely influenced by the deposited extractives.

The data of Figure 4C shows the constant benefit of cholesterol addition to the glucose generation (all measured at 72 h hydrolysis) over a wide range of enzyme loadings. In terms

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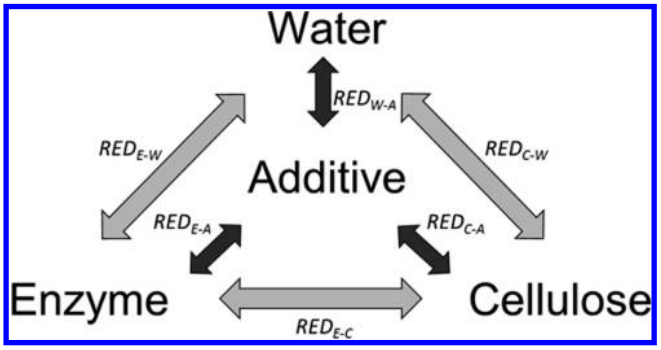
of relative effects, cholesterol increased the glucose generation by 90% at 1 FPU/g loading, whereas the benefit was only 19% for 16 FPU/g loading. This agrees with the aforementioned view regarding the irreversible adsorption of enzymes and highlights that, in the case of high enzyme loadings, the sites of strong adsorption are being depleted by the available excess of enzymes, causing lower need for cholesterol to block these sites. The benefits of cholesterol are higher in the case of low enzyme loadings, when higher proportion of the added enzymes can be inactivated by the strongly adsorbing sites on cellulose. Such depletion of the inhibitory sites by high enzyme loadings fits with the description of tight binding inhibition where stable enzyme−inhibitor complexes are being formed.[24,25] The accumulated data for cellulose hydrolysis in this work suggests what is described as a slow-tight type inhibition mechanism,[24,25] which clearly describes the situation that is commonly denoted as irreversible or unproductive binding in the field of biomass conversion. It is also worth noting that enzyme costs are a major contributor to the overall costs of the biochemical process for making sugars and biofuels.[26,27] Thus, there is a strong economic incentive to work at low enzyme loadings, e.g., 2−3 FPU, and this is the range where these extractives show the greatest benefits.

2.4. The Use of Hansen Solubility Parameters Aimed at Describing the Observed Adsorption Phenomena. The Hansen solubility parameter (HSP) provides an empirical framework that is useful for characterizing solvent/solute and solvent/surface interactions and semiqualitatively describe adsorption phenomena.[11] In this section we use the HSP framework to describe the effects of a series of extractive model compounds on cellulase and cellulose interactions. Specifically, the HSP methodology is used to examine the interactions occurring within the ternary system of cellulose, cellulose and models for biomass extractives. (If water is explicitly included in the analysis, this becomes a quaternary system.) Our approach was based on the use of the relative energy distance numbers (RED) between the interacting species (see eq 2 and Table 1), e.g., enzymes and extractives. Then we applied the corresponding RED numbers to calculate an affinity parameter (θ) that we here introduce to describe the combined effects of multiple simultaneous adsorption interactions. Details for this calculation are described in the experimental section eq 3. The affinity parameter approach is based on competing interactions and adsorption phenomena within this system. The primary interaction and adsorption phenomena are schematically represented in Scheme 1.

In this specific case, the actual deposition of hydrophobic extractives on a cellulose surface is a prerequisite for them to affect the enzyme activity on its surface. Therefore, in the HSP affinity model, a beneficial extractive compound should have an $RED_{C\text{-}A}$ number close to or below 1. In our experiments, the $RED_{C\text{-}A}$ factor applies also to the extractive deposition from acetone solutions, while other RED parameters describe the aqueous medium of hydrolysis. The $RED_{E\text{-}A}$ number between the additive and the enzyme (HSP data for zein protein used to model the cellulose enzyme) should also be close to 1 in order to prevent the treated surface from repelling the enzyme. All $RED_{E\text{-}A}$ values within the data set of Table 1 were larger than the $RED_{E\text{-}C}$ value describing the interaction of between cellulose and the cellulase enzyme. The model suggests that the enzymes adsorb on an extractive treated surface, while the modified surface prevents the enzymes from becoming irreversibly bound by reducing its adhesion energy. It is

Scheme 1. A Schematic Representation of the Prevailing Adhesion Interactions Operating during Cellulolytic Enzymatic Hydrolysis of Cellulose[a]



[a]The grey arrows represent a normal hydrolysis system, while the black arrows point to additional interactions caused by the addition of the hydrophobic extractive/model compounds.

anticipated that the hydrophobic additives are aggregated on the cellulose surface leaving a significant amount of the cellulose surface available for the well characterized cellulase binding domain and subsequent hydrolysis. It is to be noted that this model does not differentiate among specific interactions occurring between the extractive/model compounds and specific sites on the cellulose surface, e.g., different crystal faces or crystalline/amorphous transitions.

