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Elucidating Structural Characteristics of Biomass using Solution-State 2D NMR with a Mixture of Deuterated Dimethylsulfoxide and Hexamethylphosphoramide

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Recent developments of NMR methods for characterization of lignocellulosic biomass allow improved understanding of plant cell-wall structures with minimal deconstruction and modification of biomass. This study introduces a new NMR solvent system composed of dimethylsulfoxide (DMSO-d_6) and hexamethylphosphoramide (HMPA-d_{18}). HMPA as a co-solvent enhanced swelling and mobility of the biomass samples; thereby it allowed enhancing signals of NMR spectra. The structural information of biomass was successfully analyzed by the proposed NMR solvent system (DMSO-d_6/HMPA-d_{18}; 4:1, *v*/*v*) with different biomass. The proposed bi-solvent system does not require derivatization or isolation of biomass, facilitating a facile sample preparation and involving with no signals overlapping with biomass peaks. It also allows analyzing biomass with a room-temperature NMR probe instead of cryo-probes, which are traditionally used for enhancing signal intensities.

Recent attention has focused on lignocellulosic biomass as a potential substitute for petroleum in fuels and chemicals production. Lignocellulosic biomass is a complex biopolymer mainly composed of cellulose, hemicellulose, and lignin with other minor components, such as ash, protein, and other extractives. Cellulose is a linear polysaccharide with repeating glucose units linked by β-1,4 glycosidic linkages.[1] Hemicellulose is a more complicated component, because it is composed of heteropolysaccharides containing different pentoses and hexoses. It is another important carbohydrate source in biomass usually with the presence of highly branched structure and substitution of acetyl groups at C2 and/or C3 positions.[2] Lignin is the largest non-carbohydrate fraction in the biomass. It is a phenolic polymer of phenylpropane units including coniferyl, sinapyl, and *p*-coumaryl alcohol linked through aryl ether bonds and carbon–carbon bonds. It gives structural strength, cell-wall hydrophobicity, and protection of the plants from pathogenic attacks and other environmental factors.[3] This polymer is not only considered a convertible resource, but also a biomass utilization inhibitor; therefore, a deeper understanding the characteristics of biomass can provide fundamental insight into how to select, grow, and convert lignocellulosic biomass feedstocks into fuels and chemicals. The chemical compositions of biomass vary depending on its species, growing environments, harvesting periods, and even regions of the same plant. In addition, characteristics of products and residues after conversion of biomass could be changed by conversion methods, reaction parameters, and other factors. Therefore, characterization of raw biomass and its products is essential for understanding the effects of each method. For these reasons, many biomass analysis methods have been developed along with the investigation into biomass conversion technologies.

Diverse biomass analyses by degradative methods (i.e., alkaline nitrobenzene oxidation, thioacidolysis, derivation followed by reductive cleavage) and non-destructive methods with spectroscopies, such as ultraviolet (UV), Fourier transform infrared spectroscopy (FTIR), and nuclear magnetic resonance (NMR), have been introduced.[4] Among many analysis methods, NMR has been extensively used to elucidate the structural fingerprint of polysaccharides and lignin in plant cell walls. Fractionation of the biomass components through complicated separation methods was historically necessary for traditional solution-state NMR methods. Lignin was usually needed to be isolated from biomass as a form of milled wood lignin (MWL), cellulolytic enzyme lignin (CEL), or enzymatic mild acidolysis lignin (EMAL) for dissolving in NMR solvents such as deuterated acetone and dimethyl sulfoxide (DMSO-d_6) with minimal modification of lignin structures.[5] Solution-state NMR with these isolated lignins can provide detailed information of lignin structure, including linkages in the cell walls. Hemicellulose and cellulose in the plant cell walls have also been characterized by NMR analysis.[6] However, several factors are among the challenges for these methods, such as various yields of the isolated components (especially low yields for isolated representative lignin), accompanied modification of chemical struc-

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tures, and time-consuming isolation process. On the other hand, Lu et al. proposed a non-degradative derivatization method in DMSO–tetrabutylammonium fluoride (TBAF) or DMSO–*N*-methylimidazole (NMI) with solution-state NMR analysis method instead of isolation of target components.[7] This procedure can avoid laborious isolation steps, however it can potentially result in missing of some structural information. For example, pre-acetylation of biomass samples would mask natural acetylation in the cell walls.[4b]