The influence of water (i.e., the hydrophobic effect) can also be taken into account in the calculation of the affinity parameter (θ). This factor acts as an additional driving force for the enzyme binding, despite the fact that the influence of $RED_{W\text{-}A}$ to the value of θ was small compared to $RED_{C\text{-}A}$ and $RED_{E\text{-}A}$. Interestingly, however, our experimental data (see section 2.5) implied that more pronounced effects may actually arise from hydrophobic interactions. The complex hydration characteristics of cellulose fibrils may also be of importance, as the various faces of the fibrils have been shown to have different hydration densities.[28] Interestingly, such a feature can be expected to create variations in the site specific adsorption energies for both the extractives and the enzyme, as anticipated on the basis of the present effort. Overall, the effect of water is complex to model and should be the subject of further studies.

The RED numbers and the affinity parameters (θ) were calculated for each model extractive system (Table 1) plotted against the sugar release from the enzymatic hydrolysis. The resulting correlation shows a good correlation with an R^2 of 0.82 (Figure 5).

Consistent with our hypotheses, the data of Figure 5 suggest that hydrophobic extractives are deposited on the cellulose surface and reduce the irreversible binding between the cellulase enzymes and the cellulose surface.

However, it should be noted that the HSP approach includes the following limitations and assumptions:

(1) the HSP are based on group contributions which are based on an extensive series of correlations, but like any series of correlations does not accurately predict compounds with unique structures, e.g., diol and triols,
(2) the HSP does not consider solution properties as Zeta potential or the specific effects of pH, e.g., ionization of carboxyl groups, and
(3) the HSP do not take into account the spatial distribution of the functionalities along the carbon backbone of the

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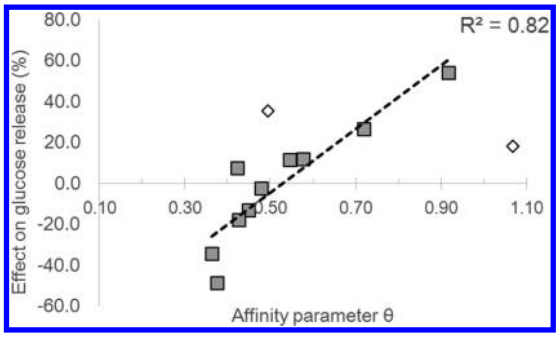

Figure 5. Correlation between the calculated affinity parameter and the observed effects on enzymatic hydrolysis. Cholestane triol and deoxycholic acid (shown as open diamonds) were excluded from the regression due to known anomalies associated with the calculated HSP of compounds bearing multiple hydroxyls.[29]

molecules examined, which is most problematic for compounds that can aggregate or have amphiphilic character.

More detailed modeling of the donor−acceptor characteristics of hydrogen bonding and experimental measurements of this suite of model compounds should add more precision to these preliminary calculations. Notably, the data of Figure 2, shows that the most beneficial compounds displayed a high hydrogen bond donor character, while the inhibitory compounds displayed acceptor character or have limited H-bonding capacity.

2.5. Quartz Crystal Microgravimetric Analysis. QCM analyses were carried out to gain additional insights into the interactions between the extractive model compounds and cellulase enzymes. Specifically, thin films were prepared with docosanol, cholesterol, docosane, and cholestene and subjected to enzyme adsorption studies (Figure 6).