Kim et al. developed a gel-state NMR method for effectively analyzing plant cell walls in DMSO-d_6 without isolation of components and chemical modification of biomass structure.[4b] This method allows shortening the aforementioned sample preparation steps. Recently, they also reported an advanced solution-state NMR method using DMSO-d_6/pyridine-d_5 solution for improving a resolution of NMR spectra and for easy sample handling.[8] For effective whole cell-wall (WCW) NMR analysis, dissolution and swelling of biomass is important. Kim and his co-workers reported that the soluble fraction of biomass samples produced high resolution NMR spectra.[4b] They also found that enhancement of biomass swelling remarkably improved signal intensities and resolution of the NMR spectra.[8] DMSO was reported as a strong swelling agent for cellulose.[9] It has a function as both a soft base (sulfoxide sulfur) and a hard base (sulfoxide oxygen).[10] To enhance the dissolution of cellulose or biomass, TBAF, pyridine, SO_2/diethylamine, paraformaldehyde, and other solvents were introduced as a co-solvent with DMSO.[11] $POCl_3$ was also tested as a swelling and dehydration agent in DMSO systems.[11a] In this study, a new solution-state NMR analysis method using a bi-solvent system comprising of DMSO-d_6 and deuterated hexamethylphosphoramide (HMPA-d_{18}) is successfully developed.

HMPA was introduced as a highly polar aprotic solvent and good medium for reduction reactions.[12] The DMSO–HMPA solvent mixture was investigated in previous studies.[13] Rounaghi and Popov used this solvent system for complexation of a cesium ion with macrocyclic ligands.[13a] Tamura and his co-workers also discussed the thermodynamic properties, such as excessive enthalpy, excessive isobaric heat capacity, and speed of sound of HMPA with DMSO and other polar solvents.[13b] However, applications of the solvent mixture on biomass analysis have not been reported. The swelling of ball-milled poplar samples in HMPA-d_{18}, DMSO-d_6, and their mixtures are presented in Figure 1. The ball-milled poplar samples were not dissolved and/or swollen in HMPA-d_{18} alone, whereas DMSO-d_6 swelled the substrate and formed a gel-state as described in the previous study.[4b] Interestingly, addition of HMPA-d_{18} in DMSO-d_6 improved the swelling effect on biomass. As shown in Figure 1, the DMSO-d_6/HMPA-d_{18} mixture notably swelled biomass with larger volume of solution-state samples comparing to the biomass dissolved in DMSO-d_6 alone. In addition, HMPA-d_{18} also appeared to enhance the mobility of the prepared cell-wall sample in the mixture (Figure 1 d), thus handling of the samples becoming easier. The P–O bond of HMPA is highly polarized and has a strong negative charge on the oxygen atom.[14] It is expected that the addition of HMPA-d_{18} interrupted some of the hydrogen bonds in the cellulose

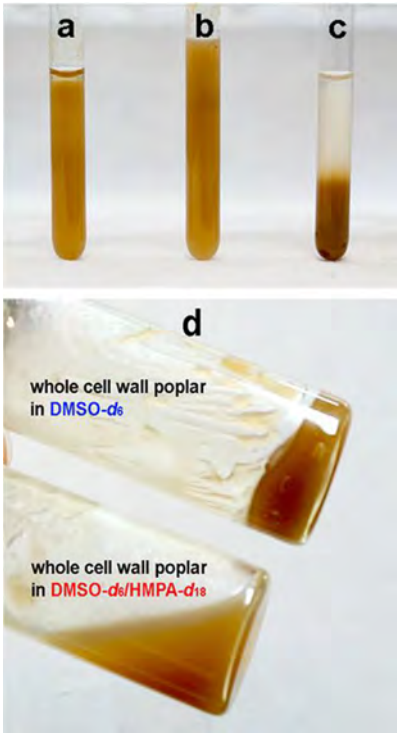

Figure 1. Ball-milled WCW poplar samples in different NMR solvents: a) DMSO-d_6, b) DMSO-d_6/HMPA-d_{18} (4:1), c) HMPA-d_{18}, and d) comparison of the mobility of WCW samples in DMSO-d_6 (upper) and DMSO-d_6/HMPA-d_{18} (lower) solvents.