The actual enzyme masses adsorbed onto films of the model compounds from a 10 mFPU/mL solution of Ctec2 enzyme cocktail (determined by QCM) were 72, 120, 154, and 192 ng/cm^2 for docosanol, cholesterol, docosane, and cholestene, respectively. For comparison, adsorption of cellulase enzymes onto a nanofibrillar cellulose film is estimated to be 195 ng/cm^2 (data not shown). Adsorption of the enzymes onto the extractive film was found to be strongest for the hydrocarbon model compounds, cholestene and docosane. The addition of the hydroxyl groups within the structure of the model substance clearly reduced its adsorption when compared to the hydrocarbon homologues. Regardless of the extremely low water solubility of all examined extractive models, the presence of hydroxyl groups in their structure could influence the hydration of the extractive-water interface. Further support to such interpretation is provided by the positive correlation shown between the $RED_{W\text{-}A}$ and the adsorbed mass of enzymes (see Figure 6 and Table 1). Based on the principles of HSP, once $RED_{W\text{-}A}$ becomes larger than unity, the cohesive interaction with water becomes increasingly unfavorable, and the enzyme adsorption should increase. Theories about surface hydration and associated effects on protein adsorption[30−32] are in agreement with the present data.

It is also to be noted that the water mediated effects should correspond only to a fraction of the total binding energy that dictates the enzyme adsorption onto cellulose.[33] In this respect, it is surprising that the films prepared from the relatively hydrophobic extractive model compounds showed enzyme adsorption behavior similar to that of cellulose.

3. CONCLUSIONS

This work documents that specific fractions of extractives, found in native wood, promote the activity of cellulase enzymes on cellulose substrates.

Detailed evaluations using various model compounds representative of wood extractives confirmed the initial results and also highlighted the complexity of the system. The amphiphilic effects created by the presence of hydroxyl groups

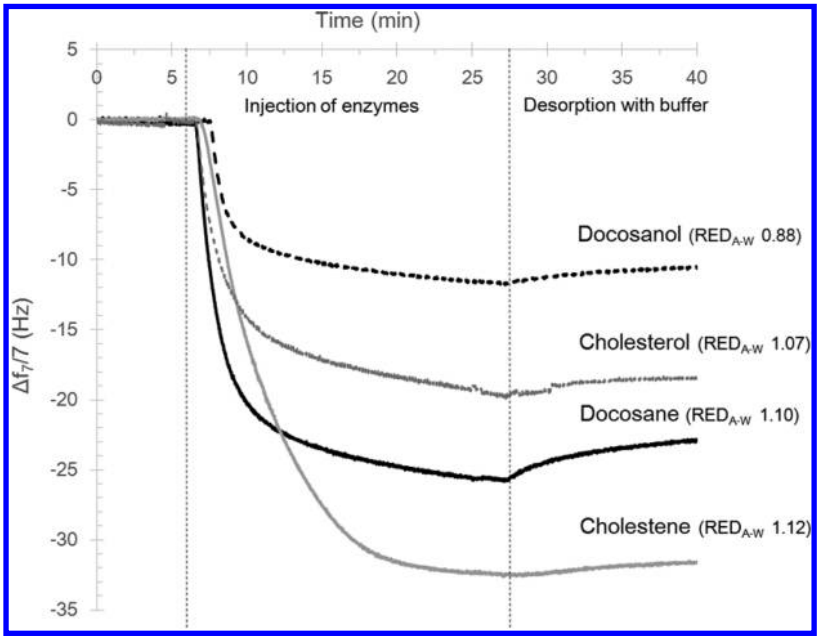

Figure 6. Enzyme adsorption from 10 mFPU/mL buffer solution onto extractive model films determined by QCM. Resonance frequency Δf is inversely proportional to the mass increase on the sensor. The $RED_{W\text{-}A}$ values describing the compounds hydrophobicity have been included for comparison.

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on some of the model compounds significantly increased the effectiveness of cellulases on cellulose when compared to pure hydrocarbon homologues. The amphiphilic character of the model systems also influenced their capability to interact with cellulosic surfaces. It was hypothesized that the amphiphilic character of such compounds prevents a tight, nonproductive binding of the cellulase enzymes to the cellulose surfaces.

The Hansen solubility parameter framework was then used to estimate the various interactions occurring in the ternary system that included the cellulose substrate, the cellulase enzyme, and model extractives. These calculations showed that the adsorption of enzymes on cellulose surfaces, treated with amphiphilic hydrocarbon molecules, remains favorable. These calculations also showed that the interaction between amphiphile treated cellulose and enzymes is weaker in comparison to the case of untreated cellulose.

It is likely that the effects seen here can be attributed to the extractives and model compounds eliminating some high energy sites on the cellulose substrate, which in turn reduces the irreversible, unproductive binding of cellulase enzymes to the cellulose substrate.

Quartz crystal microgravimetry measurements showed that strong binding occurs between real enzymes and the surfaces of extractives. Films prepared from amphiphilic compounds resulted in lower adsorption of enzymes than pure cellulose film and films prepared from the hydrocarbon analogues. The hydrophobic model compounds (devoid of amphiphilic character) allow for unfavorable strong binding between extractive and enzymes via hydrophobic interactions and results in a decrease in the overall cellulose hydrolysis.

These data needs to be followed by additional studies that couple detailed studies of the nature of the cellulose surface with varying amounts and types of extractives and/or models. These studies should lend insight into the interactions between the extractives/models and the heterogeneous cellulose substrates, particularly as it pertains to the dispersion or aggregation of the extractives/models on heterogeneous surfaces.

In conclusion, this work suggests the need for a better understanding of the role the presence and the chemical nature of biomass extractives can play on the progress of cellulolytic hydrolyses. It also highlights the significant commercial potential for fractions of wood-based extractives to promote the enzymatic hydrolysis of cellulosic biomass and lower the overall economics of the process.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Rowe, J. W., Ed. *Natural Products of Woody Plants II: Chemicals Extraneous to the Lignocellulosic Cell Wall*; Springer-Verlag: Berlin, Germany, 1989.

(2) Zheng, Y. Application of nonionic surfactants on lignocellulosic bioethanol production. In *Non-ionic Surfactants*; Wendt, P. L., Hoysted, D. S., Eds.; Nova Science Publishers, Hauppauge, NY, 2010.

(3) Eckard, A. D.; Muthukumarappan, K.; Gibbons, W. A review of the role of amphiphiles in biomass to ethanol conversion. *Appl. Sci.* **2013**, *3*, 396−419.

(4) Feng, Y.; Jiang, J.; Zhu, L.; Yue, L.; Zhang, J.; Han, S. Effects of tea saponin on glucan conversion and bonding behaviour of cellulolytic enzymes during enzymatic hydrolysis of corncob residue with high lignin content. *Biotechnol. Biofuels* **2013**, *6*, 161.

(5) Belgacem, M. N., Gandini, A., Eds. *Monomers, Polymers and Composites from Renewable Resources*; Elsevier: Oxford, U.K., 2011.

(6) Lindsey, K.; Johnson, A.; Kim, P.; Jackson, S.; Labbé, N. Monitoring switchgrass composition to optimize harvesting periods for bioenergy and value-added products. *Biomass Bioenergy* **2013**, *56*, 29−37.

(7) Tammelin, T.; Johnsen, I. A.; Osterberg, M.; Stenius, P.; Laine, J. Adsorption of colloidal extractives and dissolved hemicelluloses on thermomechanical pulp fiber components studied by QCM-D. *Nord. Pulp Pap. Res. J.* **2007**, *22*, 93−101.

(8) Quddus, M. A.; Rojas, O. J.; Pasquinelli, M. A. Molecular Dynamics Simulations of the Adhesion of a Thin Annealed Film of Oleic Acid onto Crystalline Cellulose. *Biomacromolecules* **2014**, *15*, 1476−1483.

(9) Da Silva Perez, D.; Ruggiero, R.; Morais, L. C.; Machado, A. E.; Mazeau, K. Theoretical and experimental studies on the adsorption of aromatic compounds onto cellulose. *Langmuir* **2004**, *20*, 3151−3158.

(10) Barton, A. F. *CRC Handbook of Solubility Parameters and Other Cohesion Parameters*; CRC Press: Boca Raton, FL, 1991.

(11) Hansen, C. M. *Hansen Solubility Parameters:A User's Handbook*; CRC Press: Boca Raton, FL, 2007.

(12) Hansen, C. M. *Three Dimensional Solubility Parameter and Solvent Diffusion Coefficient - Their Importance in Surface Coating Formulation*. Doctoral dissertation; Danish Technical Press: Copenhagen, Denmark, 1967.

(13) Ahola, S.; Turon, X.; Osterberg, M.; Laine, J.; Rojas, O. J. Enzymatic hydrolysis of native cellulose nanofibrils and other cellulose model films: effect of surface structure. *Langmuir* **2008**, *24*, 11592−11599.

(14) Kumagai, A.; Lee, S. H.; Endo, T. Thin film of lignocellulosic nanofibrils with different chemical composition for QCM-D study. *Biomacromolecules* **2013**, *14*, 2420−2426.