matrix and resulted in the swelling of biomass. To verify the effect of HMPA for NMR analysis of biomass, the poplar samples were prepared in DMSO-d_6 alone and in the bi-solvent system of DMSO-d_6/HMPA-d_{18}, respectively. The 2 D ^{13}C–^{1}H heteronuclear single quantum coherence (HSQC) spectroscopy and ^{13}C NMR analyses were conducted with the ball-milled poplar samples loaded in DMSO-d_6 and DMSO-d_6/HMPA-d_{18}. The volume ratio of DMSO-d_6/HMPA-d_{18} was optimized for the best resolution and signal intensities with biomass. According to our preliminary tests, a 4:1 *v*/*v* ratio of DMSO-d_6/HMPA-d_{18} showed the best result with poplar. In general, lignin from hardwood poplar mainly consists of syringyl (S) and guaiacyl (G) units. A gel-state NMR analysis with poplar in DMSO-d_6 without co-solvent already presented major components, such as methoxyl group, β-ether (β-O-4) linkage, and lignin subunits [S, G, and *p*-hydroxybenzoate (PB) units; Figure 2 a]. Addition of HMPA-d_{18} into the NMR solvent mixture enhanced signal intensities of the HSQC spectra of the poplar sample as presented in Figure 2 b. The enhanced resonance peak intensities from lignin units and side chains were clearly observed in the DMSO-d_6/HMPA-d_{18} bi-solvent system. In particular, correlation peaks from polysaccharides in both aliphatic (lignin side-chain and polysaccharide) and polysaccharide anomeric regions became more apparent. Improvement in the correlation peak intensity of the ^{13}C NMR spectra was also observed with poplar in DMSO-d_6/HMPA-d_{18} compared with the sample in DMSO-d_6 alone (Figure S2 in the Supporting Information). Addition of HMPA-d_{18} did not result in significant changes in the chemical

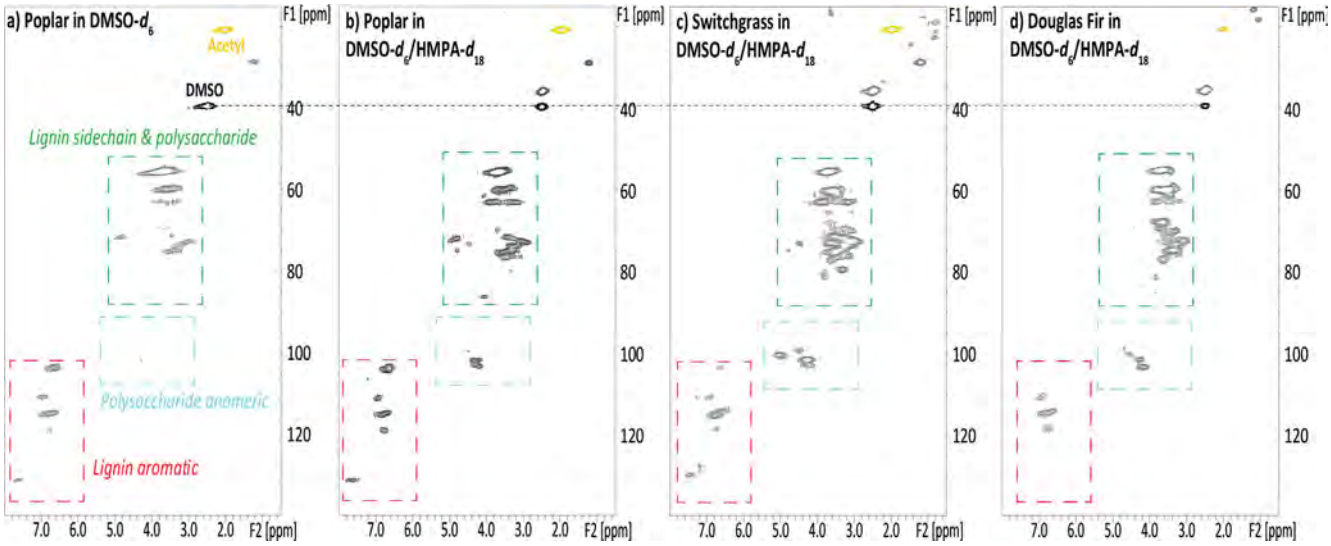

Figure 2. HSQC spectra of WCWs: a) ball-milled poplar cell wall in DMSO-d_6, b) ball-milled poplar cell wall in DMSO-d_6/HMPA-d_{18}, c) ball-milled switchgrass cell wall in DMSO-d_6/HMPA-d_{18}, and d) ball-milled Douglas fir cell wall in DMSO-d_6/HMPA-d_{18}.

shifts of lignin or polysaccharides in the cell-wall biomass, and it was confirmed by comparing the NMR spectra with a model compound (coniferyl alcohol) in DMSO-d_6 and DMSO-d_6/HMPA-d_{18} (Figure S3 and Table S1). Moreover, the signals of HMPA-d_{18} were not overlapped nor did they interfere with resonance peaks of the biomass components in the WCW NMR spectra.

Besides poplar (hardwood), different types of lignocellulosic biomass, such as switchgrass (energy crop) and Douglas fir (softwood), were also analyzed by 2D HSQC spectroscopy with DMSO-d_6/HMPA-d_{18} mixture (4:1, *v*/*v*). Compared with poplar, switchgrass has another lignin subunit [*p*-hydroxyphenyl (H)] and structural moieties *p*-hydroxycinnamates, such as ferulate (FA) and *p*-coumarate (*p*CA), in the lignin aromatic region. The well-separated correlation peaks of C2/C6 in the H units assigned at 127.8/7.19 ppm were presented, respectively, whereas C3/C5 correlation peaks in the H units were overlapped with C5 in the G units. The *p*-hydroxycinnamates are involved in lignification of grass cell walls through acylation. Ferulates that esterify arabinoxylans are reported to involve cross-linking of lignin–polysaccharides and polysaccharide–polysaccharide, thus influencing the digestibility of polysaccharides, whereas *p*CA acylates the γ-OH of lignin side-chains.[8,15] Correlation peaks of FA_2 (110.9/7.22 ppm), FA_6 (123.6/7.19 ppm), and FA_7 (144.9/7.45 ppm) were also readily observed in the bi-solvent system, while FA_5 and FA_8 (113.8/6.39 ppm) correlation peaks were completely or partially overlapped with the G_5 correlation peak. The correlation peaks of $pCA_{2/6}$ (130/7.5 ppm) and pCA_7 (145.3/7.53 ppm) were clearly shown in the HSQC spectra. The pCA_8 (113.8/6.32 ppm) was also observed. The G unit in the WCW Douglas fir was also detected using HSQC spectroscopy.

Two differently isolated samples, lignin-enriched residue (LER, cellulolytic enzyme hydrolyzed lignin enriched residue) and cellulolytic enzyme lignin (CEL), were prepared for comparing the lignin NMR analysis to the results from the WCW biomass sample (Figures 3 and 4) using the bi-solvent system. Details on lignin isolation procedures are described in the Supporting Information (Figure S1). Ball-milled biomass was hydrolyzed using enzyme mixtures for 48 h, and then freeze-dried for NMR analysis. Significant amounts of carbohydrates and other components were removed during the hydrolysis providing 30–35% (yield by mass) of LER composed mainly of lignin with some amounts of cellulose and hemicellulose. The prepared LER was easier to swell and/or dissolve in DMSO-d_6/HMPA-d_{18} mixtures, whereas there was no significant difference between the correlation peaks of lignin from LER and those of lignin from WCW analysis except phenylcoumaran (LB_α, Figures 3 and 4). Although α-position of phenylcoumaran (β-5) correlation peaks from WCW poplar were much smaller than the correlation peaks from LER, these correlation peaks were still shown at lower contour levels close to the noise level. The other lignin, called cellulolytic enzyme lignin, was also prepared through further purification of LER using 96% dioxane for 48 h. This lignin was completely dissolved in DMSO-d_6, and provided much clearer lignin spectra with ^{13}C NMR analysis, whereas the HSQC spectra were still similar to the spectra of LER and WCW samples (Figures 3 and 4).

Besides hardwood (poplar), grass (switchgrass) and softwood (Douglas fir) samples were also prepared in two different ways (WCW and CEL) for NMR analysis of lignin structural features. Douglas fir showed similar quantitative results in both WCW and CEL lignin. Switchgrass also had similar overall lignin monolignol compositions even though CEL had lower content of H units than the WCW sample of switchgrass. Table 1 shows the relative contents of lignin subunits (S, G, H, FA, *p*CA, and PB) and their linkages, including aryl ether bonds and carbon–carbon bonds. Quantification of volume integrals from lignin substructures was conducted with $S_{2/6}$, G_2, $H_{2/6}$, FA_2, $pCA_{2/6}$, and $PB_{2/6}$ contours. The relative percentage of each lignin subunit from WCW, LER, and CEL was similar and resulted in a similar S/G ratio in poplar (1.36, 1.46, 1.51), switchgrass (0.56, -, 0.52) and Doulas fir (0, –, 0), respectively.