(15) Martín-Sampedro, R.; Rahikainen, J. L.; Johansson, L. S.; Marjamaa, K.; Laine, J.; Kruus, K.; Rojas, O. J. Preferential adsorption and activity of monocomponent cellulases on lignocellulose thin films with varying lignin content. *Biomacromolecules* **2013**, *14*, 1231−1239.

(16) Song, J.; Salas, C.; Rojas, O. J. Role of textile substrate hydrophobicity on the adsorption of hydrosoluble nonionic block copolymers. *J. Colloid Interface Sci.* **2015**, *454*, 89−96.

(17) Leskinen, T. *Analytical Approaches to Understanding the Role of Non-carbohydrate Components in Wood Biorefinery*. Doctoral dissertation; Raleigh, USA, 2015, in press.

(18) Stefanis, E.; Panayiotou, C. Prediction of Hansen solubility parameters with a new group-contribution method. *Int. J. Thermophys.* **2008**, *29*, 568−585.

(19) Stefanis, E.; Panayiotou, C. A new expanded solubility parameter approach. *Int. J. Pharm.* **2012**, *426*, 29−43.

(20) Rodahl, M.; Höök, F.; Krozer, A.; Brzezinski, P.; Kasemo, B. Quartz crystal microbalance setup for frequency and Q-factor measurements in gaseous and liquid environments. *Rev. Sci. Instrum.* **1995**, *66*, 3924−3930.

(21) Ximenes, E.; Kim, Y.; Mosier, N.; Dien, B.; Ladisch, M. Inhibition of cellulases by phenols. *Enzyme Microb. Technol.* **2010**, *46*, 170−176.

(22) Haberland, M. E.; Reynolds, J. A. Self-association of cholesterol in aqueous solution. *Proc. Natl. Acad. Sci. U. S. A.* **1973**, *70*, 2313−2316.

(23) Ma, A.; Hu, Q.; Qu, Y.; Bai, Z.; Liu, W.; Zhuang, G. The enzymatic hydrolysis rate of cellulose decreases with irreversible adsorption of cellobiohydrolase I. *Enzyme Microb. Technol.* **2008**, *42*, 543−547.

DOI: 10.1021/acs.biomac.5b00889
Biomacromolecules XXXX, XXX, XXX−XXX

(24) Morrison, J. F. The slow-binding and slow, tight-binding inhibition of enzyme-catalysed reactions. *Trends Biochem. Sci.* **1982**, *7*, 102−105.
(25) Szedlacsek, S. E.; Duggleby, R. G. Kinetics of slow and tight-binding inhibitors. *Methods Enzymol.* **1994**, *249*, 144−180.
(26) Gonzalez, R. W.; Treasure, T.; Phillips, R. B.; Jameel, H.; Saloni, D. Economics of cellulosic ethanol production: Green liquor pretreatment for softwood and hardwood, greenfield and repurpose scenarios. *BioResources* **2011**, *6*, 2551−2567.
(27) Gonzalez, R.; Treasure, T.; Phillips, R.; Jameel, H.; Saloni, D.; Abt, R.; Wright, J. Converting Eucalyptus biomass into ethanol: Financial and sensitivity analysis in a co-current dilute acid process. Part II. *Biomass Bioenergy* **2011**, *35*, 767−772.
(28) Gross, A. S.; Chu, J. W. On the molecular origins of biomass recalcitrance: the interaction network and solvation structures of cellulose microfibrils. *J. Phys. Chem. B* **2010**, *114*, 13333−13341.
(29) Konstam, A. H.; Feairheller, W. R. Calculation of solubility parameters of polar compounds. *AIChE J.* **1970**, *16*, 837−840.
(30) Vogler, E. A. Structure and reactivity of water at biomaterial surfaces. *Adv. Colloid Interface Sci.* **1998**, *74*, 69−117.
(31) Janecek, J.; Netz, R. R. Interfacial water at hydrophobic and hydrophilic surfaces: Depletion versus adsorption. *Langmuir* **2007**, *23*, 8417−8429.
(32) Gong, P.; Grainger, D. W. Nonfouling Surfaces: a review of principles and applications for microarray capture assay designs. *Methods Mol. Biol.* **2007**, *381*, 59−92.
(33) Georgelis, N.; Yennawar, N. H.; Cosgrove, D. J. Structural basis for entropy-driven cellulose binding by a type-A cellulose-binding module (CBM) and bacterial expansin. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 14830−14835.

DOI: 10.1021/acs.biomac.5b00889
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