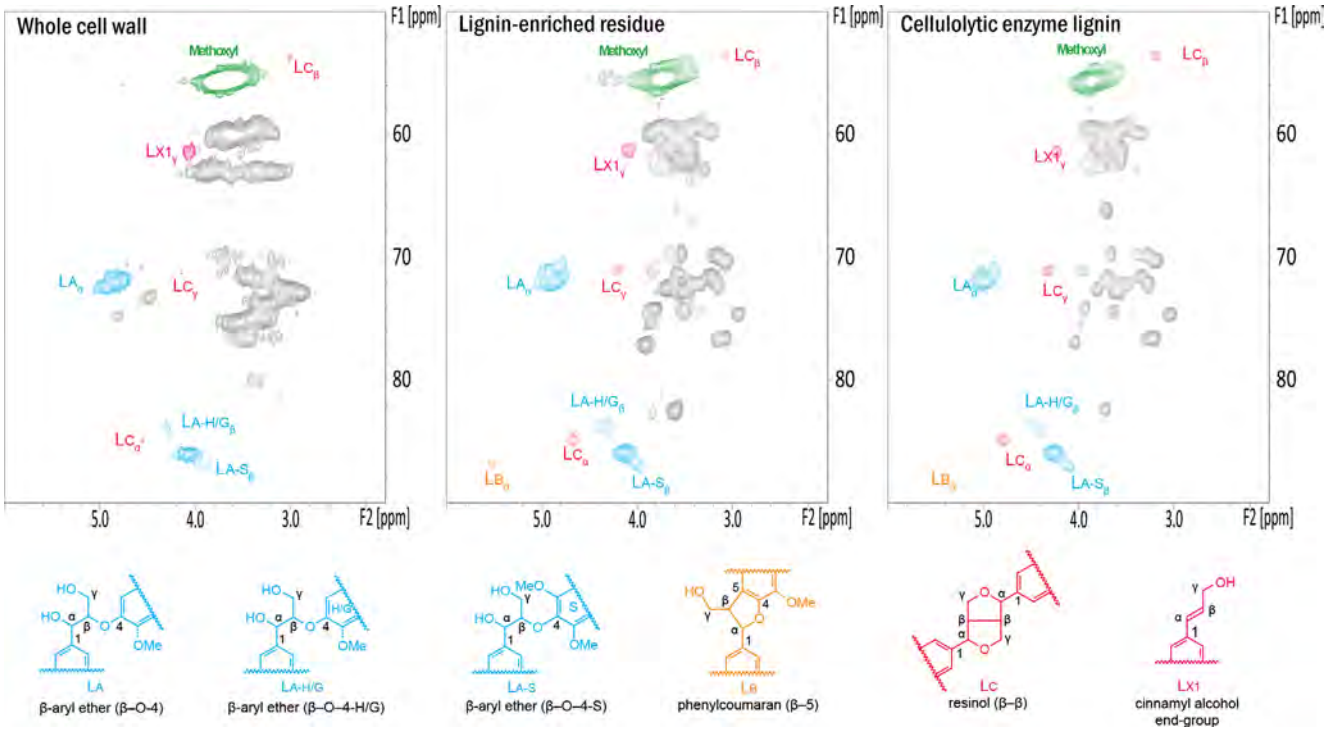

Figure 3. Lignin side-chain and polysaccharide regions of HSQC spectra of WCWs and isolated lignins (lignin-enriched residue and cellulolytic enzyme lignin) in DMSO-d_6/HMPA-d_{18} (CEL was dissolved in DMSO-d_6) from poplar.

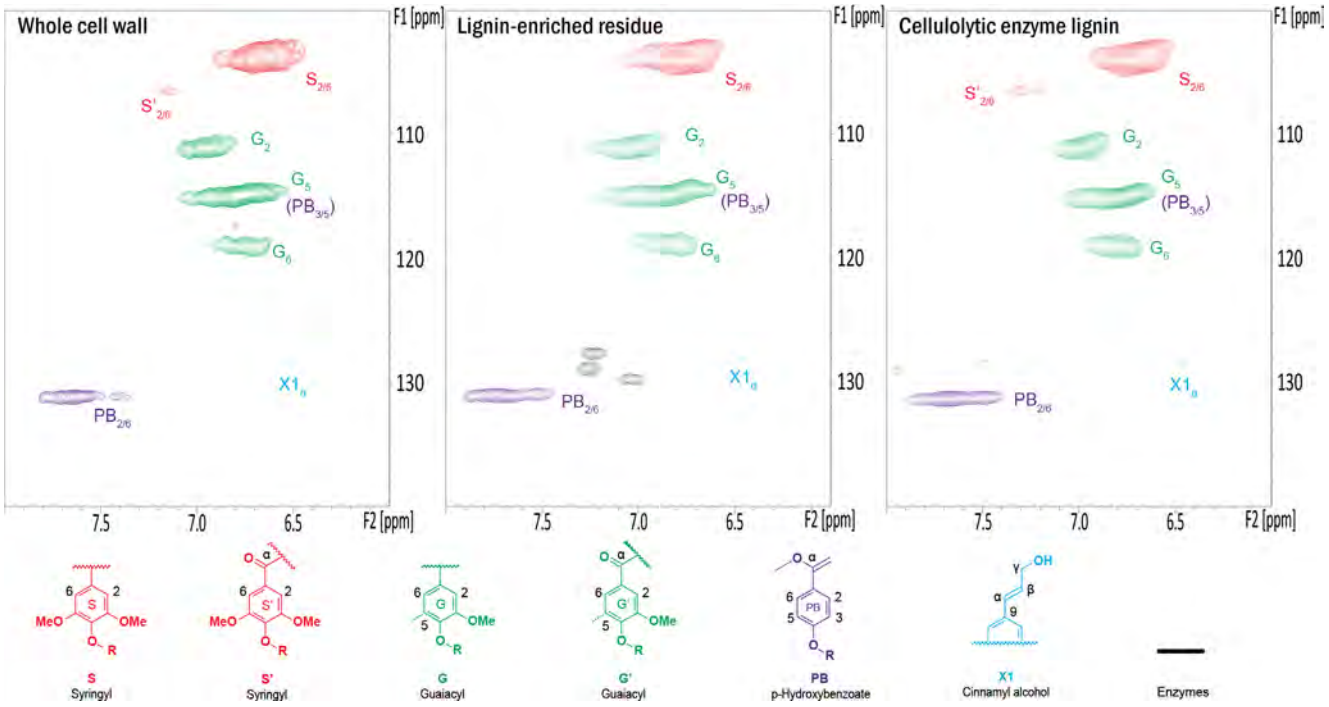

Figure 4. Lignin aromatic regions of HSQC spectra of WCWs and isolated lignins (lignin-enriched residue and cellulolytic enzyme lignin) in DMSO-d_6/HMPA-d_{18} (CEL was dissolved in DMSO-d_6) from poplar.

For quantifying lignin inter-linkages, the α-position of each unit was used. All the contents of lignin inter-linkages were similar in both LER and CEL, while C–C linkages (β-β and β-5) contents observed from WCW were less than those from the enriched/isolated poplar lignin samples. There was no significant difference observed from the relative abundance of inter-linkages in WCW and CEL from other biomass sources (switchgrass and Douglas fir). The NMR results demonstrate that the

Table 1. Quantitative information for lignin subunits and linkages in poplar by HSQC spectroscopy with WCW, lignin-enriched residue (LER), and cellulolytic enzyme lignin (CEL) from three different biomass sources: poplar, switchgrass, and Douglas fir.

Biomass	Sample type	Composition % lignin subunits						inter-linkages		
		S	G	H	PB[a]	*p*CA[a]	FA[a]	LA[b]	LB[b]	LC[b]
Poplar	WCW	57.6	42.4	–	14.7	–	–	87.1	3.4	9.5
	LER	59.5	40.5	–	13.9	–	–	82.8	6.2	11.1
	CEL	60.2	39.8	–	16.8	–	–	82.0	6.3	11.7
Switchgrass	WCW	31.1	55.4	13.4	–	26.3	15.4	77.8	14.3	7.9
	CEL	32.3	63.4	5.3	–	24.8	9.5	80.2	10.7	9.1
Douglas fir	WCW	–	100.0	–	–	–	–	67.6	21.8	10.6
	CEL	–	100.0	–	–	–	–	68.3	22.5	9.3

[a] PB, *p*CA, and FA levels are expressed as a fraction of S + G + H. [b] LA: β-O-4, LB: β–5, LC: β–β.

DMSO-d_6/HMPA-d_{18} bi-solvent system can be used for characterization of lignin structural information. The simpler and shorten preparation of cell-wall samples compared with other lignin isolations for NMR analysis makes it an attractive characterization method as discussed in the previous studies.[4b,8,16]

WCW NMR spectroscopy does not remove polysaccharides from biomass samples, thus it can also provide the structural information of polysaccharides in biomass. In Figure 2, WCW poplar in DMSO-d_6/HMPA-d_{18} showed higher intensity of polysaccharide spectra than the biomass in DMSO-d_6. For elucidating more structural information of polysaccharides in biomass and comparing the DMSO-d_6/HMPA-d_{18} WCW analysis, holocellulose was isolated using peracetic acid and compared with WCW poplar analysis (Figure 5). Similar to the comparison with isolated lignin, even though the contours of polysaccharides from WCWs were weaker than the contours from holocellulose, the WCW method was still able to provide most of structural information. In the anomeric regions, internal cellulose [(1→4)-β-D-Glc*p*], xylan [(1→4)-β-D-Xyl*p*], and mannan [(1→4)-β-D-Man*p*] units; cellulose non-reducing end [(1→4)-β-D-Glc*p* (NR)]; acetylated xylosyl residues at C2 (2-O-Ac-β-D-Xyl*p*); 2,3-di-O-methyl-α-D-glucuronic acid (2,3-di-O-MeGlcA; MGA); and 4-O-methyl-α-D-glucuronic acid (4-O-MeGlcA) were observed from both samples, whereas α-D-mannopyranoside (α-D-Man*p*) and the reducing-terminal-end of β-D-Glc*p* and β-D-Xyl*p* units were only detected from holocellulose sample. The correlation peaks of polysaccharides in the non-anomeric region except MGA_4 and xylan non-reducing end (XNR_4) were also detected from both samples. As the missing correlation peaks were observed at noise level, the analysis of polysaccharide in the biomass can be improved by further optimization. The isolated cellulose and hemicellulose from holocellulose by further purification using hydrochloric acid and sodium hydroxide were also dissolved in DMSO-d_6/HMPA-d_{18} for NMR analysis (Figure S4; detailed isolation methods are described in the Supporting Information). Internal cellulose units (Cl_2, Cl_3, Cl_4, Cl_5, and Cl_6), some cellulose non-reducing ends (CNR_3 and CNR_5), xylan internal units (XI_2, XI_3, XI_4, and XI_5), and xylan reducing/non-reducing end units ($XR\beta_2$, $XR\beta_4$, and XNR_2) were observed in the non-anomeric regions from the isolated cellulose and hemicellulose, respectively. In the anomeric regions of cellulose and hemicellulose, internal cellulose [(1→4)-β-D-Glc*p*] and internal xylan [(1→4)-β-D-Xyl*p*] signals were observed.

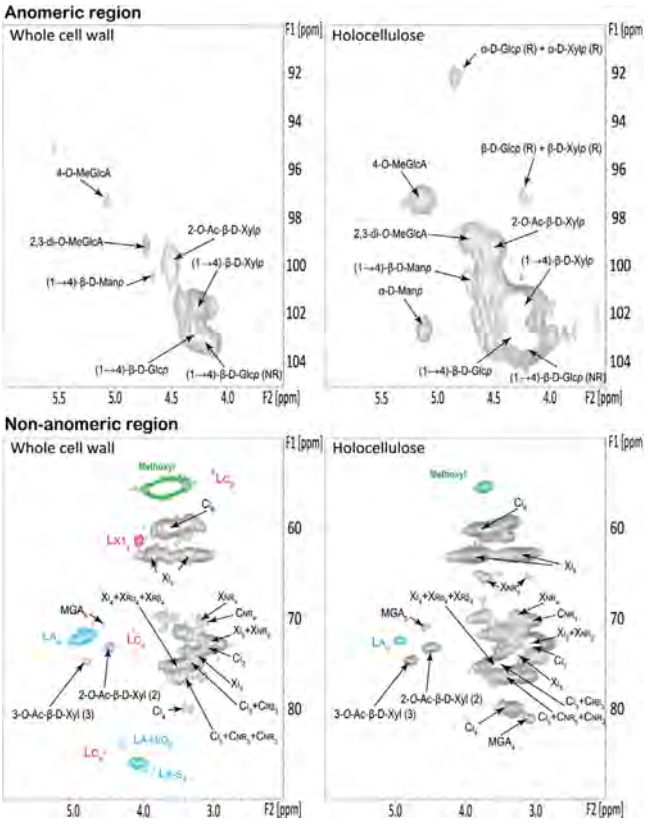

Figure 5. Anomeric regions of HSQC spectra of WCWs and isolated holocellulose from poplar in DMSO-d_6/HMPA-d_{18}.

WCW poplar analysis (Figure 5). Similar to the comparison with isolated lignin, even though the contours of polysaccharides from WCWs were weaker than the contours from holocellulose, the WCW method was still able to provide most of structural information. In the anomeric regions, internal cellulose [(1→4)-β-D-Glc*p*], xylan [(1→4)-β-D-Xyl*p*], and mannan [(1→4)-β-D-Man*p*] units; cellulose non-reducing end [(1→4)-β-D-Glc*p* (NR)]; acetylated xylosyl residues at C2 (2-O-Ac-β-D-Xyl*p*); 2,3-di-O-methyl-α-D-glucuronic acid (2,3-di-O-MeGlcA; MGA); and 4-O-methyl-α-D-glucuronic acid (4-O-MeGlcA) were observed from both samples, whereas α-D-mannopyranoside (α-D-Man*p*) and the reducing-terminal-end of β-D-Glc*p* and β-D-Xyl*p* units were only detected from holocellulose sample. The correlation peaks of polysaccharides in the non-anomeric region except MGA_4 and xylan non-reducing end (XNR_4) were also detected from both samples. As the missing correlation peaks were observed at noise level, the analysis of polysaccharide in the biomass can be improved by further optimization. The isolated cellulose and hemicellulose from holocellulose by further purification using hydrochloric acid and sodium hydroxide were also dissolved in DMSO-d_6/HMPA-d_{18} for NMR analysis (Figure S4; detailed isolation methods are described in the Supporting Information). Internal cellulose units (Cl_2, Cl_3, Cl_4, Cl_5, and Cl_6), some cellulose non-reducing ends (CNR_3 and CNR_5), xylan internal units (XI_2, XI_3, XI_4, and XI_5), and xylan reducing/non-reducing end units ($XR\beta_2$, $XR\beta_4$, and XNR_2) were observed in the non-anomeric regions from the isolated cellulose and hemicellulose, respectively. In the anomeric regions of cellulose and hemicellulose, internal cellulose [(1→4)-β-D-Glc*p*] and internal xylan [(1→4)-β-D-Xyl*p*] signals were observed.

In summary, HMPA was introduced as a co-solvent for improving NMR analysis of biomass. The proposed DMSO-d_6/HMPA-d_{18} bi-solvent system gave effective swelling of biomass; therefore, it successfully revealed biomass structural information with a good resolution of NMR spectra. The analysis was available with a NMR system that is equipped with room-temperature NMR probes and Z-gradient features, without access to cryo-probes for enhancement of signal intensities. In addition, the solvents facilitated sample handling by enhancing the mobility of NMR sample mixtures. WCW NMR analysis in this solvent system provided structural information of both polysaccharides and lignin with a simple ball-milling step. The reliability of NMR analysis of WCW in the DMSO-d_6/HMPA-d_{18} solvent was verified by comparing the analysis results to the isolated cellulose, hemicellulose, lignin, and holocellulose from the same species.

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Keywords: biomass · characterization · hexamethylphosphoramide · NMR spectroscopy · whole cell wall

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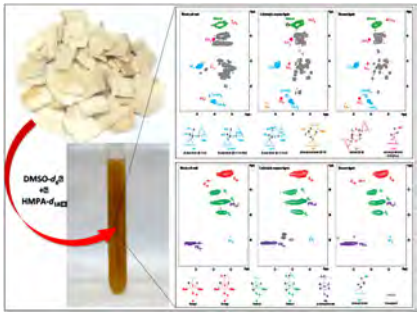
Open structures: NMR analysis is a powerful characterization method for understanding biomass structures. Here, we introduce a novel solvent system composed of dimethylsulfoxide (DMSO-d_6) and hexamethylphosphoramide (HMPA-d_{18}) for effective cell-wall NMR analysis by improving dissolution/swelling of biomass.



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Elucidating Structural Characteristics of Biomass using Solution-State 2D NMR with a Mixture of Deuterated Dimethylsulfoxide and Hexamethylphosphoramide